AIM AND OUTLINE OF THE THESIS

The principal and final aim of the thesis is to gain more insights into the pathological basis of animal neurodegenerative diseases, and compare to human beings, in order to contribute to the understanding of the pathogenesis of animal neurodegenerative disorders that might course with neuronal loss and loss of presynaptic terminals. For such a global objective, several sub-objectives are required:

1. To contribute to the knowledge in veterinary medicine of the specific animal disorder, and not only classify them by the presence of non-disease specific-neuropathologic features with a specific distribution as occur in animal NADs.

2. To study the synaptic proteins involved in membrane trafficking, synaptic vesicle docking, membrane fusion and exocytosis in order to determine a pre-synaptic terminal loss in such disorders.

3. To study if neuronal loss is due to an activation of the apoptotic pathway performing the TUNEL method and studying some proteins that promote or inhibate apoptosis.

4. To give insights to contribute to the knowledge of the pathogenesis of neurodegenerative disorders.

5. To compare results with its human neurodegenerative disorder counterparts, in order to achieve possible animal models for such diseases.

In an effort to reach the sub-objectives, four animal diseases of the CNS were chosen, taking into account two criterions: all diseases had to show neurodegeneration although they were presented in different species, and NAD has to be the hallmark of the disease.

The different works base on the following diseases are described in the materials and methods section:

- Neuroaxonal Dystrophy in a young Rottweiler (comparing results with 5 aged dogs).
- Experimental Murine Scrapie in 10 C57BL mice intraperitoneally inoculated with the Rocky Mountain Laboratory isoform.
- Equine Degenerative Myeloencephalopathy in two Arabian horses.
- Spinal Muscular Atrophy in 4 Holstein-Friesan calves.

In an effort to reach the final and global objective, all results are summarized and discussed at the discussion section, resulting in a view on the specific neuropathological basis in each disease, its contribution to the undertanding of the pathogenesis in neurodegenerative disorders of the CNS, and its similarities or differences when compare to human counterparts.

STUDIES

Study 1.

Juvenile neuroaxonal dystrophy in a Rottweiler: accumulation of synaptic proteins in dystrophic axons

Study 2.

Abnormal synaptic protein expression and cell death in murine scrapie

Study 3.

Abnormal synaptic protein expression in two Arabian horses with equine degenerative myeloencephalopathy

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Abnormal synaptic protein expression in two Arabian horses with equine degenerative myeloencephalopathy

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Abstract

Numerous ballooned neurons and multiple dystrophic axons were observed in the nuclei gracillis and cunneatus of two male Arabian horses, one a 6-month-old and the other a 1.5-year-old, with equine degenerative myeloencephalopathy, Ballooned neurons and dystrophic axons showed synaptophysin, synaptosomal-associated protein of 25 kDa, syntaxin-1 and α-synuclein immunoreactivity. Moreover, dystrophic axons were strongly ubiquitinated and showed immunopositivity against the anti-phosphorylated 200 kDa neurofilament protein. Abnormal expression of integral synaptic vesicle, synaptic vesicle-associated presynaptic plasma membrane and cytosolic proteins, which participate in the trafficking, docking and fusion of the synaptic vesicle to the plasma membrane, suggest that severe disruption of axonal transport plays a crucial role in the pathogenesis of dystrophic axons in equine degenerative myeloencephalopathy.

Key words: equine degenerative myeloencephalopathy, Arabian horse, dystrophic axons, synaptophysin, SNAP-25, syntaxin-1, α-synuclein

Introduction

Neuronal axonal dystrophies (NADs) are a group of inherited (primary or endogenous) or acquired (secondary) neurodegenerative diseases described in animals and humans that have in common the formation of dystrophic axons as the chief substrate of the disease process (Jellinger, 1973). In human beings, primary NADs include infantile, late infantile, juvenile and adult types, neuroaxonal leukoencephalopathy, juvenile and adult Hallervorden-Spatz syndrome, Nasu-Hakola disease, and senile or physiological NAD (Jellinger, 1973; Lowe *et al*., 1997). Endogenous NADs in animals have been reported in horses, sheep, rabbits, cats and dogs (Summers *et al*., 1995).

Equine degenerative myeloencephalopathy (EDM) is a specific form of NAD in which lesions are confined strictly to the cuneate and gracillis nuclei (Miller and Collatos, 1997). EDM, which is one of the most common causes of spinal cord diseases in horses (Mayhew, 1989; Nappert, 1989, Dill *et al*, 1990), is a neurodegenerative disorder of the central nervous system (CNS) of unknown origin that affects young horses between the ages of two months and three years (Mayhew *et al*., 1977; Beech *et al*., 1987; Baumgärtner *et al*., 1990; Blythe *et al.,* 1991; Summers *et al*., 1995). Horses with EDM develop an insidious onset of symmetrical spasticity, ataxia, and paresis of the four limbs, although the pelvic limbs are more commonly affected (Mayhew *et al.*, 1977; Mayhew, 1989; Cox *et al*., 1995; Miller and Collatos, 1997). Neuroaxonal dystrophic changes in EDM may be especially prominent in the thoracic nuclei of the spinal cord and in the cuneate and gracile nuclei of the caudal portion of the medulla oblongata, and they are associated with vacuoles and astrocytic response (Beech, 1984; Mayhew, 1989; Baumgartner *et al.*, 1990; Cox *et al.,* 1995; Summers *et al.*, 1995).

The cause or causes of EDM are still unknown (Dill *et al*., 1990), although it has frequently been described as a familial degenerative disease in Morgans (Beech and Haskins, 1987) and Appaloosas (Blythe *et al.*, 1991), while in other breeds such as Mongolian wild horses (Liu *et al.,* 1983) and Haflinger horses (Baumgärtner *et al*., 1990) EDM has been associated with a vitamin E deficiency.

Neuronal cytoplasmic accumulation of presynaptic proteins has been described in primary NAD and in secondary NAD (Newell *et al*., 1999). Moreover, in brains of aged humans that show physiologic NAD, a selective regional synapse loss has been suggested (Honer *et al,* 1992). Accumulation of α-Synuclein, a synaptic protein, has been observed in dystrophic axons in human NAD (Newell *et al*., 1999). Moreover, an aberrant localization of integral synaptic vesicle, synaptic vesicle-associated presynaptic plasma membrane, and cytosolic proteins, has recently been described in a Rottweiler with juvenile NAD (Sisó *et al*., 2001). Consequently, impaired axonal transport, which has been suspected of playing a crucial role in the pathogenesis of dystrophic axons (Sandbank *et al*., 1970), is a suspect in canine NAD (Sisó *et al*., 2001).

Synaptophysin is an integral membrane protein of presynaptic vesicles that interacts with other synaptic proteins and participates in exocytosis (Jahn and Südhof, 1994). SNAP-25, a plasma membrane protein

which is present in presynaptic nerve terminals, axons and growth cones (Oyler *et al.,* 1989), is thought to be essential for synaptic vesicle exocytosis (Oyler *et al*., 1989; Hess *et al*., 1992), too. Syntaxins are integral plasma membrane proteins that are implicated in the docking of synaptic vesicles at presynaptic actives zones (Bennett *et al.,* 1992). α-synuclein (α-SN), a 140 amino acid cytosolic protein enriched at presynaptic terminals, plays a role in protein-protein interactions, synaptic vesicle trafficking and turn-over, and synaptic plasticity (Clayton and George, 1998).

The purpose of the study reported here is to determine whether an accumulation of presynaptic proteins in dystrophic axons, together with a disruption in axonal transport, occurs in EDM.

Materials and Methods

Two male Arabian horses, 6 months (case 1) and 1 year old (case 2) with a history of ataxia in the posterior limbs, were suspected to have EDM and were euthanised. A complete necropsy was performed. A 1.5-year-old male horse with no neurological clinical history was included in the study as a control.

Samples from different visceral organs and brain of the three horses were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin wax, and sections, 5-µm-thick, were cut. Routine microscopic examination was carried out in each case.

Immunohistochemistry was carried out using the avidin-biotin-peroxidase method (ABC kit, Vector, Vectastain) or a modified labeled avidin-biotin (LAB) technique (DAKO LSABâ 2 System Peroxidase). De-waxed sections were first treated with 2% hydrogen peroxide and 10% methanol diluted in tris-buffered saline (TBS 100 mM, pH=7.4) for 15 min. After blocking endogenous peroxidase, sections were firstly incubated with 5% normal horse serum (monoclonals) or 20% normal goat serum (polyclonals) for 2 hours, and then incubated overnight at 4ºC with the primary antibodies. A rabbit polyclonal antibody to ubiquitin (Dako, dilution 1:100) and a mouse monoclonal antibody to phosphorylated/dephosphorylated 200-kDa neurofilaments (clone N52, Sigma, 1:800) were used. Astrocytes were evaluated with the polyclonal antibody against glial fibrillary acidic protein (GFAP, Dako, 1:600). Sections were later incubated with specific biotinylated secondary IgG antibody (Dako) at a dilution of 1:100 and, finally, with the avidin-biotin-peroxidase method (ABC kit, Vector, Vectastain) at a dilution of 1:100 for 1 hour at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Samples were counterstained with haematoxylin. Microglial cells were labelled with the biotynilated lectin *Lycoperiscon esculentum* (Sigma, L-0651) used at a dilution of 1:100 for 2 hours at room temperature and then visualised with the previously described modified labelled avidin-biotin technique.

To visualize synaptic changes, several antibodies against the synaptic vesicle (synaptophysin, syntaxin-1) and the pre-synaptic plasma membrane (SNAP-25 and α-synuclein) were also used. A monoclonal anti-synaptophysin (SYN, Dako, 1:100) and polyclonal antibodies to the synaptic-associated protein of 25,000 mol. Wt (SNAP-25, Sternberger, 1:1000), syntaxin-1 (Calbiochem, 1:25) and α-synuclein (α-SN,

Chemicon, 1:300) were used. For α-SN, samples were pretreated with 98%formic acid for 5 minutes. A modified labeled avidin-biotin (LAB) technique (DAKO LSABâ 2 System Peroxidase) was applied in all synaptic antibodies. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. All sections were counterstained with haematoxylin.

Results

a. Neuropathological findings

Microscopically, both ataxic horses showed large numbers of dystrophic axons of varying size restricted to the central nervous system (CNS). No lesions were observed in the peripheral nervous tissue (spinal roots, spinal ganglia, peripheral nerve) or skeletal muscles.

Interestingly, the pattern of lesioning showed regional differences between the two horses. Case 1 showed lesions in the posterior brain stem and in the spinal cord, while in case 2, lesions were restricted to the spinal cord. Nevertheless, lesions in both horses were bilateral and symmetrical.

Case 1 showed large numbers of swollen dystrophic axons and spongiosis in the gracillis, cuneatus and accessory cunneatus nuclei. In these nuclei neuronal loss was suspected, and the remaining neurons were pale and swollen (Fig. 1A). Moreover, small swollen axons were also observed in the trigeminal nerve spinal tract nuclei. In all these regions, axonal abnormalities were accompanied by astrocytic gliosis, as confirmed with GFAP immunohistochemistry (Fig. 1C). In this horse, a glial nodule was observed near the glossopharyngeal nucleus. In the spinal cord, low numbers of dystrophic axons were found closer to the thoracic nucleus. Vacuolar changes were minor in some spinal segments, and absent in others.

In contrast, case 2 revealed no lesions in the brain. Large numbers of spheroids and pale, swollen neurons affected the ventral horn of the cervical (Fig 1B), thoracic and lumbar segments of the spinal cord. These abnormalities were also observed in numerous neurons of the thoracic nucleus. Vacuolar changes predominated in segments with lesions. A severe astrocytic reaction confirmed by GFAP-positive cells was present in the ventral horn. A discrete perivascular cuff of mononuclear cells was present next to the pontine nuclei.

In both cases, astrocytes were hypertrophied and enlarged, while no changes in microglial cells were observed.

Dystrophic axons showed strong immunopositivity against the anti-phosphorylated neurofilament (Fig. 1H) and the anti-ubiquitin (Fig. 1I) proteins in both horses. By contrast, swollen neurons revealed a marked increase in ubiquitin-immunopositivity only when they were compared to normal neurons. Curiously, in the spinal cord, the nuclei of some neurons as well as large numbers of round glial cells in the white matter, presumably oligodendrocytes, were ubiquitinated.

The control horse showed no lesions in the CNS.

b. Synaptic protein immunohistochemical results

A severe generalized diminution in synaptic protein expression was observed in both affected horses when presynaptic terminals were assessed with SYN, SNAP-25, syntaxin-1 and α-SN. Overall, an altered staining of the perikarya of the swollen neurons and dystrophic axons was evident. SYN-immunoreactivity in the gracille and cuneate nuclei showed a diffuse but severe diminution in the neuropil. Swollen neurons and dystrophic axons (Fig. 1D) in these nuclei showed SYN-immunopositivity. The same results were observed in the spinal cord of case 2.

Neuropil immunopositivity to SNAP-25 was markedly reduced in affected horses. Cytoplasmic SNAP-25 immunoreactivity was observed, forming coarse punctate granular aggregates in swollen neurons and dystrophic axons (Fig. 1E), while normal-appearing neurons revealed mildly reduced immunopositivity.

A slight increase in positivity was observed in dystrophic axons (Fig. 1F) and ballooned neurons for syntaxin-1 immunohistochemistry when they were compared to normal neurons.

The nuclei gracillis and cuneatus of affected horses showed a reduction of α-synuclein-positivity in the neuropil, accumulation within dystrophic axons (Fig. 1G) and a coarse perineuronal deposition in lower motor neurons.

Discussion

The pattern of lesion distribution in the CNS encountered in both affected horses, and the presence of NAD features in the cuneate and gracillis nuclei, confirmed a diagnosis of EDM as previously described (Miller and Collatos, 1997). In our study, the type of lesion is identical in the two Arabian horses but the regional distribution is quite different. Pale and swollen neurons and some dystrophic axons restricted to the ventral horn were observed in one horse (case 2), suggesting a possible involvement of lower motor neurons in this animal. In spite of this, we considered the lesion to be non- disease-specific, as involvement of lower motor neurons was not described; the clinical signs and localization of the lesions suggest that EDM is a type of upper-motor neuron disease (Mayhew *et al*., 1977; Liu *et al.*, 1983; Beech, 1984; Mayhew, 1989; Cox *et al*., 1995). The intense immunoreactivity to ubiquitin observed in dystrophic axons in our two horses is similar to that observed in the infantile form of human NAD, called Seitelberger's disease (Moretto *et al.,* 1993). This result implies a local activation of the ubiquitin system in EDM, leading to lysis of altered proteins within swollen neurons and dystrophic axons in response, probably, to a variety of stressful states. This is likely as ubiquitin plays a critical role in cellular catabolic processes, where it conjugates to altered proteins, thus marking them for lysosomal or nonlysosomal degradation (Mayer *et al*., 1991). Ubiquitinated swollen axons have also been reported in horses with equine cervical compressive myelopathy (Jortner *et al*., 1996). We did not attach importance to the

staining with anti-ubiquitin of the nuclei of some neurons in the spinal cord of affected horses because it was also observed in neurons of pontine nuclei and in the spinal cord of the control horse.

We did not observe accumulation of lipofucsin, although accumulation of this lipopigment has been described as a frequent feature in EDM (Nappert *et al*., 1989; Miller and Collatos, 1997), suggesting lipid peroxidation of cell membranes.

Dystrophic axons in Arabian horses with EDM revealed low expression of phosphorilated neurofilaments (Borràs *et al*., 1997). By contrast, in our study dystrophic axons disclosed accumulation of phosphorylated/ non-phosphorylated neurofilaments (Fig. 1H), suggesting a possible impaired axonal transport.

The diffuse diminution in the neuropil with synaptophysin in the gracille and cuneate nuclei implies a severe diminution of terminal synaptic boutons in affected nuclei. Swollen neurons and dystrophic axons revealed synaptophysin immunopositivity in their cytoplasm, suggesting that an aberrant localization of this presynaptic vesicle protein occurs in EDM. Although dystophic axons were markedly positive to SNAP-25, syntaxin-1 and α-SN, and reduction in immunoreactivity against these presynaptic membrane proteins was appreciated in the neuropil of affected nuclei, the changes observed in the expression of the synaptohysin protein were more marked (Fig. 1D). In our opinion, as presynaptic proteins such as synaptophysin, synaptosomal-associated protein of 25 kD (SNAP-25), syntaxin-1 and α-synuclein are felt to participate in the trafficking, docking and fusion of the synaptic vesicle to the plasma membrane, facilitating synaptic transmission and exocytosis of neurotransmitters (Jahn *et al*., 1994; Südhof *et al*., 1995; Volknandt, 1995), these results, taken together, suggest severe axonal transport impairment in dystrophic axons of equine NAD in EMD. Interestingly, the altered patterns of presynaptic terminal attachment to ballooned neurons and accumulation of synaptic proteins within dystrophic axons in affected nuclei are similar to those patterns reported in both juvenile and aged canine NAD (Sisó *et al*., 2001). To our knowledge, this is the first report of loss of synapse and accumulation of synaptic proteins in dystrophic axons in horses with EMD.

All these results suggest that more studies in EDM are needed in order to determine if different forms of EDM can co-exist.

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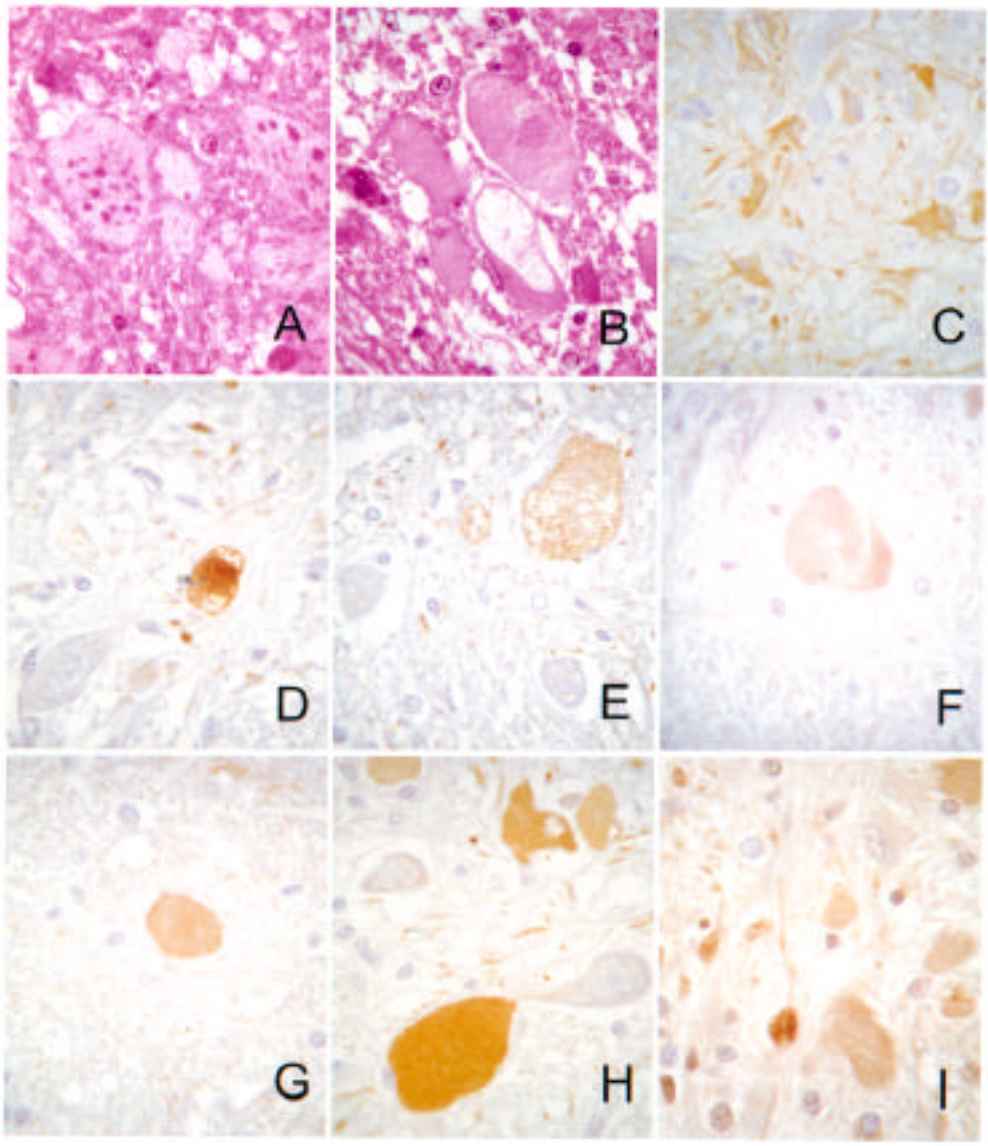

Figure 1. Equine degenerative myeloencephalopathy in two horses showed neuronal loss in the nuclei gracillis and cunneatus. Remaining neurons in both nuclei were pale and swollen (A), or vacuolated (B, arrowhead), and were associated with large numbers of swollen dystrophic axons (arrow) (B). Axonal abnormalities were accompanied by astrocytosis (C, GFAP immunohistochemistry). The majority of dystrophic axons were immunoreactive with synaptophysin (D), SNAP-25 (E), syntaxin-1 (F) and α-synuclein (G) epitopes. The neuropil in affected nuclei were slightly labelled with anti-synaptic proteins (data not shown) when compared with non-affected nuclei. Normal-appearing neurons (arrows) in affected nuclei were rarely immunolabelled with antibodies to synaptic proteins (D, E) and neurofilament epitopes (H). Most dystrophic axons (empty arrows) and some degenerated neurons (arrowhead) displayed strong neurofilament (H) and ubiquitin (I) immunoreactivities. Paraffin sections: haematoxylin and eosin-stained sections (A, B); immunohistochemistry (IHC) following the avidin-peroxidase method (ABC kit Vector, Vectastain); IHC with the modified labelled avidin-biotin (LAB) technique (DAKO LSAB2 System Peroxidase). A-I: x 1000.

Study 4.

Decreased synaptic protein expression and cell death in Holstein-Friesan calves with spinal muscular atrophy

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Decreased synaptic protein expression and cell death in the ventral horn of Holstein-Friesian calves with spinal muscular atrophy.

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Abstract

Neuropathological study of Holstein-Friesian calves with spinal muscular atrophy (SMA) has shown decreased numbers of motor neurones in the brachial and lumbo-sacral regions of the spinal cord, together with swelling and accumulation of phosphorylated neurofilaments, and neuronophagia in most of the remaining motor neurones. The pyramidal tracts, motor cortex and thalamus were not affected. Synaptophysin immunohistochemistry disclosed marked reduction of punctate terminals only around swollen neurones, thus suggesting de-afferentation at advanced stages of the degenerative process. The immunohistochemical study of proteins linked with cell death and cell survival has shown reduced expression of Fas, Fas-L, Bcl-2 and Bax in swollen motor neurones. Punctate cytochrome C immunoreactivity, consistent with mitochondrial localisation, vanished in the soma of swollen motor neurones. Finally, not a single cell was stained with anti-cleaved (active) caspase-3 (17 kDa), thus showing a lack of evidence for the involvement of the apoptotic pathways in motor neurone death. Taken together, the present findings point to necrosis as a major cause of motor neurone death in advanced stages of SMA in Holstein-Friesian calves.

Key words: spinal muscular atrophy, lower motor neurone disease, calves, Fas, Fas-L, Bcl-2, Bax, cytochrome c, caspase, apoptosis, necrosis.

Introduction

Spinal muscular atrophy (SMA) is a motor neurone disease (MND) characterised by degeneration and loss of motor neurones of the spinal cord and motor nuclei of the brain stem, and denervation and atrophy of the striated muscle. MND has been reported in several species such as dogs, cats, mice, horses, goats, rabbits, cows and humans (Oppenheimer and Esiri, 1992; Summers et al., 1995). Human MNDs include amyotrophic lateral sclerosis (ALS), infantile spinal muscular atrophy or Werdnig-Hoffmann disease (WHD) and lower motor neuron disease (LMND). SMA has been described in Horned Hereford calves (Rousseaux et al., 1985), red Danish calves (Agerholm et al., 1994), brown Swiss cattle (El-Hamidi et al., 1989; Troyer et al., 1993) and Holstein-Friesian calves (Pumarola et al., 1997).

Immunohistochemical and electron microscopical studies have shown abnormal connectivity on spinal motor neurons in ALS (Ikemoto and Hirano, 1996; Matsumoto et al., 1994; Sasaki and Maruyama, 1994b; Sasaki and Iwata, 1996a), WHD (Ikemoto et al, 1996; Yamanouchi et al, 1996) and LMND (Ikemoto et al, 1994; Matsumoto et al, 1994a; Sasaki and Maruyama, 1994a). The majority of studies have suggested that loss of synapses occurs later in the course of the disease, once motor neurones show signs of degeneration. Lower motor neurone degeneration as a result of loss of afferent contacts has no support on the basis of neuropathological studies in human MNDs. Yet no similar studies have been carried out in animal SMA.

Loss of motor neurones and chromatolytic cells filled with phosphorylated neurofilaments are important pathologic findings in the ventral horn in human (Leigh et al., 1989; Muñoz et al., 1988; Sobue et al., 1990) and animal (Summers et al., 1995; Pumarola et al., 1997) MNDs. Yet the cause of death of motor neurones is not merely the result of neurofilament accumulation.

Several studies in human MND have suggested apoptosis as the main cause of motor neurone death in ALS (Yoshiyama et al., 1994; Ekegren et al., 1999; Martin, 1999) and WHD (Simic et al., 2000). This assumption is based mainly on positive staining with the method of in situ end-labelling of nuclear DNA fragmentation. Yet other studies have shown that motor neurone death in ALS is not apoptotic (Migheli et al., 1997; He and Strong, 2000). Similarly, no evidence of apoptosis has been observed in post-natal motor neurones in WHD (Ferrer, 1996; Hayashi et al., 1997), but it has been detected during foetal development in type I SMA human spinal cord (Soler et al., 2002). Complementary observations have demonstrated abnormal expression of the proteins Bcl2 and Bax, which regulate apoptosis, in the anterior horn of ALS cases (Mu et al., 1996; Ekegren et al., 1999; Martin, 1999; Sathasivam et al., 2001). Similar changes have been observed in the anterior horn of SOD1 transgenic mice used as a model of ALS (Vukosavic et al., 1999). Moreover, caspase-1 and caspase-3 are up-regulated in ALS transgenic mice, and inhibition of caspase-3 delays disease onset and mortality in these animals (Li et al., 2000).

SMA in Holstein-Friesian calves is associated with motor neurone loss, together with a pale and swollen appearance of the remaining cells (Pumarola et al., 1997). Degenerating cells in bovine SMA are not

stained with the method of in situ labelling of nuclear DNA fragmentation. Moreover, degenerating neurones do not show c-Jun/AP-1 (N) immunoreactivity, which recognises major products of caspase-3 activation, suggesting that apoptosis is not the main mechanism of cell death in this SMA (Pumarola et al., 1997).

The present study examines the expression of the synaptic vesicle-associated glycoprotein of 38-kD synaptophysin (Jahn and Südhof, 1994; Südhof, 1995) in motor neurones of spinal cord in Holstein-Friesian calves with SMA. Then immunohistochemistry to Fas, Fas-L, Bcl-2, Bax, cytochrome C and active caspase-3 is analysed to learn about the possible role of apoptotic proteins (Desagher and Martinou, 2000) in motor neurone death in these animals.

Materials and Methods

The study was carried out on the spinal cords of 4 calves, aged between 15 days and 2 months, with spinal muscular atrophy. The animals showed locomotor difficulties at the age of 15 days that progressed to paraparesis and tretraparesis (Pumarola et al., 1997). The animals were killed with an intravenous injection of sodium pentothal. The brain and spinal cord were immediately fixed by immersion in 4% paraformaldheyde for two weeks. Then selected samples were embedded in paraffin. Dewaxed sections were stained with haematoxylin and eosin, luxol fast blue-Klüver Barrera and processed for immunohistochemistry.

Immunohistochemistry was carried out following a labelled avidin-biotin (LAB) technique (DAKO LSAB2 System Peroxidase). The monoclonal antibody to synaptophysin (Dako) was used at a dilution of 1:100. The monoclonal antibody to phosphorylated neurofilaments of kDa (clone RT97, Boehringer Mannheim) and the rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP) (Dako) were used at a dilution of 1:800 and 1:600, respectively. Microglial cells were stained with the biotynilated lectin *Lycoperiscon esculentum* (Sigma, L-0651) used at a dilution of 1:100. The rabbit polyclonal Fas (C-20) and Fas-L (N-20) (sc-715 and sc-834, Santa Cruz Biotechnology) antibodies were used at dilutions of 1:50 and 1:40, respectively. The rabbit polyclonal Bcl-2 (N-19) and Bax (N-20) antibodies (sc-492 and sc-493, Santa Cruz Biotechnology) were used at a dilution of 1:75. The cytochrome C monoclonal antibody (Pharmingen) was used at a dilution of 1:500. The cleaved caspase-3 (17 kDa) rabbit polyclonal antibody (96661S, Cell Signaling) was used at a dilution of 1:100. For synaptophysin immunohistochemistry, the sections were pre-treated with saponin for 30 min. For Fas, Fas-L, Bcl-2 and Bax immunohistochemistry, the sections were first boiled in citrate buffer. All the sections were first treated with 2% hydrogen peroxide and 10% methanol. After blocking endogenous peroxidase, the sections were incubated with normal serum for 2 hours, and then incubated overnight with the primary antibodies. Then the sections were incubated with prepared DAB + substrate-chromogen solution (3,3'-diaminobenzidine chromogen solution in buffered

substrate solution, pH 7.5, containing hydrogen peroxide 20 µl/ml) for 5 min. The sections were counterstained with haematoxylin.

Results

a. General neuropathological and immunohistochemical findings

Severe motor neurone loss was found in the brachial and lumbo-sacral regions of the spinal cord. Axonal degeneration was an associated feature in the ventral spinal roots. Large numbers of the remaining motor neurones of the spinal cord were pale and swollen; some neurones were dying and scattered figures of neuronophagia were common in every case (Fig. 1). Mild demyelination was seen in the anterior horns and ventral roots, but the pyramidal tracts were preserved. Astrocytic gliosis, as shown by increased GFAP immunoreactivity, was observed in the ventral horns. Dying motor neurones were infiltrated with mononuclear phagocytes, as revealed by specific lectin histochemistry (data not shown). Swollen neurones were filled with phosphorylated neurofilaments, the immunoreactivity to which was still detectable in phagocyted neurones (Fig. 2). No abnormalities were seen in other regions of the brain, including the motor cortex and thalamus.

b. Synaptophysin immunohistochemistry

In normal appearing neurones, synaptophysin immunoreactivity decorated the periphery of the soma and main dendrites as a fine perineuronal punctate precipitate. (Fig. 2 A and B). Decreased synaptophysin immunoreactivity, almost to the point of disappearance, was noted only in swollen neurones of the ventral spinal cord (Fig. 2 C-F). Punctate synaptophysin immunoreactivity was also decreased in the neuropile of the most severely damaged regions (Fig. 2 A-B, and C-F).

c. Immunohistochemistry to proteins involved in cell death and cell survival.

Fas immunoreactivity was very low in spinal motor neurones and the expression was decreased in swollen neurones (Fig. 4 A). Fas-L immunoreactivity was very low, too, in spinal motor neurones, and this immunoreactivity decreased with neurodegeneration (Fig. 4 B). Bcl-2 immmunoractivity recognised a fine granular precipitate, which was consistent with the mitochondrial localisation of the protein, in the neuropile and in preserved motor neurones. Yet the granular precipitate vanished in swollen cells (Fig. 4 C). Bax expression was manifested mainly as a diffuse precipitate, consistent with the cytosolic distribution of the protein, in preserved neurones. Bax immunoreactivity was practically absent in swollen motor neurones (Fig. 4 E). Cytochrome C was expressed as punctate precipitates in the soma of preserved motor neurons, in agreement with the expected mitochondrial localisation of this protein (Fig. 4 E). Cytochrome C immunoreactivity disappeared in swollen neurones (Fig. 4 F). Finally, not a single motor

neurone was stained with antibodies to cleaved (active) caspase-3 (17 kDa) (data not shown), although the antibody is effective in detecting apoptosis in other paradigms (Puig and Ferrer, 2002).

Discussion

The present study has shown loss of motor neurones, swelling of most remaining motor neurones, astrocytic gliosis and neuronophagia in the ventral horn of the brachial and lumbo-sacral regions of the spinal cord in Holstein-Friesian calves affected by SMA. Similar findings have been reported in brown Swiss cattle (El-Hamidi et al., 1989; Troyer et al, 1993). In addition, axonal degeneration and demyelination of the descending tracts of the spinal cord, and degeneration in the thalamus, midbrain and motor cortex, have been described in this breed (Troyer et al., 1992). Yet no similar changes have been noted in Holstein-Friesian calves (Pumarola et al., 1997; present observations). These findings indicate a phenotype reminiscent of ALS in brown Swiss cattle and a phenotype resembling WHD in Holstein-Friesian calves.

Synatophysin immunohistochemistry has demonstrated decreased expression on swollen motor neurones of the spinal cord in Holstein-Friesian calves, thus suggesting progressive de-afferentation of damaged motor neurones. Similar findings have been reported in WHD and LMND (Ikemoto et al., 1994; Matsumoto et al., 1994; Sasaki and Maruyama, 1994a; Ikemoto et al., 1996; Yamanouchi et al., 1996). Therefore, similar mechanisms are probably linked to synaptic loss in human and animal MNDs.

Modifications in the expression of Bax and Bcl-2, and activation of the apoptotic pathway have been described in human MNDs (Yoshiyama et al., 1994; Mu et al., 1996; Ekegren et al, 1999; Martin, 1999; Pedersen et al., 2000). Activation of apoptosis has also been described in a transgenic mouse model of familial ALS (Vukosavic et al., 1999). Yet degenerating cells in bovine SMA are not associated with increased apoptotic signals, including increased Fas and Fas-L expression, decreased Bcl-2 and increased Bax immunoreactivity. Moreover, apoptosis has been considered a cause of cell death in WHD (Simic et al., 2000), although the percentage of cells stained with the method of in situ end-labelling of nuclear DNA fragmentation (0.2-6.4%) exceeds the amount expected for a subacute degenerating disease. Neuronal death with morphological characteristics of apoptosis is enhanced, and it begins during the foetal development in WHD (Soler et al., 2002). Yet apoptosis has not been observed in post-natal spinal cords in the same group (Ferrer, 1996; Soler et al., 2002) in agreement with other reports (Hayashi et al., 1998).

Cytosolic translocation of cytochrome C, pivotal in the mitochondrial-dependent caspase activation pathway, and activation of the caspase pathway, have both been described in ALS and animal models (Guégan et al., 2001). Yet no immunohistochemical evidence supporting the occurrence of cytochrome C translocation in motor neurones of spinal cords has been found in bovine SMA. Caspase-1 and caspse-3 activation has a pivotal role in mediating cell death in an ALS transgenic mouse model (Li et al., 2000).

However no evidence of cleaved (active) caspase-3 expression has been found in motor neurones of the spinal cord in Holstein-Friesian calves with SMA. Taken together, our findings do not support apoptosis as a major cause of cell death in advanced stages of bovine SMA. Rather, our data suggest that necrosis is an important mode of cell death in bovine SMA. In this line, it is important to stress that the contribution of a non-apoptotic mode of cell death such as necrosis in the overall demise of spinal cord motor neurones in end-stage transgenic mSOD1 mice cannot be ruled out (Vukosavic et al., 2000).

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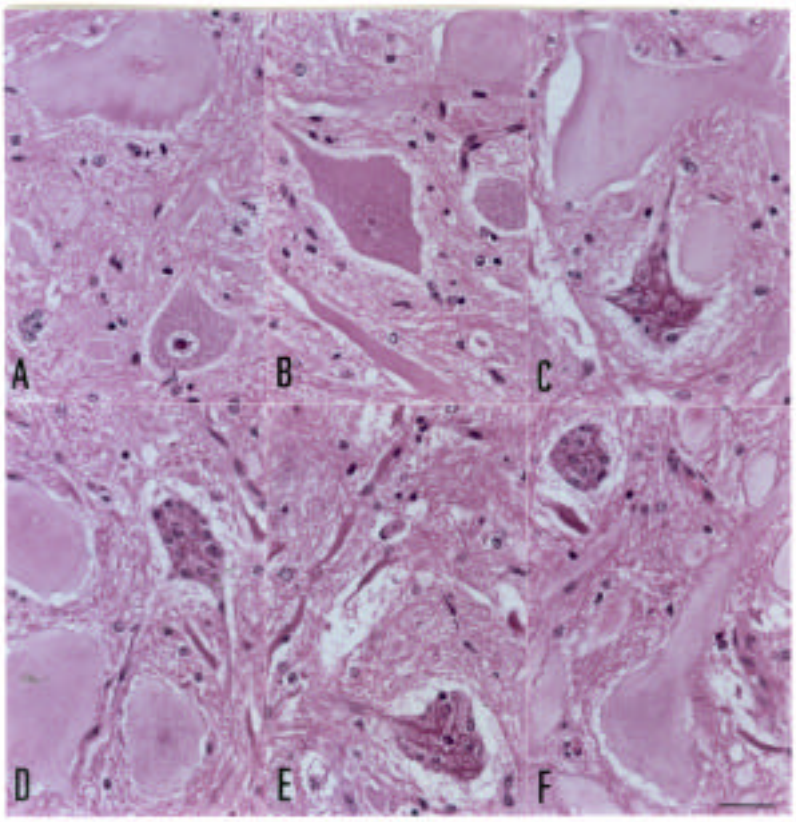

Fig. 1: Motor neurones of the spinal cord in Holstein-Friesian calves with spinal muscular atrophy (SMA). Preserved motor neurones are characterised by the cytoplasm filled with Nissl granules and the large, clear nucleus with a prominent nucleolus (A). Ballooned neurones show swollen, homogeneous cytoplasm and disappearance of Nissl granules (A-D, F). Dying cells do not have recognisable organelles and are infiltrated by mononuclear phagocytes (C-F). Paraffin sections stained with haematoxylin and eosin, bar = 25 µm.

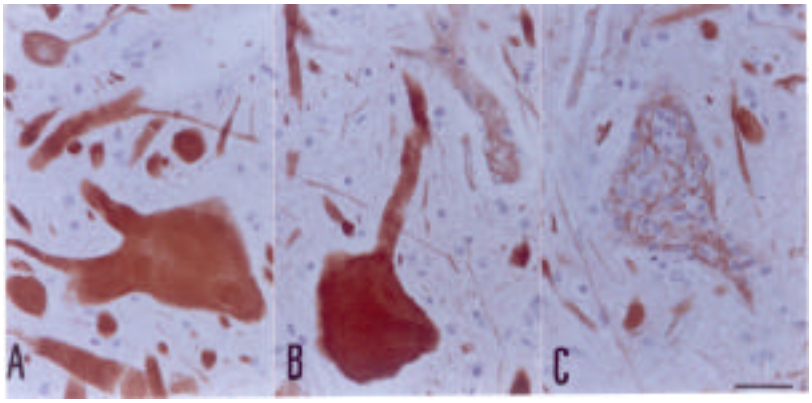

Fig. 2: Swollen neurones in the spinal cord of Holstein-Friesian calves with SMA are filled with phosphorylated neurofilaments of 200 kDa (A, B). Neurofilaments are still visualised in dying cells infiltrated by mononuclear phagocytes (C). Paraffin sections, RT97 immunohistochemistry, slight haematoxylin counterstaining, bar = 25 µm.

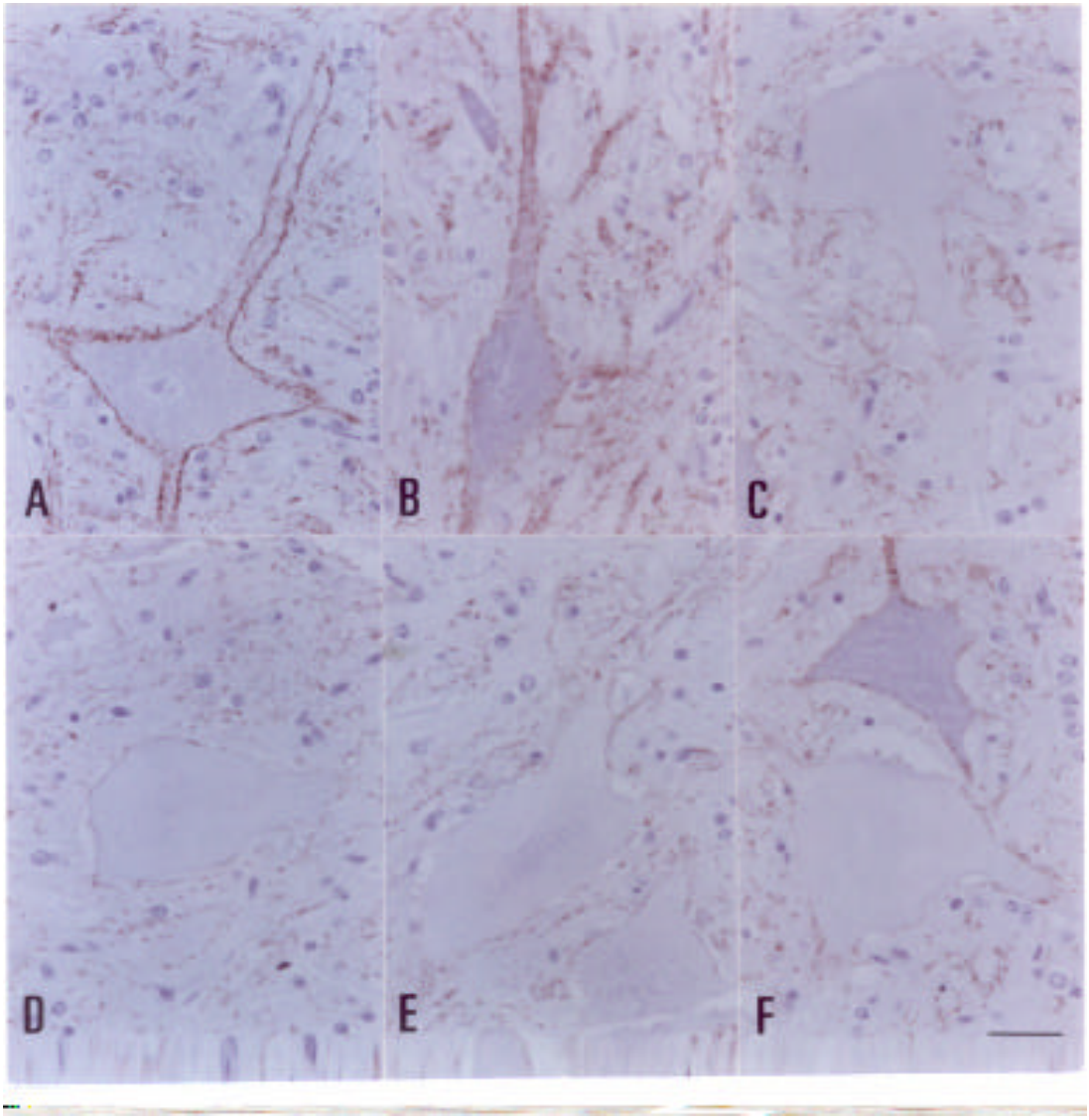

Fig. 3: Synaptophysin immunohistochemistry of motor neurones in the spinal cord of Holstein-Friesian calves with SMA. Punctate synaptophysin immunoreactivity is found around the cytoplasm and main dendrites in preserved neurones (A, B). Reduced, almost absent, synaptophysin immunoreactivity is seen only in swollen neurones (C-F). Paraffin sections slightly counterstained with haematoxylin, bar = 25 µm.

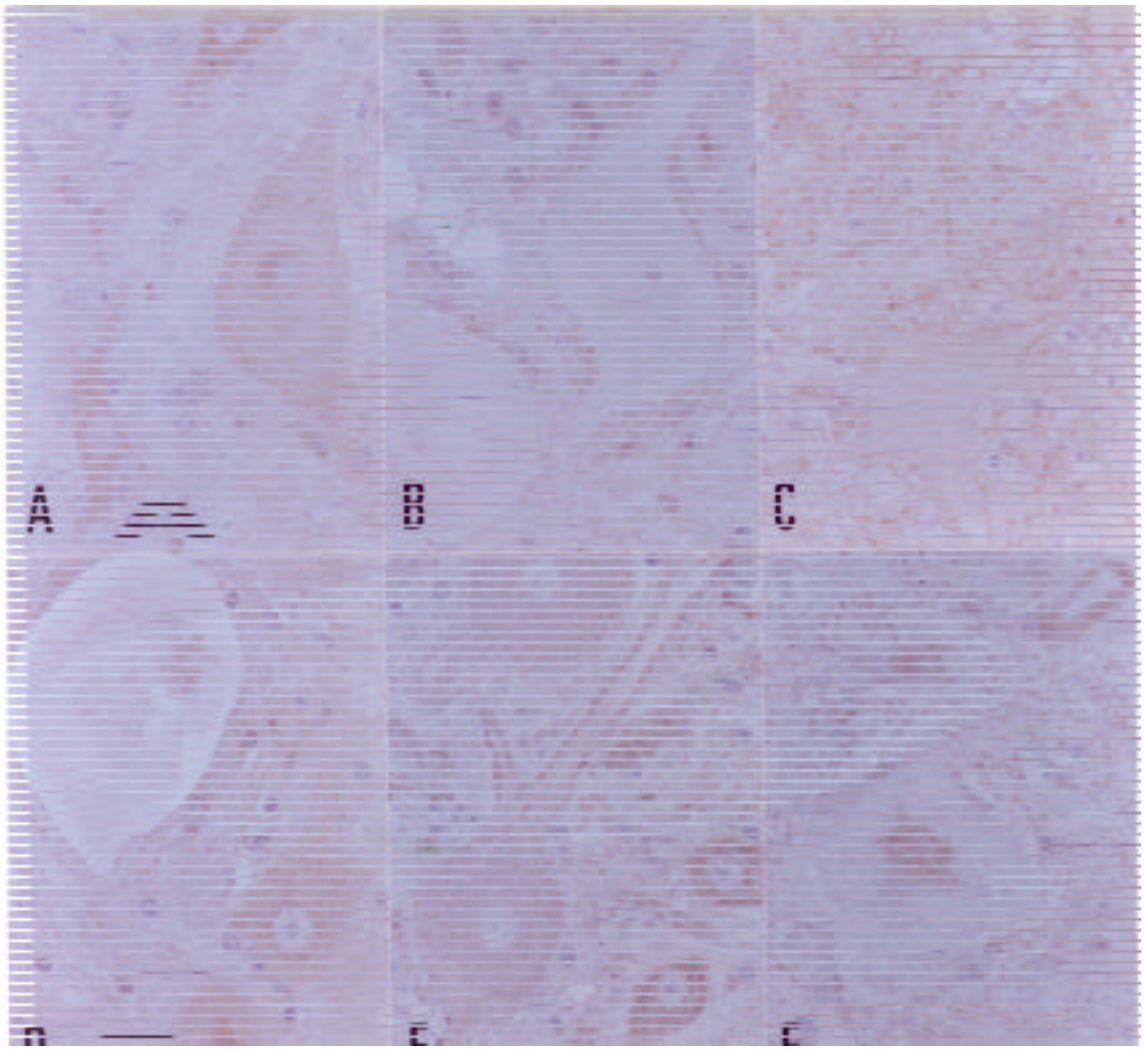

Fig. 4: Fas (A), Fas-L (B), Bcl-2 (C, Bax (D), and cytochrome C (E, F) immunoreactivity in motor neurones of the spinal cord in Holstein-Friesian calves with SMA. Reduced Fas-L, Bcl-2 and Bax immunoreactivity is found in swollen neurones (B, C, D). Cytochrome C expression, characterised by a fine punctate precipitate in preserved neurones (E), practically disappears in swollen motor neurones (F). Paraffin sections slightly counterstained with haematoxylin, bar = 25 µm.

DISCUSSION

Introduction

Although neurodegenerative disorders have characteristics in common, it is difficult to unify four different diseases.

We have grouped the discussion in six parts: 1) neuropathology: features of NAD, 2) synaptic pathology, 3) neuronal cell death, 4) stress response, 5) pathogenesis and, 6) comparative pathology: animal model systems.

Each part of the discussion includes the Rottweiler with juvenile NAD (Study 1), nine scrapie-inoculated mice at terminal stage of the disease (Study 2), two ataxic horses with EDM (Study 3) and four tetraparetic calves with SMA (Study 4), in order to find similarities between these disorders.

1. Neuropathology: features of NAD

General common features that are found in most animal and human neurodegenerative disorders by conventional morphological techniques are neuronal loss associated with astrocytosis [194, 218, 238]. Although each disorder can be separately associated to a distinct lesional pattern, neuronal cell loss and astrocytic change is observed in canine NAD, EDM and bovine SMA. Scrapie mice also shows spongiosis, astrocytosis and neuronal cell loss.

Dystrophic axons or spheroids ,which are swellings of the distal segments of axons, are the hallmark of NAD [201].

In Study 1, dystrophic axons of various size were more apparent in the gracillis, cunneatus and accessory cunneatus nuclei in the brain of the Rottweiler dog as has been described [238]. In the human and canine aged brain, the gracile and cuneate nuclei are common regions that show NAD changes [183, 190, 234]. In Study 3, neuroaxonal dystrophic changes in both horses were especially prominent in the thoracic nuclei

of the spinal cord and in the gracillis and cunneatus nuclei of the caudal portion of the medulla oblongata as have been described in EDM [178, 179, 180, 182, 212, 224]. In Study 4, dystrophic axons appeared in the ventral horn of the brachial and lumbosacral spinal cord regions as have been previously reported in Holstein-friesan calves [231] and with similarities as previous descriptions in the red Danish [176, 197] and brown Swiss calves [189, 241].

In Study 2, we did not observe NAD features probably because 1) the genetic of the animal model and the Rocky Mountain Laboratory isoform provoking severe and an early spongiotic change of the brain could have interfered and, 2) the inoculated-mice were at terminal stage and it has been suggested that NAD in murine scrapie become apparent in the early stages of the disease before spongiosis [208].

Neurodegenerative disorders such as canine NAD, EDM, bovine SMA and scrapie in mice might be consider to be different forms of NAD with similar lesional pattern but distinct CNS regional distribution. Immunohistochemical studies may help to typed each animal disease, labelling neuroaxonal changes with specific antibodies and trying to classify better these disorders by looking for similarities with well typed diseases in human beings and to contribute to give insights about their pathogenesis.

2. Synaptic pathology

The present thesis based on immunohitochemical semi-quantification of several synaptic proteins shows that presynaptic terminals are altered in a Rottweiler with juvenile NAD (Study 1), nine scrapie-inoculated mice at terminal stage of the disease (Study 2), two ataxic horses with EDM (Study 3) and four tetraparetic calves with SMA (Study 4).

Abnormal synaptic protein labelling of the perikarion of degenerated neurons and dystrophic axons, were prominent features in the Rottweiler with primary NAD and in horses with EDM, suggesting abnormal localization of the synaptic proteins and, severe and aberrant axonal transport impairment. By contrast, diffuse decreased in synaptic protein expression was prominent in scrapie-inoculated mice and calves with SMA while only the perikarion of a few neurons (see *Study 2*) and some degenerated axons (see *Study 4*) were abnormally labelled.

In Study 1, the accumulation of synaptic proteins within dystrophic axons was also observed in geriatric dogs, aged between 14 and 18 years old, with physiologic NAD and cognitive impairment suspected. Moreover, those aged dogs also showed diffuse synaptic loss. By contrast, no immunoreactivity against synaptophysin has been detected in other NADs such as human iNAD and experimental iNAD in mice [177]. Results of Study 1 suggest that diffuse and severe synapse loss might be probably associated to cognitive alterations, as has been correlated in Alzheimer's disease [240].

We only have studied some small synaptic vesicle proteins, synaptic plasma membrane proteins and synaptic mitochondria membrane proteins. Anti-synaptophysin immunohistochemistry evidenciated synaptic abnormalities in all four Studies. The rest of synaptic proteins have given different results in the 4 studies. For example, in the Study 1, we observed that most of the dystrophic axons displayed strong synaptophysin immunoreactivity but it was just in half of them that showed immunoreactivirty against synapsin-1 and Rab-3a proteins. No immunoreactivity with anti-syntaxin-1 was observed in the canine brain. In the Study 3, immunoreactivity in dystrophic axons against the synaptophysin protein was stronger than with SNAP-25, syntaxin-1 or α-synuclein. No immunoreactivity with anti-sinapsin-1 was observed in the equine CNS. In the Study 2 and Study 4, reduction in synapthophysin immunoreactivity was greater than with other synaptic antibodies used. No changes in immunoreactivity with anti-Rab3A was observed in the brains of scrapie-inoculated mice and no immunoreactivity with anti- α-synuclein was observed in the spinal cord of calves.

All this data supports that synaptic proteins are not equally implicated in different neurodegenerative diseases, and that synaptophysin is a useful and sensitive protein to determine synaptic abnormalities.

Furthermore, the α-synuclein protein has been suggested to play a role in dystrophic axons of human primary NAD and in experimental animal models [228]. In Study 1, dystrophic axons of the Rottweiler with juvenile NAD showed α-synuclein accumulation resembling the immunoreactivity present in dystrophic axons in primary human NAD and in axonal swellings (spheroids) following secondary NAD [228]. Therefore, the neuronal cytoplasmic accumulation of α-synuclein might results from an axonal injury supporting axonal transport impairment in primary NAD of Rottweiler dogs. In Study 2, abnormal small aggregates of α-synuclein were observed in the neuropil of main lesionated areas in terminal scrapie-infected mice. In Study 3, perineuronal aggregates of α-synuclein in neurons of the spinal ventral horn were also present in one horse with EDM. Moreover, dystrophic changes affected lower motor neurons in that horse. All this data, suggest a role for α-synuclein in animal neurodegeneration.

In veterinary neurodegeneration, electron microscopy have been the most used tool for detecting synaptic changes until today; for example, 1) A disruption of the presynaptic terminals associated to enlarged axons was suspected in a Jack Russell terrier pup with neuroaxonal dystrophy when synapses could not be visualized on electron microscopic examination [233] and, 2) the loss of synapses associated with abnormal PrP was suggested to precede neuronal degeneration in the scrapie-infected murine hippocampus [200]. The present thesis confirms that the immunohystochemistry technique is a useful tool to evaluate synapse damage in buffered formalin fixed tisse and paraffin embeeded sections.

3. Neuronal cell death.

In a large number of human neurodegenerative diseases there is evidence that the mechanism of neuronal cell death may involve apoptosis [198] although it is unclear whether the onset of the pathological manifestations *in vivo* is due to neuronal loss by apoptosis, or with functional neuronal damage [229].

In veterinary medicine, there are only a few neurodegenerative disorders in which apoptosis have been investigated. On one hand, vacuolated cells of two Rottweilers with a neuronal vacuolation disorder showed no involvement of the apoptotic pathway [232]; by the other hand, TUNEL-labelled cells have been detected in the canine aged brain [184, 204]. Nevertheless, it is unclear whether neuronal nuclear DNA fragmentation in aging is due to neuronal apoptosis because of the TUNEL-positive cells did not show the classical morphological hallmarks of apoptosis and, a positive correlation between the density of labelled cells and the degree of tissue autolysis was detected [184].

We did not detect TUNEL-positive cells in Study 1, Study 3 nor Study 4. By contrast, in Study 2, the method of *in situ* end-labeling of nuclear DNA fragmentation demonstrated a few positive cells with extreme chromatin condensation in the thalamus, pons, hippocampus and cerebellar white matter and, the cleaved caspase-3 IHC disclosed a few positive cells in the same regions.

Although, there are many authors that support that cell death in murine scrapie and in CJD is due to apoptosis [193, 195, 219, 230, 243], most of these studies [193, 195, 219, 243] were based on the method of *in situ* end-labeling of nuclear DNA fragmentation. We have already mentioned (see *chapter 6 of the introduction*) that there is evidence that the TUNEL method has limitations and do not permit, by its own, to confirm if neuronal cell death is due to apoptosis. Moreover, double-labeling examinations with the method of *in situ* end-labeling of nuclear DNA fragmentation and cleaved caspase-3 immunohistochemistry disclosed no co-localization in our scrapie-inoculated mice. Recent data showed that enhance expression of proteins linked to putative cell death pathways is not associated with apoptosis in Purkinje cells in CJD [230].

In conclusion, all these data might support the statement that cell death in prion diseases can be mediated by apoptosis, although maybe have been "*over-detected*", and that cell death pathways can have functional roles in neurodegeneration different from those related with apoptosis.

In contrast to human MNDs [187, 196, 220, 242] , degenerating cells in bovine SMA (Study 4) did not exhibit increased expression of Bax protein nor decreased expression in Bcl-2 protein, supporting no recruitment of the mitochondrial-dependent apoptotic pathway. In a previous study in Holstein-Friesan calves with

SMA [231] degenerating neurons did not stain with the method of in situ end-labelling of nuclear DNA fragmentation and did not show c-Jun immunoreactivity supporting that cell death in SMA differs from programmed cell death in the CNS during development. Moreover, calves with SMA showed marked neuronophagia suggesting that cell death could be mediated by necrosis.

Our results show that apoptosis is not the main mechanism of cell death in terminal stage murine scrapie and that cell loss in canine NAD, EDM or SMA in calves is not mediated by an apoptotic mechanism. In conclusion, the little available data in veterinary medicine, together with our results, supports that neuronal cell death in some animal neurodegenerative disorders, may not involved apoptosis, at least, as a main mechanism of cell death.

4. Stress response.

As we introduced the neurofilament, ubiquitin, and αB- crystallin proteins in the same chapter of the introduction, we will discuss all proteins together.

The accumulation of intracellular inclusions [92] is a common feature of human neurodegenerative disorders. These inclusions are mostly formed by protein aggregates that usually are difficult to unfold or degrade [202] and difficult to detect. Moreover, anti-ubiquitin is the ideal tool to detect neuronal inclusions in the majority of cases of sporadic and familial ALS, [186, 213] in the brains of scrapie-infected mice [214] as well as in cerebellar plaques in CJD [237] and Gerstmann-Straussler Scheinker (GSS) syndrome [227] patients.

In veterinary medicine, presence of ubiquitin-immunoreactive free granules and intracytoplasmic globules in glial cells and macrophages was observed in old dogs with primary white matter degeneration [191] .

None of the diseases studied in this thesis have shown the presence of intracellular inclusions in neurons nor glial cells even when the anti-ubiquitin immunohistochemistry was performed. The fact that neuronal inclusions were not detected in our scrapie inoculated mice when they have been already described [214] could account for various factors like the isoform inoculated isoform (*Rocky Mountain Laboratory*) or the route of inoculation (intraperitoneally).

Abnormalities in high-molecular-weights subunits of neurofilaments may provoke alterations in the organization of the neuronal cytoskeleton [218]. In the Study 1, dystrophic axons of bigger size did not showed immunoreactivity for phosphorylated high molecular weight neurofilament (PHNF) epitopes while did it with anti-ubiquitin. Similarly to previous descriptions [183], dystrophic axons in the canine aged brains displayed ubiquitin immunopositivity while neurofilament accumulation was not observed. Studies in infantile human NAD (iNAD) and experimental models of iNAD in mice (BPAU-induced DA) demonstrate

that dystrophic axons were intense ubiquitinated [177]. All these data suggest that ubiquitin plays a role in catabolic processes involved in the pathogenesis of dystrophic axons in primary NAD. In the Rotweiler with juvenile NAD, dystrophic axons were rarely immunoreactive with phosphorylated neurofilament epitopes as have been described in physiologic NAD associated to aging [183], suggesting altered axonal retrograde transport.

In Studies 3 and 4, all dystrophic axons that displayed immunoreactivity against the PHNF also did for anti-ubiquitin, suggesting that immunoreactivity of espheroids is likely to be the result of immunoreactivity present in lysosome-related dense bodies. Moreover, swollen neurones mostly observed in the EDM and the bovine SMA showed ubiquitin immunoreactivity, as have been described in most neurodegenerative disease [236], especially motor neuron diseases such in infantile motor neuron disease [207], Werdnig-Hoffmann disease [203] and in an animal model of motor neuron degeneration [225]. Dystrophic changes in the ventral horn of the spinal cord revealed strong anti-PHNF immunoreactivity in Study 4 confirming that SMA in Holstein-Friesan calves is a MND with neurofilament accumulation.

Accumulation of 200 kDa neurofilament protein within dystrophic neurites have been observed in the Creutzfeldt-Jakob disease (CJD) suggesting axonal transport impairment [211]. In our experimental scrapie model, Study 2, we did not observed dystrophic changes even with anti-neurofilament immunohistochemistry.

Taken toghether these results, it might be notorius that, 1) a local activation of the ubiquitin system leading lysis of altered proteins is present in dystrophic axons in response probably to a variety of stressful states and, 2) filamentous inclusions are not present in none of our studies and, 3) PHNF abnormal accumulation in Study 3 and Study 4, implies impairment of the axonal transport.

We did not attached importance to the staining with anti-ubiquitin of the nuclei of some neurons in the spinal cord of horses with EDM because it was also observed in neurons of pontine nuclei and in the spinal cord of the control horse. Although, this staining of the nuclei is due to a major component of normal chromatin which is ubiquitinated histone H2A [216], it is interesting to point the observation that positive immunostaining of nuclei with anti-ubiquitin have been reported in human fresh tissue preparations but not usually seen after formalin fixation and paraffin processing [216].

Recent work has also shown that neurons that show ubiquitin staining are immunoreactive with antisera to αB- crystallin [215].

In our studies we only obtained αB- crystallin immunoreactive cells in mice in Study 2, although with interesting results. αB- crystallin immunoreactivity decorated the cytoplasm of oligodendroglia in control

and "scrapie" brains as did with the ubiquitin antisera (data not shown), but also αB- crystallin immunoreactivity was found within hypertrophic astrocytes in the lateral thalamus, pons, cerebelllar peduncles, and in the hippocampus of only inoculated scrapie mice. Moreover, the expression of αB-crystallin in oligodendrocytes was reduced in mice following prion infection. This was not likely the mere result of decreased expression of αB-crystallin, since this protein was over-expressed in reactive astrocytes in the same scrapie-inoculated mice. Therefore, the present results sustain the observation that scrapie prions modify the stress response [239], and point to oligodendroglial cells as putative targets of PrP^{res}. This observation is in line with previous electron microscopical studies showing oligodendroglial inclusions, and vacuolation and degenerative changes in the white matter myelin sheaths in CJD and in mice experimentally infected with the Fujisaki strain of CJD [188, 209].

5. Pathogenesis.

The pathogenesis of almost all neurodegenerative disorders is not well understood. In the last past 10 years, many studies have focus their attention to the synapse and to the cell loss, in order to contribute to the knowledge of the different events that may occur in aging [185, 221] and during the disease [198, 222]. Recent results showed that the aging process and many neurodegenerative disorders courses with synapse loss and neuronal death [222].

Despite this thesis is based on four different diseases in four different species, all have charachteristics in common: changes in synaptic protein, cell loss and activation of the stress response.

We first will focus on Study 2 as large number of investigations have been carried out in prion diseases. Although the pathogenesis of dystrophic axons in NAD is unclear, our results suggest that axonal transport impairment and synaptic abnormalities play an important role in the pathogenesis of animal neurodegenerative disorders as have been suggested in CJD [210]. If these events leads to eventual death of the neuronal cell body and neuronal loss remains unknown. Nevertheless, it seems that apoptosis is not the main mechanism of neuronal cell death in the animal model that we have studied.

Our results show that ubiquitin may have an important role in lysosomal function in dystrophic axons and ballooned neurons probably due to the accumulation of abnormal proteins as have been described in CJD [205, 206, 223, 226] and in axonal transport disruption [181, 192]. Moreover, experiments in prion diseases have suggested that this take place early in the time course of the process [217]. We don't know if it is an early event in the animal neurodegenerative disorders we studied because most of the animals were at terminal stage of the disease.

Nevertheless, the results in αB-crystallin sustain the observation that scrapie prions modify the stress response as have been previously suggested [239], and point to oligodendroglial cells as putative targets of PrP^{Sc}.

It has been suggested that there was no relationship between extracellular fibrilar accumulation of abnormal PrP, mainly in the form of PrP amyloid, and synaptic pathology [199] although recent electron microscopical observations have shown that loss of synapses associated with abnormal PrP precedes neuronal degeneration in the scrapie-infected murine hippocampus [200]. These findings, together with our results strongly support the concept that non-fibrilar PrP^{Sc} has deleterious effects on pre-synaptic structures in the form of a reduction of synaptic protein expression and loss of synapses as the accumulation of PrP^{sc} was regional correlated with the synaptic loss.

Moreover, there is enough evidence to believe that signalling events ocurring in synaptic terminals play important roles in either promoting (e.g., activation of glutamate receptors in postsynaptic spines) or preventing (e.g., activation of neurotrophic factors in presynaptic terminals) neuronal cell death [222].

Primary jNAD in the Rottweiler and in the two Arabian horses shown no TUNEL-positive cells. Furthermore, no changes were observed in several apoptotic signals in calves with SMA and low numbers if cleaved-3-caspase and TUNEL-positive cells were observed in terminal scrapie-inoculated mice. All this data, supports that apoptosis is not as important as the accumulation of synaptic proteins and the synaptic loss for the pathogenesis in these animal disorders and suggests that a different programmed cell death from apoptosis may be is involved in these disorders.

However, if apoptosis is blocked by a defect in energy metabolism as have been suggested for some neuropathologicla syndromes [235], remains unknown from our studies.

Although it seems that cell loss is an early feature of the disease in ALS, Study 4 shows reduced synaptic protein expression in end-terminal calves with SMA and described this synaptic change as a delayed event in the disease, in contrast to the substantial synaptic alteration that take place in the early stages in the ALS. Furthermore, it is not known in our disorders such as in Alzheimer's disease, whether cell loss is secondary to synaptic injury and axonal transport impairment [198] suggesting that abnormal synaptic protein expression might appear as an early feature in these diseases. All these results, suggest the existence of different types of motor neuron disease in veterinary medicine.

In conclusion, all these data evidenciated that pathogenesis of CNS disorders is complex and that maybe there are involved many different mechanisms that are still unknown. Furthermore, each disease has its own pathogenesis and it is difficult to unify them.

6. Comparative pathology: animal model systems.

The pathological investigation on neurodegenerative diseases has been transformed in the recent years through insights gained by using animal models [218]. It is well known that mouse animal models are useful tools for studying prion diseases as our results also show.

Our results show that the juvenile NAD of the Rottweiler has similarities with infantile NAD in humans and that calves with SMA revealed similarities with Werdnig-Hoffmann disease.

Despite EDM has similarities with primary NAD in the Rottweiler, the variability obtained in the results suggests that further studies are needed in order to 1) improve the knowledge of this disease and, 2) propose this disease as an animal model. If it is a MND, a primary NAD or a secondary NAD resulting from a deficiency in vitamin E remains unknown. Nevertheless, we suspect that similar to bovine SMA, different syndromes can be present.

As commented previously, the pathogenesis of neurodegenerative diseases is not understood because there is still too much to discover about the nervous system and most of the questions focused on molecular mechanisms can not still be answered. We hope that this thesis will help to clarify some unresolved issues and bring some new ideas and tools, that help to understand in someway the pathogenesis of NAD, MND and prion diseases.

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CONCLUSIONS

1. Neurodegenerative disorders such as primary NAD of Rottweilers, EDM, bovine SMA and scrapie in mice might be consider to be different forms of NAD with similar lesional pattern but distinct CNS regional distribution. Immunohistochemical studies labelling neuroaxonal changes with specific antibodies are helpful in order to typed each animal disease .

2. Abnormal synaptic protein expression is a common feature in primary NAD of Rottweilers, in canine aging, equine neurodegenerative myeloencephalopathy, bovine spinal muscular atrophy and murine scrapie.

3. Synaptic proteins are not equally implicated in diferent neurodegenerative diseases, and that synaptophysin is a useful and sensitive protein to determine synaptic abnormalities.

4. α-Synuclein, a marker for human neurodegeneration is also useful in animal neurodegenerative diseases.

5. Neuronal cell death in animal neurodegenerative disorders, may not involved apoptosis, at least, as a main mechanism of cell death suggesting that cell death pathways can have functional roles in neurodegeneration different from those related with apoptosis.

6. In animal neurodegenerative disorders, axonal transport impairment plays a role in the pathogenesis of dystrophic axons resulting in aberrant accumulation of neurofilaments and synaptic proteins.

7. Ubiquitin activates protein metabolism resulting in accumulation of lysosomes in dystrophic axons and ballooned neurons.

8. Only in the experimental murine scrapie αB-crystallin showed that the stress response was modified.

9. In experimental murine scrapie astrogliosis is a common feature. We observed that oligodendroglial cells were resuced in inoculated mice suggesting that these cells are putative targets of PrP^{Sc}.

10. In experimental murine scrapie, PrP^{Sc} has deleterious effects on pre-synaptic structures in the form of a reduction of synaptic protein expression and loss of synapses.

11. The juvenile NAD of Rottweilers might be a useful animal model for infantile NAD in humans.

12. SMA in Holstein-Friesan calves might be a useful animal model for human SMA.

13. Scrapie-inoculated mice are as expected a useful animal model for prion diseases.