CHAPTER 5 : SUMMARISING DISCUSSION

In this study the clinical signs and pathology of five plant poisonings and a mycotoxicosis affecting the nervous system of domestic ruminants in southern Africa are described. For comparative purposes, an inherited storage disease (β-mannosidosis) and a drug-induced neurotoxicosis (closantel overdosage) are also presented. Chapter 1 outlines the aims of this thesis, namely

- to document the pathology of those conditions where detailed descriptions were lacking,
- to study the light and transmission electron microscopical lesions from a differential diagnostic perspective, and,
- to consider the possible causative mechanisms of the nervous lesions.

Lysosomal storage diseases (Chapter 2)

A short summary on the classification of the lysosomal storage diseases in domestic animals is given in Chapter 2.1. In Chapter 2.2 a novel lysosomal storage disease in goats induced by the plant Ipomoea carnea is reported [17]. Analysis of plant material confirmed the presence of swainsonine and two glycosidase inhibitors, namely, calystegine B2 and calystegine C1. The storage disease caused by I. carnea is analogous to the lysosomal storage disease in ruminants induced by ingestion of locoweeds (Astragalus and Oxytropis spp.) and poison peas (Swainsona spp) [19,23,41,58]. These plant species also contain swainsonine, an indolizidine alkaloid, which is an inhibitor of lysosomal α-mannosidase and mannosidase II [18,36]. This group of plant-induced storage diseases closely mimics inherited α-mannosidosis in animals. In Chapter 2.3 β-mannosidosis is documented in Hereford calves, a breed not previously known to be afflicted by this inherited disorder. The condition was biochemically confirmed by an absence of lymphocyte β-mannosidase activity in one of the affected calves.

Comparison of the two storage diseases described in this thesis highlighted two important aspects pertinent to their recognition and diagnosis. Firstly, they are a diverse group of multisystemic disorders with variable phenotype. The Hereford calves with β-mannosidosis manifested severe clinical disease, hydrocephalus and evidence of myelin deficit in the brain. Similar findings in β-mannosidosis in Salers calves [1,8,35] and Nubian goats [29,37] have been reported. In contrast, goats exposed to I. carnea showed less severe clinical signs while specific gross lesions were restricted to asymmetry and atrophy of the cerebellum in 1/6 animals. It has been documented that the severity of disease expression is influenced by the severity of the enzyme deficit [36]. If a mutant gene defect is such that mutant enzyme is not synthesized as in β-mannosidosis, the absence of enzyme activity will result in severe, early onset disease. In the plant-induced storage diseases there is most likely some residual enzyme activity resulting in less severe disease.
Secondly, in β-mannosidosis and *I. carnea* intoxication, storage vacuoles are empty in routinely prepared sections for light microscopy, indicating that the storage material is soluble in water and/or organic solvents. It is therefore not possible to diagnose these conditions based on morphological or staining characteristics of the storage material only. Confirmation of the diagnosis of these disorders therefore depends on biochemical analysis to determine the enzyme defect and the stored substrate. In the case of the plant-induced storage diseases, identification of the chemical compound(s) biosynthesized by the plant provides valuable additional information to support the diagnosis.

This study showed that in chronic *I. carnea* intoxication there is degeneration and loss of cerebellar Purkinje neurons, which probably accounted for the irreversible clinical signs in some of the chronic cases. Purkinje neurons are particularly vulnerable to injury in other storage diseases [56]. It is not known how the accumulation of lysosomal substrate perturbs cell function. Possible explanations that have been put forward are mechanical impairment of intracellular transport; a cytotoxic effect of the accumulated substrate; spheroid formation that affects inhibitory γ-aminobutyric acid (GABA)ergic fibres; and disturbances in synaptic function [56].

‘Maldronksiekte’ in cattle, caused by ingestion of the plant *Solanum kwebense*, is characterised by degeneration, necrosis and loss of cerebellar Purkinje cells and, to a lesser extent, neurons in cerebellar nuclei. Affected Purkinje neurons were either swollen, pale and finely vacuolated or shrunken and more eosinophilic than normal. In the present study, some Purkinje cells and neurons in cerebellar nuclei stained strongly with *Canavalia ensiformis* (ConA) agglutinin and weakly with *Triticum vulgaris* (WGA) and succinyl-WGA (S-WGA) agglutinins (Chapter 2.4). Ultrastructurally, small numbers of membranous bodies were found in the cytoplasm of degenerated Purkinje cells. Low levels of calystegines but not swainsonine and other known glycosidase inhibitory alkaloids were detected in one sample of plant material collected from one of the incriminated farms. The condition is clinically and pathologically virtually identical to *S. fastigiatum* poisoning, a condition believed to be a lysosomal storage disease (probably a gangliosidosis) based on the presence of membranous cytoplasmic bodies in Purkinje cells [48]. When compared to other storage diseases, two pathological features of intoxication by *S. kwebense* and *S. fastigiatum* are, however, unusual, namely the predominance of lesions in Purkinje cells and the degeneration and loss of most Purkinje cells in the cerebellum of affected cattle. ‘Maldronksiekte’ is therefore classified as an acquired cerebellar cortical degeneration morphologically resembling cerebellar and cerebral cortical degeneration in miniature Poodles [14]. The condition may indeed represent a plant-induced intoxication that does not result from an enzymatic lysosomal defect but in which Purkinje cells are unable to metabolise a plant toxin or cellular substrate [56]. Acquired cerebellar cortical degeneration induced by toxic plants is not restricted to cattle. A syndrome characterised by degeneration and loss of Purkinje cells has been associated with the ingestion of *Solanum cinereum* in goats in Australia [7].
The question is why Purkinje cells are the predominantly cell type affected in cerebellar cortical degeneration. Some studies in domestic animals revealed striking morphological abnormalities in degenerated Purkinje cells but the pathogenesis for the selective involvement of these neurons remains unclear. Morphological changes in injured Purkinje cells in cerebellar cortical degeneration comprised accumulations of tubulovesicular profiles of agranular ER in Aberdeen Angus cattle [5] and cytoplasmic vacuoles and lamellar bodies originating from ER and stacked cisternae of presumed ER-origin in dogs [14,44]. In spinocerebellar ataxia type 6 (SCA6) in humans, a condition in which Purkinje cells are particularly affected, a molecular basis of selective Purkinje neuron degeneration has been reported [33,49]. This condition is an autosomal dominant neurodegenerative disease associated with the expansion of trinucleotide (CAG) repeat coding polyglutamine in the alpha 1A (P/Q-type)-voltage dependant calcium channel. The mutant proteins which are produced form cytoplasmic aggregates and altered protein-protein interactions result in excessive channel activity, intracellular Ca\(^{2+}\) overload and cell death. The term ‘channelopathy’ has been coined for this condition [57]. Whether or not apoptosis is involved in neuronal degeneration in cerebellar cortical degeneration needs further study.

**Toxic myelinopathies (Chapter 3)**

In this chapter four toxic myelinopathies are described; three are induced by plants (*Helichrysum argyrosphaerum, Ornithogalum prasinum* and *O. saundersiae* and *Crotalaria sparthioides*) and one is caused by overdosage of the anthelmintic compound closantel. As an encompassing term, myelinopathies denote non-inflammatory conditions of the central or peripheral nervous system in which the primary event relates to some disorder of myelin formation, maintenance, or stability [38]. In Chapter 3.1 a classification of myelinopathies based on morphological criteria in domestic animals is given and explained. Chapter 3.2 documents the clinical syndrome and pathology induced by the plant *Helichrysum argyrosphaerum* in sheep and a goat and in Chapter 3.3 the effects of overdosage by the halogenated salicylanilide closantel in sheep and a goat are described. The two intoxications are clinically and pathologically indistinguishable and are characterised by blindness, ataxia, nystagmus, widespread spongy changes (status spongiosis) in the central nervous system, optic neuropathy and retinal degeneration. The status spongiosis is caused by splitting of myelin lamellae at the intraperiod line, which is consistent with myelin oedema. Oedema of myelinated fibres leads to compression of the optic nerves in the bony canal and eventually results in Wallerian degeneration and fibrosis of the nerves. In both poisonings, toxic retinal degeneration was present. There have been some conflicting interpretations of the site of retinal injury in salicylanilide poisoning. Necrosis of the ganglion cells [6,9,47] and degeneration of the outer retinal layers [26,52] have been reported as primary lesions. Results of our study showed involvement of the photoreceptor layer in both conditions possibly targeting the outer segments in poisoning with *H. argyrosphaerum* and of the rods in salicylanilide overdosage.
Chinkerinchee poisoning (poisoning by *Ornithogalum prasinum* and *O. saundersiae*) is described in Chapter 3.4. Diarrhoea was consistently present in cattle and sheep, while blindness occurred in a high proportion (4/11 and 3/3) of cattle. The blindness is associated with a central visual pathway lesion characterised by bilateral status spongiosis of the cerebral white matter especially affecting the optic radiation and internal capsule. Transmission electron microscopy revealed splitting of myelin lamellae at the intraperiod lines and swollen astrocytic processes in the affected white matter. It would appear that this intoxication is a unique example of white matter myelin oedema causing blindness in cattle.

Toxicity with *Crotalaria sparthioides* in cattle induced hepatic encephalopathy secondary to cirrhosis (Chapter 3.5). The pyrrolizidine alkaloid senkirkine was isolated from toxic plant material. Brain lesions were bilateral symmetrical and most profound at the junction of the white and grey matter (consistently affecting the cerebral cortex, cerebellar white matter and peduncle, thalamus, basal ganglia and internal capsule) in three natural and one experimentally intoxicated bovines. There are few published reports on the ultrastructural changes in hepatic encephalopathy in domestic animals. In one experimental study in which calves were infused with ammonia, swelling of myelin sheaths and splitting of myelin lamellae at the intraperiod lines was demonstrated [11].

The pathogenesis of hepatic encephalopathy is still largely unclear although there is agreement on the important role of neurotoxins, especially ammonia [3,45]. In human medicine, much research is directed to study alterations of astrocyte morphology and function and it has been proposed that hepatic encephalopathy is an example of an ‘astrocytopathy’ or ‘gliopathy’ [45]. Astrocytes are the only cells in the brain to contain glutamine synthetase and are accordingly a major site of cerebral ammonia detoxification. It is proposed that astrocyte swelling, induced by amongst others ammonia, hyponatraemia, some neurotransmitters, tumour necrosis factor-α and benzodiazepines, is an early event in hepatic encephalopathy [4,28,45]. An increase in astrocyte water content may then activate extracellular regulated protein kinases, elevates intracellular calcium concentration, upregulates the peripheral type benzodiazepine receptor and affects multiple ion channels and amino acid transport.

Status spongiosis denotes spongy vacuolation of nervous tissue noticeable by light microscopy [2]. Transmission electron microscopy is often required to determine the morphological basis of the vacuolation [56]. The vacuoles usually contain fluid and may be present within myelin sheaths, processes in the neuropil, or the cytoplasm of astrocytes or oligodendrocytes [2]. In the four toxic myelinopathies described in this thesis (Chapter 3), vacuoles within the nervous system were formed within myelin sheaths due to splitting of myelin lamellae the intraperiod lines. This is the most location of fluid in status spongiosis [40]. The intramyelinic vacuoles can be considered to occur in the embryonic extracellular space since the intraperiod line is formed by apposition of the external surface of the plasma membrane of the myelin forming cells [46]. Experimentally it has been
shown that this space can be penetrated by various markers [46]. The integrity of the intraperiod line is believed to be maintained by proteolipid protein (PLP) [20] but whether splits can be produced by an over- or an underproduction of PLP is not clear [10]. The distribution of the vacuolation varied despite having a similar morphological basis. The vacuolation was mainly periventricular in *H. argyrosphearaeum* poisoning and overdosage with closantel, located predominantly in the optic radiation and internal capsule in chinkerinchee poisoning, and involved myelinated bundles of fibres that are interspersed with the gray matter in hepatic encephalopathy.

**Aspergillus clavatus neuromycotoxicosis (Chapter 4)**

In Chapter 4.1 the light and transmission electron microscopical changes of acute *Aspergillus clavatus* intoxication in cattle is reported. In this neuromycotoxicosis there is chromatolysis particularly of motor neurons in the ventral horns throughout the spinal cord and of neurons in selected brain stem nuclei including the spinal tract nucleus of the trigeminal nerve and the dorsal nucleus of the vagus nerve. At the ultrastructural level, changes in chromatolytic neurons were characterised by disintegration of Nissl bodies, abundant free ribosomes and increased numbers of mitochondria and dense bodies consistent with the axonal reaction. Swollen axons were present in close proximity to affected nerve cell bodies and they contained axoplasmic debris and degenerated organelles compatible with Wallerian degeneration. Wallerian degeneration was more prominent in proximal than in distal portions of the peripheral nerves. The nature and distribution of lesions in the nervous system in this condition indicate a neuronopathy/axonopathy. In Chapter 4.2 chronic *Aspergillus clavatus* intoxication in cattle fed sorghum beer residues is described. Lesions were characterised by extensive, bilaterally symmetrical Wallerian degeneration in ascending and descending tracts throughout the length of the spinal cord and focal neuronal degeneration in the spinal cord grey matter.

The question is why there is a selective vulnerability of specific populations of neurons, in this case motor neurons, to injury. Recent studies on the pathogenesis of motor neuron diseases in humans, especially amyotrophic lateral sclerosis (ALS), shed some light on factors that render neurons more vulnerable. Various potential contributors to disease have been proposed and are briefly discussed:

- **Damage to the cytoskeleton:** motor neurons are amongst the largest cells in the nervous system. They often have long axonal processes and those supplying the muscles of the distal lower limb in humans may be up to one metre in length [53]. Such neurons would not only have high-energy demands and a high metabolic rate, but also need a robust cytoskeleton with a high neurofilament content. Neurofilament proteins maintain cell shape and axonal calibre and play a significant role in axonal transport. Abnormal assembly and accumulation of neurofilaments in cell bodies and proximal axons in motor neurons in ALS are characteristic. It is not known however, whether accumulation of neurofilament protein occurs as a consequence of axonal transport blockage or whether the neurofilament abnormality directly causes...
impairment of axonal transport. Evidence to support the latter hypothesis has come from studies with transgenic mice: animals which overexpressed two of the three neurofilament subunit proteins of different weights (neurofilament [NF]-light and neurofilament [NF]-heavy) developed motor neuron disease as did mice which expressed mutations in one of the subunits of neurofilaments [39].

Several motor neuron diseases exhibiting neurofilament accumulation in neurons have been reported in domestic animals. These include spinal muscular atrophy in Brittany Spaniels [12] and Rottweilers [55] and motor neuron disease in Yorskshire [30] and Hampshire pigs [43] and in horned Hereford [51] and brown Swiss cattle [22]. The condition in Brittany Spaniels, which is inherited as an autosomal dominant trait, has been considered as a model for the study of ALS. Cork and co-workers [13] found inappropriate phosphorylation of neurofilaments in neuronal perikarya in six diseases associated with neurofilament accumulation, namely, spinal muscular atrophy in Brittany Spaniels, motor neuron disease in horned Hereford (‘Shaker’ calves), pigs and rabbits, ‘swayback’ in lambs, and myelopathy in zebras. The significance of this cytoskeletal abnormality remains unclear. Some neuron diseases in animals, however, do not display neurofilament accumulation and despite detailed studies the pathogenesis is still unclear in conditions such as progressive neurogenic muscular atrophy in Pointers [32] and spinal muscular atrophy in German Shepherds [15].

- **Excitotoxicity**: Glutamate is the principal excitatory neurotransmitter in the nervous system. Glutamate is normally released into the synaptic cleft where postsynaptic receptors are activated. The excitatory signal is terminated by removal of the neurotransmitter by transported proteins located on glial and neuronal cells. Excessive stimulation of glutamate receptors may induce derangement of intracellular calcium homeostasis and the production of free radicals – the term ‘excitotoxicity’ has been coined for this process - leading to cellular degeneration and necrosis [50].

  Why then are motor neurons selectively affected if the glutamate neurotransmitter system is widely distributed in the human central nervous system? An important finding is that in motor neurons which are vulnerable to degeneration, impairment of the excitatory amino acid transporter 2 (EAAT2) is evident which may lead to abnormally high levels of glutamate in extracellular and cerebrospinal fluid [50].

- **Oxidative stress**: Mutations in the gene encoding the enzyme Cu/Zn superoxide dismutase have been reported in patients suffering from motor neuron disease [39,54]. This enzyme catalyses the conversion of intracellular superoxide radicals, produced during normal cellular metabolism, to hydrogen peroxide, which is then eliminated by other free radical scavenging enzymes. The mutant enzyme may metabolise biochemical compounds such as hydrogen peroxide and peroxynitrate abnormally, resulting in increased formation of highly damaging hydroxyl radicals and the formation of nitrotyrosine residues.
on intracellular proteins. Cu/Zn superoxide dismutase is an ubiquitous enzyme but has high expression in motor neurons compared to other cells of the nervous system. Why then are motor neurons more susceptible to injury in motor neuron disease? It has been proposed that cells such as neurons in which this enzyme has a high level of expression are more vulnerable to genetic or post-translational alterations interfering with the function of these proteins [53,54].

- **Calcium binding proteins**: Calcium is essential for many normal cellular processes including neurotransmission and the regulation of intracellular second messenger systems. Intracellular regulation is maintained by a complex system of compartmentalisation and transportation involving binding to calcium buffering proteins, including parvalbumin and calbinding D28k. These buffering proteins play an important role in protecting neurons from calcium-mediated injury following for example activation of glutamate receptors [50,53]. Human motor neurons that are vulnerable in ALS do not express these calcium-binding proteins while less susceptible motor neuron groups do [50]. Deficiency of calcium binding proteins therefore may play an essential role in the pathogenesis of neuronal degeneration in motor neuron disease.

**Vacuolation of nervous tissue and cells in veterinary neuropathology**

A histological feature of all the conditions that are described in this thesis is the presence of vacuolation of varying severity in neurons and/or neuropil in the central nervous system of affected animals. In veterinary neuropathology, the diagnostic significance of spongy changes in the neuropil and intracytoplasmic neuronal vacuolation, irrespective of their cause, has been highlighted by the recent epidemics of spongiform encephalopathies in bovine, feline and exotic ungulates [60]. The initial diagnosis of these conditions often relies on histopathology, as was the case in the bovine spongiform encephalopathy (BSE) outbreak in the United Kingdom [61]. Histological examination of brain material of suspect cases is used as an essential method of surveillance for BSE [16].

Accurate interpretation of vacuolation in nervous tissue may be difficult since spongiform changes in the neuropil can be readily obscured by autolysis [21] and by disruption of tissues during routine processing [59]. It may even be caused by certain tissue processing procedures [62]. Perikaryal vacuolation, in contrast, is usually preserved even in the presence of tissue artefacts [59].

Other conditions in ruminants besides those reported in this thesis that may exhibit neuronal vacuolation. In cattle, neuronal vacuolation and spongiform changes of the white matter has occasionally been reported in rabies [24]. Idiopathic neuronal vacuolation was described in a 6-day-old calf [27]. The red nucleus and habenular nucleus in cattle may exhibit non-specific, perikaryonal vacuolation. These vacuoles are single or multiple and empty and are regarded as incidental findings in healthy cattle [25,42].

In a study on the differential diagnoses of BSE, non-specific vacuolation of the substantia nigra in 47/225 cattle brains was noted. Of these 16 had additional
lesions such as abscessation, non-suppurative meningoencephalitis or chronic hippocampal atrophy. In some cases the vacuolation extended rostrally to involve the thalamic radiation and the internal capsule, particularly the globus pallidus, or the cerebellar roof. The vacuoles were of different size and located at the junction of the grey and white matter, indicating myelin oedema [34]. In a subsequent report, white matter spongy changes resembling intramyelinic vacuolation was seen in 25.5% (129/506) symptomless 7-year-old cattle. The vacuolation was most often present in the substantia nigra and in 15.4% (78/506) the vacuolation was bilaterally symmetrical [25]. Idiopathic brainstem neuronal chromatolysis and hippocampal sclerosis in cattle is a differential diagnosis for A. clavatus neuromycotoxicosis. Non-specific white matter and neuronal vacuolation has also been described in sheep [31,63].

References


