

Post-anesthetic equine myelopathy: characterization of neuronal reaction and axonal injury using beta-amyloid precursor protein immunohistochemistry

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Case Report

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Abstract

A 2-year-old female Clydesdale horse was anesthetized in dorsal recumbency for an elective surgical procedure to treat osteochondritis dissecans involving the intermediate ridge of the right tibia. Following surgery, the horse lost motor and sensory function in the hind limbs and was unable to stand. The status deteriorated and the horse was euthanized 20 h post-surgery. Histopathologic examination revealed mild to moderate acute myelopathy of the spinal cord between T14 and S3. The lesions were bilateral, with one side being slightly more affected, and more severe in the distal lumbar and sacral spinal cord segments. Immunohistochemistry for beta-amyloid precursor protein (β -APP) was compared to other fast axonal transport proteins such as neurofilament heavy protein, synaptophysin, and ubiquitin. In the presented case, the β -APP immunohistochemistry revealed positively-labeled, multiple, segmentally-swollen axons (axonal bulbs) and rarely neurons. The positively-stained areas overlapped with the lesions seen on HE stained slides including areas in which changes were poorly discernible by routine histologic exam. Amongst the fast axonal transport proteins detected via immunohistochemistry, β -APP was considered adequate to use as a potential sensitive biomarker for axonal injury in this case of post-anesthetic myelopathy. β -APP immunohistochemistry may be a useful tool to study and diagnose axonal injuries of the central and peripheral nervous system in veterinary medicine.

Introduction

Equine post-anesthetic myelopathy is a rare and fatal complication of prolonged general anesthesia. Several cases have been reported mostly in young horses anesthetized in dorsal recumbency (Ragle C 2011). The principal clinical signs after anesthesia are inability to stand in recovery, hind limb paralysis, and absence of panniculus reflex (middle to caudal thorax) (Ragle C 2011). The microscopic findings include variable amounts of edema, hemorrhage, and necrosis of the spinal cord; with the grey matter being predominantly affected (Ragle C 2011). However, a sensitive biomarker of axonal injury has not been utilized in any reported case of equine post-anesthetic myelopathy.

Beta-amyloid precursor protein (β -APP) is a 695-770 residue neuronal peptide and ubiquitous membrane glycoprotein synthesized in normal neurons. Although the precise biological functions of this protein are unknown, β -APP exerts an array of biological activities involving neuroprotection and tissue repair in mammals (Goodman and Mattson 1994; Schubert et al. 1991; Sheetz et al. 1998; Smith and Anderton 1994). This neuropeptide has also been shown to play a physiological role in cell adhesion and endogenous neuroprotection in response to injury (Blumbergs et al. 1995; LeBlanc et al. 1992; Mattson 1997). It is transported anterogradely via fast axoplasmic transport by binding to the kinesin light chain subunit of kinesin-1 (Sherriff et al. 1994a).

In human medicine, β -APP is recognized as an early and sensitive marker of axonal injury (Geddes et al. 2000). The protein accumulates proximal to the site of axonal injury within axonal retraction bulbs following a disruption in axoplasmic transport, thus allowing immunohistochemically detectable levels to accumulate (Blumbergs et al. 1994; McKenzie et al. 1996; Sherriff et al. 1994b). Although β -APP-positive immunohistochemistry definitively indicates damage of axons, its accumulation is not specific for a traumatic etiology or hypoxic-ischemic injury (Graham et al. 1988; Graham et al. 2004; Leclercq et al. 2002). Current histological techniques are unable to discriminate between axonal injury due to these two processes. Axons labelled with β -APP antibody were described after traumatic brain injury, in neurodegenerative diseases and around infarcts, abscesses, and neoplastic metastases (Sherriff et al. 1994a; Sherriff et al. 1994b). It is hypothesized that β -APP accumulates in conditions in which the cytoskeleton breaks down (Li et al. 1995). β -APP is upregulated and can be detected immunohistochemically in the brain as early as 30 minutes after various traumatic and ischemic insults and before manifestation of cellular injury in routine HE stained sections (Hortobagyi et al. 2007; Van den Heuvel et al. 1999). In the laboratory rat, β -APP immunoreactivity was present in a few swollen axons after 4 h of compression injury of the spinal cord (Li et al. 1995).

In diagnostic veterinary medicine, β -APP immunohistochemistry to detect early axonal damage in spinal cord and brain injury has been utilized in domestic animal, such as dogs (Bock et al. 2013), pigs (Finnie et al. 2003), guinea pigs (Finnie et al. 2000b), and sheep (Finnie et al. 2000a; Finnie et al. 2001; Lewis et al. 1996), but not in a post-anesthetic myelopathy case. We present a case herein of post-anesthetic myelopathy in a horse, including utilization of β -APP immunohistochemistry to characterize early histopathologic changes in the spinal cord.

Case History

A 2-year-old, 594 kg, female Clydesdale horse was admitted to the Veterinary Health Center at the University of Missouri for an elective surgical procedure to address osteochondritis dissecans of the intermediate ridge of the right tibia. The horse's pre-operative clinical exam revealed normal vital parameters. The horse was sound at a walk and showed no evidence of ataxia or proprioceptive deficits. No cranial nerve deficits were evident. Moderate effusion was present in the right tibiotarsal joint and mild effusion was present in the left tibiotarsal joint.

The day following admission the horse was anesthetized for tibiotarsal joint arthroscopic surgery. Sedation was achieved with xylazine (1.1 mg kg^{-1} IV), and anesthesia was induced with ketamine (2.2 mg kg^{-1} IV) and midazolam (0.067 mg kg^{-1} IV) and maintained with isoflurane in oxygen. Routine monitoring included direct arterial blood pressure measurement, ECG, and arterial blood gas analysis. Mean arterial blood pressure (MAP) was maintained between 65 and 80 mmHg with dobutamine given to effect. The

MAP fell to 60 mmHg during two brief episodes lasting less than 10 minutes. Four serial arterial blood gas analyses performed at equal intervals gave arterial oxygen tensions of 133, 101, 64, and 91 mmHg. Anesthetic duration was 3 hrs 27 minutes. In recovery, the horse appeared to have complete neurologic dysfunction of the hindlimbs. Although the horse was able to attain a sternal position with the use of her head and front limbs, the hind limbs remained immobile with no motor or sensory function. Patellar and anal reflexes were absent. There were no upper motor neuron signs (e.g. spasticity of muscles, overreactive reflexes) to hindlimbs. Cutaneous trunci reflex could not be elicited caudal to the 15th thoracic vertebrae. Supportive care was instituted but no improvement was evident and the horse was humanely euthanized 20 hours post-surgery.

The carcass submitted to the Veterinary Medical Diagnostic Laboratory, University of Missouri (Missouri, USA) for necropsy. At necropsy, the left gluteal skeletal muscles were diffusely mildly pale and edematous. The trachea contained red froth and the lungs were edematous. The spleen and liver were congested. No gross lesions were present in the other organs, including brain and spinal cord. Tissue samples were collected and fixed in 10% neutral buffered formalin, trimmed and embedded in paraffin. Paraffin sections (4 µm in thickness) were stained with hematoxylin and eosin (HE). In addition, after deparaffinization, sections of the spinal cord were immunohistochemically stained with a rabbit polyclonal anti-beta-amyloid precursor protein (β-APP) (Invitrogen, 51-2700), mouse monoclonal anti-neurofilament heavy protein (NF-H, 200 KDa, BioLegend, 801702), mouse monoclonal anti-synaptophysin (DAKO/Agilent, M7315), rabbit polyclonal anti-ubiquitin (DAKO/Agilent, Z0458), and the EnVision™+ System (DAKO) was used for antigen detection.

Results

Histopathologic examination was consistent with mild to moderate acute myelopathy of the spinal cord between T14 and S3. Multifocal coalescing and sometimes locally extensive spaces containing edema fluid separated the dorsal column of grey matter from the dorsal funiculus of white matter. Vacuolation of neuropil was prominent in the ventral horns. Occasional neurons in the ventral horn exhibited central chromatolysis and cytoplasmic eosinophilia occasionally with nuclear pyknosis. The axons in the affected areas were multifocally swollen. The blood vessels in the adjacent grey and white matter were dilated and contained a few monocytes. Occasional foci of acute hemorrhage occurred in the white matter of the affected sections of spinal cord. The lesions occurred bilaterally but severity was slightly greater on one side. The ventral grey matter and lateral and ventral funiculi were most affected. These changes were most severe in the distal lumbar and sacral spinal cord.

Immunohistochemistry for beta-amyloid precursor protein (β-APP) demonstrated several positively-labeled segmentally-swollen axons (axonal bulbs and spheroids) at the interface of the grey and white

matter at S1 level. The axonal spheroids were more abundant at the ventral portion of the spinal cord. β -APP positive axonal swellings were common in the areas of vacuolated neuropil, but the axonal injury located in the corresponding areas on HE slides was sometimes minimal or undetectable. The neuronal cell bodies showed minimal to quasi absent immunoreactivity with the anti- β -APP. Serial sections were obtained to compare β -APP staining pattern with other fast-transport proteins. Immunoreactivity with neurofilament heavy protein (NF-H) resulted in a strong immunopositivity of most spheroids but abundant cellular bodies were immunolabeled (nonspecific for the lesion). The immunolabeling of synaptophysin at S1 level was different from the β -APP and NF-H immunostaining patterns. The segmentally-swollen axons stained positively amid widespread staining of the normal white matter. The injured axons were very faintly immunolabeled with anti-ubiquitin, quasi undistinguished from the granular staining of the white matter.

Discussion

In the presented case, the horse could not stand up but was able to stay in sternal recumbency. The paralysis accompanied by sensory loss in the hindlimbs, absence of anal tone, loss of patellar reflexes, and absence of cutaneous trunci reflex caudal to the 15th thoracic vertebrae are consistent with a lesion localized in the thoracic, lumbar and sacral segments of the spinal cord, specifically between T14 and S3. A lesion located in the cervical (C1-C5), cervical intumescence (C6-T2), and thoracolumbar (T3-L3) regions will not cause lower motor neuron functional effects (e.g. muscles atrophy, flaccid paralysis, decreased reflexes) in the pelvic limbs. There was no upper motor neuron associated signs (e.g. spasticity of muscles, overreactive reflexes) in the pelvic limbs, which rule out a lesion located in the cervical (C1-C5), cervical intumescence (C6-T2), and thoracolumbar (T3-L3) spinal cord segments. A lesion strictly confined to the thoracolumbar (T3-L3) region would not be sufficient to explain the lower motor neuron signs in the pelvic limbs and loss of anal sphincter tone, although the cutaneous trunci reflex would have been absent caudal to the spinal cord lesion. Euthanasia was elected because of the rapid progression of neurological signs. Presence of fecal incontinence and lower motor neuron dysfunction in the urinary bladder (flaccid, distended, easy to express, dribbling urine) were not assessed in this case. The presence of lower motor neuron dysfunction in the pelvic limbs indicate a lesion present in the cranial lumbosacral (L4-S1) region. It is mainly the loss of perineal reflex that is indicative of a lesion affecting the caudal lumbosacral segment (S1-S3) of the spinal cord.

The classical pathological findings in cases of post-anesthetic equine myelopathy are hemorrhage, edema, and malacia of the grey matter of thoracic, thoracolumbar, lumbar, and/or lumbosacral to sacral regions of the spinal cord. In our case, a large proportion of the spinal cord was affected, prominently involving the grey matter with less effects in the white matter. Wallerian degeneration in the affected lower motor neurons in the spinal cord ventral horns was very subtle in HE sections. A few motor neurons of the grey matter were swollen with eosinophilic cytoplasm (chromatolysis) and karyolytic nuclei. In general, the morphologic changes of axonal degeneration in HE stained sections may present various

degrees of swelling with differences in topology which also depend on the severity and chronicity of the lesion.

IHC staining at the site of malacia allows detection of protein changes in distribution and expression beyond that detected with HE stained sections. The nervous tissue lesions involve post-translational modification will be detectable with IHC using primary antibody that detects, for example, a covalently modified protein (e.g. phosphorylation, nitrosylation) or a cleaved protein. These biochemical modifications are not visible in the HE stained sections. Immunohistochemical technique can be useful in confirming axonal injury of the spinal cord in veterinary medicine. The enlarged and swelling axons form spheroids and accumulation of protein occur at site because of disruption of antero- and retrograde transportation. Sensitive biomarker of axonal injury are the proteins of fast axoplasmic transport such as β -APP, neurofilament proteins, synaptophysin, and ubiquitin. However, these biomarkers are non-indicative of a particular etiology.

Immunostaining for β -APP accumulation (i.e., axonal swelling/bulb) is a well-established technique to identify traumatic axonal injury within the brain in human medicine and laboratory animal research (Graham et al. 2000). β -APP has been detected by immunohistochemistry within 35 min after traumatic brain injury (Hortobagyi et al. 2007). A previous study conducted in laboratory rats with compression trauma to the spinal cord demonstrated β -APP-immunopositive accumulation in nerve cell bodies and swollen axons of the grey matter 4 h after compression with the dorsal segment exhibiting more pronounced accumulation versus the ventral segment (Li et al. 1995). In this rat study, the accumulation of β -APP occurs rapidly and is long lasting after compression injury (9 days) with the magnitude of accumulation positively correlating with increasing severity of the lesions (Li et al. 1995). Herein, β -APP immunoreactivity occurred in cellular bodies of neurons affected with chromatolysis denoting areas of degenerated neurons and axonal swelling but many lesions noted in IHC were subtle or not detectable by HE staining. Proteins involved in axonal transport are used as markers of axonal injury. Proteins such as β -APP accumulate when the cytoskeleton breaks down. β -APP IHC is superior in detecting early or subtle neuronal lesions in this specific case of equine post-anesthetic malacia. The usefulness of β -APP IHC will require further studies with quantitative techniques and more case numbers.

The pathogenesis of post-anesthetic myelopathy is not fully understood. The development of neuronal changes and axonal injury are posited to involve ischemic-hypoxic injury to the spinal cord, executed by a number of different mechanisms, including a sequel to the dorsal recumbency (Ragle C 2011). In this interesting case of post-anesthetic myelopathy, vacuolation at the interface of the grey and white matter was present laterally and ventrally. These vacuoles represent myelinated fiber degeneration and loss. A few spheroids were perceptible on HE slides. The β -APP-positive axonal swellings were common in areas of spongy change at the interface of the grey and white matter. The positively-stained areas overlapped with the lesions seen on HE slides (lesions visible), but also occurred in adjacent areas without visible HE

section alterations, suggesting enhancement of sensitivity with this immunohistochemical procedure beyond that provided by routine HE stains. The β -APP expression in this myelopathy case were rarely observed in neuronal cellular bodies. It is hypothesized that widespread expression of β -APP in neuronal perikarya represent an acute phase response to trauma rather than an irreversible damage (Van den Heuvel et al. 1999). The time of elapsed after the anesthesia was 20 h in this case. The microscopic changes most likely involved axonal injury beyond the possibility for axon recovery.

The pattern of β -APP labeling was compared with other fast axonal transport proteins such as neurofilament heavy protein (NF-H), synaptophysin, and ubiquitin. Many axons and cellular bodies were labeled with NF-H. The neurofilament network consists of two neurofilament pools: a stationary pool forms the skeleton (phosphorylated neurofilament subunits) of the axons and a moving pool contains newly synthesized non-phosphorylated units (Dahl et al. 1989; Nixon 1993). Immunohistochemistry with NF-H facilitates the identification of damaged swollen axons and blubs, and does not specifically stain damaged axons. Staining of the normal white matter was observed with monoclonal antibody raised against synaptophysin. Almost all axons (normal and injured) stained positively which indicate a lack of specificity. This non-specific staining make the immunohistochemistry interpretation very challenging. Consequently, synaptophysin would not be an ideal biomarker of axonal injury in post-anesthetic myelopathy cases. Concerning the ubiquitin immunohistochemistry, the injured axons were stained pale and inconsistently stained, and sometimes quasi undistinguished from the granular background staining in white matter. In a previous study, ubiquitin-immunopositive axonal injury was apparent at 6 hours post injury (Oemichen M 2006). This protein would be an adequate biomarker of axonal injury because the swollen degenerating axons are often heavily ubiquitinated. However, this feature diminish as Wallerian degeneration progresses to fiber loss (Jortner et al. 1996). For this potential biomarker, time of necropsy would be critical in order to detect heavily ubiquitinated axons at the early stage of axonal injury. In the later stage, the chance to observe a positive signal with anti-ubiquitin antibody is diminished. The reason behind that is the swollen axons undergoing catabolic and degeneration processes. In our case, necropsy has been performed at later stage, which would explain the faint staining obtained with anti-ubiquitin.

To conclude, we presented a classical case of post-anesthetic myelopathy with the characteristic the axonal swelling and Wallerian degeneration noted in ventral and lateral white matter in the spinal cord between T14 and S3 segments. Using a primary antibody that labels proteins of fast axonal transport such as β -APP, neurofilament heavy protein (NF-H), synaptophysin, and ubiquitin, we demonstrated the extent and intensity of intra-axonal immunohistochemical reaction in the stage of axonal swelling. NF-H, synaptophysin, and ubiquitin stain normal neurons and axons, but not all of them. These antibodies do not specifically stain damaged axons. However, they are useful to follow the tracts and identify damaged areas of blebs/bulbs and spheroids. β -APP has been used to investigate traumatic axonal injury of the brain, brain stem and spinal cord injuries in humans, laboratory animals, and domestic animals but has

not been commonly used as a sensitive methodology to determine axonal injury in equine post-anesthetic myelopathy. In this report, β -APP immunohistochemistry was considered adequate to investigate the presence and distribution of axonal injury in the spinal cord of a horse, providing evidence that β -APP immunostaining can detect axonal damage attributed to prolonged anesthesia and dorsal recumbency. β -APP immunohistochemistry may be a useful tool to study or diagnose traumatic injury of spinal cord in large animals.

Declarations

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Conflicting of interest

The authors have no conflict of interest to disclose.

Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is an investigation of an animal at post-mortem examination.

Contributions

JSF drafted the manuscript, JK and AB provided important information on the case and DYK carried out the histopathologic examination. All authors read, critically revised and approved the final manuscript.

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Figures

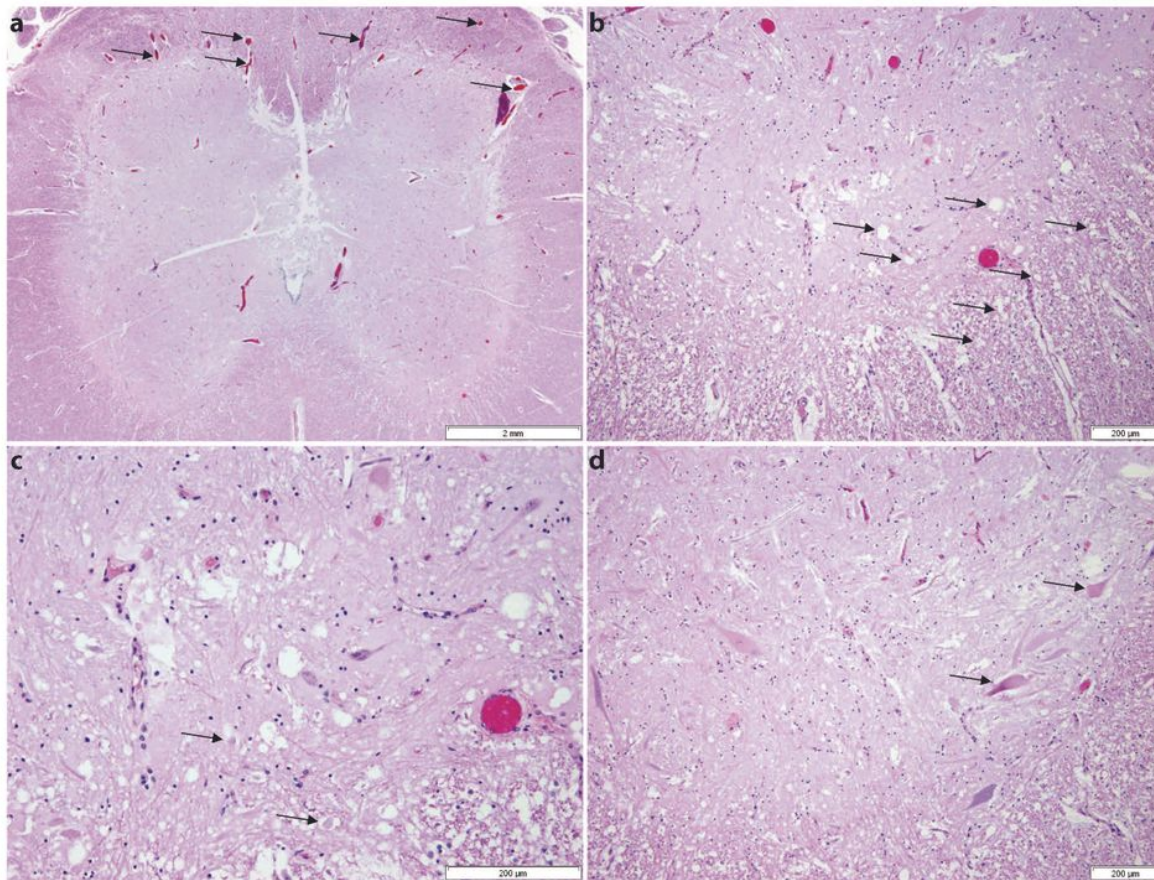


Figure 1

Transverse sections of the equine spinal cord from level S1 stained with hematoxylin and eosin (H&E). (a) At low magnification, congestion is noticeable in the dorsal part of the white and grey matter, where vascularization is abundant (arrows). (b) At higher magnification, the ventral and lateral region of grey and white matter contains numerous vacuoles (arrows). The vacuolation is more prominent at the interface of the grey and white matter. (c) A few spheroids are perceptible (arrows). (d) A few neurons in the dorsal horns are angular and exhibit various degrees of chromatolysis including initial swelling of the cell body, peripheral margination of the nucleus and cytoplasmic eosinophilia.

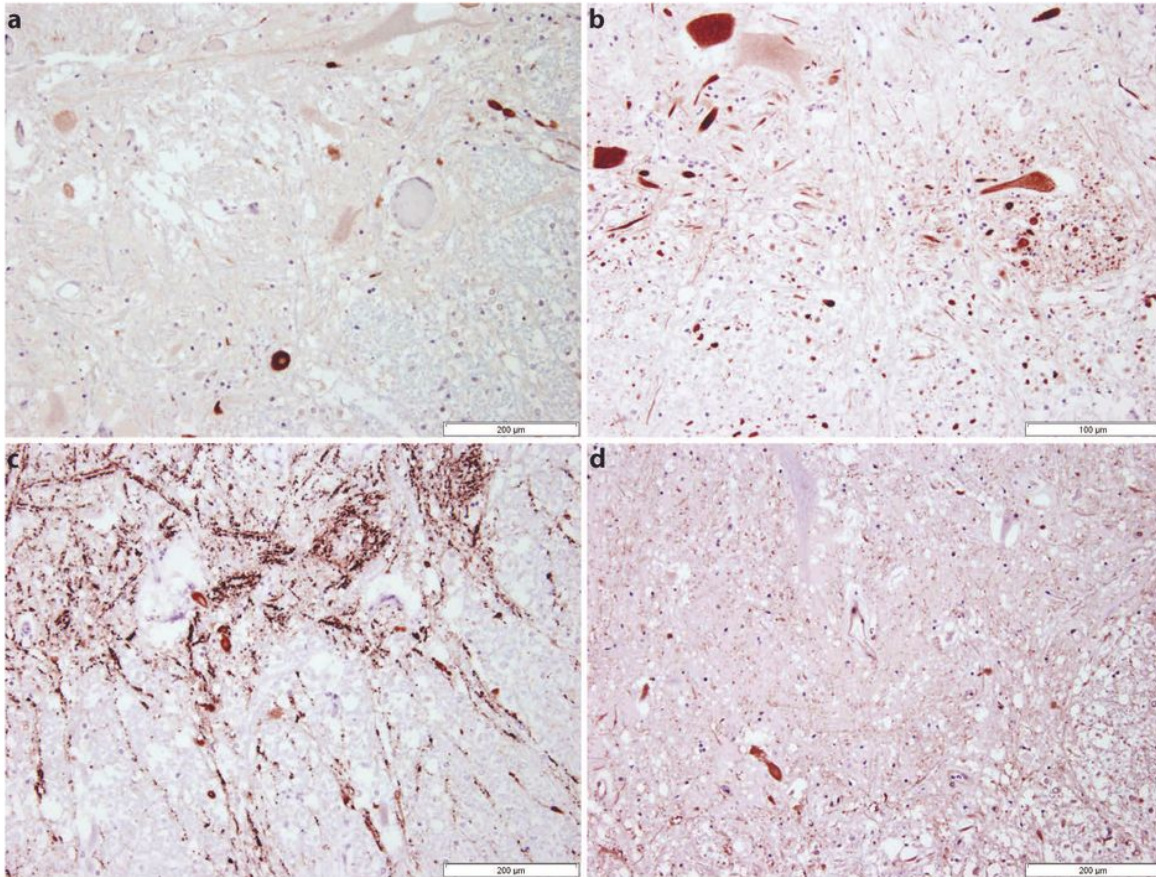


Figure 2

Comparison of axonal injury labelling using antibodies in adjacent regions of the equine spinal cord at level S1. (a) β -amyloid precursor protein (β -APP): the segmentally-swollen axons (axonal bulbs, spheroids) stained strongly at the white-to-grey matter interface, where faintly perceptible in the hematoxylin and eosin (H&E) stained sections. (b) Neurofilament heavy protein (NF-H, 200 KDa): many axons and cellular bodies were labeled strongly. (c) Synaptophysin: the segmentally-swollen axons stained strongly, but normal white matter stained positively. (d) Ubiquitin: there were pale labeling of injured axons and granular staining in white matter.