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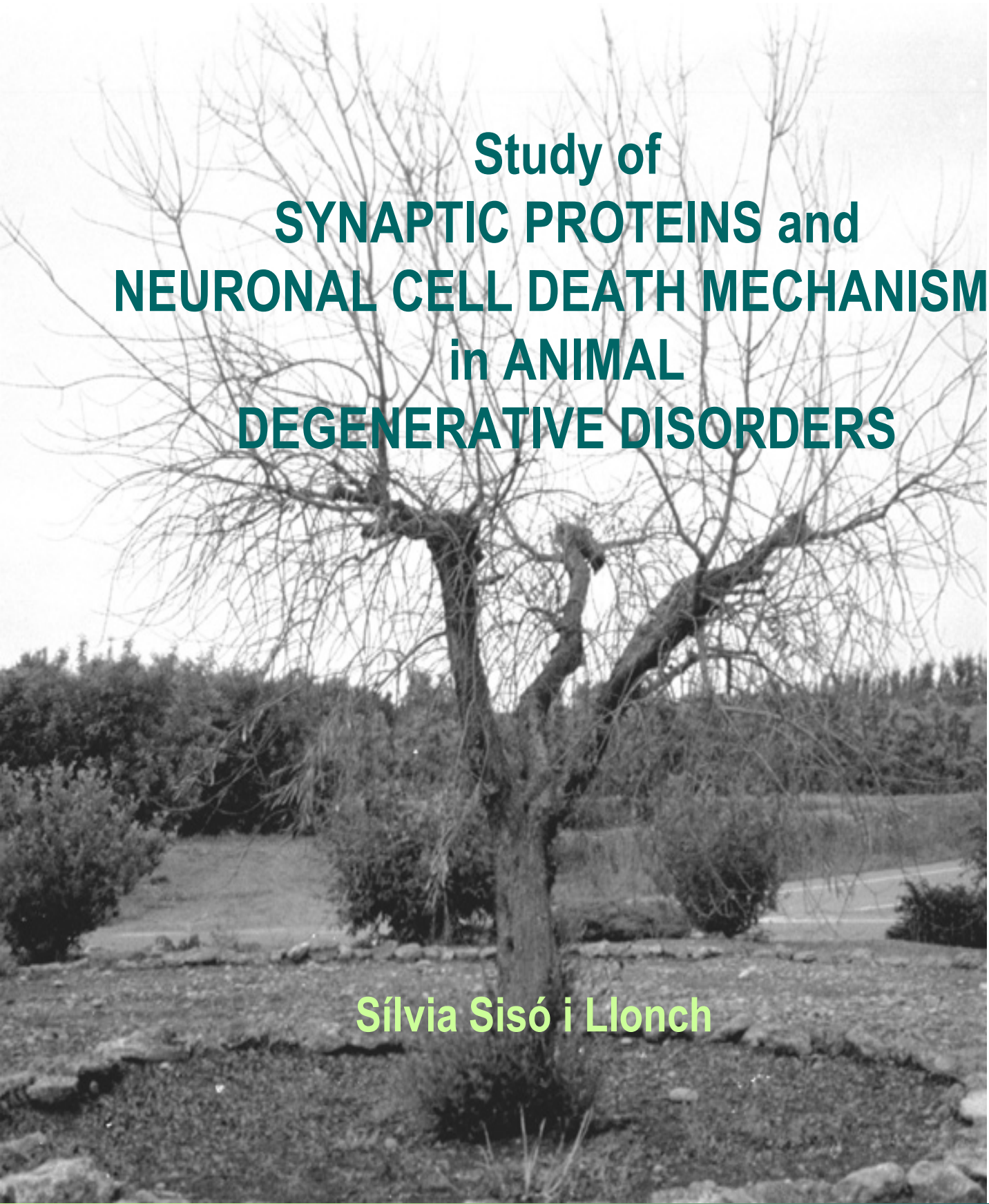

Study of SYNAPTIC PROTEINS and NEURONAL CELL DEATH MECHANISMS in ANIMAL DEGENERATIVE DISORDERS

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TESI DOCTORAL

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Juny 2002

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Tesi que presenta Sílvia Sisó i Llonch
per optar al títol de doctora.

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Bellaterra, juny de 2002

Sílvia Sisó i Llonch va gaudir
d'un contracte associat al projecte europeu FAIRJ-CT 98-6022,
titulat "*Role of PrP in prion spread and stablishment of central nervous system*",
durant el període comprès entre gener 1999-gener 2001.

Summary

Preface

Neuropathology has become from the last hundred years or so a specialist and multidisciplinary science (*Foreword in the Greenfield's Neuropathology textbook*). It was dominated initially by neurologists and psychiatrists, and to a large extend ignored by general human pathologists. Progress in neuropathology has been rapid due to the interaction between clinicians and laboratory scientists and through the insights gained by application of molecular biological techniques and animal model system. In the last past 15 years, many studies have focused their attention in the aging process and many neurodegenerative disorders, in which despite all these previously commented advances, the pathogenesis remains, still today, uncertain.

In veterinary science, neuropathology emerged later than in human medicine, probably due to veterinary pathologists were discouraged from entering in a field of complexity, the necessity to use specialized technical methods in its study, the low number of clinical cases, and the difficulty in achieving interaction between clinicians and pathologists. For that reason, most sections of the textbook of animal neuropathology, *Veterinary Neuropathology*, are introduced by describing first the comparable disorder in humans. Some CNS disorders are highly comparable between animals and humans, some share some points of similarity, and others are limited to humans or to individual animal species or animal breeds.

This differences between human and veterinary medicine are reflected when you review how degenerative disorders are classifyied in the last editions of the *Greenfield's Neuropathology* and of the *Veterinary Neuropathology*. Descriptions of animal degenerative diseases of the Central Nervous System (CNS) are grouped in metabolic and circulatory disorders, intoxications and toxicoinfectious diseases, nutritional diseases, and the last group includes hereditary, familial, and idiopathic degenerative disorders. By contrast, in *Greenfield's Neuropathology* we can find that some human neurodegenerative disorders, as opposed to its animal disease counterparts which are group together, are classify in a specific chapter.

Greenfield's and, *Summers et al,* described human and animal neurodegenerative disorders as all diseases that show neuronal loss associated with astrocytosis by conventional morphological techniques.

This thesis focuses on neurodegenerative disorders includes in the part of hereditary, familial, and idiopathic degenerative disorders of the *Veterinary Neuropathology* textbook. It is based on the study of four animal neurodegenerative disorders that show neuronal cell death and astrocytosis: juvenile neuroaxonal dystrophy (jNAD) of the Rottweiler, the experimental murine scrapie, the equine degenerative myeloencephalopathy (EDM) and the bovine spinal muscular atrophy (SMA). They are sporadic and rare diseases which means that a low number of descriptions exist in veterinary medicine except for scrapie

which has been study in depth over the past 20 years. All of them have its own counterparts in human medicine where they have been study in depth.

Light microscopic examination of four animal disorders studied in this thesis showed the presence of degenerated axons, also named spheroids or dystrophic axons. *Jellinger* described in 1973, that 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 (DA) as the chief substrate of the disease process.

In the neuroaxonal dystrophy section of the *Veterinary Neuropathology* textbook, the jNAD of the Rottweiler and the EDM are included, the former as a primary NAD whereas the later is not well stablished if it is a primary or a secondary NAD. Moreover, there are several descriptions of distrophic axons in prion diseases and human motor neurons diseases (including spinal muscular atrophy).

In conclusion, all four diseases were considered to be studied as different types of NAD. Juvenile neuroaxonal dystrophy (jNAD) of the Rottweiler (Study 1), the experimental murine scrapie (Study 2), the equine degenerative myeloencephalopathy (EDM) (Study 3) and the bovine spinal muscular atrophy (SMA) (Study 4) are included in the STUDIES section.

The aim of the thesis is to study in depth these animal disorders using the available tools in order to study synaptic pathology and cell death at a molecular level, and compare results with human disorders. For these reasons, the introduction section of the thesis has been structured in different chapters in order to introduce the four animal neurodegenerative disorders, its human counterparts, and a review of the molecular biology and pathologic basis related with synapses and cell death.

It is our hope also to encourage veterinary neuropathology researchers, to demonstrate veterinary neurologists that pathology is a complementary and a useful tool, and that our colleagues in human neuropathology find something, even ideas, of interest in this thesis.

SSLL.

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Agraïments

Aquesta tesi és el resultat de quatre anys de feina; d'ajuda rebuda, d'ideies i experiències compartides i, d'insistència i col.laboració per part de molts. Vull ressaltar, que també en determinats moments ha estat un període d'entrebancs continus, desil.lusions i desenganys puntuals. I que sense totes aquestes fases i la participació de molta gent ara no em sentiria tan recompensada i satisfeta.

Primer m'agradaria agraïr l'esfrorç realitzat a totes aquelles persones que directament han estat implicades en aquesta tesi:

Als meus directors de tesi, Martí i Isidre, moltes gràcies per introduïr-me en aquest món tan fascinant de la patologia nerviosa. M'heu ensenyat i demostrat que les idees, el coneixement, la passió, el fracàs i la insistència són eines imprescindibles dins la investigació. Gràcies també per la paciència que heu demostrat i per haver-me animat i recolzat en tot moment.

Al laboratori del Dr. Aguzzi per proporcionar-nos els ratolins necessaris per a poder realitzar un dels estudis d'aquesta tesi; i a l'arxiu del Servei de Diagnòstic Veterinari de la Unitat d'Histologia i Anatomia Patològica per haver proporcionat el material per a dur a terme els altres estudis.

A la Berta, la Rosi i la Marga de Bellvitge, que sempre em van buscar un racó en el laboratori per poder treballar i per fer-me sentir com a casa.

A tots els companys (professors, becaris, secretàries i tècnics) de la Unitat d'Histologia i Anatomia Patològica, perquè d'alguna manera o altra han fet que aquests anys de convivència i col.laboració hagin estat molt positius per mi. Especialment a la Fina, la MJ, la Rosa i l'Alberto.

A l'Anabel, la Francesca, el Santi, el Carles i la Laura per totes les converses i experiències compartides. Avui que no estem junts, els records em fan somriure. Un petó a tots !!

Al Rafael Mendieta i al Mariano, per haver-me aconsellat i haver cregut en mi en moments crítics.

A PRIOCAT, perquè entre altres coses, m'ha permès poder acabar la tesi sense desvincular-me de la investigació.

I al Tom Yohanann, per les magnífiques correccions de l'anglès realitzades en aquesta tesi.

I vull agraïr també l'ajuda rebuda a totes aquelles persones que d'alguna manera o altra han hagut de patir les conseqüències de la tesi:

A les meves amigues, Mónica i Caro, per totes les pallisses que us ha tocat aguantar, i perquè sou fantàstiques!! I sobretot a tu, Àlex, per ajudar-me en tot moment, especialment en els dolents. Et trobaré a faltar si marxes.

I per últim, a la meva família, sobretot als meus pares, a la meva germana i a tu Willie, perquè us estimo!!

Glossary of Abreviations

AD Alzheimer's Disease
ALS Amyotrophic Lateral Sclerosis
Apaf-1 Proapoptotic protease activating factor
BPAU Parabromophenylacetylurea
BSE Bovine Spongiform Encephalopathy
CNS Central Nervous Tissue
CJD Creutzfeldt-Jakob Dsease
DA Dystrophic axons
DNA Desoxyribonucleic acid
EDM Equine Degenerative Myeloencephalopathy
FFI Fatal Familial Insomnia
FSE Feline Spongiform Encephalopathy
GSS Gerstmann-Sträussler-Scheinker
HSD Hallervorden-Spatz Disease
HSP Heat shock proteins
ICAD Inhibitor of caspase-3-activated DNase
IHC Immunohistochemistry
INAD Infantile form of NAD
ISEL *In situ end labeling*
jNAD Juvenile form of NAD
MND Motor Neuron Disease
NAD Neuroaxonal Dystrophy
NF Neurofilaments
NFH High-molecular-weight NF subunit
NFL Thelow-molecular-weight NF subunit
NFM Medium-molecular-weight NF subunit
PARP Poly ADP-ribose polymerase
PBP Progressive Bulbar Palsy
PBS Phosphate buffer saline
PCD Programmed cell death
PHNF Phosphorylated NFH
PLS Primary Lateral Sclerosis
PMA Progressive muscular atrophy
PrPc Cellular prion protein
PrPsc Scrapie prion protein
SMA Spinal Muscular Atrophy
SNAP-25 Synaptosomal associated protein of 25 kD
SNARE Soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors
TNF Tumor necrosis factor
TSE Transmissible Spongiform Encephalopathy
TSNARE Target membrane-localized SNARE
TUNEL *Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling*
VSNARE Vesicle-associated SNARE

INTRODUCTION

CNS DEGENERATIVE DISORDERS

1. Veterinary Neuropathology

Neuroaxonal Dystrophy (NAD) in Veterinary Neuropathology

As previously introduced in the *preface* it is diffcult to classify neurodegenerative diseases in a global or generalized way. Moreover, in veterinary medicine, the majority of the conditions described in domestic animals are incidental changes in the brain and spinal cord [36].

Animal neurodegenerative disorders included in this thesis were chosen and studied as a set following two basic basic criteria:

- Presence of disseminated (meaning, affecting more than one nucleus) axonal swellings (spheroids or dystrophic axons) in each animal disease. As it is the characteristic pathological finding in NAD [22] all animal diseases were suspected to be a type of NAD.
- All of them should have been described as NADs in the literature suggesting that our diseases were not incidental findings:
  - Primary canine NAD due to an autosomal recessive inheritance.
  - Physiological NAD associated to aging.
  - Equine NAD associated to the equine neurodegenerative myeloencephalopathy (EDM).

Although spinal muscular atrophy (SMA) and prion diseases in animals are not included in the section of NAD of the Veterinary Neuropathology textbook [34], features of secondary NAD are present in both diseases [11, 23, 24, 25]. For this reason we also included studies on:

- Bovine SMA
- Experimental Murine Scrapie

Our diseases might be classify as follows:

1.1. Physiologic or normal NAD is a common finding in certain parts of the aging central nervous system of animals. Dystrophic axons are a common feature of the brain of aged dogs older than 8 years old [37]. Besides, NAD of brain stem propioceptive nuclei can be found in young animals without functional impairment [34].

1..2. Primary neuroaxonal dystrophies (NADs) in animals have been reported in horses, sheep, rabbits, cats and dogs [34]. We have focused our attention in the juvenile NAD of the Rottweiler and the Equine Degenerative Myeloencephalopathy (EDM).

1.2.1. Canine neuroaxonal distrophy is an uncommon degenerative neurologic disorder discribed more frequently in the Rottweiler breed [12, 14, 21, 34], although this disorder have been discribed in other breeds such as Chihuahua [6], Jack Russell terrier [35] and Collie sheepdog [13]. Depending on the breed, NAD may differ in age of onset, accompanying neurological signs and clinical course, and neuropathological features [13]. In Rottweilers, It is clinically manifest as a progressive ataxia of the limbs and head [12, 13, 14] and it has been also associated to laryngeal paralysis in some cases [5]. Microscopically, NAD is characterized by the swelling of the distal segment of the axons in the Central Nervous System (CNS) [34].

1.2.2. The syndrome of ***equine degenerative myeloencephalopathy (EDM)*** is one of the most common cause of spinal cord diseases in horses [18, 19, 30]. It is a neurodegenerative disorder of the central nervous system (CNS) of unknown origin that affects young horses whose age is between two months and three years [2, 4, 7, 27, 34]. EDM is clinically diagnosed as a motor neuron disease [16, 34]. In EDM, horses develop an insidious onset of symmetrical spasticity, ataxia, and paresis of the four limbs although pelvic limbs commonly are more affected [27, 28, 29].

Moreover, EDM has features of NAD [34]. Neuroaxonal dystrophic changes may be especially prominent in the thoracic nuclei of the spinal cord and in the cuneate nucleus and nucleus gracillis of the caudal portion of the medulla oblongata, and are associated to vacuoles and astrocytic response [2, 3, 4, 8, 26, 27, 28]. The cause or causes of EDM are still unknown [19] although it has been frequently described as a familial degenerative disease in Morgans [4] and Appaloosas [7] while in other breeds such as Mongolian wild horses [26] and Haflinger horses [2] has been associated to a vitamin E deficiency.

1.3. Secondary NADs in animals: can be due to a large number of disorders [23].

1.3.1. Transmissible Spongiform Encefhalopathies (or Prion Diseases) have been described naturally in several animal species [34]: Scrapie in sheep and goat, Transmissible Mink Encephalopathy, Bovine Spongiform Encefalopathy (BSE), Chronic Wasting disease in mule deer, black-tailed deer, and Rocky Mountain elk, and, Feline Spongiform Encephalopathy (FSE). There are many variants of natural Scrapie, distinguishable by their disease characteristics in genetically-defined mice [9]. These variation has been well documented for experimental scrapie in mice [10, 17]. These findings argue that the distribution of PrP^{sc} is influenced by both the infecting prion and the amino acid sequence of PrP^{c} [32]. Neurological signs, although they may differ between species, usually appear in adults as prion diseases are characterized by a long incubation period. Despite this variability, the neuropathological features common to all prion diseases are spongiform degeneration of the neuropil and neurons and their processes, neuronal loss, reactive astrogliosis and abnormal protease-resistant PrP deposition [32]. Due to the long incubation period, experimental scrapie models in mice have become an important and necessary tool for the study of prion diseases.

1.3.2. Spinal Muscular Atrophy (SMA) is well documented in dogs [15], cats [39] and calves [34]. SMA in calves have been classify as a lower motor neuron disease with neurofibrillary accumulation [34]. This type of MND shows under light microscopic examination, pale and distended (or swollen) neurons in the spinal cord ventral horn with neurofilamentous degeneration [34]. This disease is apparently inherited as an autosomic recessive trait [31, 34].

SMA has been described in Horned Hereford calves [34], red Danish calves [1], brown Swiss cattles [20, 38] and Holstein-Friesian calves [33]. Affected animals show progressive muscular atrophy and become paraparetic between about 2 and 6 weeks of age, although some cases might be congenital [34]. Neuropathological findings in Holstein-Friesian [33] are only observed in the spinal cord and are similar to those reported in red Danish and brown Swiss calves, but differ from the Horned Hereford calves ones.

In conclusion, two forms of bovine SMA have been suspected [34]. The most consistent pathologic features in SMA in Holstein-Friesian calves are the loss of motor neurons, remaining neurons with marked swollen cell bodies (ballooned neurons), gliosis and neuronophagia in the ventral horn of the brachial and lumbosacral regions of the spinal cord [33]. These findings have been previously described in this breed [33], and reported in other breeds such as red Danish [1] and brown Swiss calves [20, 38].

2. Human Neuropathology

2.1. Introduction to human degenerative CNS disorders.

In human beings, there are multiple different ways to classify CNS disorders. They can be classify depending on the phenotype, cause, age, morphological features, distribution of lesions, molecular insights, etc.; so, it is difficult to make a general classification.

Following the classification made in the Greenfield's Neuropathology, neurodegenerative diseases can be grossly divided as common cause of dementia or not; for example, as occur in prion diseases and aging. In contrast, there are other disorders that principally courses with disorders of movement and system degenerations, such as motor neuron disorders. As an independent group, included in miscellaneous disorders affecting ganglia, we can found the group of neuroaxonal dystrophies and related disorders.

We focused our attention to three CNS disorders:

2.1.1. Neuroaxonal dystrophies (NAD):

NAD was first recognized by Seitelberger as a pathognomonic substrate for several neurological diseases [54, 55]. Hallervorden-Spatz disease, Nasu-Hakola disease, infantile NAD or Seitelberger's disease, late infantile, juvenile, and adult NADs, and the neuroaxonal leucodystrophy are considered primary (or endogenous) NADs [50].

Secondary or symptomatic forms ocur in the brain with ageing [43, 53] and under various conditions, e.g. chronic alcoholic encephalopathies or Parkinson's diseases.

Late infantile, juvenile and adult NAD tipically present with rigidity, ataxia, dysarthria, motor weakness, psychiatric disorders and later dementia [55]. Macroscopically and histologically, there is overlap in appearances with those described for Hallervorden-Spatz syndrome.

The morphology of the dystrophic axons is characterized by the appearance of axonal swellings, which are designated axonal spheroids. Dystrophic neuroaxonal swellings vary from 20 to 120 μm in diameter and have pale granular eosinophilic appearance, occasionally developing a more intensely stained eosinophilic core region [50]. They contain densely packed axoplasmic organelles, including tubulovesicular structures, residual and dense bodies, disintegrated mitochondria and other abnormal profiles [50]. The changes are first seen in the most distal parts of the axon and their terminals. They progress in a retrograde direction leading to demyelination, axonal disconnection and reactive astrocytosis [55]. Immunocytochemistry shows reactivity with antisera to neurofilament proteins and ubiquitin only in smaller spheroids [51]. Neurofibrillary tangles and diffuse Lewy bodies have been reported [44].

2.1.2. Motor Neuron Disorders (or Diseases) (MND):

Initial descriptions of MND were made in the latter part of the nineteenth century [40] describing different clinical features of disease and partly from histopathology. Sporadic and familial forms of amyotrophic lateral sclerosis (ALS), progressive bulbar palsy (PBP), progressive muscular atrophy (PMA) and primary lateral sclerosis (PLS) are different MND [50]. The cause of MND is unknown although some of them are suspected to be associated to some specific gene mutations (familial forms) [50].

In *Greenfield's Neuropathology* they are classify into three groups:

- Primary MND: can be subdivided in:
  - idiopathic motor neuron diseases (such as sporadic amyotrophic lateral sclerosis, ALS)
  - heritable motor neuron diseases (such as NAD or spinal muscular atrophy)
- Secondary MND: associated to infectious, neurotoxins, metabolic or autoimmune diseases.
- Motor neuron degeneration as part of another multisystem neurodegenerative disease such as in prion diseases.

In ALS or motor neuron disease (in singular), a degeneration of upper and lower motor neurons manifeted by weakness and wasting of the affected muscles, with evidence of corticospinal tract involvement while only lower motor neurons are affected in PMA or Werdhnig-Hoffman disease and only upper motor neurons in PLS [50].

a) Amyotrophic lateral sclerosis (ALS):

The main histological change in ALS is loss motor neurons with associated astrocytosis in anterior horns of the spinal cord, in the brain stem and the motor cortex. Moreover, inclusion bodies, some immunoreactive against ubiquitin [41,47] can be found in remaining neurons. Surviving neurons in the spinal

anterior horn often show swelling associated with accumulation of phosphorylated neurofilaments. Axonal spheroids are also frequently seen in the anterior horn [48]. In ALS and PLS the white matter of the spinal cord shows loss of myelin from the corticospinal tracts associated with variable astrocytic gliosis and accumulation of macrophages [50].

b) Spinal muscular atrophy (SMA):

Several types of SMA are distinguished depending on the age of onset, distribution of weakness, severity and clinical progression and pattern of inheritance. In conclusion, as an autosomal recessive form od SMA there are described 3 different types of SMA [50, 52]:Type 1. Acute infantile SMA or Werdnig-Hoffmann disease; Type 2. Chronic infantile SMA; Type 3. Chronic proximal SMA or Kugelberg-Welander disease) although there might be an overlap between these groups and classification sometimes can be difficult [52]. Type 1 causes progressive muscle weakness affecting both limbs and respiratory muscles in liveborn infants [52]. Type 2, patients often develop contractures and scoliosis and may survive until late adolescence, and type 3, is a mixed form of both anterior types [50].

Macroscopically, there is atrophy of skeletal muscles and of anterior nerve roots corresponding to loss of anterior horn cells [50]. Autonomic nuclei of the thoracolumbar cord and Onufrowicz's nucleus in the sacral cord are spared [45]. The remaining motor neurons in affected areas are characteristically swollen and contain abnormally phosphorylated neurofilaments [49] and are ubiquitinated [46].

2.1.3. Prion diseases:

Human prion diseases included sporadic or familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI), kuru and the new variant of CJD (a human disease as the result of exposure to food products contaminated with bovine spongiform encephalopathy) [42]. These disorders, togheter with the animal syndromes are called *transmissible spongiform encephalopaties* (TSE) [42]. The pathogenesis of sporadic, genetic and infectious (iatrogenic) forms of these diseases are due to abnormalities of the prion protein (PrP), a normal cell membrane protein expressed constitutively and a particularly high levels in nerve cells [42]. The term *prion* by itself designates the infectious pathogenic particle which transmits these disorders [42] and accumulates preferently within the CNS. Cases classify as CJD, regardless of whether the case has a genetic or sporadic presentation or was acquired by infection with prions, courses with rapidly progressive dementia leading to death in a few months to a few years with extrapyramidal and/or cerebellar signs, myoclonic jerks, a characteristic electroencephalogram (EEG) pattern and vacuolation of the cortical and subcortical grey matter neuropil with little or no amyloid plaque formation.

2.2. Comparative pathology

As has been previously introduced, it does not exist too much bibliography about the four animal disorders that we studied (probably, because of the majority of the conditions described in domestic animals are incidental changes in the brain and spinal cord [42].

This means that in veterinary medicine we usually have to go through human disorders in order to study an animal disease. In this thesis, we did, and we found that animal diseases were similar to some human disorders, as it is illustrated in **Table 1**.

Table 1. Correlation between animal and human degenerative disorders of the CNS

A) Veterinay Medicine	B) Human Medicine
NAD in a Rottweiler dog	Primary NAD
EDM in Arabian horses	Motor Neuron Diseases, Primary/Secondary NAD
SMA in Holstein-Friesan calves	Motor Neuron Diseases
Murine Scrapie	Human Prion Diseases

Moreover, as pathological investigation in human beings have indeed become the study of the molecular basis of the diseases, it is necessary to study such animal diseases (Table 1A) in an attempt to identify pathological alterations comparable to those encountered in the homologus human ones (Table 1B), and to contribute to the pathogenesis of such neurodegenerative disorders that most of them until now remain uncertain.

In conclusion, the comparative patholgy not only has to be useful for veterinary pathologists in contributing to the knowledge of the disease in animals, but also, for human pathologists in order to look for possible animal models.

MOLECULAR BIOLOGY AND PATHOLOGIC BASIS OF DISEASE

3. The chemical synapse

The synapse: **the point of functional contact between one neuron and another.**

Most interneuronal communication relies on the use of a chemical transmitter (neurotransmitter), secreted subsequent to action potentials by presynaptic cells to influence the activity of post-synaptic cells [85].

Neurotramsitters are concentrated in membrane-bound structures, called synaptic vesicles that dock at specialized sites called active zones along the presynaptic membrane. Vesicles not docked at the membrane are clustered behind it and associated with cytoskeletal elements [85]. Action potentials release neurotransmitter by depolarizing the presynaptic membrane and opening Ca^{2+} channels that are strategically colocalized with the synaptic vesicles in the active zones [74, 81, 85]. The local intense rise in Ca^{2+} concentrations triggers the fusion of docked vesicles with the plasma membrane and exocytosis[74]. After exocytosis, some vesicles may rapidly reclose whereas others fuse fully with the plasma membrane and are recovered by endocytosis (also regulated by presynaptic Ca^{2+}) [74, 81, 85]. Maintaining a constant supply of vesicles requires the efficient recycling of synaptic vesicles [85].

The synapse: a cascade of protein-interactions:

The synaptic vesicle-exo-endocytic cycle include different steps [74, 81, 85]:

- *Transmitter uptake from the cytoplasm*
- *Cytoskeletal and intervesicular anchoring*
- *Plasma membrane docking*
- *Membrane fusion (exocytosis)*
- *Endocytosis and recycling*

The synaptic vesicle-exo-endocytic cycle, specially targeting, attachment and fusion of vesicles, is mediated by the sequential action of protein complexes [85]. The components of the synaptic vesicle membrane are initially synthesized in the cell body before transported to nerve terminals by fast axonaplasmic transport [60, 85]. Diverse synaptic proteins are distributed in various subcellular compartements of the synapse [60, 85].

3.1.1. Transmitter uptake from the cytoplasm. The synaptic vesicle:

Synaptic vesicles in the mammalian CNS are small and uniform organelles with a diameter averages approximately 40-50 nm [67]. A restricted set of proteins are required for all synaptic vesicle functions; mainly a) proteins, involved in neurotransmitter uptake and storage, and b) proteins involved in vesicular membrane trafficking [67].

a) Proteins involved in neurotransmitter uptake and storage:

In neurotransmitter uptake are implicated:

- the electrogenic proton ATPase, which is necessary for driving neurtransmitter against a concentration gradient [67]. It is purified from vesicles that derived from recycled synaptic vesicles [70] named the clathrin-coated vesicles [65].
- ion channels and electron transporters that allow for charge compensation or provide reduction equivalents [67].
- carriers specific for individual neurotransmitters differentially distributed in the CNS [67].

b) Proteins involved in vesicular membrane trafficking:

The putative trafficking proteins that plays a role in protein-protein interactions [78] or protein-membrane interactions during vesicular membrane traffic, can be grouped into:

- *Integral membrane proteins*: such as synaptotagmin [73], synaptophysin [71], synaptobrevin [64] and syntaxin [58, 59]. All of them remain on the vesicle during its entire cycle [67].
- *Peripheral membrane proteins*: such as synapsin [63, 67, 79, 83], rab3A [67, 76], the synaptosomal-associated protein of 25 kDa (SNAP-25) [66, 72] and synucleins [61, 68].

All these proteins are represented in **Figure 1**.

3.1.2. Cytoskeletal and intervesicular anchoring

Movement of synaptic vesicles within neurons, to and from nerve terminals, as well as within the nerve terminal require interaction with cytoskeletal elements [67] such as actin and tubulin microfilaments. *Synapsin-1*, a cytosolic protein enriched in presynaptic terminals can bind reversibly to both actin microfilaments and synaptic vesicles [60, 85]. Both binding activities are regulated by phosphorilation of

synapsin [67, 81, 85]. This phosphorilation is mediated by a calmodulin dependent kinase-II [81]. Phosphorilated synapsin allows reserve synaptic vesicles to be mobilized through active zones of the presynaptic membrane while non-phosphorilated synapsin leads these synaptic vesicles to be transport down the axon by fast axonal transport and retains them in the presynaptic terminal, creating a reserve pool for next stimulation with local calmodulin-activated kinases [67, 81, 85].

3.1.3. Plasma membrane docking

When synaptic vesicles are free-mobilized a vesicle binding to the presynaptic plama membrane occurs [67, 81, 84, 85]. This is mediated by integral vesicle membrane proteins such as *synaptotagmin* and *synaptophysin* that bind directly to putative components (receptors) of the presynaptic membrane: *neurexins* (such as the α-latrotoxin) [73], and *physophilin* [80], respectively.

3.1.4. Membrane fusion (exocytosis)

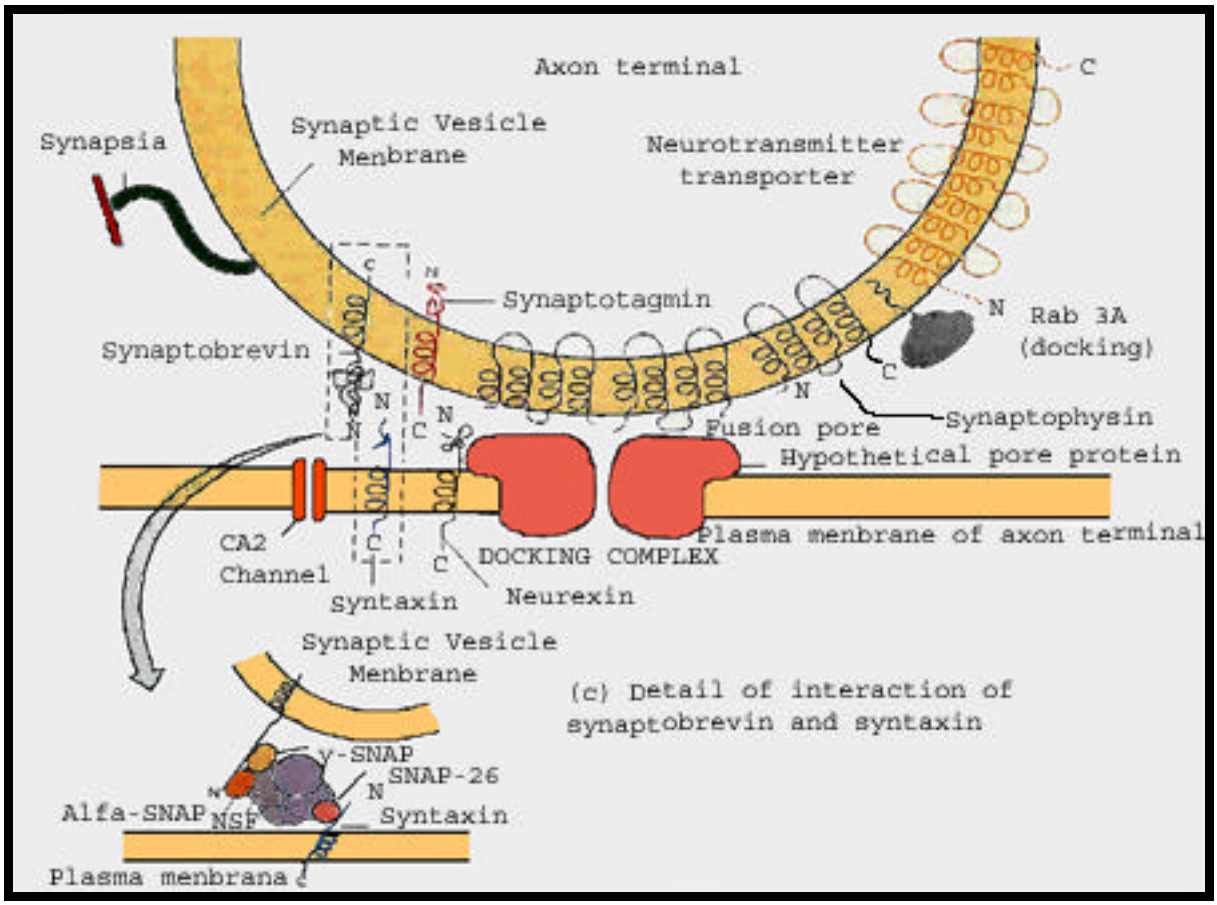
The mechanism of membrane fusion is still unclear [67]. After docking, exocytosis of synaptic vesicles require the formation of a fusion complex consisting of the synaptic vesicle protein *synaptobrevin* (vesicle-associated membrane protein, or VAMP) and the plasma membrane proteins *syntaxin* and *SNAP-25* [57, 60, 85]. All these proteins are commonly referred to as *SNAREs* [soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors] [56, 57, 60, 85] and are receptors for soluble Golgi trafficking proteins [75]. Vesicle-associated SNARE (v-SNARE) include synaptobrevin and, target membrane-localized SNARE (t-SNARE) the syntaxin protein as well as the SNAP-25 [56, 57].

Recent evidence indicates that the SNARE complex is only one of the several protein complexes involved in vesicle targeting and fusion [56, 75]. It is well stablished that synaptobrevin also can bind to synaptophysin resulting in a complex which is upregulated during neuronal development and only takes place in adult synaptosomes, concluding that the *synaptophysin-synaptobrevin complex* is a hallmark of synaptic vesicle maturation [57]. Synaptobrevin bound to synaptophysin can not enter the SNARE complex and, conversely, synaptobrevin which is a part of the SNARE complex, can not interact with synaptophysin [64]. In conclusion, although this synaptophysin-synaptobrevin complex is not essential for exocytosis, provides a reserve pool of synaptobrevin for exocytosis that can be recruited during periods of high synaptic activity [57].

The SNARE complex is required for irreversible docking of vesicles [82] and induces a state consistent with hemifusion not driving full membrane fusion [62]. It is believed that vacuole calcium stores are required for fusion, that calcium is released from vacuoles after docking and that synaptic calcium channels interact

with t-SNAREs [56]. It is also suggested that Ca^{2+} dependent proteins such as *synaptotagmin* may interact with the SNARE complex in presence of Ca^{2+} influx [56]. Although it seems clear that calcium flux may play a role in membrane fusion, it is thought that additional regulators are required in order to increase the precision of exocytosis at the synapse. Candidates for the regulation of the formation of prefusion complex previously commented, are GTP-binding proteins [67]. *Rab3* is a low-molecular-weight GTPase, that acts as a molecular switch [60, 85]. It is associated to the synaptic vesicle in its GTP-bound state, but is recycled as a soluble intermediate after GTP hydrolysis, leading in its GDP-bound form the SNARE complex formation [60, 85].

Figure 1. Synaptic proteins that are required for vesicular membrane trafficking, docking and exocytosis.



Tresguerres, 1999 (Ref. 82)

3.1.5. Endocytosis and recycling

After exocytosis, the components of the synaptic vesicle must be recovered from the presynaptic plama membrane [77]. Two main mechanisms take place [85]:

a) A reversal of the fusion process: a fusion pore opens to release neurotransmitters and rapidly closes to re-form a vesicle [85].

b) Endocytosis: is mediated by vesicles coated with the protein *clathrin* associated to some accessory proteins (that may interact with *synaptotagmin*) [70]. The *dynamin* protein (a GTPase, whose activity is regulated by calcium phosphorilation and dephosphorilation) recover the vesicle membrane, then takes place the fusion of coated vesicles with the endosomal cisternae and finishes with the formation of synaptic vesicles from endosomes [85].

4. Programmed Cell Death (PCD)

***Apoptosis* (derived from the greek meaning "dropping of leaves off a tree"):**

PCD or apoptotic cell death is an active process, for which protein synthesis is required. Pathological features of apoptosis include a number of attributes morphologically and biochemically distinguishing the process from necrosis (see **Table 2**). Apoptosis is the end-point of an energy-dependent cascade of molecular events, initiated by certain stimuli and consisting of four separable but overlapping components [86, 88, 90, 92, 94]:

- Signalling pathways that initiated apoptosis
- Control and integration stage
- An execution phase
- Removal of dead cells

All these phases are illustrated in **Figure 2**.

Table 2. Differences in the pathological features of neurons by apoptosis and necrosis *

	Pathological features	
	Apoptosis	Necrosis
Pattern of death	Individual single cells	Whole group of cells
Cell shape changes	Cell shrinkage	Cell swelling and lysis
Plasma membrane changes	Membrane preservation	Early membrane breakdown
Organelle changes	Involution, "apoptotic bodies"	Organelle swelling,disruption
Nuclear changes	Chromatin condensation and fragmentation	Karyolysis, pyknosis (or Karyorrhexis)
DNA breakdown	Internucleosomal DNA Fragmentation, free 3'-ends	Diffuse and random DNA degradation
Cell degradation	Phagocytosis without cell Infiltration or inflamation	Marked inflamation, with macrophage invasion

** Honig and Rosenberg, 2000 (Ref.88)*

4.1. Signalling pathways (causative factor and induction)

Apoptotic stimuli (*hormone or growth factor deprivation, death ligand binding to plasma membrane receptors, free readicals*,...) generate either transmembrane signals that are transmitted to intracellular

regulatory molecules (mediated by receptor-ligand interactions; e.g., *Fas-Fas ligand, TNF- TNF receptor*) or intracellular signals that are directly addressed at targets present within cell [88, 93].

4.2. Control and integration state (the initiator phase)

This state is performed by specific proteins that connect death signals to the execution program. These proteins are crucial in determining "commitment" or "abortion" of the apoptotic process. On one hand, subplasmalemmal association of cytoplasmic receptor domains and adaptor proteins (e.g., the Fas-Fas ligand model) [88, 93]. By the other hand, involvement of proteins that regulate mitochondrial permeability. These regulatory proteins are mainly members of *the Bcl-2 family* that are located in the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope [93]. Many of this family inhibit apoptosis (*Bcl-2*) while other family members (*Bax*) promote apoptosis [93]. In apoptosis, alteration of permeability in the inner mitochondrial membrane causes reduction of membrane potential and mitochondial swelling, and in the outer mitochondrial membranes releases *cytochrome c* from the mitochondria to the cytosol [87, 88, 93]. Cytochrome *c* release precedes the morphologic changes of apoptosis, showing that it occurs early and, consistent with a regulatory function [93]. Released cytochrome *c* disrupts the binding between Bcl-2 and the *pro-apoptotic protease activating factor* (Apaf-1) that induces caspase activation [87, 93].

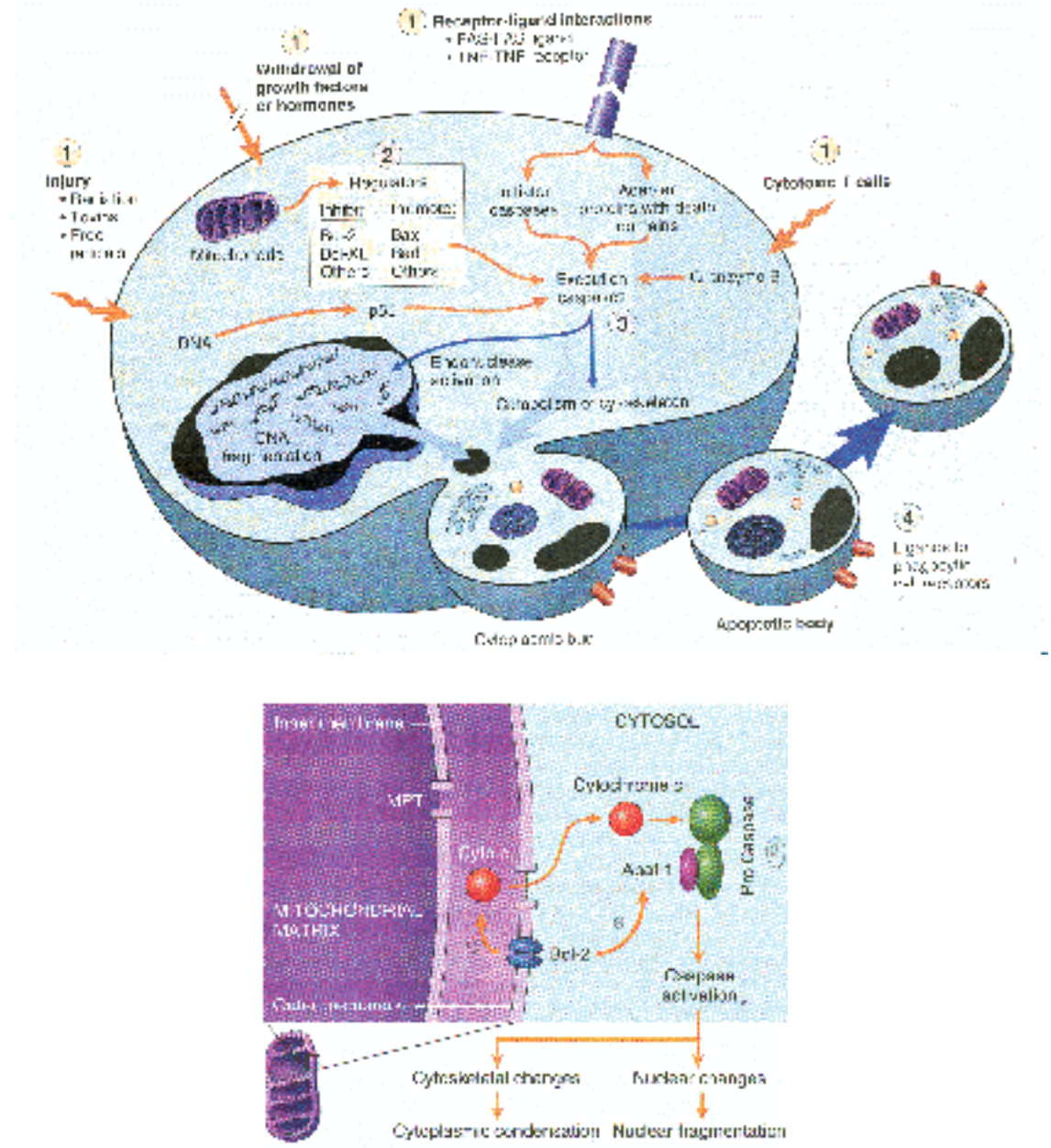
4.3. The execution phase

The final pathway is a proteolytic cascade in which proteases belonging to the *caspase family* trigger and mediate the execution phase [88, 91]. This enzyme family has two catalytic properties: "c" reflects a cysteine protease mechanism, and "aspase" refers to their unique ability to cleave after aspartic acid residues [91]. Initiator caspases, include *caspase-9*, which binds to Apaf-1, and *caspase 8*, which is triggered by Fas-Fas ligand interactions [87, 93]. These initiator caspases then act subsequently to cleave "down-stream" or effector *procaspases 3, 6, 7*, that finally lead to *cleaved-caspase 3*, which in turn cleaves a variety of cytosolic proteins such as DNA repair enzymes [e.g., *PARP* or poly (ADP-ribose) polymerase], structural proteins such as *actin* and *nuclease inhibitors* (ICAD) [88].. This terminal phase leads to *endonuclease activation* resulting in the cell degradation phase [88, 93].

4.4. Removal of dead cells

Apoptotic cells and their fragments have marker molecules on their surfaces that facilitate early recognition by phagocytes for phagocytic uptake. The process is so efficient that dead cells disappear without inflammation [93].

Figure 2. General molecular elements of apoptotic cascade.



** Robbins, 1999 (Ref. 93)*

5. The role of intermediate filament, ubiquitin, and αB- crystallin

A relationship between ubiquitin, αB- crystallin and intermediate filaments not aggregated into inclusions [98, 101] has been suggested. It is thought that these proteins are related and that their function is part of a cytoprotective cell response to eliminated damaged components [102].

Because of inclusions seen in the CNS in different neurodegenerative diseases are based on intermediate filament, ubiquitin, and αB- crystallin [102], we have introduced these proteins in the same chapter.

5.1. Neuronal cytoskeleton is composed of three interconnected filaments: the actin microfilaments, tubulin microtubules and intermediate filaments [95]. **Neurofilaments** are the major type of internmediate filaments formed from three subunits with variable molecular weight: high-molecular-weight subunits (NFH), from 180 to 200 kDa; from 130 to 170 kDa for the medium subunit (NFM); and, from 60 to 70 kDa for thelow-molecular-weight subunit (NFL) [95]. Although neurofilaments do not appear to be necessary for nervous system development, deficiencies in neurofilament proteins are not completely inocuous [103]. Absence of the medium and high-molecular-weights subunits increases the velocity of transport for slow components in the axon [100] and provokes alterations in the organization of the neuronal cytoskeleton [103]. An important characteristic of neurofilaments is that the NFH and NFM subunits are high phosphorilated in their carboxy terminus [95, 103]. The apparent greater plasticity of the neurofilament network in regions like perikarion, initial segment and nodes along the axon may provide some insights into the vulnerability of these regions in neurofibrillary disease [106].Moreover, it is consider that phosphorylation may control the major events in the life of newly synthesized neurofilament proteins, namely their assembly, axonal transport, turnover and integration into the axonal cytoskeleton [106].

5.2. Ubiquitin is a cell stress protein that can be involved in controlling multienzyme pathways for destroying abnormal and damage proteins, and in apoptosis [107]. Its physiological roles are multiple: DNA repair, cell cycle control, and stress response [97]. Ubiquitin is transported exclusively with the same slow component that carries cytoskeletal and cytoplasmic proteins [102]. Its distribution in a subset of lysosomal-related dense bodies have shown to be a site of generation of abnormal peptides in both Alzheimer's disease and prion encephalopathies [104, 105].

5.3. αB- crystallin is a small heat shock protein (HSP) of the crystallin family. Its function is to activate the heat-shock/stress response as a consequence of intracellular accumulation of abnormally folded proteins. It is mainly expressed in oligodendroglia and astrocytic cells [99]. It is constitutive in oligodendroglia in mice under stress conditions and their function *in vivo* remains unclear [96].

6. Neuronal Proteins: basic and applied aspects in human neurodegenerative diseases.

As pathology is well in the era of molecular biology, has indeed become the study of the molecular basis of the disease. It has been shown that proteins not only are involved in the formation of structural lesions associated with major diseases of the central nervous system, but also are useful markers for the detection and study of these lesions [118].

One possible method to study proteins is the *immunohistochemistry (IHC) technique,* as a diagnostic tool as well as for research.

Human medicine has focused its studies in different sets of proteins related to:

- Synaptic function
- Axonal transport
- Cell death
- Glial cell proliferation

Results show that different proteins that interact in the synapses in order to release neurotransmitters, and several different molecules that participate in the intracellular pathways that leads to apoptosis, are involved in pathological changes of neurons in neurodegenerative diseases and, that its phosphorilation state must influence in disease.

6.1. Synaptic proteins:

6.1.1. Introduction

The functional and structural stability of neuronal microcircuitry depends on its capacity to exchange information across the synapse junction [113]. This complex sets of functions is regulated by diverse synaptic proteins, distributed in different subcellular compartments of the synapse (see Fig. 1 in 3.1.1.b).

The basic mechanisms by which these synaptic proteins could be altered under abnormal conditions are [150]:

a) sustained chronic or acute injury to cell body or neuritic processes with subsequent anterograde degeneration of the synaptic terminal (trauma, ischemia, or metabolic disorders)
b) direct injury to the synaptic site by specific neurotoxins, viruses, prions,..
c) failure of the neuronal cell apparatus to maintain synaptic activity and survival due to decreaed production of neurotrophic factors or altered signal transduction (protein kinase C, *fos*)

6.1.2. Role in neurodegeneration

For the purposes of the present thesis we will only review human neurodegenerative disorders described in Table 1.

· *Aging and Alzheimer's Disease (AD):*

In humans, during aging there is a 15-20% reduction in synaptophysin immunolabeled terminals [149, 150] in the frontal cortex, and not in temporal or occipital cortex, suggesting a selective, regional synapse loss [125]. A hippocampal synaptic loss was assume when levels of synapsin-I were measured by radioimmunoassay in AD cases and compared to controls [157]. Moreover, a fragment of α-synuclein has been found in senile plaques [169] and is especially abundant in the central portion of mature plaques [151] as well as synapsin-I and synaptophysin [134, 150].

· *Neuroaxonal dystrophies (NAD):*

Abnormalities at presynaptic terminals have been studied in depth in infantile NAD [124, 160]. Synaptophysin did not react with dystrophic axons in an experimental model of NAD induced by parabromophenylacetylurea (BPAU) [108]. α-Synuclein immunoreactivity was present in dystrophic axons in primary NAD and in axonal swellings (spheroids) following secondary NAD suggesting that the neuronal cytoplasmic accumulation of this presynaptic protein results from an axonal injury [157]. By contrast, almost all spheroids found in one case of Hallervorden-Spatz disease (HSD) were negative for α-synuclein [171].

· *Prion diseases:*

Reduced expression of proteins linked to exocytosis and neurotransmission has been reported in human and animal prion diseases [116, 130, 133]. In the granular cell layer of the cerebellum of Creutzfeldt-Jakob disease (CJD) patients, reduced synaptophysin, synapsin-I, SNAP-25, syntaxin-I and Rab3A was reported [117]. Interestingly, in other cerebellar areas this reduction was not observed [117]. Moreover, synaptophysin and Rab3A were accumulated within axon torpedoes and Purkinje cells, respectively [117].

· *Motor Neuron Diseases (MND):*

Loss of spinal anterior horn presynaptic terminals have been well documented in lower motor neuron disease [126, 129, 162], lateral amyothropic sclerosis [127, 152, 163] and Werdnig-Hoffmann disease [128, 174]. Moreover, it has been demonstrated that these changes take place during the process of neuron degeneration in patients with ALS [163, 164], Werdnig-Hoffmann disease [174] and lower motor neuron disease [162].

6.2. Proteins involved in cell death

6.2.1. Introduction

Actually, the apoptotic pathway can be investigated using antibodies against a large number of pro-apoptotic and anti-apoptotic proteins (illustrated in Fig.2 of chapter 4). In the last 5 years, the most used method was the *in situ* end-labeling technique of nuclear DNA fragmentation *(or TUNEL method)* that has been described as a specific technique to detect apoptosis-related DNA fragmentation [119]. Neuronal DNA fragmentation, as revealed with the TUNEL method, has been reported in both animal and human brains in normal aging and in several neurodegenerative diseases. In the past few years, some authors have expressed doubts about the use of the TUNEL method as apoptosis-specific for different reasons [109, 115, 121, 144, 165]:

- DNA fragmentation also occurs in the late stages of necrosis, although in a random manner [121, 144],
- the post-mortem delay and fixation of samples (in the processing of samples) affects DNA integrity [164],
- TUNEL-positivity in neurons in which none of the classical morphological hallmarks of apoptosis is detected [109],
- prion inactivation in brains of scrapie mice immersed in 96% formic acid for 15 minutes resulted in large numbers of TUNEL-positive cells as affects DNA integrity (our own observations concerning apoptosis in murine scrapie),
- and, TUNEL method showed positive labeling in models of apoptosis, necrosis, and autolysis in rat liver cells [121].

All these data support that the TUNEL method has limitations and do not permit, by its own, to confirm if neuronal cell death is due to apoptosis. For this reason we believe that the immunohistochemistry technique may complement the TUNEL method in apoptosis research.

6.2.2. Role in neurodegeneration

· *Prion diseases:*

It has been suggested that cell death in murine scrapie and in CJD is due to apoptosis [120, 122, 143, 159, 172]. Most of these studies are based on the method of *in situ* end-labeling of nuclear DNA fragmentation (ISEL

technique or TUNEL). As previously mention in part 3 of chapter 2, 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. Nevertheless, the expression of proteins linked to signaling pathways have been studied in the cerebellum of CJD patients [159]. Microscopic examination showed that granule cells were specially vulnerable while Purkinje cells were relatively resistent in those patients [159]. Moreover, this study shows strong activation of caspase-3 in Purkinje cells and a few positive cells in the granular and molecular layers of the cerebellum [159]. Interetingly, Taken together these controversial results they conclude that the enhanced expression of several proteins linked to putative cell death pathways is not associated with apoptosis in Purkinje cells [159].

· *Motor Neuron Diseases (MND):*

There is direct evidence for DNA lesions specifically in motor neurons of ALS patients [114, 148, 175] as well as in individuals with Werdnig-Hoffmann disease [167]. It seems that apoptosis is an important mechanism of cell death in human MND. ALS patinets showed decrease Bcl-2 expression whereas Bax expression is increased [114, 123, 148]. Moreover, activation of caspase-9 and translocation of cytochrome c from the mitochondria to the cytosol has been demonstrated in the spinal cords of transgenic mSOD1 (mutant superoxide dismutase-1) mice [123]. It seems that only in end-stage transgenic mSOD1 mice is the downstream caspase-7 activated and the inhibitor of apoptosis, XIAP, cleaved [123]. Recent data in transgenic mSOD1 mice [170] supports that the contribution of a non-apoptotic mode of cell death such as necrosis can not be excluded in this model of ALS.

6.3. Proteins involved in "stress" and axonal transport

6.3.1. Introduction

Immunochemical staining to detect ubiquitin has become an essential technique in evaluating neurodegenerative processes due to its role as a cell stress protein, its evolution conservation, its participation in destroying abnormal or damage proteins and its roles in programmed cell death [166]. Anti-ubiquitin also identifies both filamentous and lysosomal structures in neuronal processes as well as in some swollen neurones. Moreover, as it has an important role in lysosomal function, it is distributed in a sub-set of lysosomal-related dense bodies recently shown to be a site of generation of abnormal peptides in both AD and prion diseases [153].

6.3.2. Role in neurodegeneration

Intermediate Neurofilament

Disruption of neurofilament organization is a hallmark of pathology in many neurodegenerative diseases such as motor neuron diseases [110, 131]. In infantile neuroaxonal dystrophy (Seitelberger's disease),

reactivity to neurofilament was restricted to espheroids of small size [156]. In experimental CJD, neurofilament accumulation within affected neurites has been demonstrated [138]. Ballooned neurons in infantile motor neuron disease [136] and in Werdnig-Hoffmann disease [132] showed accumulation of neurofilaments. This abnormal accumulation in the perikarion of motor neurons may not only impair neurofilament transport into the axon [112, 147] but also provoke neuronal death [131] in ALS and related motor neuron diseases. The molecular nature of the filaments making up the inclusions in ALS remains uncertain although it has been proposed to be underlyied by the neurofilament protein [161].

Ubiquitin and αB- crystallin.

Ubiquitin-immunoreacive granules occur in normal human aging , Parkinson's disease, Alzheimer's disease, or motor neuron disease [137, 146]. Swollen neurones (or ballooned neurons) and espheroids in many neurodegenerative disease may show ubiquitin immunoreactivity [108, 142, 168] as well as intraneuronal inclusions in cortical regions in sporadic ALS [173].

In *Seitelberger's disease*, reactivity to ubiquitin was restricted to espheroids of small size [156].

Swollen neurones in most neurodegenerative disease may show ubiquitin immunoreactivity [168]. Recent work has also shown that such neurons are immunoreactive with antisera to αB- crystallin [140].

Ballooned neurons of *MNDs* such as in infantile motor neuron disease [136], in Werdnig-Hoffmann disease [132] and in an animal model of motor neuron degeneration [154] are immunoreactive with antisera to ubiquitin. The finding of specific ubiquitin-immunoreactive inclusions in the majority of cases of sporadic and familial ALS has been a major advance as they are not easily detected by other [111, 139].

In *prion diseases*, ultrastructural examination of the brain has shown colocalisation of PrP and ubiquitin in lysosome-related membrane-bound vesicles [135] and experiments have suggested that this take place early in the time course of infection [141]. This is in accord with data showing that the lysosomal system is a major site for processing of PrP in prion encephalopathies [155] and suggests that the lysosomal compartment, involving ubiquitin, is centrally involved in disease pathogenesis [153] although the function of ubiquitin in this setting is unknown.

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