MORPHOLOGIC AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF PRIMARY GRANULE CELL DEGENERATION OF JACK RUSSELL TERRIERS

by

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(Under the direction of Karen Paige Carmichael)

ABSTRACT

Cerebellar cortical degeneration with primary Purkinje cell degeneration is common in dogs. However, primary granule cell degeneration without the Purkinje cell loss is extremely rare and has only been reported in a few cases. The purpose of this study was to characterize a spontaneously occurring inherited neurologic disorder in Jack Russell Terriers. In this disorder, there is extensive granule cell degeneration without Purkinje cell loss. I intended to determine the type of cell death and examine the underlying causes of this cell death in the ataxic Jack Russell Terriers. Apoptosis is known to play an important role during the normal development of cerebellum and in some neurodegenerative disorder. However, apoptosis has not been investigated in dogs with cerebellar cortical degeneration. Using *in situ* end labeling and electron microscopy in a group of ataxic and non-ataxic dogs, I identified the means of cell death in granule cells as apoptosis. Two of the important mediators of apoptosis, Bax and Bcl-2, were investigated immunohistochemically. Bax was found to be overexpressed in granule cells in the ataxic dogs. There was no difference in the expression of Bcl-2 between the ataxic and non-ataxic dogs. Immunohistochemical examination for neurotrophins, nerve growth factor (NGF) and neurotrophin (NT)-3, and the common neurotrophin receptor $p75^{NTR}$ showed that the number of granule cells with increased $p75^{NTR}$-immunoreactivity in the internal granule cell layer increases in the ataxic dogs, while the number of nerve growth factor immunoreactive granule cells in the molecular layer decreases in these dogs. NT-3 expression does not show any difference between the ataxic and non-ataxic dogs. The overall results indicate that apoptosis is the mean of cell degeneration in the ataxic Jack Russell Terries, and this apoptosis is mediated by the action of pro-apoptotic molecule, Bax. A decrease in the NGF or overexpression of $p75^{NTR}$, which is also known to mediate apoptotic cell death in certain occasions, might be responsible for the initiation of granule cell death in the Jack Russell Terriers.

INDEX WORDS: Cerebellar cortical degeneration, Apoptosis, Bax, Bcl-2
Nerve Growth Factor, Neurotrophin-3, $p75^{NTR}$

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DEDICATION

This dissertation is dedicated to my wife, my family, friends, and my country, Republic of Turkey for supporting me throughout my doctoral program.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Significance

Inherited cerebellar defects have been reported in all domestic animals with varying frequency (Baird and Mackenzie, 1974; Cork et al., 1981; Jaggy and Vandevelde, 1988; Woodman et al., 1993; Aye et al., 1998; Willoughby and Kelly, 2002). Most defects can be grouped under two broad categories, cerebellar hypoplasia and cerebellar abiotrophy. Cerebellar hypoplasia can be the result of a variety of in-utero nervous system insults such as toxins and viral infections. Occasionally, the hypoplastic cerebellum may appear normal grossly, and the pathologic findings can only be recognized on microscopic examination. Loss of Purkinje cells and granule cells can be identified.

Cerebellar abiotrophy describes premature neuronal death of usually Purkinje cells and granule cells. More specifically, the term abiotrophy describes inherited degeneration or atrophy of cells that are formed normally but are unable to survive due to an intrinsic metabolic defect. The term cerebellar cortical degeneration has been used commonly to describe cerebellar abiotrophies. Cerebellar cortical degeneration has been reported in many canine breeds (Björk et al., 1957; de Lahunta and Averill, 1976; Nesbit and Ueckermann, 1981; Steinberg et al., 1981; Carmichael et al, 1983; Montgomery and Storts, 1983; Cummings and de Lahunta, 1988; Chieffo et al., 1994; Bildfell et al., 1995; Carmichael et al., 1996; Higgins et al., 1998). Clinically, these dogs have abnormal gaits characterized by truncal ataxia, forelimb, or hindlimb hypermetria and wide base stance.

Nystagmus and intention tremors have also been described commonly. Ataxia in these cases is usually progressive. The onset of the clinical signs varies from breed to breed and from few weeks to months. Histologically, the most consistent finding is cerebellar cortical cell loss with Purkinje cell degeneration and depletion followed by secondary granule cell degeneration and resultant thinning of internal granule cell layer (IGL). A few reports of primary granule cell degeneration with little or no involvement of Purkinje cells have been reported in Beagles, Brittany Spaniels, Rough Coated Collie and Border Collie dogs (Hartley et al., 1978; Tago et al., 1993; Tatalick et al., 1993; Sandy et al., 2002). Most of these have been single case reports with no attempt to determine etiology or pathogenesis.

Recently, a neurodegenerative disorder characterized by loss of granule cells in the IGL of the cerebellar cortex was reported in Jack Russell Terriers (JRT) (Carmichael et al., 2002). Affected puppies showed clinical signs of intention tremor, head bobbing, hypermetria, and oscillatory spontaneous nystagmus and truncal ataxia as early as two weeks of age. Histologically, thinning of cerebellar folia and severe depletion of granule cells in the IGL without Purkinje cell loss were recognized. Some Purkinje cells were vacuolated especially in the dorsal and caudal vermis. Many granule cells in the IGL had dense hyperchromatic nuclei.

In the cerebellum, as well in other parts of the central nervous system (CNS), normal neural cell development and survival require certain cell-to-cell interactions, gene expressions, and neuroprotective proteins such as neurotrophins. Normal development of cerebellum necessitates neural migration and maturation of progenitor cells. Programmed cell death, also known as apoptosis, plays an important role during this development. In

certain human neurodegenerative disorders and some murine mutants, apoptosis has been shown to be the cause of neuronal cell death (Chang et al., 1993; Dragunow et al., 1995; Migheli et al., 1995; Norman et al., 1995; Portera-Cailliau et al., 1995; Wüllner et al., 1995; Friedlander et al., 1997; Ohgoh et al., 2000). The cause of neuronal apoptosis in these cases has been proposed to involve excitotoxicity and/or depleted neurotrophin expression (Matsui et al., 1990; Ryo et al., 1993; Portera-Cailliau et al., 1995).

Most of the current information about neurotrophins and their receptors has been obtained from murine studies. Currently, there is little data on the potential affects of neurotrophins on the development, migration, and survival of cerebellar granule cells in other species. Using our unique animal model, I planed to determine the role of apoptosis in JRT with primary granule cell degeneration (PGCD) and the involvement of neurotrophins in the regulation of the apoptosis. Neurotrophins in certain human neurodegenerative diseases have been shown to have potential therapeutic applications (Sofroniew et al., 2001). This might be the case in JRT with PGCD as well as other neurological disorders in animals. The project will also add knowledge to the poorly described cerebellar development of dogs.

Background

Ataxia in animals

Neurologic dysfunctions in animals are being recognized with increasing frequency (Steinberg et al., 2000; Schild et al., 2001; van der Merwe and Lane, 2001; Barone et al., 2002; Willoughby and Kelly, 2002). In many of these cases, cerebellar involvement has been documented. The cerebellum, together with the basal ganglia, is the center for coordination of movements. Basically, it co-ordinates one movement with

the previous one. Therefore, any defect in cerebellum is seen as a dyscoordination in movements, called ataxia. Diverse defects in the cerebellum have been recorded as a cause of ataxia especially in young and neonatal animals of various species (Kortz et al., 1997; Aye et al., 1998; Mrissa et al., 2000). The defects include complete or partial agenesis, diffuse hypoplasia and cerebellar abiotrophy. Cerebellar agenesis is rare. It is usually due to unknown causes although there have been cases of cerebellar agenesis caused by in utero bovine diarrheal virus infection (personal communication to Carmichael). Cerebellar hypoplasia can be caused by in utero viral infections, toxins and idiopathic insults. Although the cerebellum can be reduced in size, often there is no gross change and the abnormality is only evident histologically.

The most common cerebellar defect is cerebellar abiotrophy, which is usually characterized by reduced size of the cerebellum with depletion of Purkinje cells and granule cells. In dogs, cerebellar cortical degeneration with occasional involvement of the spinal cord is common and has been recognized in a variety of breeds (Street et al., 1957; de Lahunta and Averill, 1976; Nesbit and Ueckermann JF, 1981; Steinberg et al., 1981; Carmichael et al, 1983; Montgomery and Storts, 1983; Cummings and de Lahunta, 1988; Yasuba et al., 1988; Cummings et al., 1991; Chieffo et al., 1994; Bildfell et al., 1995; Carmichael et al., 1996; Higgins et al., 1998). Degeneration and loss of Purkinje cells are the most common lesions present in cerebellar abiotrophy. Changes in the structure and number of these cells eventually lead to secondary depletion of granule cells. However, reports of granule cell degeneration with little or no involvement of Purkinje cells have been documented. Mild, non-progressive tottering starting at 5 months of age was reported in a Beagle (Tago et al., 1993). In this report, there was severe depletion of the

internal granule cell layer, while the Purkinje cells were normal in both number and distribution. Ultrastructurally, single membrane bound vacuoles were described in some of the Purkinje cells. Similar lesions of granule cell degeneration with no Purkinje cell loss or vacuolation were reported in a Brittany Spaniel dog with the onset of clinical signs at 2.5 years old (Tatalick et al., 1993). Clinically, this dog showed forelimb hypermetria, truncal ataxia and intention tremors. Depletion of granule cells followed by secondary Purkinje cell degeneration in the cerebellum, and Wallerian degeneration in the brain stem and spinal cord were described in a 6-week-old Rough Coated Collie puppy (Hartley et al., 1978). Clinically, this dog had intention tremors and was reported to fall frequently.

Primary granule cell degeneration in cerebellum was reported in JRT (Carmichael et al., 2002). Affected puppies start to show clinical signs of truncal ataxia, forelimb hypermetria, intention tremors and oscillatory spontaneous nystagmus as early as 2-3 weeks of age. Severe spasticity and dyscoordination especially in the pelvic limbs are recognized. The puppies have difficulty in standing and frequently fall to either side. Most of the clinical signs are progressive although it seems that with time the dogs learn to compensate. On gross examination, the only significant finding is reduced cerebellar size and weight (Figure 1.1). In a normal dog, the cerebellum weighs 10 to 12% of the total brain weight while in the ataxic JRT, this ratio is between 4 to 9% (Carmichael et al., 2002). Microscopically, there is severe depletion of the granule cells in the IGL and thinning of this layer is observed. There is no reduction in Purkinje cell number, however some show intracellular vacuolation especially those in the dorsal and caudal vermis.

Astrocytosis and astrogliosis occur in the IGL of younger puppies (Carmichael et al., 2002).

This ataxic disorder in JRT differs from previously described forms of ataxia in this breed. In one form, widespread Wallerian degeneration of the brain and spinal cord, together with focal symmetrical demyelination of the dorsalateral and ventromedial columns of the cord were recognized with onset of clinical signs at 2 to 6 months of age (Hartley and Palmer, 1973). In the other report, absence of the septum pellucidum, hypoplasia of the corpus callosum and marked bilateral hydrocephalus with neuroaxonal dystrophy in the brain stem and especially in the sensory nuclei were reported in a 9-week-old JRT (Sacre et al., 1993).

In humans, a rare disorder known as granule cell type cerebellar atrophy (Norman, 1940) and an ataxic form of Creutzfeldt-Jacob disease (Brownell and Oppenheimer, 1965) were described as causes of primary granule cell degeneration. In both cases, stellate bodies were often observed in the molecular layer. These bodies have been suggested to be spines of Purkinje cell dendrites, and formed as a result of degeneration of granule cells and the parallel fibers (Oyanagi, 1991).

The known causes of cerebellar degeneration in animals are very limited. Certain viral diseases such as hog cholera, feline panleukopenia, bovine viral diarrhea and Border disease are known to cause cerebellar hypoplasia in pigs, cats, cattle and sheep, respectively (Emerson and Delez, 1965; Kahrs et al., 1970; Kilham et al., 1971; Barlow, 1980). Canine herpes virus was also suggested to be responsible for cerebellar degeneration (Percy et al., 1971). In some of the viral infections, selective necrosis of the external granular layer was described (Sullivian, 1985).

Certain toxins have also been shown to cause cerebellar degeneration. Trichlorfon, an antiparasitic agent, is known to cause cerebellar degeneration in pigs during development (Knox et al., 1978). Selective degeneration of granule cells was reported with cycasin (Hirano et al., 1972) and organic mercury poisoning (Hunter and Russell, 1954). Experimental application of X-irradiation was also shown to cause depletion of granule cells (Altman and Anderson, 1972).

The etiology in most cases of cerebellar degeneration in dogs has never been investigated. However, most have been either proven or suspected to be hereditary with undetermined genetic defects. An autosomal recessive inheritance in most cases has been shown or proposed (Björk et al., 1957; Steinberg et al., 1981).

Several genetic murine mutants with cerebellar granule cell degeneration have also been established. These include the *weaver*, s*taggerer*, and *reeler* mice. The *weaver* mutant mouse has severe ataxia, fine tremors and occasional tonic clonic seizures (Rezai and Yoon, 1972; Rakic and Sidman, 1992). In *weaver* mice, massive granule cell loss in cerebellum, particularly in vermis, occurs prenatally. Most of the degenerating granule cells are located in the external granular layer (EGL) and EGL-molecular layer interface. In addition, partial Purkinje cell degeneration and dislocalization have been seen in *weaver* mutants (Smeyne and Goldowitz, 1989). It has been shown that a Gly to Ser mutation in the Girk2 gene is responsible for weaver mice (Patil et al., 1995). Girk2 gene encodes a G-protein-activated inwardly rectifying potassium channel. The mutation impairs the Na^+/K^+ homeostasis, and causes a marked depolarization (Tong et al., 1996).

The murine mutant, *staggerer*, is characterized by the degeneration of granule cells and the very delayed development of Purkinje cells (Herrup, 1983). Another murine

mutant, *reeler*, is characterized by reeling ataxic gait, tremor, and dystonic posture. Histologically, malposition of various neurons, including Purkinje cells and pyramidal cells and progressive decrease in the number of granule cells have been reported (Matsui et al., 1990).

Cerebellar degeneration with primarily Purkinje cell involvement was described in some murine mutants, such as *lurcher, leaner,* and *ax* (Lyon, 1955; Phillips, 1958; Herrup and Wilczynski, 1982). In the *leaner* mice, a mutation in the P/Q type $á_{1A}$ calcium channel gene has been reported (Fletcher et al., 1996). Expansion of a CAG repeat in the gene encoding the same channel was also reported to be the cause of late-onset dominantly inherited ataxic, spinocerebellar inherited ataxia type 6 in humans (Zhuchenko et al., 1997).

Histogenesis of cerebellum

During the second half of gestation, the cerebellum evolves from the rhombencephalic metencephalon. The external granular layer neuroepithelium, which originates from the germinal trigone, develops first and later gives rise to granule cells, basket cells and stellate cells. Within the EGL, two different zones, outer proliferative zone and inner differentiating or premigratory zone, can be seen. Cells in the outer zone are densely packed and have a roundish or cuboidal shape. In the inner zone, cells are located more loosely and have a roundish shape in the translobular plane but are spindle shaped in the parlobular plane. While most of the cells in the outer zone are mitotically active, only a few mitotic cells are located in the inner zone.

The majority of the progenitor cells in the EGL give rise to granule cells, which are the most abundant cell type in the cerebellum. It is still not known if every progenitor

cell has the capability to become granule cell or if there are selective progenitor cells that can develop into granule cells (Altman and Bayer, 1997).

Granule cells start to migrate prenatally from the EGL through molecular layer and down into the IGL where they reside for the rest of their life. Migration of these cells continues during early postnatal life. The age of cerebellar maturation varies from species to species and is the age at which granule cell migration is completed. The migration process is completed within the first couple of weeks after birth in murine models while in dogs it can continue until 8 to 9 weeks.

During migration, granule cells go through a series of changes both morphologically and physiologically. In the early stages of migration, they are bipolar in shape extruding two thin fibers in the parlobular plane. Later, as they descend into the molecular layer, these cells rotate about 90^{o} and continue to descend to the IGL. Once in the IGL, granule cells continue to their maturation by forming synaptic arborization with mossy fibers that originate from various precerebellar nuclei in the spinal cord, medulla and pons (Altman and Bayer, 1997).

Migration of granule cells is a complicated but regulated process. Certain cell-to-cell interactions, development and organization of Bergmann glial fibers, certain gene expression, and presence of adequate neurotrophins and their receptors are required to accomplish the ordered series of events for the migration (Kuhar et al., 1993; Gao et al, 1995; Lindholm et al., 1997).

Not all granule cell precursors in the EGL migrate. A proportion of these cells undergo apoptosis as a part of normal development (Segal et al., 1997). This apoptosis takes place in the EGL and partially in the superficial molecular layer.

Granule cells are the only excitatory cells in the cerebellar cortex, and axonal processes of these cells, known as parallel fibers, form synapses with Purkinje cells, basket cells, Golgi cells, and stellate cells. Because of the widespread web of communication and the regulatory functions of these cells, degeneration and dysfunction of granule cells lead to functional dyscoordination.

Apoptosis

The term apoptosis was first used by Kerr et al. (1972) to describe certain morphologic changes occurring during the normal turnover of hepatocytes. Programmed cell death is a broader term and might be correctly used to describe cell death occurring as a normal part of the life of nematode *Caenorhabditis elegans* (Yuan and Horvitz, 1990; Hengartner et al., 1992). Although the terms apoptosis and programmed cell death are commonly used interchangeably, different forms of programmed cell death have been described (Clarke, 1990; Sperandio et al., 2000).

Apoptosis differs from necrosis, which is non-programmed cell death (formerly 'oncosis'), by both microscopic and molecular changes. Necrotic cells are recognized by swelling and then lysis. Loss of membrane integrity, disintegration of cellular organelles and random degradation of DNA are the characteristic features of necrosis (Majno and Joris, 1995). This type of cell death causes significant inflammatory response and the necrotic cells are mainly phagocytosed by macrophages.

Apoptosis, in contrast, is characterized by the condensation of both the nucleus and the cytoplasm. In the nucleus, dense chromatin masses appear and increase in number, and the nucleus becomes pyknotic. The cytoplasmic membrane is convoluted and drawn deep into the cytoplasm and forms membrane blebbings. Cell membrane blebs

form apoptotic vesicles, which contain mostly unaffected cellular organelles. Subsequently, a loss of ribosomes from polysomes and rough endoplasmic reticulum is seen (Schweichel and Merker, 1973; Wyllie, 1981; Clarke, 1990; Majno and Joris, 1995). Apoptotic cells are usually phagocytosed by adjacent cells without causing an inflammatory response (Kerr et al., 1972).

One of the most characteristic features of apoptosis is the fragmentation of DNA into 180 to 200 bp oligonucleotides (Wyllie, 1980). In this type of fragmentation, DNA is broken at the linker region between histones on chromosomes. On agarose gels, DNA from apoptotic cells forms a classical laddered appearance, which is considered a hallmark of apoptosis. However, large DNA fragmentation of DNA to 50 to 300 kbp (Gromkowski et al., 1986; Oberhammer et al., 1993) and multiple single strand breaks (Peitsch et al., 1993) have also been described in apoptosis, and anti-ssDNA antibodies are now becoming a new tool for investigating apoptotic cells (Kumamoto and Ooya, 2001).

Membrane integrity is preserved in the apoptotic cells, however translocation of phosphatidylserine from the cytoplasmic to the outer side of the cell membrane takes place. Translocation of phosphatidylserine also occurs in necrosis, however membrane integrity is not preserved in necrotic cells. Annexin V can be used to detect translocated phosphotidylserine in conjunction with a dye exclusion test in apoptotic cells in culture (Koopman et al., 1994; Vermes et al., 1995).

The molecular basis of apoptosis has been investigated in detail; initial studies on apoptosis started in *C. elegans* (Ellis et al., 1991; Hengartner and Horvitz, 1994a). It has been shown that the gene products of *ced-3* and *ced-4* promote apoptosis while *ced-9*

inhibits apoptosis in this nematode. Ced-4 binds to Ced-3 and promotes its activation while Ced-9 binds to Ced-4 and prevents it from activating Ced-3. Caspase-9, Apaf-1, and Bcl-2 were shown to be the mammalian homologues of Ced-3, Ced-4, and Ced-9, respectively (Hengartner and Horvitz, 1994b).

Ced-3 is similar to the members of cysteine proteases, which also include human interleukin-1â-converting enzyme. Later a series of similar enzymes have been discovered (Cohen, 1997). All of the apoptotic enzymes have the amino acid cysteine in their active site and cleave their targets at specific aspartic acid residues, therefore are collectively called caspases.

Caspases are synthesized as inactive zymogens comprised of a large domain and a small domain. Activation of procaspases takes place by cleavage at an aspartic acid residue, usually by another caspase or self-cleavage (Thornberry and Lazebnik, 1998). Caspases can be grouped as initiator caspases and affecter caspases. For example, procaspase-8, an initiator caspase, is usually activated by binding of Fas-ligand to Fas, a transmembraneous cell surface receptor (Varfolomeev et al., 1998). Fas contains an intracytoplasmic death domain, which can bind to the similar death domains present on the apoptosis adaptor protein, FADD (Fas-associating death domain). This eventually leads to self-cleavage and activation of procaspase-8 via a process called 'induced proximity' (Ashkenazi and Dixit, 1998). Then, activated caspase-8 can cleave and activate other caspases such as caspase-3, -6, and –7, which are the affecter caspases.

Many targets of caspases have been discovered. Among them, PARP (Poly ADP ribose polymerase) and DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) are important DNA repair proteins, while U1-70kDa plays an important part in splicing of

precursor mRNA (Cryns and Yuan, 1998). A well-documented mechanism for iCAD (inhibitor of caspase activated DNase) has been established. In healthy cells, CAD (caspase activated DNase) is normally inactivated by iCAD (Enari et al., 1998). Cleavage of iCAD by caspases during apoptosis releases CAD, which causes the internucleosomal DNA cleavage. Some targets of caspases, such as lamin, a major structural protein of nuclear envelope, gelsolin, a mediator of actin filament formation, and fodrin (spectrin), a membrane associated cytoskeletal protein, clearly reveals the reason for changes in the cytoskeletal structure during apoptosis (Takahashi et al., 1996; Kothakota et al., 1997). Some other targets of caspases have been described as protein kinase C ä, *mdm2* proto-oncogene product, focal adhesion kinase (FAK), replication factor C and histone H1 (Cohen, 1997; Rudel and Bokoch, 1997; Wen et al., 1997).

The other important mediators of apoptosis are Bcl-2 family proteins (Merry and Korsmeyer, 1997; Adams and Cory, 1998; Chao and Korsmeyer SJ, 1998). These proteins share at least one of four conserved regions, called Bcl-2 homology domains (BH1-4). These domains are responsible for the dimerization and functions of these proteins. Most members of the Bcl-2 proteins contain the BH3, death domain. While some members of this family, such as Bcl-2 and Bcl-x_L (the only two members that have BH4 domains) are anti-apoptotic, other members, such as Bax, Bad, and Bid are pro-apoptotic (Chao and Korsmeyer, 1998). Currently known members of Bcl-2 superfamily are listed in Table 1.1.

The *bcl-2* gene encodes a 26-kDa intracellular membrane associated protein. Most of the Bcl-2 family of proteins are known to function on mitochondrial membranes. Bcl-2, as well as Bcl-x_L and Bax can form homodimers and heterodimers (Sattler et al., 1997)

as well as ion-conductive pores in artificial membranes (Minn et al., 1997). It has been suggested that during apoptosis such pores form and act as vehicles for the translocation of cytochrome c from mitochondria. Cytochrome c is a part of the apoptosome, which also includes procaspase-9 and Apaf-1. Cytochrome c binding to Apaf-1 leads to association of Apaf-1 and procaspase-9 through death domains. This association activates caspase-9, which then activates effector caspases. Several mechanisms have been established with involvement of molecules of the Bcl-2 family. For example, activated procaspase-8 cleaves the pro-apoptotic protein, Bid, and the truncated-Bid is translocated to the mitochondrial membrane where it forms heterodimers with Bax (Nijhawan et al., 2000). In another mechanism, Bad, another pro-apoptotic protein, is normally present in the phosphorylated form, which can bind to 14-3-3 proteins, a family of phosphoserine/phosphothreonine-binding molecules that control the function of a wide array of cellular proteins (Zha et al., 1996). However, dephosphorylation of Bad takes place when survival factors, such as IL-7, are withdrawn. Then the dephosphorylated Bad binds to Bcl-x_L inhibiting its function (Raff, 1998). It has been proposed that the relative proportions of anti- and pro-apoptotic molecules determine the extent to which apoptosis is suppressed or activated (Oltvai et al., 1993). The activity of Bcl-2 family members can also be regulated by an increase or decrease in the expression of the genes that encode them.

Role of apoptosis in health and sickness

Life requires death. All mammalian cells die either during the lifespan or at the end of the life of the creature in which they reside. Cell death is largely mediated by necrosis. Necrosis can be considered an accidental type of cell death. However, there are

certain types of cell death that are genetically programmed. Apoptosis plays an important part in eliminating those cells that are excessive or no longer needed. It mediates various morphogenetic changes such as separation of digits, elimination of the tail in tadpoles, and elimination of newly produced lymphocytes and neutrophils, and overall turnover of epithelial cells (Raff, 1992; Hensey and Gautier, 1999). Elimination of certain virally infected cells and cancer cells also involves apoptosis. In these cases apoptosis is certainly warranted.

In certain situations, unwanted apoptosis can take place. Apoptosis has been shown to play part in Acquired Immune Deficiency Syndrome (AIDS), toxin-induced liver disease, and ischemic injuries such as myocardial infarction, stroke and reperfusion injury (Mattson, 1997). It is also the mean of cell death in certain neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa and some forms of cerebellar degeneration (Dragunow et al., 1995; Portera-Cailliau et al., 1995).

During morphogenesis of cerebellum, precursor cells in the EGL are abundant, and most of these cells migrate to the IGL. However, some cells that do not migrate remain in the EGL where they are eliminated by mean of apoptosis. This type of cell death is also seen partially in the superficial molecular layer. A form of programmed cell death does take place in the IGL. In this form, very few dying cells are detectable (Wood et al., 1993; Muller et al., 1995). This type of cell death was described as autophagic degeneration and considered to be part of the final organization of the cerebellum.

Neurotrophins and their receptors

Survival of cells largely depends on factors and signals provided by neighboring cells. Therefore, cell-to-cell communication forms the basis of cell survival even after the nutritional requirements are met. In many cases, survival of some cells requires sacrifice of others due to presence of a limited amount of survival factors. In the nervous system, neurotrophic factors are largely responsible for many of these cell-to-cell interactions necessary for maintenance of neurons.

Neurotrophic factors are target derived trophic factors that play important roles in survival and maintenance of neurons. More specifically, they regulate cell fate decisions, axon growth, dendrite pruning, the patterning of innervation and the expression of proteins crucial for normal neuronal functioning, such as neurotransmitters and ion channels (Coffey et al., 1997; Mamounas et al., 2000; Huang and Reichardt, 2001; Sofroniew et al., 2001). In the mature nervous system, they control synaptic function and synaptic plasticity (Arvanov et al., 2000).

Among neurotrophic factors, neurotrophins are a select group of extracellular molecules that share similar sequence and structure. There are six known neurotrophins; nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5, NT-6 and NT-7 (the last two are only in fish). Neurotrophins mediate their special functions by binding two different classes of cell membrane bound receptors; Trk (Tropomyosin related kinase) family of receptor tyrosine kinases and $p75^{NTR}$. Three different Trk receptors, namely TrkA, TrkB, and TrkC, have been described (Glass and Yancopoulos, 1993). In general, NGF preferentially binds to TrkA, while BDNF and NT4/5 bind to TrkB, and NT-3 to TrkC. These receptors have been

erroneously called the high-affinity receptors whereas $p75^{NTR}$ has been named the low-affinity receptor of neurotrophins. It has been shown that neurotrophins bind both type of receptors with almost the same affinity (Lee et al., 2001). In addition, all neurotrophins have been proposed to have the capability to bind any of the Trk receptors because of the highly conserved sequences on these receptors. For example, it has been shown that NGF and NT-3 can bind TrkA. Similarly, BDNF, NT-3, and NT-4 can bind TrkB (Bibel et al., 1999).

Binding of a neurotrophin to its Trk receptor causes receptor tyrosine phosphorylation. Several tyrosines have been shown to be responsible for the activation of different pathways in cytoplasm (Loeb et al., 1994; Middlemas et al.,1994). Receptor tyrosine phosphorylation causes activation of adaptor proteins that triggers intracytoplasmic pathways, finally leading to activation of certain genes. Two such adaptor proteins, Shc and FRS-2/SNT (Fibroblast growth factor receptor subunit 2/Suc associated neurotrophic factor induced tyrosine phosphorylated target) have been described (Kouhara et al., 1997). It has been shown that Shc mediated pathways are largely responsible for the transient activation of the ERK family of MAP kinases while the FRS-2/SNT mediated pathway causes prolonged activation of these kinases.

Several pathways have been described in the Trk mediated control of apoptosis (Sofroniew et al., 2001). For example, NGF binding to TrkA causes activation of Shc. Then, Shc-mediated activation of secondary messengers, Grb2 and Sos, takes place. Sos is a Ras exchange factor and causes the activation of Ras, which then can activate phosphatidylinositol-3-kinase (PI3K). PI3K can also be directly activated by Grb2-mediated activation of Gab1 (Sofroniew et al., 2001). Activated PI3K can then

phosphorylate and activate Akt1/2. Phosphorylated Akt1/2 can phosphorylate Bad. Phosphorylated Bad binds to 14-3-3 and is sequestered in the cytoplasm (Masters et al., 2002). It is known that dephosphorylation of Bad frees it, triggering pro-apoptotic mechanisms on the mitochondrial membrane.

The other neurotrophin receptor, $p75^{NTR}$, does not have intrinsic tyrosine kinase activity, and is proposed to be a co-receptor for Trk receptor kinases. $p75^{NTR}$ is a member of the TNFR superfamily, and contains a death domain. Therefore, it has been proposed that this receptor might play a role in apoptotic cell death in neurons (Chao, 1994; Frade et al., 1996). However, no known apoptotic mediators that bind to its death domain have been discovered (Lee et al, 2001). Lately, several adaptor proteins that bind to $p75^{NTR}$ have been discovered. Among these, neurotrophin receptor interacting factor (NRIF) (Casademunt et al., 1999), neurotrophin receptor-interacting MAGE homolog (NRAGE) (Salehi et al., 2000), and $p75^{NTR}$-associated cell death executor (NADE) (Mukai et al., 2000) promote apoptosis, by yet an unknown mechanism, while tumor necrosis factor receptor-associated factors (TRAF) (Khursigara et al., 1999), Schwann cell factor-1 (SC-1) (Chittka and Chao, 1999), and Rophillin A (RhoA) (Yamashita et al., 1999) play a role in the non-apoptotic stimulation of neurons.

$p75^{NTR}$ is expressed in high levels especially during development and after CNS lesions. Expression of Trk receptors also changes with development. For example, early granule cells in the cerebellum predominantly express TrkB while more mature cells express TrkC (Tessarollo et al., 1993; Lindholm et al., 1997). It has been shown that proportional expression of TrkB to TrkC is 5:1 at 3 weeks of age while this ratio is 1:3 at the time EGL disappears in mice (Segal et al., 1995). Similar to the pattern of expression

of the receptors, neurotrophins are expressed differently during development. Different levels of expression of neurotrophins and their receptors suggest that they might regulate granule cell migration, survival, and development.

Expression of TrkC has been detected in Purkinje cells (Lamballe et al., 1991). NT-3 has also been determined in the Purkinje cells, however its mRNA expression has not been found in these cells, suggesting that Purkinje cells provide NT-3 from granule cells anterogradly (Ernfors et al., 1992; Zhou and Rush, 1994; Neveu and Arenas, 1996).

NGF was detected in the cerebellar granule cells, however expression of its receptor, TrkA was not determined in these cells (Ernfors et al., 1992; Muller et al., 1994). In the granule neurons, NGF can act in autocrine fashion. Expression of NGF has not been determined in Purkinje cells, but its receptor, TrkA, was also not expressed in these cells (Ernfors et al., 1992).

NT-4/5 expression has not been yet determined in either granule cells or Purkinje cells, however TrkB mRNA and protein expression has been detected in the both types of cells. (Merlio et al., 1992; Lindholm et al., 1993).

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FIGURE 1.1 Macroscopic comparison of cerebellum from a 6-week-old ataxic Jack Russell Terrier (JRT) to that of the same age non-ataxic JRT. Note the reduced cerebellar size in the ataxic dog.

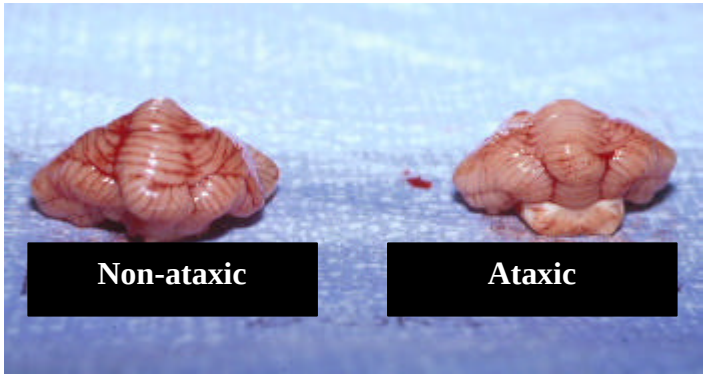

TABLE1.1 Currently known Bcl-2 superfamily members.

Bcl-2 superfamily	
Anti-apoptotic	**Pro-apoptotic**
Bcl-2	Bax
Bcl-x_L	Bak
Bcl-w	Bok
Mcl-1	Bik
A1	Blk
NR-13	Hrk
Boo	BNIP3
BHRF1	Bim_L
LMW5-HL	Bad
ORF16	Bid
KS-Bcl-2	EGL-1
E1B-19K	Bcl-x_S
Ced-9	DP5
	Bld

CHAPTER 2

PRIMARY GRANULE CELL DEGENERATION IN THE CEREBELLUM OF ATAXIC JACK RUSSELL TERRIERS[1]

[1] Özen H. and K.P. Carmichael. To be submitted to *J Comp Neurol*.

ABSTRACT

The objective of this study was to characterize the type of cerebellar granule cell death in Jack Russell Terriers (JRT) with primary granule cell degeneration (PGCD). To do so, we examined formalin-fixed paraffin embedded cerebellar sections from ataxic and non-ataxic dogs using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)-assay. We also examined granule cells from ataxic and non-ataxic dogs ultrastructurally. To determine the probable involvement of Purkinje cells in the process of granule cell degeneration, we studied, immunohistochemically, Purkinje cell morphology using Calbindin D-28K and the synaptic arborization using synaptophysin in cerebellar cortical neurons of the ataxic dogs and compared the findings to that of non-ataxic dogs. Numbers of TUNEL-stained cells in the ataxic dogs were higher than in non-ataxic dogs. Distribution of TUNEL-stained cells in different layers of cerebellum was determined and most of the dying granule cells were found in the internal granule cell layer (IGL). Electron microscopic examination of these cells showed increased condensation of both nucleus and cytoplasm. Cellular boundaries of these cells were preserved, and the cytoplasm contained many membrane-bound vesicular structures interpreted to be apoptotic bodies. Calbindin D-28K immunoreactivity was confined to Purkinje cells while synaptophysin immunoreactivity correlated with the normal synaptic anatomy of the cerebellum. In these cases, no differences between the ataxic JRT and non-ataxic dogs were observed. Collectively, these findings indicate that the type of cell death in the ataxic JRT is apoptosis, and most of the apoptotic cell death takes place in the IGL. They also suggest that Purkinje cells do not play an important role in the process of granule cell degeneration in PGCD.

Indexing terms: cerebellar abiotrophy; apoptosis; calbindin D-28; synaptophysin

Cerebellar cortical degeneration is commonly reported in dogs (Björk et al., 1957; de Lahunta and Averill, 1976; Nesbit and Ueckermann, 1981; Steinberg et al 1981; Carmichael et al., 1983; Montgomery and Storts, 1983; Cummings and de Lahunta, 1988; Chieffo et al., 1994; Bildfell et al., 1995; Carmichael et al., 1996; Higgins et al., 1998). Affected animals have progressive ataxia characterized by abnormal gait, forelimb and/or hindlimb hypermetria, intention tremors and occasional nystagmus. The onset of the clinical signs varies from case to case but in most cases first clinical signs are seen during the first few months of life. Microscopically, Purkinje cell degeneration and loss with secondary granule cell degeneration are the main findings. Only a few cases of granule cell degeneration without concurrent Purkinje cell loss have been described in dogs (Hartley et al., 1978; Tago et al., 1993; Tatalick et al., 1993). Recently, a cerebellar cortical degeneration with predominant granule cell loss has been reported in Jack Russell Terriers (JRT) (Carmichael et al., 2002). Ataxic puppies had progressive clinical signs of forelimb hypermetria, intension tremors and nystagmus beginning at 2 to 3 weeks of age. Microscopically, granule cells were severely degenerated and depleted, and many pyknotic cells were located in the internal granule cell layer (IGL) of the cerebellar cortex. Purkinje cells were not decreased in number, however Purkinje cell vacuolation was noted especially in the caudal and dorsal vermis. Granule cell degeneration is seen as early as 5 weeks of age in affected JRT. The IGL is markedly reduced when compared to age matched non-ataxic dogs and by 16 months of age, the IGL is almost completely devoid of granule cells.

In this study, we propose to determine the mechanism of granule cell death in ataxic JRT.

MATERIALS AND METHODS

TUNEL technique. Formalin-fixed paraffin-embedded cerebellar sections from 16 ataxic Jack Russell Terriers (JRT), aged between 5 weeks to 14 months, were used for the *in situ* apoptosis assay to reveal free 3'-OH ends of fragmented DNA, typically shown by apoptotic cells. Nine of these dogs were female, five were male, and two were unknown sex. For the control group, cerebella of three JRT and five dogs of various breeds, ranging from 4 weeks to 12 months old, with no known cerebellar disorders were used. All animals were treated in strict compliance with the policy of the National Institutes of Health on the care of humans and laboratory animals.

Following euthanasia, cerebella of the dogs were immediately immersion fixed in 10% neutral buffered formalin for no longer than 48 hours, and paraffin embedded. Five ìm sections of cerebella were cut and processed with ApopTag ® Plus In Situ Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, MD). For TUNEL staining, sections were rinsed in 0.1 M PBS, and placed in Oncor protein digesting enzyme for 15 minutes. Following rinses in distilled water, endogenous peroxidase activity was blocked by 3% H_2O_2 for 5 minutes. Sections were placed in equilibration buffer for 15 seconds and then incubated with reaction buffer containing digoxigenin-dUTP and terminal deoxynucleotidyl transferase (TdT), for 1 hour in a humidified chamber at 37^{o}C. To stop the reaction, the samples were incubated in stop/wash buffer at room temperature for 10 minutes. Sections were then rinsed in 0.1 M PBS and exposed to the undiluted solution of

anti-digoxigenin peroxidase-conjugated antibody for 30 minutes at room temperature. After rinsing with 0.1 M PBS, peroxidase activity in the samples was revealed with a solution of diaminobenzidine/H_2O_2 for 6 minutes. Finally, sections were rinsed in distilled water and counterstained with methyl green for 10 minutes. Following rinses in distilled water, sections were placed in three changes of xylene for 2 minutes, and coverslipped in Permount (Fisher). For positive control, sections of rat mammary glands obtained at the fourth day after weaning were used. Negative controls were provided by incubating the sections in water instead of TdT enzyme.

Stained cells were counted under a light microscope in 50 randomly selected fields at 40X magnification. All counted fields contained all of the cerebellar cortical layers. Numbers of TUNEL-reactive cells were determined in the external germinal layer (EGL), molecular layer and the internal granule cell layer (IGL).

Statistical analyses. Numbers of TUNEL-stained cells from the ataxic and non-ataxic dogs were analyzed using two-tailed Student's *t* test. Differences on the numbers of TUNEL-stained cells among the three cerebellar layers were compared using Kruskal Wallis test. P values < 0.05 were considered significant.

Electron microscopy. Cerebella of three ataxic JRT were used for the morphologic examination of granule cells at the electron microscopy level. Two of the puppies were 8 weeks old and one was 6 weeks old. For control, two 6-week-old JRT puppies with no known cerebellar disease were used. Freshly obtained cerebellar tissues were immediately immersion fixed in a solution composed of 2% glutaraldahyde, 2% paraformaldahayde, and 0.2% picric acid in 0.1 M cacodylate buffer overnight. The tissues were then rinsed in 0.1 M cacodylate buffer for 15 minutes and post fixed in 1%

OsO_4 prepared in 0.1 M cacodylate buffer for 1 hour. Following fixation, the tissues were rinsed with deionized water for 10 minutes three times and stained with 0.5% uranyl acetate for 1 hour. Thereafter, the samples were rinsed again and dehydrated in graded series of ethanol starting from 50% through absolute, and then placed in 100 % acetone and finally in 100 % propylene oxide. The tissues were embedded in Epon-Araldite and the polymerization of the embedding media was done at 75^{o}C oven for at least 24 hours. 50 nm sections were cut in a microtome (Reichert Ultracut S Ultramicrotome, Leica, Inc., Deerfield, IL) and placed on nickel grids. Finally, the sections were stained with 5% methanolic uranyl acetate and 10% lead citrate sequentially and observed under a JEM 1210 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at 80 Kv.

Immunohistochemistry. For the immunohistochemical detection of proteins of interest, a total of 15 ataxic dogs and 10 non-ataxic dogs were selected. Cerebella from five 6-week-old JRT, five 7-week-old JRT and five 8-week-old ataxic JRT were examined. In the control group, three 6-week-old, two 7-week-old, and five 8-week-old puppies were examined.

Freshly obtained tissues were immediately immersion fixed in 10% neutral buffer formalin for no longer than 48 hours, and paraffin embedded. Five ìm sections were cut and deparaffinized in three changes of Hemo De (Fisher Scientific, Pittsburgh, PA). The sections were rehydrated in reducing concentrations of ethanol from absolute to 70% then washed under running tap water. After rinsing the sections with 0.1 M phosphate-buffered saline containing 0.3% Triton-X-100 (PBS-T), pH 7.4, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 for 20 min. Non-specific antibody binding was prevented with 10% normal goat serum for 30 min at room

temperature. Thereafter, rabbit anti-Calbindin D-28K (Chemicon International Inc., Temecula, CA), and mouse anti-synaptophysin (Calbiochem, San Diego, CA) were applied to the sections in dilutions of 1:1000 and 1:500, respectively, and the sections were incubated overnight at 4^{o}C. Two sets of control slides were prepared and incubated with either normal serum from the animal that the primary antibody made in or PBS-T. Following the incubations, excess antibodies were washed off by rinsing the sections with PBS-T three times, 5 minutes each. For the detection of bound antibodies, the sections were incubated for 30 min in biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) for Calbindin D-28K, and in biotinylated anti-mouse IgM (Vector Laboratories Inc., Burlingame, CA) for synaptophysin. Both of the secondary antibodies were diluted at 1:200 with PBS-T. Following another 30 minutes of incubation in strepavidin-biotin complex (ABC, Vector) visualization of antibody binding was provided by a 2-5 minute reaction in 3,3'-diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA). The sections were then rinsed several times with PBS-T and counterstained with hemotoxylin for 2 minutes, washed under running tab water for 5 minutes, coverslipped with Permount (Fisher Scientific, Pittsburgh, PA) and observed under a light microscope.

RESULTS

TUNEL technique. Cell counts from the TUNEL study revealed that there were substantial differences in stained cells between the ataxic and non-ataxic dogs. The highest number of stained cells was found in 8-week-old ataxic puppies (Figure 2.1). There were an average of 94.3 TUNEL-stained cells in these dogs. Compared to 8 weeks

old dogs, the number of TUNEL-positive cells in 7 weeks old puppies was greatly decreased. These puppies had an average of 34.7 stained cells. Interestingly, there was great variation within the group of 5-week-old puppies with a count range of 8-54. Cell counts from the older dogs (4 months and older) showed less TUNEL-positive cells than the most of the younger dogs. The 4-month-old group had approximately less than 20 stained cells in all fields counted and the rest of the older dogs tested had virtually no TUNEL-stained cells.

In the ataxic JRT, stained cells were located in three cerebellar layers; EGL, molecular layer, and IGL. There were no stained cells in the Purkinje layer and the cerebellar white matter. At low magnification, it was evident that most of the stained cells were located within the IGL. However, stained cells were not preferentially located within any specific part of the IGL. Stained cells were present throughout all lobes of the cerebella with no apparent preference for either the vermis or the lateral hemispheres. In all of the sections tested, most of the stained cells were diffusely scattered although, rarely, they were present in small clusters of 2-3 cells. Again, these clusters were not preferentially located in any specific part of the cerebellum.

Morphologically, TUNEL stained cells in the IGL usually had Pyknotic nuclei (Figure 2.2). Occasionally, the nucleus was eccentrically located. In few cases, stained cells had no visible nucleus. Occasionally, some of the stained cells were similar in size and shape to normal granule cells. In the IGL, TUNEL-positive cells were predominantly similar in size and shape to non-stained granule cells suggesting that they were granule cells.

There was relatively less variation in the number of TUNEL-positive cells among non-ataxic dogs than ataxic ones, with the exception of 4 weeks old non-ataxic dog. This dog had canine distemper infection and majority of the stained cells were located in the white matter. Among the other non-ataxic dogs tested, the highest number of stained cells was seen in a 6-week-old puppy in which there were total of 13 stained cells. In other two puppies with the same age, each had only 1 stained cell. In the only 4-week-old puppy, there were 4 stained cells and the puppies aged 8 and 9 weeks had 3 and 6 stained cells, respectively. There were no stained cells in the 4-month-old non-ataxic dog. There was only 1 stained cell in the 12-month-old dog.

Total number of granule cells in the internal granule cell layer in the ataxic dogs were visibly less than in the non-ataxic dogs. Therefore, the ratio of the stained cells to total number of cells was smaller for ataxic JRT compared to non-ataxic dogs.

To determine where the majority of stained cells were located in ataxic dogs and non-ataxic dogs, cell counts from individual cerebellar cortical layers were performed. We calculated the percentage of the total cell count in each cerebellar cortical layer (Figure 2.3).

In the ataxic dogs (ages, 8 weeks to 4.5 months), the majority of stained cells (mean 92.84%) were located within the IGL. The percentages of the number of stained cells in the EGL and the molecular layer were significantly less (mean 2.14% and mean 5.02%, respectively) than that of IGL ($p < 0.05$).

In non-ataxic dogs of all ages, there were too few cells for meaningful percentage to be calculated. It was noted, however that the few TUNEL-positive cells were located in the IGL.

Electron microscopy. The morphologic features of cerebellar granule cells from non-ataxic dogs were similar to those described in the literature (Altman and Bayer, 1997). These cells were round to ovoid and ~6-8 ìm in diameter. Cytoplasmic membranes of the granule cells were smooth and in close contact with 2 or 3 other granule cells. They had a single, round or ovoid nucleus that was approximately 5-6 ìm in diameter. Nuclear chromatin was mostly located peripherally. There were usually one or few large heterochromatic foci. Cytoplasm of these cells was scant and seen as a narrow rim around the nucleus. Few small mitochondria with intact inner and outer membranes were recognizable within the cytoplasm (Figure 2.4A).

Affected or dying granule cells from the cerebellum of ataxic JRT were characterized mainly by condensation of nuclear chromatin (Figure 2.4B-C). There were many heterochromatic foci located within the nuclei of the affected granule cells. The number of these foci was markedly increased as compared to that of normal granule cells. A rim of heterochromatin was also present in the affected cells. Besides these heterochromatic foci, overall heterochromacity of the nuclei of these cells were greatly increased. The level of chromatin condensation varied among the affected granule cells. In some of these cells, the nuclei were diffusely heterochromatic while in others chromatin was clumped in dark globules. The nuclei of the granule cells from ataxic dogs were moderately to markedly shrunken and the shape of nuclei varied from ovoid to irregular. A few indentations of the nuclear membrane were recognizable in some of the affected cells.

The cytoplasm of affected granule cells was moderately to markedly condensed. Cytoplasmic membrane of the dying cells was intact. Many variable sized, vesicular-

membrane bound spaces were present within the cytoplasm of these cells. A few mitochondria were present in these cells. They were usually larger and more vesicular than those from non-ataxic dogs. Cisternal membranes of these mitochondria were usually deformed. In almost all of the affected granule cells, normal looking mitochondria were present in conjunction with the abnormal appearing mitochondria.

Calbindin D-28K immunohistochemistry. Calbindin immunoreactivity was strictly confined to Purkinje cells in both non-ataxic (Figure 2.5A) and ataxic dogs (Figure 2.5B). Perikarya and the dendritic processes of these cells were strongly immunoreactive for calbindin. In the IGL, immunoreactive segments of Purkinje cell axonal processes were visible. This staining was continued in the white matter reaching to the deep nuclei. Granule cells, Golgi cells, basket cells and stellate cells were clearly unreactive for calbindin.

In all of the sections tested, almost all of the Purkinje cells were recognized to be immunoreactice for calbindin. A few unstained cells did not show any regional preference, and seemed to be located randomly between the immunoreactive cells. Stained Purkinje cells always showed strong immunoreactivity, and there seemed no difference in the level of staining among the cells in the same sections. In the ataxic dogs, where there were Purkinje cells with vacuolated cytoplasm, there seemed no difference in the staining intensity between these cells and the other stained Purkinje cells. There was no difference between the ataxic and the non-ataxic dogs in the immunostaining intensity.

Synaptophysin immunohistochemistry. Staining distribution of anti-synaptophysin antigen was confined to the axons, dendrites and some of the Purkinje cells in both non-ataxic (Figure 2.6A) and ataxic dogs (Figure 2.6B). The molecular layer showed mostly

diffuse strong immunostaining. Purkinje cell membranes were also strongly labeled with the antibody. Although cell membranes at the soma of Purkinje cells had distinct staining, nuclei were clearly unresponsive to antibody. Primary and secondary dendrites of Purkinje cells also showed strong immunoreactivity.

In the internal granule cell layer, dendritic arborization of granule cells (mossy fiber rosette) in this layer had strong immunoreactivity to synaptophysin. Clumps of these stained dendrites were scattered throughout the IGL. However, granule cell soma did not show any staining. Synaptophysin immunoreactivity was not present on the Golgi cell membranes.

There was no synaptophysin immunoreactivity in the cerebellar white matter. However, neurons of the cerebellar deep nuclei showed profound cytoplasmic staining.

There was no difference in the staining pattern or staining intensity between ataxic and non-ataxic dogs.

DISCUSSION

Using two completely independent techniques, we have shown that the type of cell death in degenerating granule cells in the cerebellum of ataxic JRT is apoptosis. Apoptosis is known to be the main type of programmed cell death in many morphogenetic processes and various neurodegenerative disorders such as amyotrophic lateral sclerosis, ischemic stroke, Alzheimer's disease, Huntington disease, and Parkinson's disease (Dragunow et al., 1995; Portera-Cailliau et al., 1995; Friedlander et al., 1997; Mattson, 1997). Apoptosis is also extremely important in the normal development of cerebellum. During the histogenesis of the cerebellum, cells located in

the external granular layer (EGL) migrate and form the IGL. Some of the cells in the EGL fail to do so and are eliminated by apoptosis in the EGL. Some apoptotic cell death can also be seen in the superficial molecular layer.

In general, apoptotic cells are recognized as pyknotic in routine hemotoxylin and eosin (H&E) stained histologic sections (Janowsky and Finlay, 1983). However, distinguishing apoptosis from necrosis is not always possible in H&E sections and further investigation is usually necessary.

Apoptotic cells show certain morphologic and biochemical changes that distinguish them from necrotic cells. They are recognized mainly by the condensation of the nucleus and cytoplasm, and the presence of membrane bound vesicles while preserving the membrane integrity. One of the most pathognomic features of apoptosis is the fragmentation of DNA into 180-200 bp fragments. These DNA fragments can be detected by agarose gel electrophoresis where they form what is classically called the ‘apoptotic ladder’. Insertion of synthetic nucleotides to the 3’-OH end of the fragmented DNA allows for detection of these nucleotides by antibodies. Although DNA fragmentation is considered to be the hallmark of apoptosis, in certain situations, DNA fragmentation has not been detected even though there was apoptotic morphology. On the other hand, internucleosomal DNA fragmentation has been detected in rare necrotic cell deaths (Grasl-Kraupp et al., 1995). DNA fragmentation with non-apoptotic morphology in the pyramidal layer of the hippocampus of aged dogs has been described (Borràs et al., 1999). Therefore, TUNEL assay might not always detect apoptotic cells. Another limitation of TUNEL-assay is that since DNA fragmentation is an early event in apoptosis, the test may not detect cells in later phases of apoptotic cell death.

In the current study, the TUNEL assay detected many degenerating granule cells in the cerebellum of ataxic JRT. Cell counts on stained sections revealed that the numbers of stained cells were significantly higher in the ataxic dogs compared to the non-ataxic dogs. The highest number of TUNEL-stained cells were determined in 8 weeks old puppies, indicating that peak degenerative changes probably takes place at this age. There was moderate variability in the numbers of TUNEL-positive cells in all age groups. This is to be expected. In a given litter with more than one ataxic puppy, it has been recognized that siblings show clinical signs at slightly different ages and the severity of the clinical ataxia varied from one dog to the other. This variation possibly corresponds to the histologic variability of granule cell degeneration. Differences in the numbers of the TUNEL-stained cells, therefore might explain the variations in the severity of clinical signs seen in the ataxic JRT. The TUNEL assay did not detect any stained cells in ataxic dogs that were older than 6 months. Presence of virtually no stained cells in these cases probably indicates that cell degeneration in our study ceased by this age. However, the absence of ataxic dogs between 4.5 months and 6 months in this study limited our ability to more accurately characterize the progression of granule cell death in ataxic JRT.

Quantitative analysis of TUNEL-stained granule cells in cerebellum of ataxic JRT showed that most of the cell death in the ataxic JRT took place in the IGL. In lesser degrees, cell death occurs in the EGL and the molecular layer. The overall pattern of dying granule cells in the cerebellum of ataxic JRT clearly differs from the one seen during the development of cerebellum in which the most cell death occurs in the EGL.

Presence of TUNEL-stained cells in EGL, molecular layer, and IGL of cerebellum of ataxic JRT suggests that the timing of cell death is not a function of cell

migration. During the normal development of the cerebellum, the earliest migrating cells are found in the deeper (near the white matter) layers, while later migrating cells are located more superficially (near Purkinje cell layer). Most degenerating cells were located diffusely in the IGL in the ataxic JRT. Presence of degenerating cells in all other layers clearly indicates that initiation of cell death is not dependent on early or late cellular activities.

One interesting finding from the TUNEL-assay was the rare detection of stained cells in the non-ataxic dogs. These cells were seen to be distributed in all of the cerebellar cortical layers though with higher frequency in the IGL. Presence of these cells in non-ataxic dogs is important since it is generally assumed that apoptotic cell death in cerebellum of healthy individuals only takes place in the EGL during the development. However, our study shows that this type of cell death can also occur in the IGL of the cerebellum. Again the number of these cells is few and are sparsely scattered throughout the IGL. Programmed cell death in the IGL of cerebellum has been previously reported (Wodd et al., 1993; Muller et al., 1995). In these reports the number of dying cells were shown to be very low. However, the type of programmed cell death in these reports was thought not to be apoptosis due to inability to demonstrate *in situ* end labeled positive cells. Due to the absence of DNA fragmentation, the type of cell death in these cases was called ‘apoptosis-like’. Programmed cell death in the IGL after the granule cells complete their migration was proposed to take place to balance the final number of granule cells in this layer.

One other unexpected finding was the determination of substantial number of TUNEL-stained cells in a non-ataxic dog. However, in this case the stained cells were

located in the white matter. It was reported that the dog had concurrent canine distemper. It is possible that the positive cells were either dying inflammatory cells or infected astrocytic glia.

Another important finding from the TUNEL study was that the assay was partially limited in detecting some of the presumably apoptotic cells. In H&E sections from our cases, we recognized many pyknotic cells (data not shown). In general, apoptotic cells appear pyknotic in H&E sections. It was evident that in a given case the number of TUNEL-positive cells was less than that of pyknotic cells. The reason for not detecting all the apoptotic cells might be explained by the occurrence of DNA fragmentation in the early stages of apoptosis. Therefore TUNEL assay cannot detect all dying cells with different stages of apoptosis. Besides, apoptosis is usually a fast process and the apoptotic cells are eliminated quickly by the neighboring cells. Therefore, in a given time the number of TUNEL stained cells would be less than the total number of apoptotic cells. Our results clearly show that results from TUNEL assay solely might not reflect the real level of cell death in a given system, and suggest that the pyknotic cell count from H&E sections might be included to show all the dying cells. Pyknotic cell count, for example, was used to show the presence of apoptotic cells in the EGL of cerebellum during the development (Janowsky and Finlay, 1983). However, pyknosis solely cannot be used as a criterion to show apoptotic cell death. Pyknosis was shown to take place in two different types of programmed cell death, apoptosis and autophagic cell death (Clarke, 1990). Recently the term paraptosis was suggested for autophagic cell death. Pyknosis always occurs in apoptosis, whereas it is seen in only some cases of autophagic cell death. Apoptosis can be differentiated from autophagic cell death by the presence of DNA

fragmentation or the absence of autophagic vacuoles. Pyknosis can also be sen in necrosis.

Occasionally, TUNEL-stained cells do not show apoptotic morphology. For example, TUNEL-positive cells were reported in the brain of aged dogs (Borràs et al., 2000). However, the morphology of cell death was described not to be apoptosis in this report, and called ‘apoptosis-like’ cell death. However, they suggested that classical apoptosis might also take place in brain during aging.

Apoptosis can be most definitively determined by electron microscopic examination. Visualization of membrane bound vesicles called ‘apoptotic bodies’ is the ultimate way to detect apoptotic cells. Ultrastructural examination also helps differentiating apoptosis from other programmed cell death types. Although electron microscopy is the most powerful tool in identification of apoptotic cells, it is restricted to the examination of single or few cells. It cannot be used to show the degree of cell loss in a given system.

Electron microscopic examination of degenerating granule cells was conducted to conclusively define the type of cell degeneration in cerebellum of ataxic JRT. Our findings were similar to those previously described cases of apoptosis in the literature (Kerr et al., 1972; Wyllie, 1980; Clarke, 1990).

Classically all types of cell deaths are mediated by either non-programmed cell death or programmed cell death. Non-programmed cell death is responsible for all of the non-physiological cell deaths and commonly known as necrosis. A detailed yet insufficient classification has been described for programmed cell death (Clarke, 1990). In this classification three major morphological types namely apoptotic, autophagic, and

non-lysosomal vesiculate were described. Among these apoptosis is known to be most common and most studied one. It has been shown that during the normal development of cerebellum several types of programmed cell death could be seen, and most of these are due to apoptosis (Wood et al., 1993). Our results correlate with these findings.

Apoptotic cell death also plays an important role in the degeneration of granule cells in the cerebellum of certain murine mutants namely *weaver*, *lurcher*, and *ax* (Migheli et al., 1995; Norman et al., 1995, Wüllner et al., 1995; Ohgoh et al., 2000). Primary granule cell degeneration with limited Purkinje cell involvement has been described in these mice (Smeyne and Goldowitz, 1989), however the granule cell loss occurs predominantly in the EGL. In these mutants, certain events that might interfere with the migration of granule cells have been proposed to be the cause in the ataxic mice. In ataxic JRT, degenerating granule cells are found predominantly in the IGL and this suggests that cell degeneration occurs independently of other migratory requirements. However, presence of some stained cells in the EGL and molecular layer suggests that migratory elements could play some role in the process of cell death.

In most of the cerebellar cortical degenerations in dogs and some murine mutants, such as *lurcher*, granule cell degeneration and depletion were described following the primary degeneration of Purkinje cells (Phillips, 1958) . It is well established that the survival of a cell depends on the signals provided by the neighboring cells. In the central nervous system, neurons form a complex network via their processes. Granule cells, which are the most abundant type of cells in cerebellum, make synapses with every other type of neurons in the cortex of this organ. Therefore, degeneration or malfunction of one type of cell in the cerebellar cortex might influence the survival and function of granule

cells. For example, in many cases of cerebellar cortical degeneration in dogs of various breeds, primary Purkinje cell death followed by granule cell degeneration has been described (Björk et al., 1957; de Lahunta and Averill, 1976; Nesbit and Ueckermann, 1981; Cummings and de Lahunta, 1988; Chieffo et al., 1994; Bildfell et al., 1995; Higgins et al., 1998). In these cases, loss of synaptic communication between the degenerating Purkinje cells and the granule cells might explain the depletion of granule cells. In the current study, utilizing markers for Purkinje cells and a synaptic protein, we tried to determine potential affect of Purkinje cells as well as other neurons on the granule cell death in the ataxic JRT. Immunohistochemical staining for both markers showed similar patterns between the ataxic and the non-ataxic dogs. Therefore, we would conclude, at least at the morphological level, that Purkinje cells are not involved in the process of cell degeneration of granule cells in the ataxic JRT. However, we cannot rule out that any changes in the expression of several survival factors, such as neurotrophins, provided by other cells might influence the cell death in the ataxic JRT.

In conclusion, we have shown that cell death in granule cells of ataxic JRT is due to apoptosis. The cause of this apoptosis however has not been determined, but it is unrelated to abnormal Purkinje cell structure and is likely caused by lack of an essential trophic factor necessary for granule cell survival.

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FIGURE 2.1 Distribution of TUNEL-positive cells in cerebella of ataxic Jack Russell Terriers (JRT) and non-ataxic dogs. Cerebellar sections from 16 ataxic JRT and 8 non-ataxic dogs were processed for *in situ* nick end-labeling for the detection of apoptotic cells. Total numbers of cells were determined for each case in randomly chosen 50 fields. Each age represents a single dog.

FIGURE 2.1

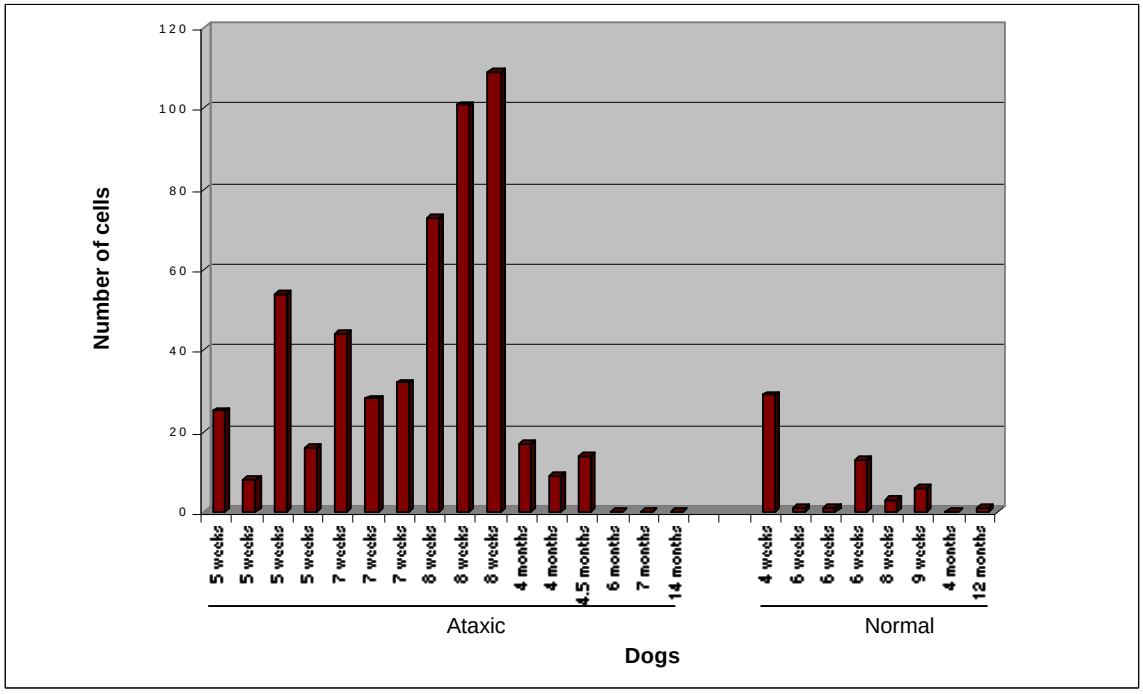

Number of cells
120
100
80
60
40
20
0
5 weeks
5 weeks
5 weeks
5 weeks
7 weeks
7 weeks
7 weeks
8 weeks
8 weeks
8 weeks
4 months
4 months
4.5 months
6 months
7 months
14 months
4 weeks
6 weeks
6 weeks
6 weeks
8 weeks
9 weeks
4 months
12 months
Ataxic
Normal
Dogs

FIGURE 2.2 Detection of TUNEL-positive cells in cerebellum of ataxic JRT.

Cerebellar sections were processed for TUNEL assay to detect apoptotic cells. Stained cells were mostly detected in the internal granule cell layer (arrows). Scale Bar = 50 ìm.

FIGURE 2.2

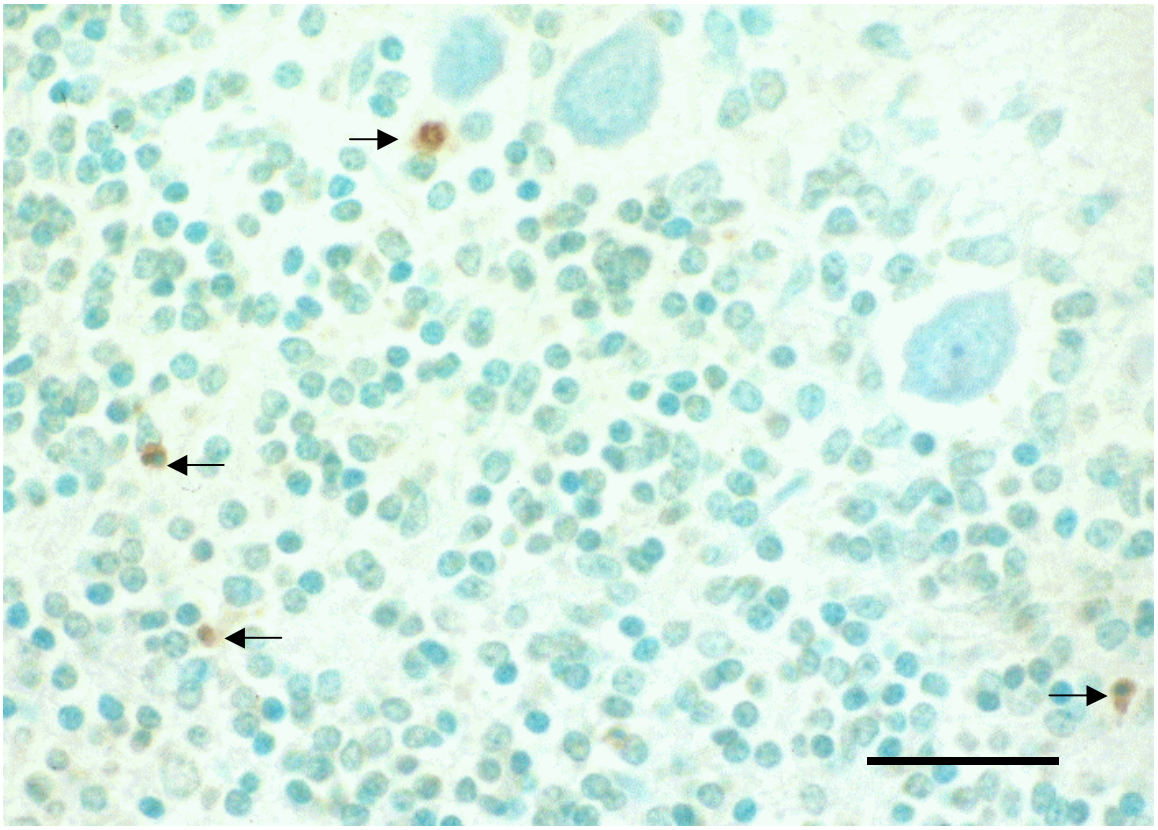


FIGURE 2.3 Distribution of TUNEL-reactive cells within the layers of cerebella of ataxic JRT. Stained cells from the TUNEL assay were counted in each layer of cerebellar sections from ataxic JRT. Distribution of total stained cells within the cerebellar layers was determined. Each age represents an ataxic JRT. $P < 0.05$. EGL, external granular layer; Mol. L., molecular layer; IGL, internal granule cell layer.

FIGURE 2.3

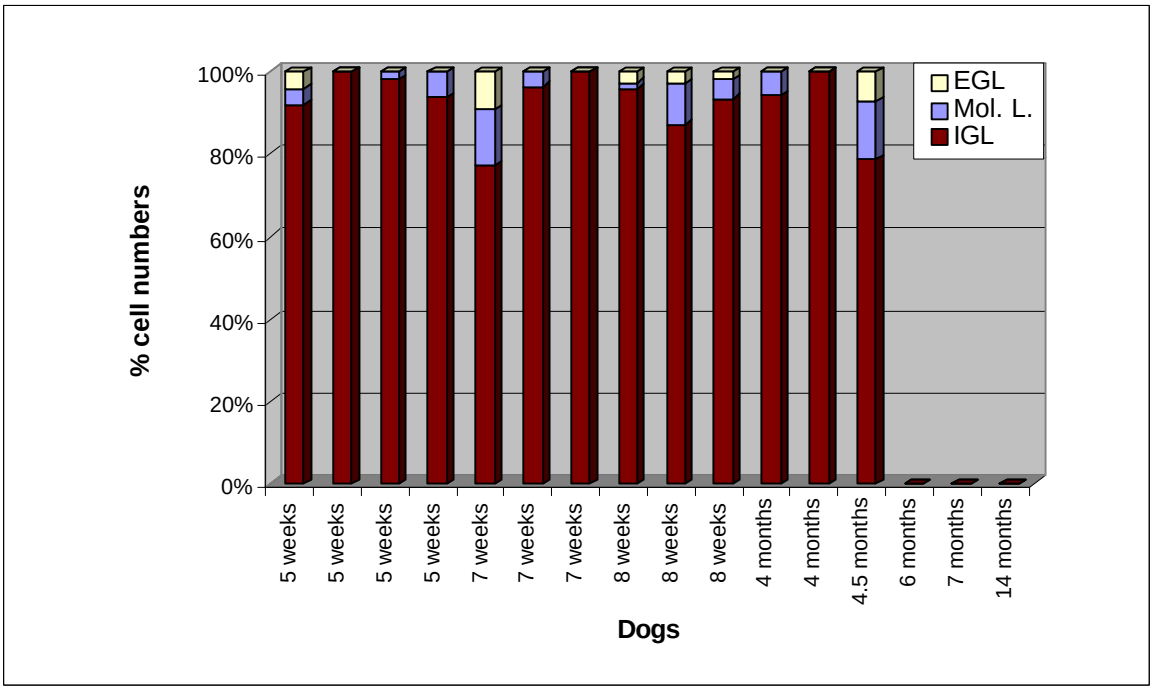

EGL
Mol. L.
IGL
100%
80%
60%
40%
20%
0%
% cell numbers
5 weeks
5 weeks
5 weeks
5 weeks
7 weeks
7 weeks
7 weeks
8 weeks
8 weeks
8 weeks
4 months
4 months
4.5 months
6 months
7 months
14 months
Dogs

FIGURE 2.4 Ultrastructural visualization of healthy granule cells (A) and comparison to dying granule cells (B and C) in ataxic JRT. Freshly obtained cerebella from ataxic and non-ataxic dogs were processed routinely to observe granule cells, ultrastructurally. In the ataxic JRT, dying granule cells had convolution of the cell cytoplasm and condensation of the nucleus and cytoplasm. Healthy granule cells had a round to slightly ovoid nucleus surrounded by a thin layer of cytoplasm containing few mitochondria. Mitochondria from normal and degenerating granule cells were presented in the insets. Bar scales; Figure 2.4A = 3 ìm, Figure 2.4B = 1.5 ìm, and Figure 2.4C = 4 ìm.

FIGURE 2.4A

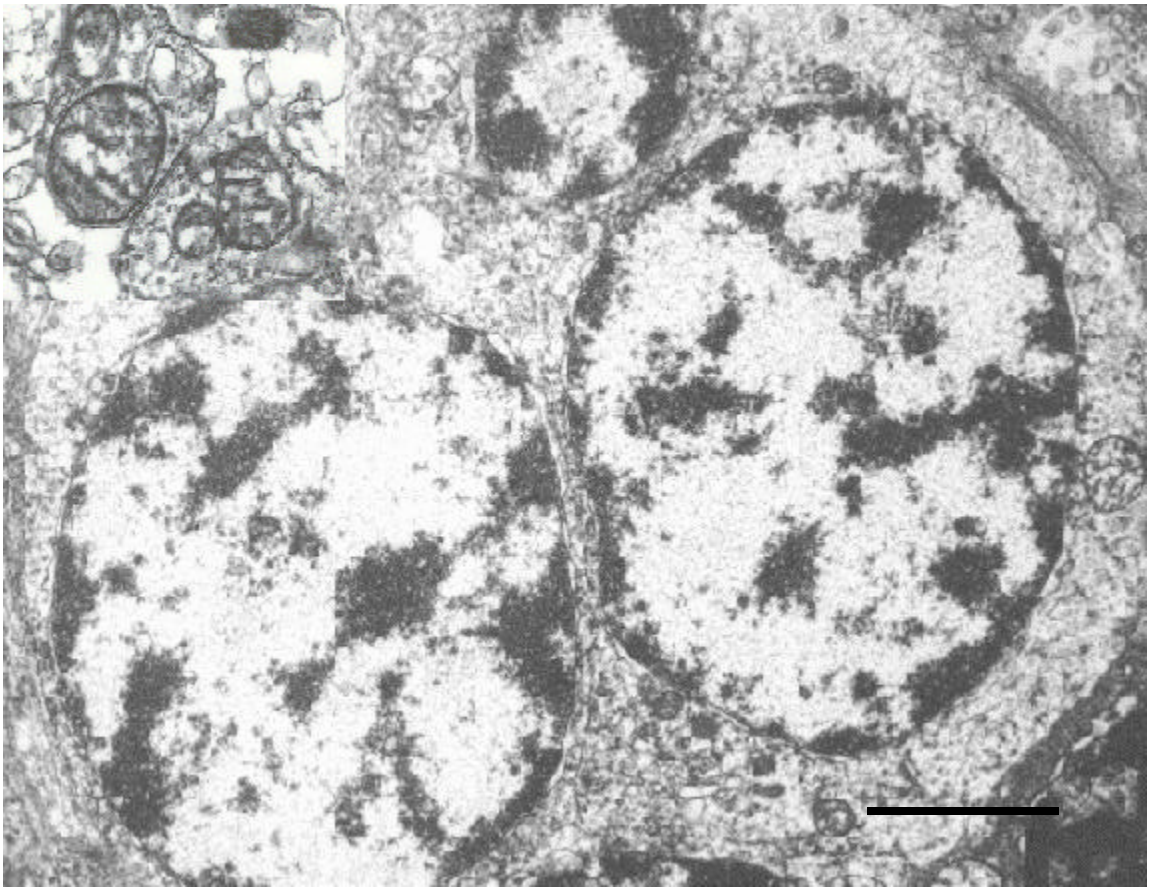


FIGURE 2.4B

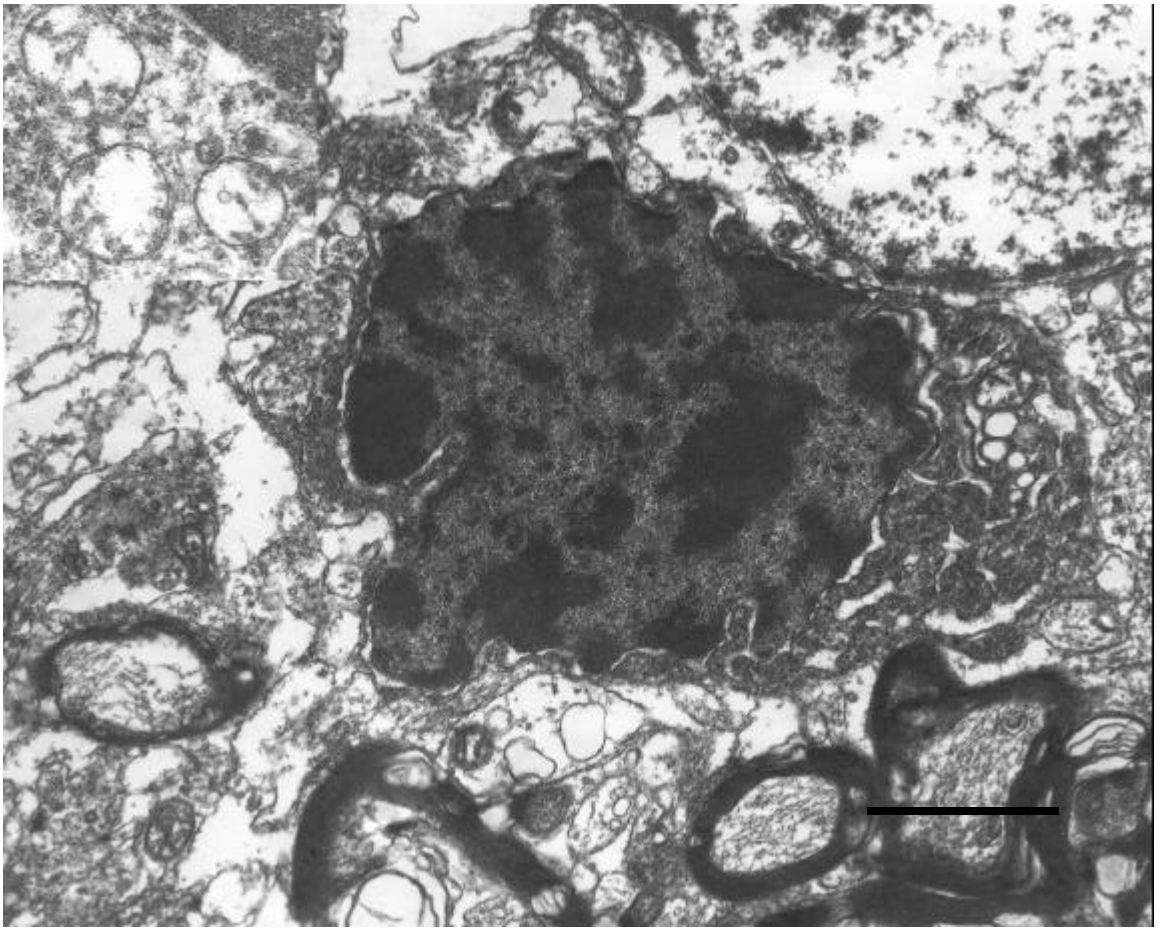


FIGURE 2.4C

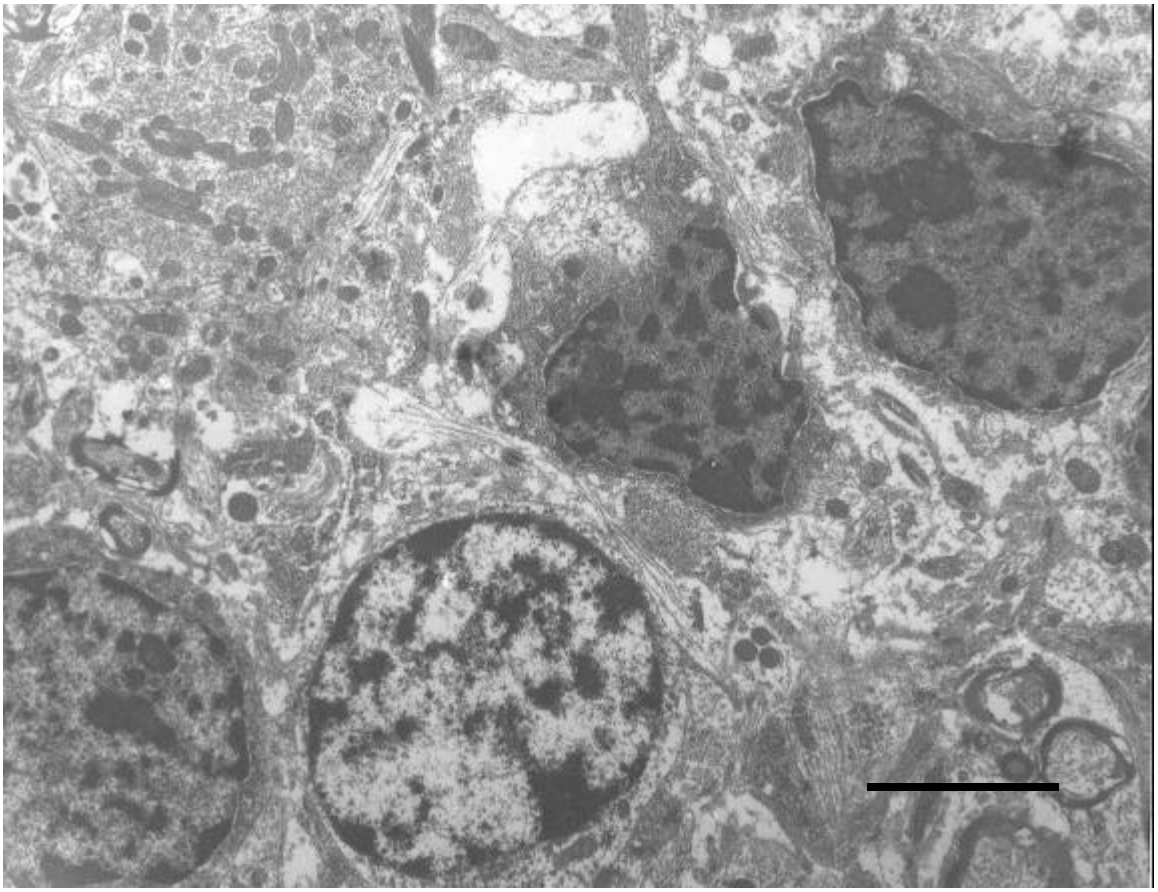


FIGURE 2.5 Immunohistochemical localization of Calbindin D-28K in cerebellar sections of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections of ataxic and non-ataxic dogs were immunolabeled for Calbindin D-28K using avidin-biotin-peroxidase method and counterstained with Hemotoxylin. Calbindin D-28K reactivity was strictly confined to Purkinje cell perikarya and dendrites. Sections of cerebella from ataxic dogs were immunostained similar to that of non-ataxic dogs. Scale bars = 200 ìm.

FIGURE 2.5A

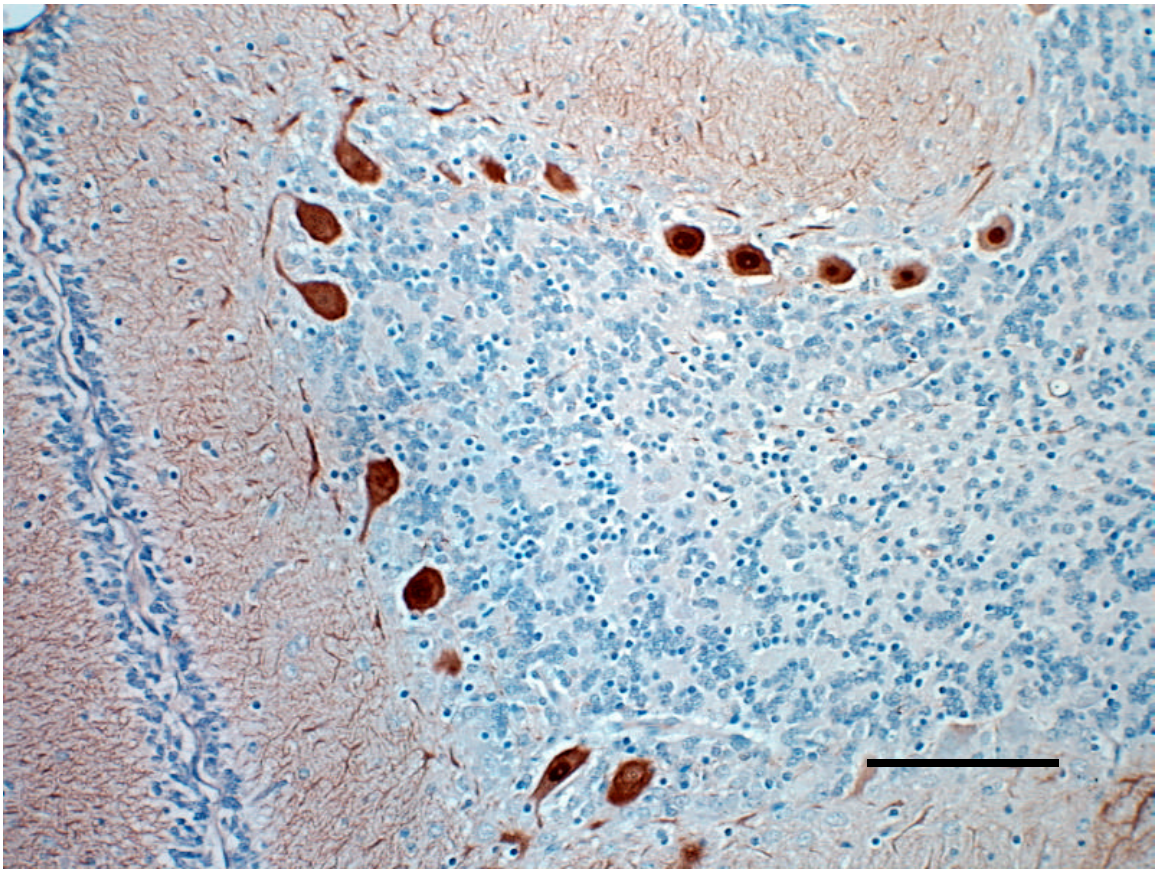


FIGURE 2.5B

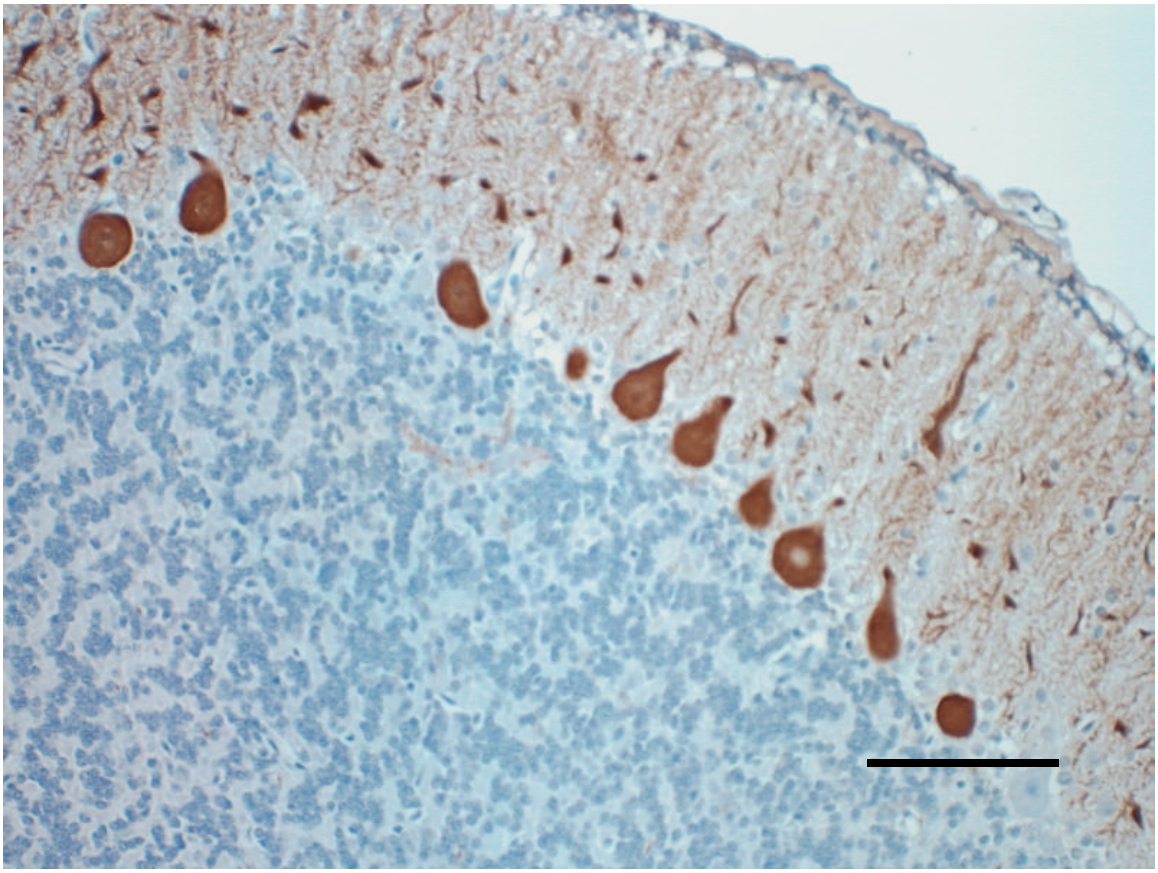


FIGURE 2.6 Immunohistochemical localization of synaptopyhsin in cerebellar sections of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections were immunolabeled for synaptophysin using avidin-biotin peroxidase method and counterstained with hemotoxylin. The staining was located diffusely in the molecular layer and on the dendritic arborization of granule cells in the internal granule cell layer. The staining pattern of synaptophysin in cerebellar sections of ataxic JRT was similar to that of non-ataxic dogs. Scale bars = 200 ìm.

FIGURE 2.6A

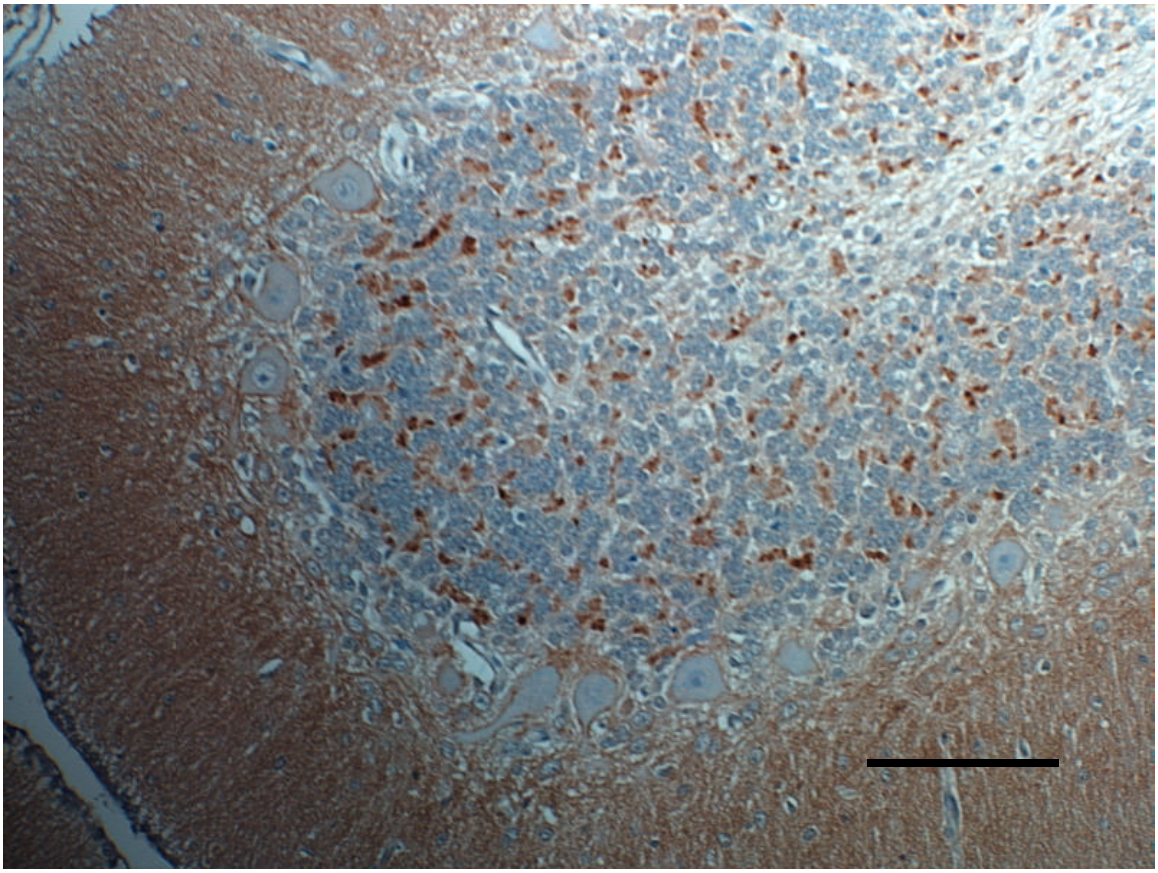
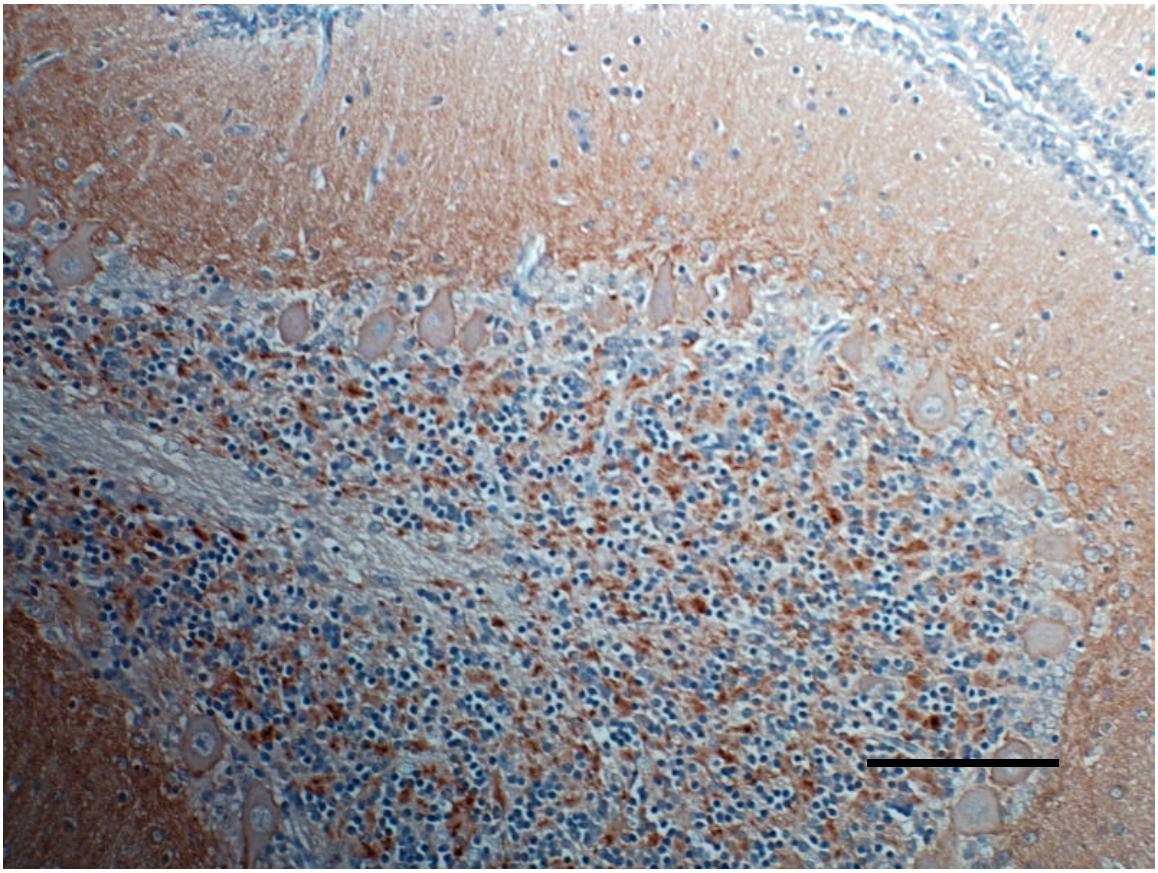


FIGURE 2.6B



CHAPTER 3

BAX OVEREXPRESSION IS ASSOCIATED WITH APOPTOTIC CELL DEATH IN PRIMARY CEREBELLAR GRANULE CELL DEGENERATION OF JACK RUSSELL TERRIERS[1]

Özen H. and K.P. Carmichael. To be submitted to *J Comp Neurol*.

ABSTRACT

In the current study, we investigated the immunohistochemical localization of two apoptotic mediators, Bcl-2 and Bax, in cerebella of ataxic Jack Russell Terriers (JRT) with primary granule cell degeneration and non-ataxic dogs. Cerebella of a group of ataxic dogs and age-matched non-ataxic dogs were immunohistochemically labeled for Bax and Bcl-2. Immunoreactive cells were counted and compared between the ataxic and non-ataxic dogs. Bcl-2-stained cells were detected throughout all layers of the cerebellum. No difference between the ataxic and non-ataxic dogs was observed. Bax-stained cells were largely detected in the internal granule cell layer of ataxic JRT, while only few Bax-immunostained cells were determined in the non-ataxic dogs. These results demonstrate that Bax mediated apoptotic pathways play a role in the cell death of granule cells in the ataxic JRT and Bcl-2 probably does not protect cerebellar granule cells against cell death in the ataxic JRT.

Indexing terms: cerebellar abiotrophy; apoptosis; Bax; Bcl-2

A neurodegenerative disorder characterized by granule cell loss in cerebellum has been described in Jack Russell Terriers (JRT) (Carmichael 2002). We have previously showed that the granule cell death in the cerebellum of ataxic JRT was due to apoptosis. Apoptotic cell death has been studied exclusively in the literature, and its role during normal development and in certain disorders has been described (Steller, 1995; Hengartner, 1998; Raff, 1998; Thompson, 1998). In particular, apoptosis has been shown to play an essential role in certain neurodegenerative disorders, such as Alzheimer's

disease, Huntington disease, and amyotrophic lateral sclerosis (Dragunov et al., 1995; Thompson, 1995; Barinaga, 1998; Nijhawan et al., 2000).

Apoptotic cell death is mediated by the actions of certain cysteine proteases that are collectively known as caspases (Cohen, 1997; Thornberry and Lazebnik, 1998). The actions of caspases are mainly regulated by the members of Bcl-2 family of proteins. In mammals, the anti-apoptotic molecules of this family include Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 whereas the pro-apoptotic members are Bax, Bad, Bak, Bid, Bim, Bok, Bid, and Bik. Most of the Bcl-2 family of proteins are located on the outer mitochondrial membranes, and to a lesser extent on the endoplasmic and nuclear membranes (Monaghan et al., 1992). Some members of the Bcl-2 family of proteins, such as Bax and Bad, are also located in the cytoplasm and during the initiation of apoptosis they are translocated onto the mitochondrial membranes where they are thought to interact with other members of the Bcl-2 family proteins. Such evidence came from the observation that Bcl-x_L and Bax can form homodimers and heterodimers (Sattler et al., 1997). It was also shown that these molecules could form ion-conductive pores in artificial membranes (Minn et al., 1997). Such pores were proposed to form during apoptosis and cause the release of cytochrome c, which then activates caspases.

Actions of Bcl-2 and Bax are known to be regulated by different mechanisms. Phosphorylation and dephosphorylation of the proteins, and changes in the *bcl-2* and *bax* gene expressions are some of the ways apoptosis is regulated (Raff, 1998). For example, overexpression of Bax accelerates apoptosis upon cytokine deprivation and this protein is redistributed from the cytoplasm to the mitochondrial membrane (Pastorino et al., 1998). Bax can also form heterodimers with Bid, and upon activation of the latter molecule, both

redistribute to the mitochondrial membrane (Nijhawan et al., 2000). Bax can also directly inhibit Bcl-2 by binding to it. Therefore, it was proposed that relative abundance of anti-apoptotic molecules to pro-apoptotic ones determine the state of which is activated or suppressed.

In this study, we intend to investigate the immunohistochemical localization of two of the important mediators of apoptosis, Bax and Bcl-2, in the cerebellar cortex of ataxic JRT. The results were quantitatively compared to that of non-ataxic dogs.

MATERIALS AND METHODS

Immunohistochemistry. For the immunohistochemical detection of proteins of interest, a total of 15 ataxic dogs and 10 non-ataxic dogs were selected. Cerebella from five 6-week-old JRT, five 7-week-old JRT and five 8-week-old JRT were examined. In the control group, three of the puppies were 6-week-old, two were 7-week-old, and the rest were 8-week-old.

Freshly obtained tissues were immediately immersion fixed in 10% neutral buffer formalin for no longer than 48 hours, and paraffin embedded. Five ìm sections were cut and deparaffinized in three changes of Hemo De (Fisher Scientific, Pittsburgh, CA). The sections were rehydrated in reducing concentrations of ethanol from absolute to 70% then washed under running tap water. After rinsing the sections with 0.1 M phosphate-buffered saline containing 0.3% Triton-X-100 (PBS-T), pH 7.4, antigen retrieval was performed. Antigen retrieval for Bax was provided by placing the sections in 0.01 M citrate buffer, pH 6.0, for 15 min at 100^{o}C in a water bath. Antigen retrieval for Bcl-2 was done by incubating the sections with 1X Trypsin/EDTA (Sigma) at 37^{o}C for 15 min.

After rinsing the sections with PBS-T, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 for 20 min. Non-specific antibody binding was prevented with 10% normal goat serum for 30 min at room temperature. Thereafter, primary antibodies, mouse anti-Bax (Zymed Laboratories Inc., South San Francisco, CA) and mouse anti-Bcl-2 (Sigma, St. Louis, MO) were applied to the sections in dilutions of 1:150 and 1:50, respectively, and the sections were incubated overnight at 4^{o}C. Two sets of control slides were prepared and incubated with either normal mouse serum or PBS-T. Following the incubations, excess antibodies were washed off by rinsing the sections with PBS-T three times, 5 minutes each. For the detection of bound antibodies, the sections were incubated for 30 min in biotinylated anti-mouse IgM diluted at 1:200 with PBS-T (Vector Laboratories Inc., Burlingame, CA). Following another 30 minutes of incubation in strepavidin-biotin complex (ABC, Vector Laboratories Inc., Burlingame, CA) visualization of antibody binding was provided by a 2-5 minute reaction in 3,3'-diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA). The sections were then rinsed several times with PBS-T and counterstained with hemotoxylin for 2 minutes, washed under running tab water for 5 minutes, coverslipped with Permount (Fisher Scientific, Pittsburgh, PA), and observed under a light microscope.

Statistical analyses. In sections that were labeled for Bax and Bcl-2, the number of stained cells was counted under a light microscope at 40X magnification from 50 randomly chosen fields in each case. In each counted field, all of the cortical layers were included. The total number of stained cells was analyzed in the ataxic and non-ataxic dogs using two-tailed Students' *t* test. Comparison of three different age groups within

either the ataxic or non-ataxic dogs were done using Kruskal Wallis test. In all of the analyses, P values < 0.05 were considered significant.

RESULTS

Bcl-2 Immunohistochemistry. In the ataxic JRT, immunolabeling with Bcl-2 revealed that most stained cells were located in the deep molecular layer and in the Purkinje cell layer-IGL interface (Figure 3.1A-B). Due to the presence of more densely stained cells in the molecular layer, all cells located in the 'interface layer' were counted within the border of molecular layer. Besides this division, the molecular layer was further divided into two equal layers, superficial and deep molecular layers, for the simplicity of describing the staining pattern.

Within the molecular layer there were heavily stained cells throughout the sections. However, there were relatively more stained cells in the deep molecular layer and most of the stained cells were in close contact with one another especially in the interface layer where they comprised a layer of 3-4 cell-thickness. Almost all of the cells located in the interface layer were labeled with Bcl-2. There was an obvious gradual increase in the number of stained cells from the more superficial to deeper portions of the molecular layer. This increase was due to the gradual increase in the total number of cells within the each zone, not due to increased immunoreactivity of cells. Throughout the molecular layer, the stained cells showed morphologic differences. Most of the stained cells in the superficial molecular layer were ovoid, with a perpendicular orientation. Most of these cells were slightly smaller than those in the deeper molecular layers. Some cells were similar in size to those in the deeper molecular layer but were ovoid not round. Cells located in the middle of the superficial and deep molecular layers were mostly

round to slightly ovoid and bigger than granule cells located in the IGL. Cells in the deep molecular layer and the interface layer were more variable in shape and size. The majority of these cells were round and larger than both those in the more superficial layers and the granule cells in the IGL. In and around the Purkinje cell layer, basket cells were clearly stained.

Cells located in the deep molecular layer had ovoid nuclei, which was surrounded by a thin layer of cytoplasm. At both sites of the cellular perikarya the bases of the cellular processes were clearly visible, but not stained with Bcl-2. In deeper zones, cells showed a slightly larger cytoplasm with the presence of an eccentric nucleus located at the IGL side. Cells in the interface layer had round nucleus surrounded by thin cytoplasm. Close association of these cells made it difficult to differentiate their process, however no evident staining on these processes was recognized. In all of the sections, cells in the molecular layer had more vesicular nuclei than granule cells of IGL.

Some, but not all of the Purkinje cells were stained with Bcl-2. There was no consistency in the staining of Purkinje cells among different lobes and part of lobes. In many cases stained and unstained cells were located side by side.

In the IGL, all of the granule cells were stained with Bcl-2. However, the staining density with the antibody was clearly less than that described for the molecular layer. Staining of cytoplasm was clearly different than those in the molecular layer, where the staining in the cytoplasm was diffuse. In the granule cells of IGL, there was a patchy staining over the cytoplasm with some areas more intensely stained than others. In both ataxic and non-ataxic dogs, there was no apparent difference between the granule cells in terms of staining pattern and the density of labeling. Since, all of the granule cells were

stained, no cell counting was performed. Golgi cells and premigratory cells of EGL were stained heavily with Bcl-2.

There were a few cells that were consistently non-immunoreactive in the molecular layer. These cells had small round nuclei and no visible cytoplasm. They were mostly located in the mid- and superficial molecular layer. Based on shape, size and the double immunolabeling with glial fibrillary acidic protein (data not shown) and Bcl-2, these cells were strong candidates for astrocytic glial cells. Similar cells with no staining were also present in the IGL.

In the non-ataxic dogs, Bcl-2 immunoreactivity in the cerebellar cortex was similar to that of ataxic JRT both in distribution and the overall staining pattern.

Since most of the heavily stained cells were located in the molecular layer, the number of stained cells was counted only in this layer both in the ataxic and non-ataxic dogs. Mean numbers of stained cells in ataxic and non-ataxic dogs were compared from each age group (Figure 3.2). In the molecular layer, mean numbers of Bcl-2 immunoreactive cells were 2633.6, 2344.6, and 2809.4 for ataxic puppies of 6, 7, and 8 weeks, respectively. In 6, 7, and 8-week-old non-ataxic dogs, means were 2549.5, 2871, and 2278.3, respectively. There was no statistical difference between the ataxic and non-ataxic dogs in the number of total stained cells in the molecular layer ($p<0.05$). There was also no statistical difference between age groups within the affected and non-ataxic dogs ($p<0.05$).

Bax immunohistochemistry. In cerebellar sections of the ataxic JRT, the majority of Bax-immunoreactive cells were located in the IGL, while only a few were in the molecular layer (Figure 3.3A). Within the molecular layer, the stained cells were mostly

located in the deep molecular layer. Occasional stained cells were also present in the middle portion of the molecular layer.

In the IGL, Bax-immunoreactivity was seen only in the cytoplasm of granule cells. Bax-reactive cells were observed in all portions of the IGL, however it seemed that there were more stained cells in the superficial and middle portions of the IGL than that of deeper portions.

Most of the immunoreactive cells had round, heterochromatic nuclei, which were surrounded by a thin layer of cytoplasm, which was completely or partially stained. Some of the stained cells had irregularly shaped and more pyknotic nuclei than the others. However, many stained cells resembled ‘healthy’ granule cells in terms of nuclear heterochromacity and size.

Basket cells and Golgi cells were not stained with Bax. While most of the Purkinje cells did not show immunoreactivity to Bax, some were stained intensely. In those dogs in which where the EGL were present, premigratory cells were mostly unreactive with Bax.

In the non-ataxic dogs, Bax-reactivity of the granule cells in all cerebellar cortical layers was virtually absent. There were only very few detectable Bax-stained cells. Most of these stained cells were located within the IGL while a few were in the molecular layer (Figure 3.3B). Because of the scarcity of stained cells, it was not possible to estimate the preferential localization of these cells within the IGL.

Bax-immunoreactivity in Purkinje cells and premigratory cells in cerebellar cortex of non-ataxic dogs was similar to those of the ataxic JRT.

In order to compare Bax-immunoreactivity in ataxic and non-ataxic puppies, the numbers of stained cells were counted both in the molecular layer and IGL, separately. In both ataxic and non-ataxic dogs, mean numbers of stained cells were calculated. In the IGL, there were 158.2, 141.8, and 117.6 immunoreactive cells in the ataxic dogs of 6, 7, and 8 weeks of age, respectively (Figure 3.4). The numbers of stained cells in the 6, 7 and 8-week-old non-ataxic dogs within the IGL were 4.7, 5, and 5.8, respectively. Statistically, the numbers of Bax-reactive cells in the IGL for ataxic puppies were significantly higher than that of non-ataxic dogs ($p<0.05$). There seemed to be a reduction in Bax-immunoreactivity among the ataxic dogs as age increased. However, this decrease was not significantly important ($p<0.05$). On the other hand, the p value in the comparison of 6 and 8 weeks-old ataxic dogs was 0.051, indicating that the decrease in the Bax-reactive cells within the IGL layer among ataxic puppies might be noteworthy. In the non-ataxic dogs, there were no significant differences among the age groups tested ($p<0.05$).

The mean numbers of Bax-reactive cells in the molecular layer of 6-, 7-, and 8-week-old ataxic dogs were 6, 4.6, and 5.4, respectively. These numbers for non-ataxic dogs were 0.3, 0.5, and 0, respectively. Therefore, the numbers of Bax-reactive cells in the molecular layer were significantly higher for ataxic dogs than that of non-ataxic dogs ($p<0.05$). There were also no statistical differences between the three age groups tested among both ataxic and non-ataxic dogs ($p<0.05$).

Although the overall numbers were low, this study clearly indicates that the number of Bax-reactive cells was significantly higher in the ataxic JRT than that of non-ataxic dogs (Figure 3.4).

DISCUSSION

We have shown that Bcl-2 immunoreactivity was present throughout the cells of cerebellum in dogs. Premigratory or precursor cells located in the EGL were shown to be highly immunoreactive and immunoreactivity was still present in the migrating granule cells. Granule cells of the IGL were also immunostained for Bcl-2, though the staining intensity in these cells were slightly less but largely uniform throughout the granule cells. In this study, the cell counts from the molecular layer and the overall staining characteristics for Bcl-2 did not show any difference between the ataxic JRT and the non-ataxic dogs. Immunoreactivity to Bax, however revealed differences between the ataxic and non-ataxic dogs. In the Bax-reactive cell counts, we detected more stained cells in the cerebella of ataxic JRT than those of the non-ataxic dogs. Most of the immunoreactive cells were localized within the IGL though few were also present in the molecular layer. Using TUNEL assay, we have previously shown that the majority of the apoptotic cells in the ataxic JRT were located in the IGL. These results correlate with the current findings indicating that Bax-mediated pathways might play an important role during the apoptotic cell death of granule cells in the ataxic JRT. In the non-ataxic dogs, the presence of occasional Bax-immunoreactive cells was correlated with the presence of scattered TUNEL-stained cells throughout the layers of cerebellum, but likewise was more frequent in the IGL.

It has been reported that during normal development of the cerebellum in mice Bax immunoreactivity in the cells of the EGL is high, and as the granule cells become more mature, Bax expression decreases (Vekrellis et al., 1997). The same report also showed the presence of Bax immunoreactivity in the Purkinje cells. These results were

partially confirmed by our study. We showed the presence of Bax immunoreactivity in the cytoplasm of Purkinje cells, though in most Purkinje cells the immunostaining was faint. On the other hand, Bax-immunoreactivity of premigratory cells in the EGL was mostly absent in both the ataxic and non-ataxic dogs. This finding indicates that there may be differences in Bax expression in the murine and canine cerebellum.

Our findings from the Bcl-2 study were similar to previous studies. It has been shown that Bcl-2 is highly expressed in the postmitotic differentiating cells of postnatal developing cerebellum of mice (Merry et al., 1994). We were also able to show high Bcl-2 immunoreactivity in the premigratory cells of the EGL. The same report also indicated that the immunoreactivity was higher in the inner differentiating zone of the EGL. Presence of only two or three layers of cells in the EGL of cerebella in the dogs in this study prevented us from identifying such a pattern. In the previous report, it was also shown that Bcl-2 activity was present in the granule cells located in the IGL, though with decreases in the expression. We also showed the presence of Bcl-2 immunoreactivity in these cells, however a slight decrease in the expression of Bcl-2 was noted. Our findings also correlate with the previous report on the subject of differential immunolabeling on the Purkinje cells.

Many reports describing the role of Bcl-2 in protecting neurons from apoptotic cell death have been published. The first evidence of such a role for Bcl-2 came from Garcia et al (1992). They showed that nerve growth factor-deprived sympathetic neurons could be rescued from apoptotic cell death by Bcl-2. A neuroprotective effect was also demonstrated for the cerebellar granule cells. It was shown that Bcl-2 is underexpressed in the apoptotic cells during the physiological elimination of these cells during migration

(Muller et al., 1997). Many reports with similar anti-apoptotic functions of Bcl-2 have been described (Batistatou et al., 1993; Mah et al., 1993; Farlie et al., 1995; Kane et al., 1995; Piñón et al., 1997). However, it was recognized that Bcl-2 was not effective in protecting apoptosis caused by certain agents (Allsopp et al., 1993, Behl et al., 1993) and it became evident that Bcl-2-dependent and Bcl-2-independent pathways existed.

Bax and Bcl-2 are two of the important apoptosis regulating proteins. While Bax possesses pro-apoptotic properties, Bcl-2 acts as an anti-apoptotic agent. It has been previously hypothesized that Bax acts on the release of cytochrome c from mitochondria via binding to BH1, BH2, and BH3 domains of Bcl-2, hence inhibiting its function (Adams and Cory, 1998). However, in certain situations it was determined that Bax and Bcl-2 might function independently. For example, it was shown that partial removal of BH domains from the *bcl-2* gene does not prevent Bax from mediating apoptosis (Simonian et. al., 1996). Similarly, mutant Bax protein with inability to bind Bcl-2 could still induce apoptosis (Zha and Reed, 1997). *bax*- and *bcl-2*- knockout mice show different phenotypes (Knudson and Korsmeyer, 1997). In *bcl-2* knockout mice, abnormalities are seen in kidneys, which have fewer nephrons. These mice also develop hypopigmentation in the hair follicules. *bax* knockout mice appear healty, however the male mice are infertile.

Presence of increased number of granule cells with Bax overexpression in face of normal expression of Bcl-2 in these cells suggests that survival of cerebellar granule cells in dogs is Bcl-2-independent. This might corralate with the previous hypothesis that relative abundance of anti-apoptotic and pro-apoptotic molecules determines if a cell survive or die (Oltvai et al., 1993). Since increased Bax expression relative to unchanged

Bcl-2 expression was detected in the ataxic JRT, the apoptotic cell death might be initiated by the action of Bax. It has been shown that some members of anti-apoptotic and pro-apoptotic members of Bcl-2 family of proteins form homodimers and heterodimers (Sattler et al., 1997). In the presence of abundant free Bax in the cytoplasm, these molecules would form homodimers and be redistributed to mitochondrial membranes. Here, as it has been suggested before, they might form channels from which cytochrome c could escape into the cytoplasm (Minn et al., 1997). Then, cytochrome c could bind Apaf-1, therefore activating caspase 9 and a cascade of other caspases that eventually would kill the cell. Formation of ion-conductive channels disturbs mitochondrial homeostasis. Changes seen in mitochondria, such as swelling and destruction of the membrane integrity, during the course of apoptosis have been shown previously (Kerr et al., 1972). Similar changes have also been determined by us in a previous study in the ataxic JRT providing additional support for the apoptotic cell death in granule cells of JRT being due to Bax mediated changes in the mitochondrial membranes.

Although not statistically significant, the number of granule cells with increased Bax-immunoreactivity in the ataxic JRT seemed to have a tendency to decrease with increasing age. Such a decrease correlates with the finding in our previous study that the number of *in situ* apoptosis test-positive granule cell numbers decrease as the age increases.

In this study, we showed that the apoptotic cell death of granule cells in the JRT is associated with Bax expression. However, the underlying factor that causes the overexpression of Bax still remains to be solved. There are various factors that are crucial for the survival and the maintenance of neurons. The best-known survival factors in the

nervous system are the neurotrophic factors. These molecules are synthesized in a cell and then act usually on another type of cell by receptor-mediated pathway. Since these factors are target-derived molecules limited amounts of neurotrophic factors are present in a given neural environment. Therefore, any reduction in the production of these factors results in the use of limited amount of trophic factor by a limited number of cells, leaving others without the supply of trophic input. Therefore, our future studies will aim to determine the expression level of neurotrophic factors and their receptors.

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FIGURE 3.1 Immunohistochemical localization of Bcl-2 in cerebella of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections were immunolabeled for Bcl-2 using avidin-biotin-peroxidase method with following hemotoxylin counterstain. Immunostained cells were located throughout the layers of cerebella both in the ataxic and non-ataxic dogs. Intensely stained cells were recognizable especially in the deep molecular layer. Granule cells in the internal granule cell layer and premigratory cells of external germinal layer were also stained. Scale bars = 200 ìm.

FIGURE 3.1A

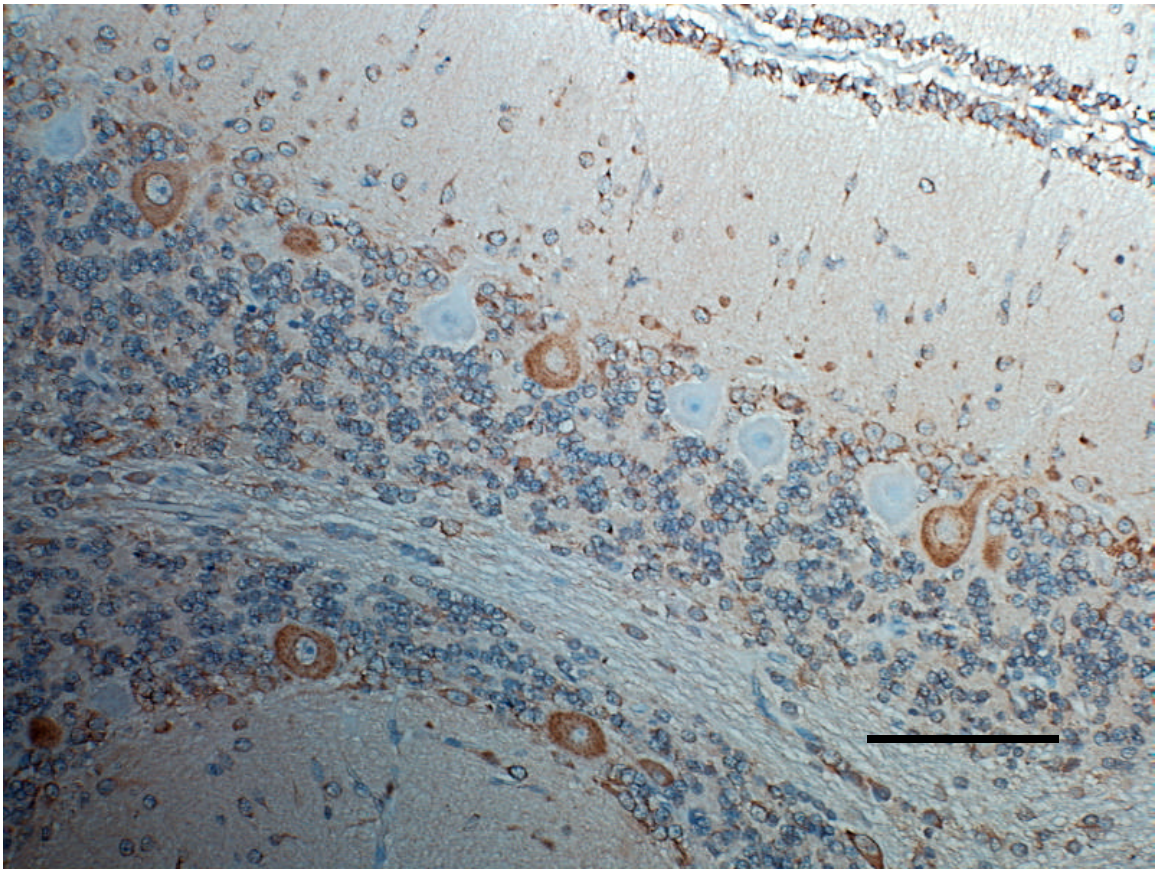


FIGURE 3.1B

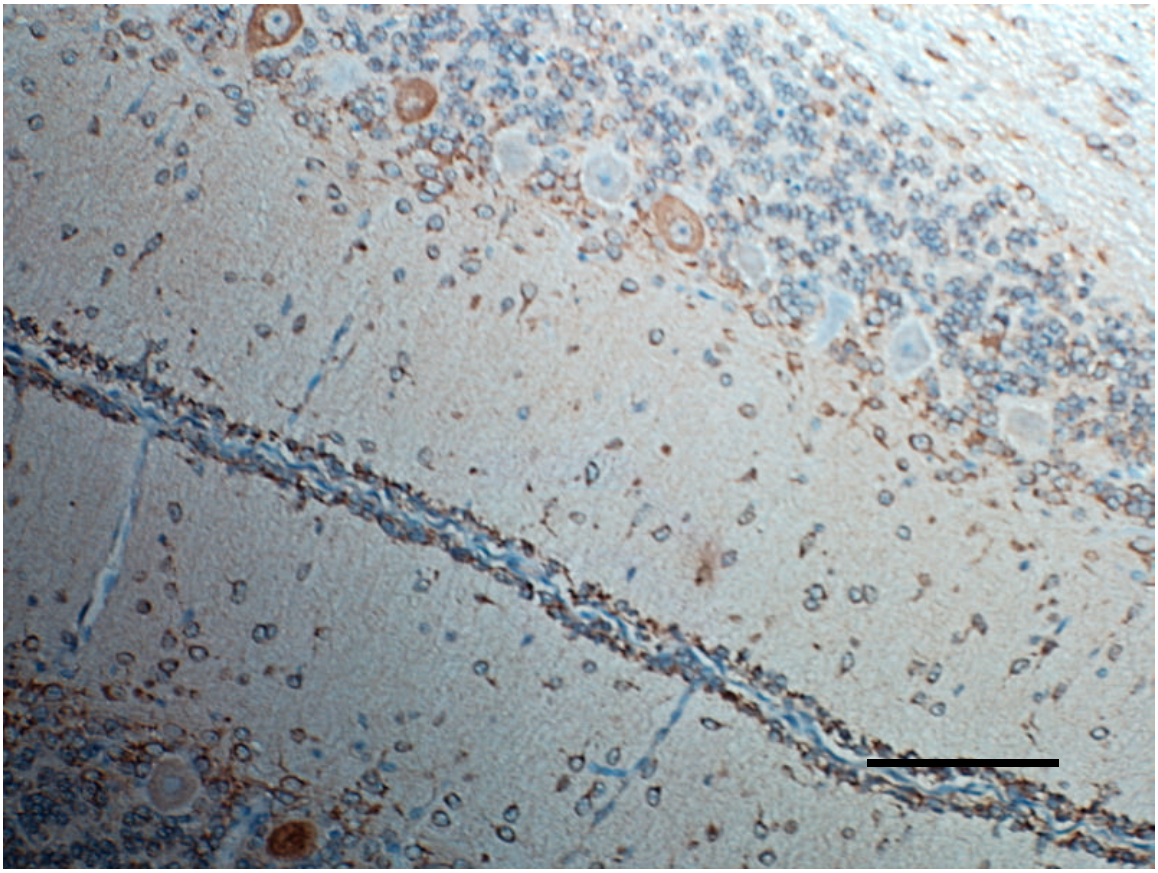


FIGURE 3.2 Comparison of number of Bcl-2-immunoreactive cells located in the molecular layer between the ataxic JRT and the non-ataxic dogs. Immunostained cells were counted in the molecular layer of cerebellar sections. Mean numbers of stained cells were calculated within the each age group of ataxic and non-ataxic dogs. No statistical difference was observed between the ataxic and non-ataxic dogs, as well as among the three different age groups. $P < 0.05$.

FIGURE 3.2

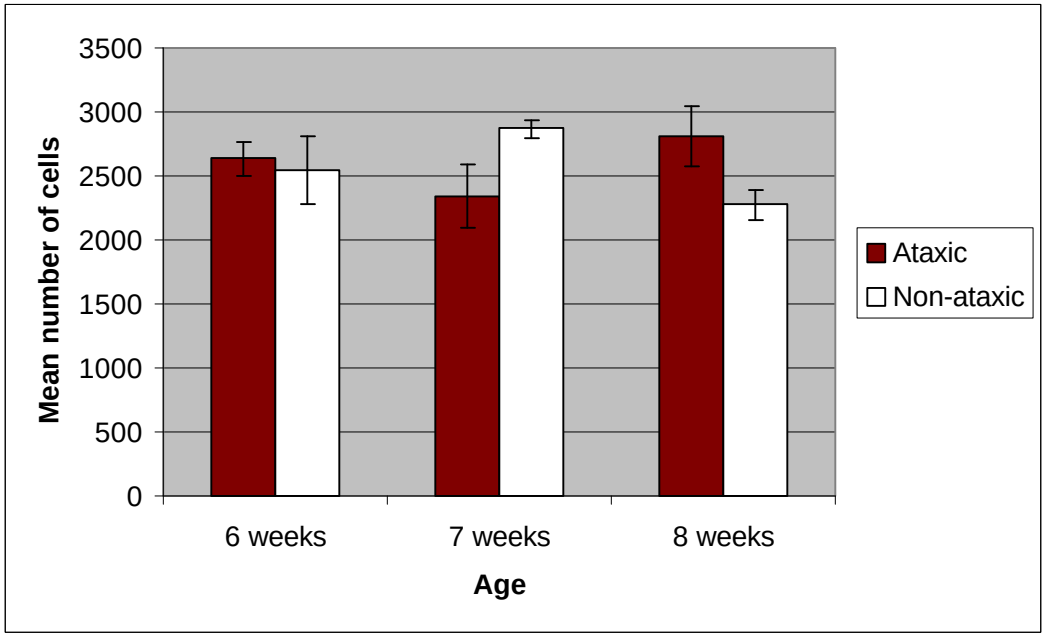

3500
3000
2500
2000
1500
1000
500
0
Mean number of cells
6 weeks
7 weeks
8 weeks
Age
Ataxic
Non-ataxic

FIGURE 3.3 Immunohistochemical localization of Bax in cerebellar sections of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections were immunolabeled for Bax using avidin-biotin-peroxidase method and counterstained with hemotoxylin. Immunoreactive granule cells were detected especially in the internal granule cell layer of ataxic JRT (arrows). Purkinje cells, basket cells, and Golgi cells were not immunolabeled with Bax. In the normal dogs, very few Bax-reactive granule cells were recognizable. Scale bars = 100 ìm.

FIGURE 3.3A

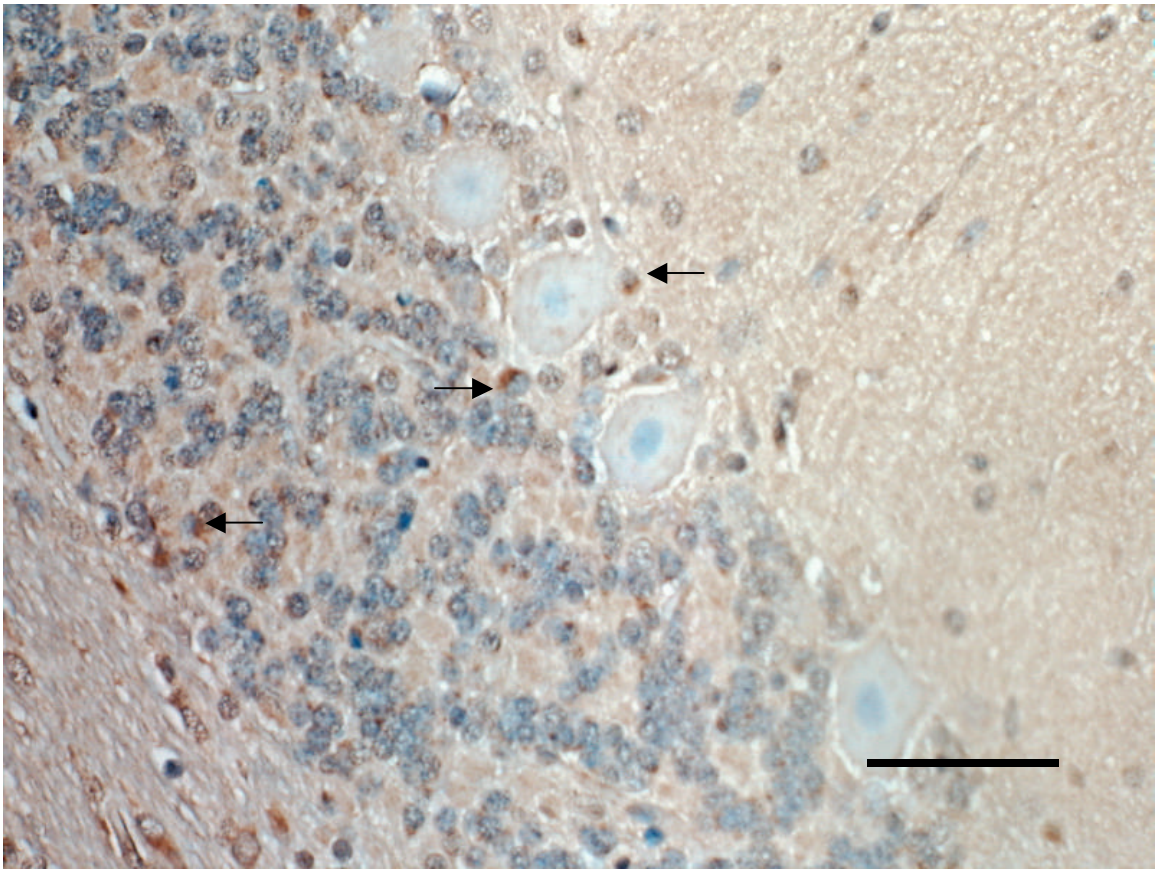


FIGURE 3.3B

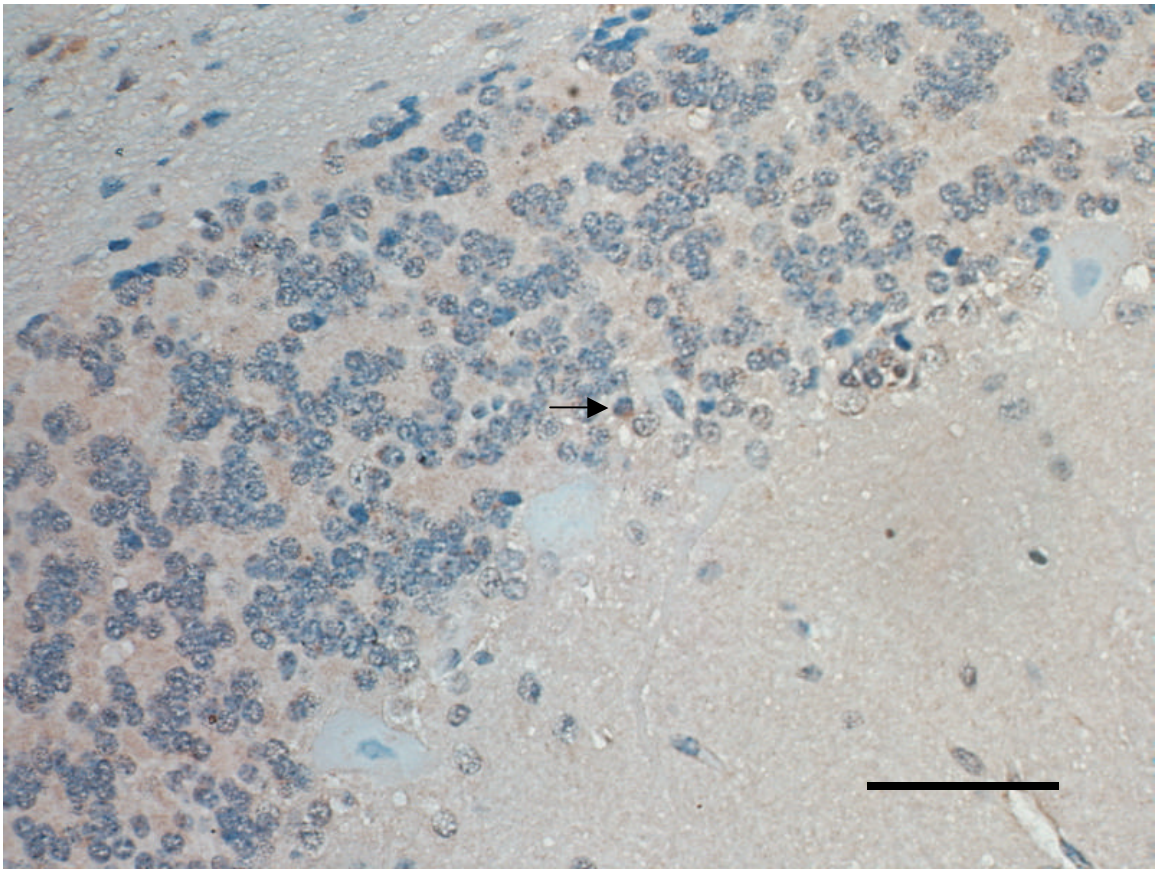
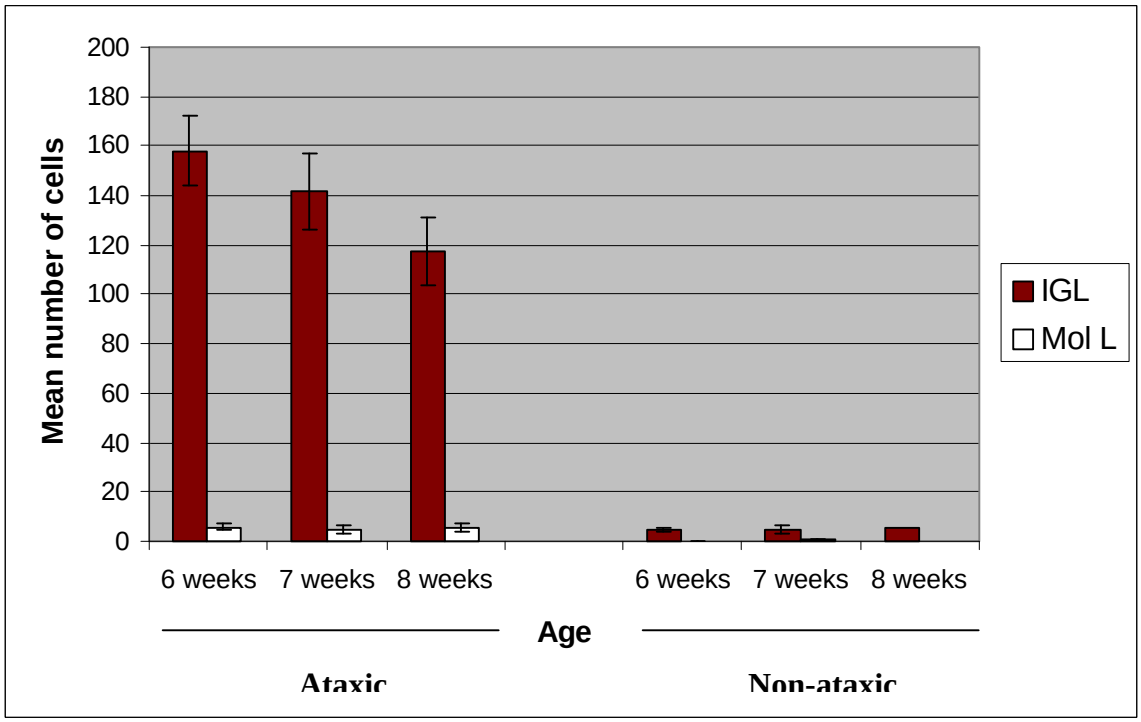


FIGURE 3.4 Detection of total Bax-immunoreactive granule cells in the ataxic JRT and normal dogs, and distribution of these stained cells in the internal granule cell layer and the molecular layer. Cerebellar sections of ataxic and normal dogs were immunolabeled for Bax, and immunoreactive cells were counted. Numbers of stained cells were determined and mean numbers of stained cells were calculated in each age group. Statistical compressions were done between the ataxic JRT and the normal dogs. Comparisons were also done among three age groups. $P < 0.05$.

FIGURE 3.4


Mean number of cells
200
180
160
140
120
100
80
60
40
20
0
6 weeks
7 weeks
8 weeks
6 weeks
7 weeks
8 weeks
Age
Ataxic
Non-ataxic
IGL
Mol L

CHAPTER 4

IMMUNOHISTOCHEMICAL LOCALIZATION OF NGF, NT-3, AND P75^{NTR} IN CEREBELLA OF JACK RUSSELL TERRIERS WITH PRIMARY GRANULE CELL DEGENERATION[1]

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ABSTRACT

Cerebellar cortical degeneration in Jack Russell Terriers (JRT) was previously shown to be due to the degeneration of granule cells via apoptosis. Neural cell survival, maintenance, and function are mainly regulated by neurotrophins. These trophic factors mediate their actions by binding specific receptors and the common neurotrophin receptor $p75^{NTR}$. $p75^{NTR}$ is known to have dual functions, neural survival and death. Therefore, we have studied localization of neurotrophins, nerve growth factor (NGF) and neurotrophin (NT)-3, and receptor $p75^{NTR}$ in the cerebellum of JRT with primary granule cell degeneration (PGCD) by immunohistochemistry. Our results showed that there were more $p75^{NTR}$ immunoreactive granule cells in the internal granule cell layer of ataxic JRT than that of non-ataxic dogs. Slightly more $p75^{NTR}$-immunoreactive Golgi cells were also present in the ataxic dogs. There were similar numbers of $p75^{NTR}$-immunolabeled cells in the molecular layer in both ataxic and non-ataxic dogs. While NGF immunoreactivity was detectable in the Purkinje cells of both ataxic and non-ataxic dogs, there was a significant reduction in the number of immunoreactive cells located in the molecular layer of cerebellum in the ataxic dogs. We did not detect any differences both in the pattern of staining throughout the cerebellum and in the number of NT-3 reactive cells between the ataxic JRT and the non-ataxic dogs. These findings suggest that increased granule cell expression of $p75^{NTR}$ might mediate initiation of cell death. Our results also suggest that decreased NGF expression during the migration of granule cells might initiate the previous changes in the maintenance of these cells that finally causes cell death.

Indexing terms: cerebellar abiotrophy; apoptosis; $p75^{NTR}$, nerve growth factor; neurotrophin-3

Neurotrophic factors are a group of extracellular molecules that are potent regulators of neuronal survival, differentiation, function, and maintenance. Among these, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5, NT-6 and NT-7 (the last two are only in fish) are derived from a common gene and are collectively named neurotrophins (Hallbook 1999). These molecules show high sequence similarity and structural resemblance.

Neurotrophins are target-derived trophic factors that can also act in an autocrine and paracrine fashions (Muller et al., 1994; Acheson et al., 1995).

Neurotrophins bind to three different neurotrophin receptors, Trk (Tropomyosin related kinase) receptor tyrosine kinase-A, -B and -C (TrkA, TrkB, and TrkC). These receptors are considered neurotrophin specific, however in certain occasions, neurotrophins can bind to more than one type of Trk receptors (Benedetti et al., 1993; Clary and Reichardt, 1994; Bibel et al., 1999). In addition to Trk receptors, all neurotrophins can also bind to common neurotrophin receptor, $p75^{NTR}$ (Lee et al., 2001). It has been proposed that $p75^{NTR}$ might function during normal nervous system development to eliminate excess neurons (Lee et al., 2001). Many studies have shown that opposing functions can be mediated by the same receptor. It has been proposed that $p75^{NTR}$ might act as a co-receptor to regulate the functions of Trk receptors. For example, it was shown that TrkA could bind both NGF and NT-3. However, in the presence of $p75^{NTR}$, TrkA could only bind to NGF (Benedetti et al., 1993). Likewise, while TrkB

could bind BDNF, NT4/5, and NT-3, in the presence of $p75^{NTR}$ TrkB could only bind BDNF (Bibel et al., 1999). Although $p75^{NTR}$ cannot directly bind Trk, such an association might be provided by another molecule. The protein called ARMS (ankyrin repeat-rich membrane spanning), which functions in the aggregation of membrane channels, has been proposed to mediate this interaction (Kong et al., 2001). It was also assumed that $p75^{NTR}$ might function as a decoy receptor to regulate the level of neurotrophins in a given time and environment. Such decoy receptors were also proposed because of the presence of truncated forms of Trk receptors with no tyrosine kinase activity (Ip et al., 1993; Barbacid, 1994). Last, $p75^{NTR}$ activation was shown to regulate polymerization of F-actin molecules, which play an important role in formation and maintenance of neuronal processes.

During normal development of the cerebellum, expression of neurotrophins and their receptors on the granule cells changes as they migrate from the external granular layer (EGL) to the internal granule cell layer (IGL). Early granule cells predominantly express TrkB, while as the cells become more mature they increase the expression of TrkC (Segal et al., 1995). It was determined that the ratio of TrkB to TrkC during the early postnatal development was 5:1 whereas this ratio changed to3:1 in adulthood (Pomeroy et al., 1997). Therefore, differential expression of BDNF and NT-3 were proposed to play essential roles in the morphogenesis of the cerebellum (Segal et al., 1997). Specific functions of BDNF in the cerebellum include promoting the survival of granule cells, signaling the differentiation of precursor cells, inducing the expression of NT-3, and, in general, increasing the axonal outgrowth (Gao et al., 1995). On the other hand, NT-3 increases fasciculation of neurons and promotes Purkinje cell survival

(Lindholm et al., 1993; Segal et al., 1995). The involvement of NGF in cerebellar development is largely unknown, and often its expression is defined as low in this organ. However, NGF and some other neurotrophins mediated apoptotic cell death was determined in several systems (Bredesen and Rabizadeh, 1997; Carter and Lewin, 1997; Bamji et al., 1998; Agerman et al., 2000).

A neurodegenerative disorder characterized by primary apoptotic granule cell loss in the cerebellum has been described in JRT with PGCD (Carmichael et al., 2002). In this study, we investigated the involvement of NGF, NT-3 and $p75^{NTR}$ in the process of granule cell death in the ataxic JRT.

MATERIALS AND METHODS

Immunohistochemistry. Primary antisera used were 1) rabbit anti-mouse p75 nerve growth factor receptor polyclonal antibody (Chemicon International Inc., Temecula, CA) diluted 1:1000; 2) chicken anti-human NT-3 polyclonal antibody (Promega, Madison, WI) diluted 1:50; 3) rabbit anti-mouse nerve growth factor polyclonal antibody (Chemicon International Inc.) diluted 1:1000. Dilutions were made in 0.1 M phosphate-buffered saline containing 0.3% Triton-X-100 (PBS-T), pH 7.4.

For immunohistochemical detection of proteins of interest, a total of 15 ataxic dogs aged between 6 to 8 weeks were used. For control, a total of 10 aged-matched normal puppies were used. Freshly obtained tissues were immediately immersion fixed in 10% neutral buffer formalin for no longer than 48 hours and paraffin embedded. Five ìm sections were deparaffinized in three changes of Hemo De (Fisher Scientific, Pittsburgh, PA) and rehydrated in reducing concentrations of ethanol from absolute to 70% and

washed under running tap water. Antigen retrievals were accomplished by placing the sections in Vector Antigen Unmasking Solution (Vector Laboratories Inc., Burlingame, CA) 10 min at 96^{o}C water bath for p75^{NTR}, 25 ìm/ml Pronas e (Sigma, St. Louis, MO) (15 min at 37^{o}C for NT-3, and 1X Trypsin-EDTA (Sigma) 15 min at 37^{o}C incubator for NGF. Those p75^{NTR}-stained sections were allowed to cool for 20 min. After rinsing all the sections with PBS-T endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 for 20 minutes, and the sections were rinsed once with the same buffer for 5 min. Thereafter, primary antibodies were applied to the sections in dilutions mentioned above, and the sections were incubated overnight at 4^{o}C. Two sets of control slides were prepared and incubated with either normal serum from the animal that the primary antibody made in or PBS-T. Following incubation, excess antibodies were washed off by rinsing the sections with the PBS-T for three times 5 minutes each. For the detection of primary antibodies, the sections were incubated for 30 minutes in biotinylated anti-rabbit IgG (Vector Laboratories Inc.) for p75^{NTR} and NGF, and in biotinylated anti-chicken IgY (Vector Laboratories Inc.) for NT-3, both diluted at 1:200 with the PBS-T, followed by another 30 minutes of incubation in strepavidin-biotin complex (ABC, Vector Laboratories Inc.). Reaction visualizations were provided by a 2-5 minute reaction in 3,3'-diaminobenzidine (DAB, Vector Laboratories Inc.). Finally, The sections were rinsed several times with ddH_2O and counterstained with hemotoxylin for 2 minutes, and coverslipped with Permount (Fisher Scientific Inc.).

Statistical analyses. For p75^{NTR} and NT-3, number of stained cells was counted under a light microscope at 40X magnification from 50 randomly chosen fields in each case. In each counted field, all of the cortical layers were included. The difference in total number

of stained cells was analyzed in the ataxic and non-ataxic dogs using two-tailed Students' *t* test. Comparison of three different age groups within either the ataxic or non-ataxic dogs were done using Kruskal Wallis test. In all of the analyses, P values < 0.05 were considered significant.

RESULTS

p75 immunohistochemistry. In cerebellar sections of ataxic JRT, $p75^{NTR}$ -immunoreactivity was detected in cells located both in the molecular layer and the internal granule cell layer (IGL) (Figure 4.1A). Within the molecular layer, most of the immunoreactive cells were located especially in the deeper portion of this layer (Figure 4.1A). There was quite a variation on the immunoreactivity level among these cells, while most had intense $p75^{NTR}$-immunostaining, some did not have visible immunolabeling. Most of the immunoreactive cells in this layer had round to ovoid vesicular nucleus, which was occasionally located eccentrically within the thin layer of cytoplasm. Cell processes of these cells were mostly stained and recognizable at the light microscope level. While there was generally no difference between the stained cells and the non-stained cells, some of the non-stained cells were smaller than the stained cells.

Double immunolabeling with $p75^{NTR}$ and glial fibrillary acidic protein (GFAP), a marker for glial cells, showed that $p75^{NTR}$-positive cells in the molecular layer were not reactive with GFAP indicating that these cells were not of glial origin (data not shown). Absence of GFAP staining and the presence of some of these cells in the upper zones of molecular layer strongly suggested that these cells were migrating granule cells.

Within the molecular layer, the basket cells were recognized to be positive for $p75^{NTR}$. These cells were clearly distinguishable from other stained cells based on size, shape and orientation.

In the IGL the most strongly stained cells were Golgi cells, but not all Golgi cells were stained with $p75^{NTR}$. Therefore, cell counting of these cells was done.

In the IGL, almost all of the granule cells were seen to be immunoreactive with $p75^{NTR}$. However, some cells were recognized with higher intensity staining (Figure 4.1A). Therefore, counting of these immunoreactive cells was included in the procedure. Stained granule cells were usually slightly smaller than healthy granule cells. However, some were about the same size as normal granule cells. $p75^{NTR}$-reactive granule cells were round to ovoid and some presented a cytoplasmic staining which gave these cells a star-like appearance. Most of these cells had a more heterochromatic nucleus than healthy cells.

In majority of the cases, most of the Purkinje cells were not reactive with $p75^{NTR}$. However, some stained Purkinje cells were occasionally determined. There was no apparent pattern in term of these cells' staining. In those sections where EGL was present, none of the premigratory cells were stained with $p75^{NTR}$.

In the non-ataxic dogs, immunolabeling with $p75^{NTR}$ revealed similar staining characteristics to those described in the ataxic dogs. In the molecular layer, $p75^{NTR}$-immunoreactive cells were mostly located in the deeper portions of this layer (Figure 4.1B). There were also unreactive cells in this layer. However, these unreactive cells were usually smaller than the stained ones. In the molecular layer, basket cells were clearly immunoreactive with $p75^{NTR}$.

In the IGL of cerebellar sections of non-ataxic dogs, all of the granule cells were $p75^{NTR}$-immunoreactive (Figure 4.1B). There seemed no difference in the level of immunoreactivity among these cells, all having diffuse immunoreactivity throughout the sections. Golgi cells were clearly immunostained with $p75^{NTR}$.

Number of stained cells in the three different age groups from both ataxic and non-ataxic puppies was counted from 50 randomly chosen locations. Mean numbers of $p75^{NTR}$ reactive cells in the molecular layer and IGL were calculated. In the molecular layer, means of stained cell numbers were 533.2, 375.6, and 498.2 for ataxic puppies of 6, 7, and 8 weeks, respectively (Figure 4.2A). Mean numbers of stained cells for 6-, 7-, and 8-week-old non-ataxic puppies were 508.7, 486.5, and 495.4, respectively. There was no statistical difference between the ataxic and non-ataxic puppies in terms of stained cells in the molecular layer ($p<0.05$). There was also no statistical difference between the different age groups among the ataxic and non-ataxic dogs ($p<0.05$).

In the IGL, two separate cell counting were done, one for Golgi cells, and the other for granule cells. Mean numbers of stained Golgi cells were 157.8, 123.6, and 188.6 for ataxic puppies of 6, 7, and 8 weeks, receptively (Figure 4.2B). These numbers for non-ataxic puppies were 66.6, 103.5, and 77.6, respectively. Comparison of ataxic dogs to normal dogs revealed a statistical difference ($p<0.05$). There were significantly more stained Golgi cells in the ataxic dogs than non-ataxic dogs.

Mean numbers of granule cells with increased $p75^{NTR}$-immunoreactivity were 43.7, 35.5, and 39.8 for ataxic puppies of 6, 7, and 8 weeks, respectively (Figure 4.2C). These numbers for normal dogs were 5, 4, and 3.6, respectively. Statistical comparison of ataxic dogs to normal dogs revealed a significant difference ($p<0.05$). Therefore, in ataxic

puppies, $p75^{NTR}$-immunoreactive granule cells were significantly higher than that of normal dogs. In both ataxic and normal dogs, there was no significant difference among each age groups tested ($p<0.05$).

NT-3 Immunohistochemistry. In the ataxic dogs, examination of sections showed that the most significantly stained cells were located in the molecular layer (Figure 4.3A). Immunoreactive cells were located throughout in this layer. However, it was recognized that most of the stained cells were located in the deeper portions of the molecular layer. In this location, most of the cells were reactive with the NT-3 antibody. In the more superficial portions of the molecular layer, more cells seemed to be unreactive with the NT-3. Immunoreactive cells showed variations in shape and size depending on the location within the molecular layer. Cells located in the deeper portions had more vesicular, round to ovoid nucleus. These cells had comparably larger cytoplasm, which stained strongly with NT-3. Those cells located in the middle portion of the molecular layer had more elliptic to ovoid nucleus that was surrounded by a thin cytoplasm. Intensity of the staining of these cells was usually lower than those located at the deeper portions of the molecular layer. There was also a variation in the staining intensity among cells located in the superficial portions of the molecular layer.

In those animals in which the EGL persisted, cells in this layer were stained. Staining intensity of these cells was greater than those located in the upper and middle portions of the molecular layer.

In most of the ataxic-puppies, a majority of the Purkinje cells was stained (Figure 4.3A). There were always some cells in this layer that did not stain or stained minimally. In other cases, a majority of the Purkinje cells did not stained, although in these cases

there were always stained Purkinje cells especially in the outer portions of the cerebellar lobes. In those cases where the Purkinje cells were stained, the staining was recognized in the cell perikarya. Primary and somewhat secondary dendrites of these cells were also stained with NT-3.

In the IGL, most of the stained cells were Golgi cells. The staining intensity of these cells was similar to those immunoreactive cells located at the deeper portions of the molecular layer. In close examination, granule cells were recognized to be immunoreactive to NT-3. Staining intensity on these cells did not show substantial changes throughout the sections, and therefore no cell count was done on these cells.

In the non-ataxic dogs, immunoreactivity to NT-3 throughout the cerebellar layer showed similar staining characteristics to those described for the ataxic JRT. In the molecular layer, most immunoreactive cells were located in the deeper portions of this layer (Figure 4.3B). NT-3 immunoreactive cells were also recognizable in the more superficial portion of the molecular layer. In the non-ataxic dogs, immunoreactivity in Purkinje cells showed variation from cell to cell, having some Purkinje cells were immunopositive (Figure 4.3B). In the IGL, granule cells were immunostained with NT-3, and the staining characteristics did not differ from those described for ataxic dogs.

Since the most highly immunoreactive cells were those cells in the molecular layer and Golgi cells in the IGL, cell count was performed for only these two cell types. Due to inability to differentiate those closely packed stained cells in the EGL, a cell count in this layer was omitted. Number of stained cells was determined in individual dogs, and the mean numbers of cells were calculated for the three age groups tested.

In the ataxic JRT, mean numbers of stained cells in the molecular layer were 1735.6, 1419.5, and 1588 in 6, 7, and 8 weeks old puppies, respectively (Figure 4.4A). There were no statistical differences among the ataxic dogs with different ages ($P<0.05$). The numbers were somewhat variable in the non-ataxic dogs. The highest number of NT-3 reactive cells was found in the 6-week-old puppies. Mean number of stained cells in this age group was 2158. In 7 and 8 week-old puppies, the number of stained cells dropped greatly to 1274 and 976, respectively. With this pattern, there was a decreasing number of NT-3 reactive cells in the molecular layer of non-ataxic dogs. In comparison of the cell counts between the same age groups, though the presence of slightly higher numbers of cells in the non-ataxic dogs, there was no statistical difference between 6-week-old ataxic and normal dogs ($p<0.05$). On the other had, there were statistical differences within the 7- and 8-week-old ataxic and non-ataxic puppies. In both age groups, the numbers of NT-3 stained cells were significantly higher in the ataxic puppies ($p<0.05$).

In the IGL, the number of stained Golgi cells was determined, and the mean numbers of stained cells were calculated (Figure 4.4B). There were 102.8, 107.5, and133.5 stained cells in the 6, 7, and 8-week-old ataxic puppies, respectively. Although the numbers seemed increasing with age there were no statistical differences among the different age groups. The numbers of stained cells were 128, 80, and 60.7 in 6, 7, and 8-week-old non-ataxic dogs, respectively. There seemed a decreasing tendency to the numbers of stained Golgi cells in non-ataxic dogs with increasing ages. Although there was a statistical difference between the 6- and 7-week-old non-ataxic dogs, no statistical difference was present between the 7- and 8-week-old non-ataxic dogs ($p<0.05$).

Comparison of ataxic vs. non-ataxic dogs revealed that there is only statistical difference between the 8 week-old ataxic and non-ataxic dogs. Therefore, only in this age group, the number of stained Golgi cells was significantly higher in the ataxic dogs.

NGF Immunohistochemistry. In the ataxic puppies, the most striking labeling was recognized on the Purkinje cells (Figure 4.5A). Perikarya, primary and secondary dendrites of these cells were intensely stained for NGF. However, it was recognized that not all Purkinje cells were immunoreactive for NGF. In most of the sections, immunopositive Purkinje cells were seen right next to immunonegative Purkinje cells. Intensity of NGF-immunoreactivity in Purkinje cells also varied from cell to cell, some had more intense immulabeling than others. This pattern of staining showed no lobular predilection.

In the ataxic dogs, some cells stained for NGF in the molecular layer (Figure 4.5A). In general, the staining intensity on these cells was less than that of Purkinje cells. These faintly stained cells were mostly located in the deep molecular layer and to a lesser extent superficial molecular layer. They had round to ovoid, vesicular nuclei, which were surrounded by a thin rim of cytoplasm. Staining was present on the perikarya of these cells, and in some cases, on the bases of the cell processes. Because of the faint labeling, in some cases it was difficult to differentiate stained cells from non-stained ones. Therefore, no cell counting was done on these cells. Rather the data was presented based on the overall staining pattern (Table 4.1).

In the IGL, only Golgi cells were immunopositive. While most of these cells were immunoreactive some did not have immunolabeling. There was no immunostaining for

NGF on the granule cells located in the IGL, and those of the premigratory cells of the EGL.

In the non-ataxic dogs, immunoreactivity to NGF was most strikingly present in the Purkinje cells. Staining characteristics for NGF on these cells were similar to those described for ataxic puppies. In the molecular layer, NGF-immunoreactive cells were abundant especially in the deep molecular layer (Figure 4.5B). In most non-ataxic dogs, immunostaining intensity on these cells was great.

In the IGL, Golgi cells were immunoreactive with NGF, while no immunostaining was observed on the granule cells in both ataxic and non-ataxic dogs.

In comparison of ataxic and non-ataxic puppies, there was generally more stained cells in the molecular layer, and the imunostaining intensity was usually greater than those of non-ataxic puppies. Due to overall minimal staining on these cells, no cell count was performed, rather a more subjective comparison was done based on the overall staining. A same approach was followed to describe the staining on the Purkinje cells and the Golgi cells.

In six out of ten non-ataxic dogs, most of the cells located in the molecular layer were immunoreactive with NT-3 while in the rest, some of these cells had immunoreactivity (Table 4.1). On the other hand, in twelve out of fifteen ataxic dogs, there were only few of these cells that had immunostaining, and in the rest of the ataxic puppies there were only some of these cells immunoreactive. Overall staining characteristics with NGF immunoreactivity on Purkinje cells and Golgi cells did not show substantial differences between the ataxic and non-ataxic puppies, though

occasional differences between individual cases were observed among both ataxic and the non-ataxic dogs (Table 4.1).

DISCUSSION

In the present study, we investigated immunohistochemical localization of NGF, NT-3, and $p75^{NTR}$ in cerebellar sections of a group of ataxic JRT and age-matched non-ataxic JRT. Our results showed that in the IGL of the cerebellum, the number of granule cells with increased immunoreactivity to $p75^{NTR}$ was significantly higher in the ataxic JRT compared to that of non-ataxic dogs. This finding indicates that $p75^{NTR}$ might play an important role in the process of granule cell death in the ataxic JRT. In previous studies, we have shown, using TUNEL assay and Bax immunohistochemistry, that granule cells die via apoptosis, and that the apoptotic processes mostly takes place in the IGL and is associated with increased Bax immunoreactivity. In the current study, the findings of increased numbers of $p75^{NTR}$ immunoreactive cells in the IGL of ataxic dogs with the absence of such a difference in the molecular layer, suggest that $p75^{NTR}$ mediated cell suicide activity might be the cause of granule cell degeneration. On the other hand, overexpression of $p75^{NTR}$ was shown to occur in many cell types that were under stress, and, therefore it was suggested that $p75^{NTR}$ might just act as a stress receptor (Dowling et al., 1999; Chang et al., 2000). Therefore, the increased number of $p75^{NTR}$-immunoreactive cells in the IGL of cerebellum of ataxic JRT might only indicate loss of cell homeostasis and occurrence of certain degenerative changes in these cells. The presence of certain degree of $p75^{NTR}$ immunoreavtivity in granule cells of IGL in both

ataxic and non-ataxic puppies suggests that $p75^{NTR}$ might be involved in cell survival or maintenance of these neurons in this layer.

Presence of $p75^{NTR}$-immunoreactive cells in the molecular layer of cerebella in both ataxic and non-ataxic dogs suggest that this receptor might function during granule cell migration and is needed for cell survival and axonal outgrowth. The neurotrophin, BDNF, is also known to provide these two functions by binding to TrkB (Segal et al., 1995). Therefore, $p75^{NTR}$ might facilitate the action of TrkB by providing specific binding of BDNF to its cognate receptor. Such facilitative functions were described for $p75^{NTR}$ (Benedetti et al., 1993; Bibel et al., 1999).

We also investigated differences in the number of stained Golgi cells between the ataxic and non-ataxic dogs. Golgi cells were recognized to be strongly immunoreactive for $p75^{NTR}$ both in the ataxic and the non-ataxic dogs. However, $p75^{NTR}$-immunoreactive Golgi cell counts revealed that in the ataxic dogs there were significantly more stained Golgi cells than that of the non-ataxic dogs. This difference did not seemed to be due to loss of these cells, but rather increased expression of this receptor. Such an increase in the expression of $p75^{NTR}$ might cause important consequences. For example, $p75^{NTR}$ can act as a decoy receptor, and bind and limit the amount of survival signals for other cells. Such a mechanism might be responsible for the degeneration of granule cells in the ataxic JRT.

Immunohistochemical investigation of NGF in our study showed that NGF expression is prominently present only in the Purkinje cells. This finding is in accordance with the finding that the NGF receptor TrkA is also expressed in the Purkinje cells (Cohen-Cory et al., 1989; Aloe and Vigneti, 1992). Therefore, these findings strongly

suggest that NGF might play important functions in the survival and/or maintenance of these neurons. mRNA expressions of NGF and NGF receptor TrkA were reported to be high in the premigratory cells of rat cerebellum (Cohen-Cory et al., 1989). However, we did not observe any prominently stained cells in the EGL, suggesting that there are differences in neurotrophin receptor distribution between the murine and canine cerebellum.

The findings from subjective comparison of those faintly NGF-immunostained cells located in the molecular layer suggest that relatively decreased numbers of these cells might be the reason for initiation of cell death processes in the ataxic JRT. Decreased level of NGF expression, in theory, might hinder survival signals provided by this neurotrophin. However, NGF was shown not to provide such prominent survival signals on the cerebellar granule cells, at least in culture. (Nonomura et al., 1996). On the other hand, presence of molecular layer cells with NGF-immunoreactivity in the non-ataxic dogs might suggest that NGF plays an important role during the development of cerebellum in dogs. These results clearly indicate the need for more research on the activity of NGF and other neurotrophins in cerebellum.

Finally, we looked at the immunolocalization of NT-3. Immunoreactivity was present throughout the cell types of cerebellum with no differences between the ataxic JRT and the non-ataxic dogs. These findings suggest that NT-3 probably does not play a significant role in the process of granule cell death.

In conclusion, we have shown that overexpression of $p75^{NTR}$ in the granule cells occur during the course of granule cell degeneration. Knowing the established function as a death receptor, we propose that $p75^{NTR}$ mediates granule cell death. Initial deprivation

of NGF during the migration of granule cells might initiate the destruction of homeostasis that would finally cause the degeneration of these neurons.

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FIGURE 4.1 Immunohistochemical localization of $p75^{NTR}$-reactive cells in the cerebellar sections of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections of ataxic and non-ataxic dogs were processed for immunohistochemical labeling for $p75^{NTR}$ using avidin-biotin-peroxidase method. Immunoreactive cells were determined throughout the layers of cerebellum. In the molecular layer, stained cells were recognized especially at the bottom portion of this layer. In the internal granule cell layer, Golgi cells and granule cells had p75-immunoreactivity. Note that in the ataxic dogs stained Golgi cells (arrowhead) and more intensely-stained granule cells (arrows) were more abundant compared to the normal dogs. Scale bars = 100 ìm.

FIGURE 4.1A

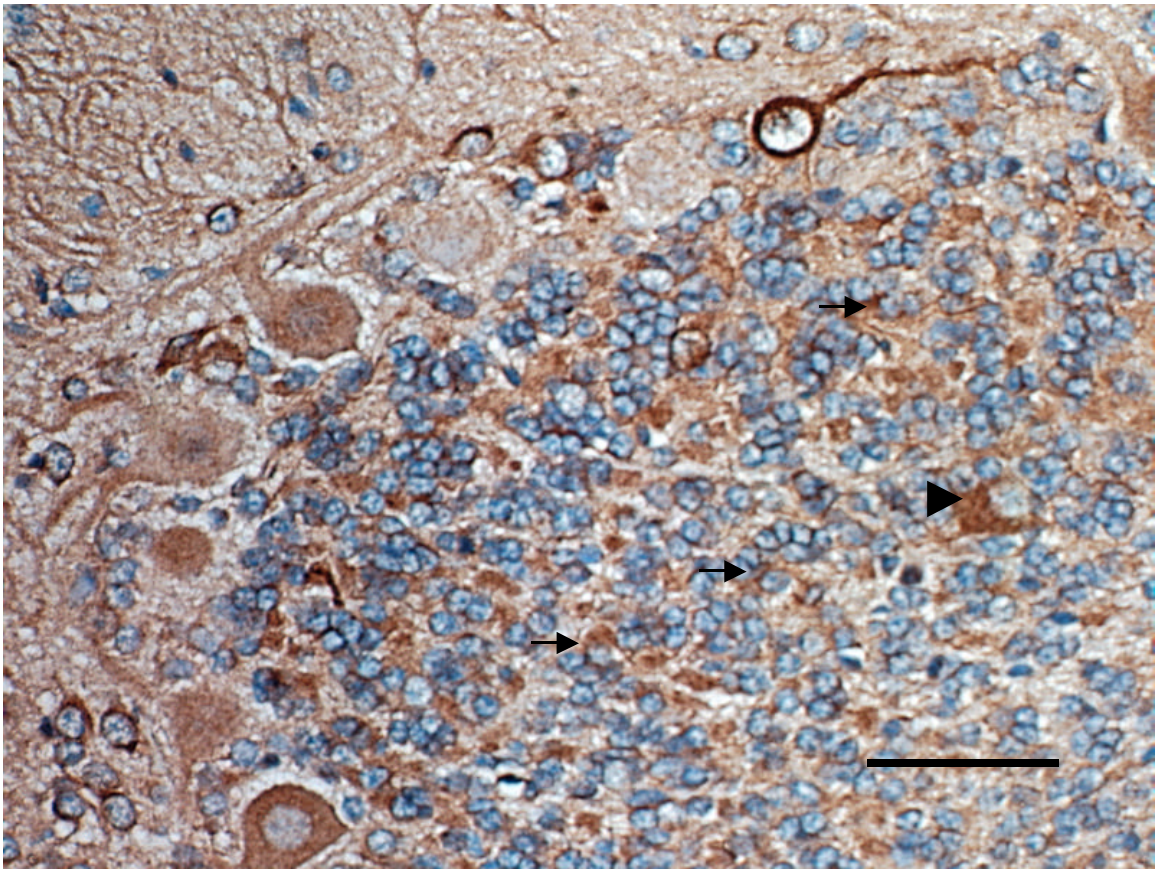

FIGURE 4.1B

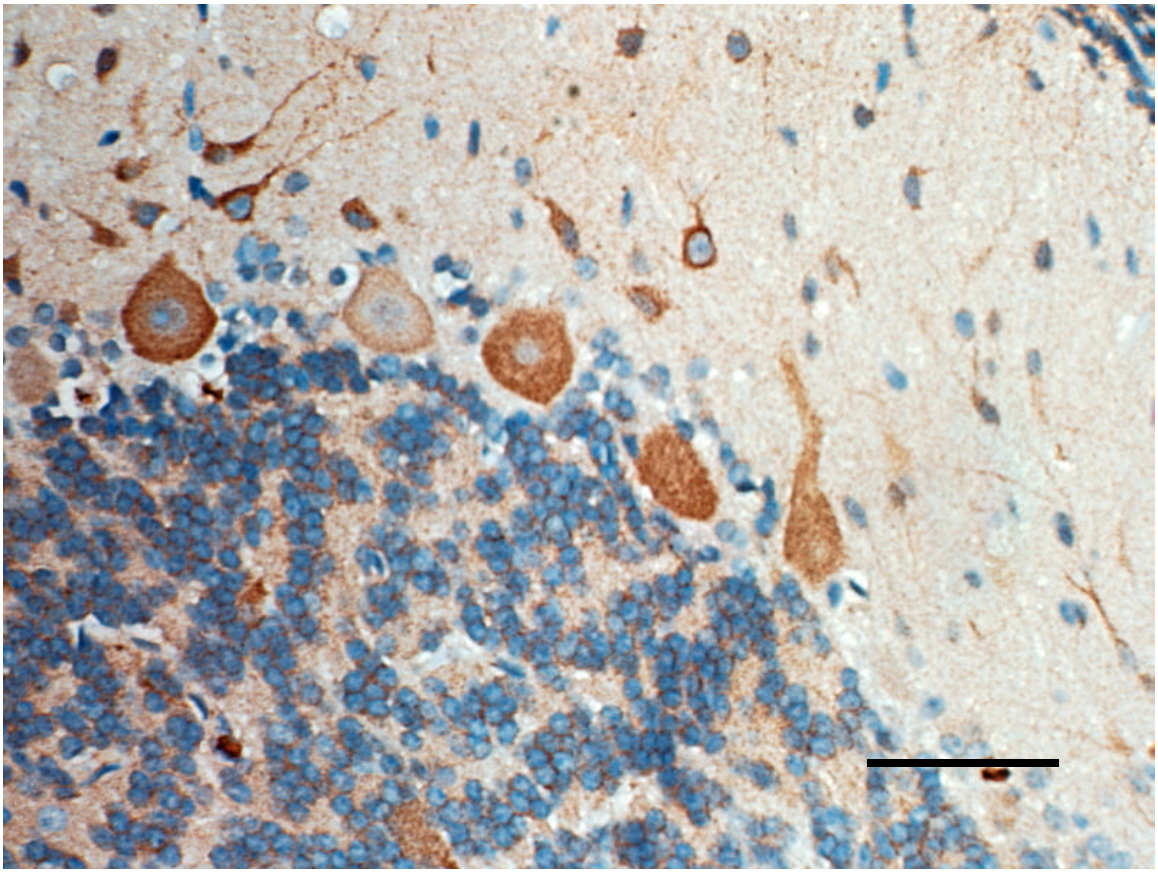

FIGURE 4.2 Distribution of $p75^{NTR}$-immunoreactive molecular layer cells (A), Golgi cells (B), and granule cells (C) in cerebella of ataxic JRT and non-ataxic dogs. Cerebellar sections were immunohistochemically labeled for Bcl-2. Immunoreactive cells were counted in the molecular layer and the internal granule cell layer. Mean numbers of stained cells in the molecular layer were calculated. Similarly, mean numbers of stained Golgi cells and more intensely stained granule cells were determined. Statistical comparisons were made between the ataxic and normal dogs, as well as among the three different age groups. $P < 0.05$.

FIGURE 4.2A

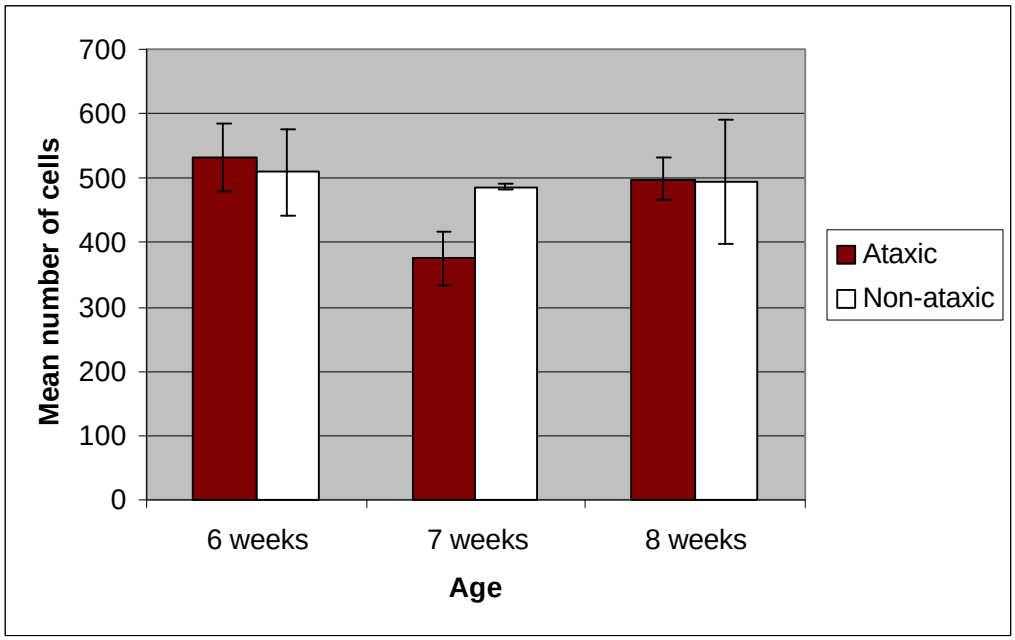
700
600
500
400
300
200
100
0
Mean number of cells
6 weeks
7 weeks
8 weeks
Age
Ataxic
Non-ataxic

FIGURE 4.2B

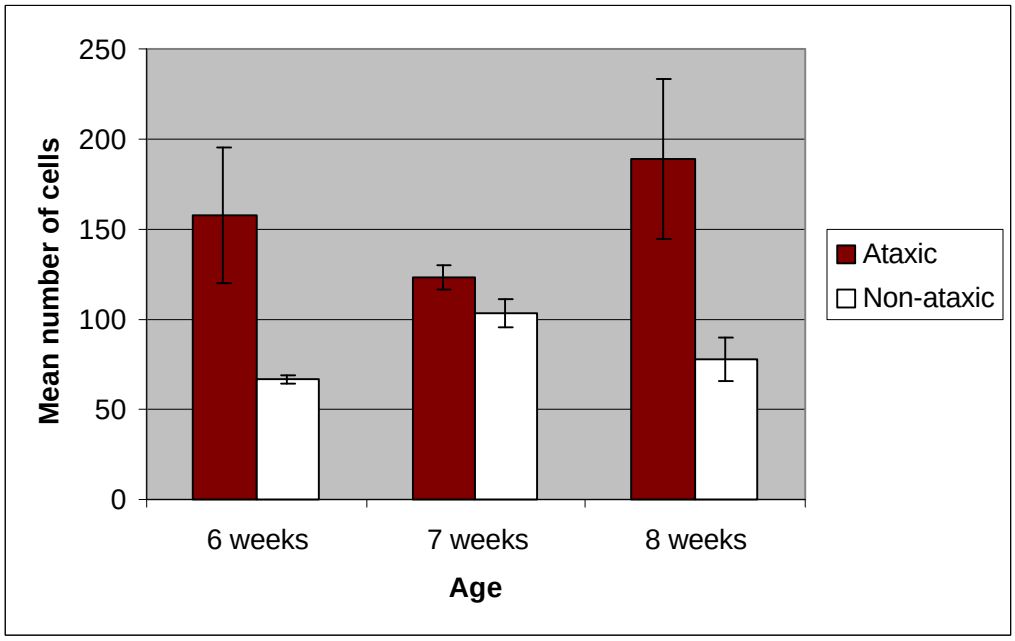
250
200
150
100
50
0
Mean number of cells
6 weeks
7 weeks
8 weeks
Age
Ataxic
Non-ataxic

FIGURE 4.2C

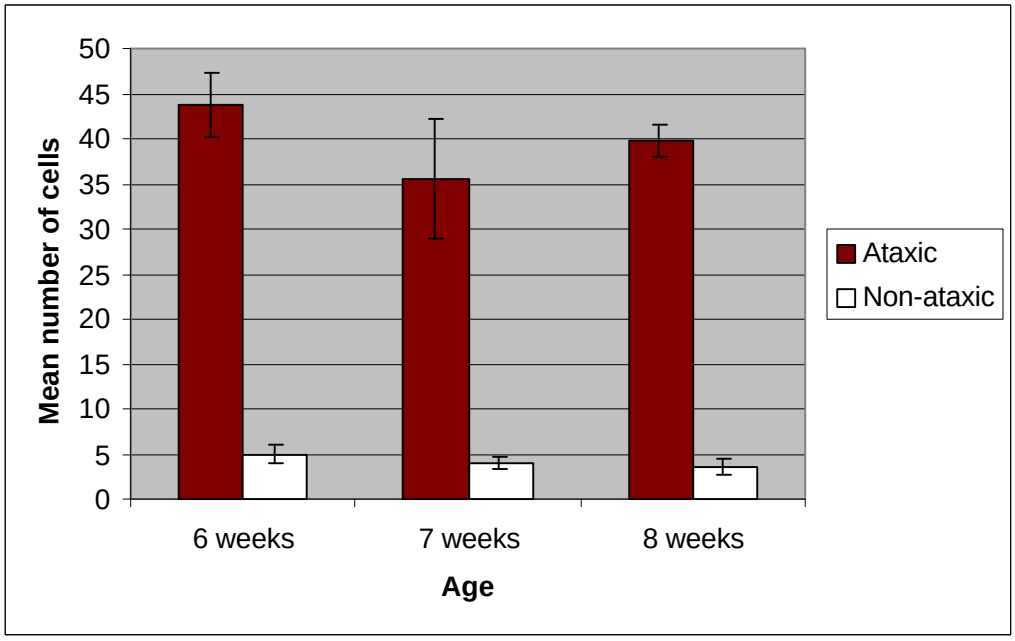
Mean number of cells
50
45
40
35
30
25
20
15
10
5
0
6 weeks
7 weeks
8 weeks
Age
Ataxic
Non-ataxic

FIGURE 4.3 Immunohistochemical localization of NT-3 in cerebellar sections of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections were immunolabeled for NT-3 using avidin-biotin-peroxidase method. Most of the stained cells in the molecular layer were confined at the bottom part of this layer in both ataxic JRT and normal dogs. In the internal granule cell layer, Golgi cells and granule cells were immunoreactive for NT-3. Scale bars = 100 ìm.

FIGURE 4.3A

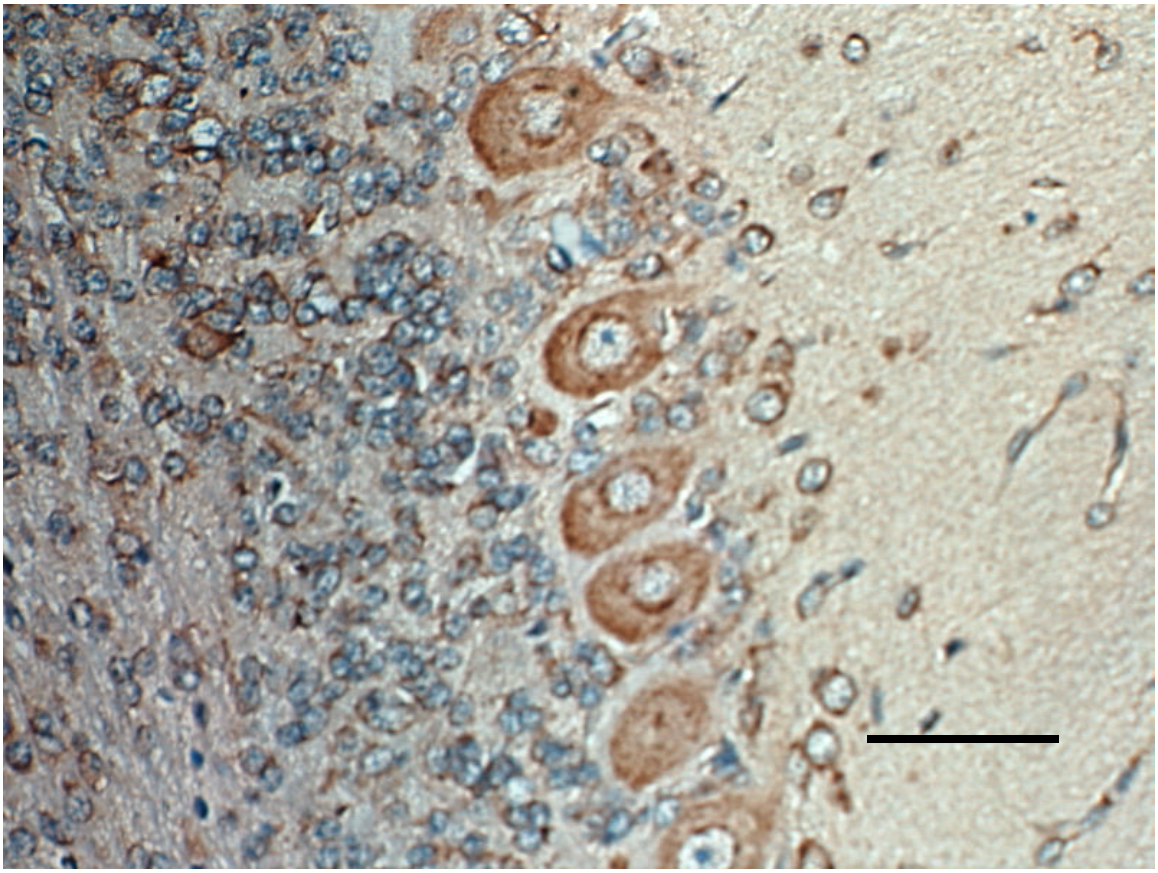

FIGURE 4.3B

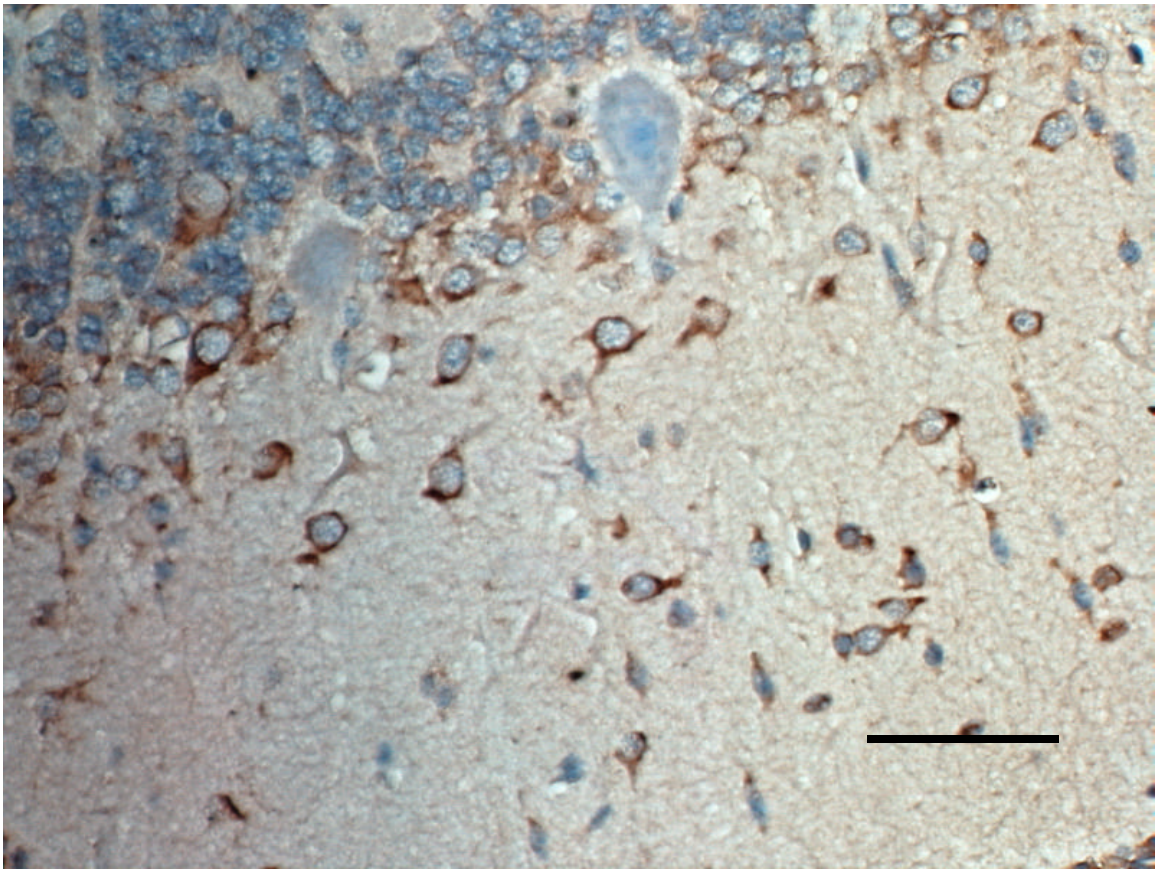

FIGURE 4.4 Comparison of NT-3-immunoreactive molecular layer cells (A) and Golgi cells (B) between the ataxic JRT and non-ataxic dogs. Cerebellar sections of ataxic and normal dogs immunolabeled for NT-3 and stained cells were counted. Mean numbers of immunoreactive cells in the molecular layer and Golgi cells in the internal granule cell layer were determined within the three age groups tested. Statistical comparisons were done between the ataxic and the normal dogs as well as among the three age groups. $P < 0.05$

FIGURE 4.4A

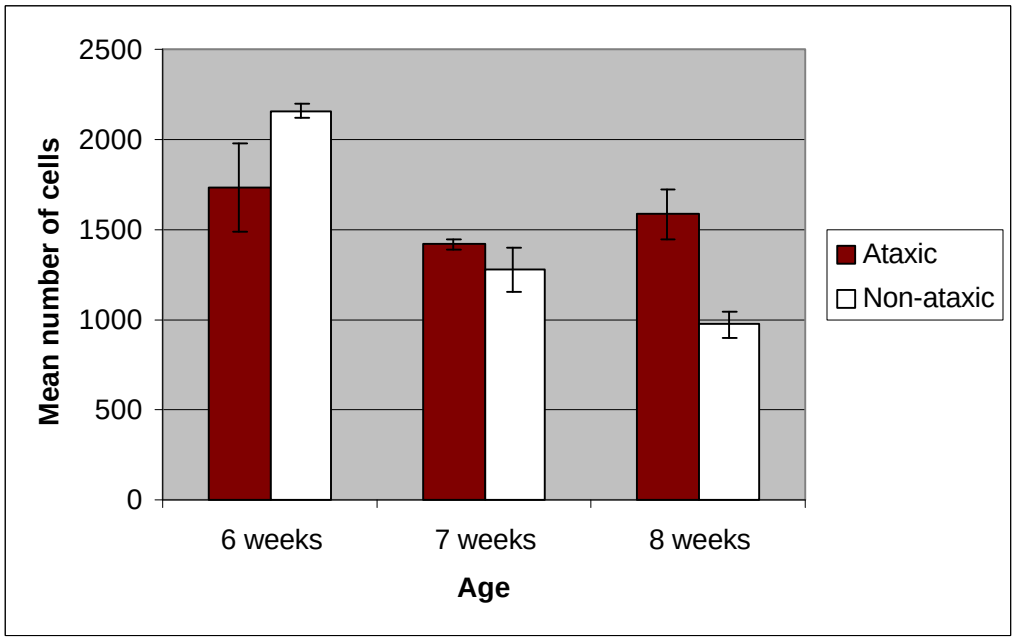
2500
2000
1500
1000
500
0
Mean number of cells
6 weeks
7 weeks
8 weeks
Age
Ataxic
Non-ataxic

FIGURE 4.4B

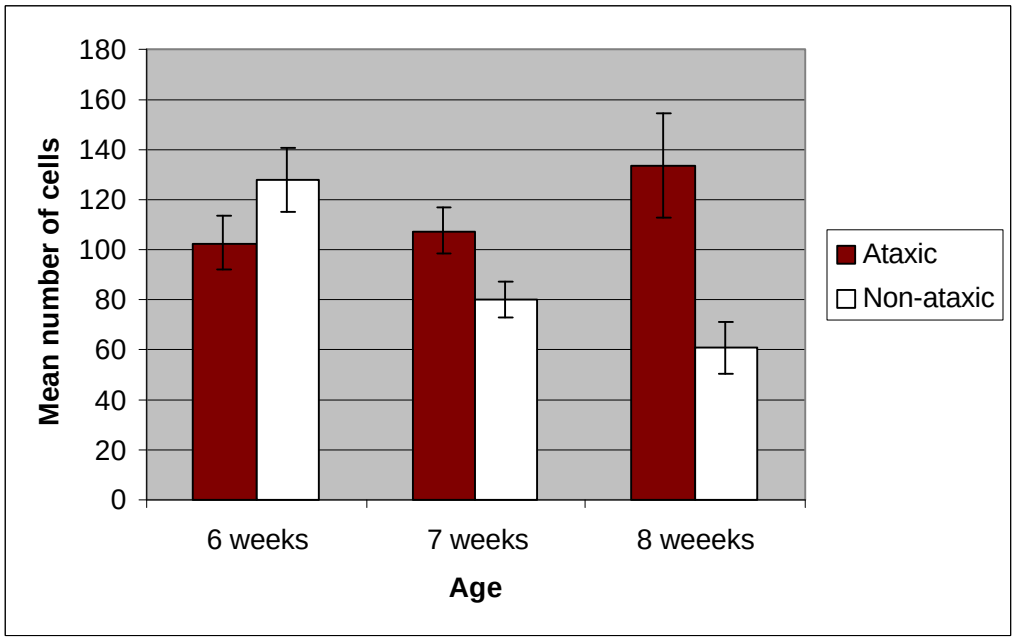
180
160
140
120
100
80
60
40
20
0
Mean number of cells
6 weeks
7 weeks
8 weeeks
Age
Ataxic
Non-ataxic

FIGURE 4.5 Immunohistochemical localization of NGF in cerebellar sections of ataxic JRT (A) and non-ataxic dogs (B). Cerebellar sections were immunolabeled for NGF using avidin-biotin-peroxidase method and counterstained with Hemotoxylin. In both ataxic and normal dogs, Purkinje cells were the only intensely stained cells. In the molecular layer some faintly stained cells were also recognized especially at the bottom portion of this layer. Note that more of these cells were stained in the ataxic JRT. Scale bars = 100 ìm.

FIGURE 4.5A

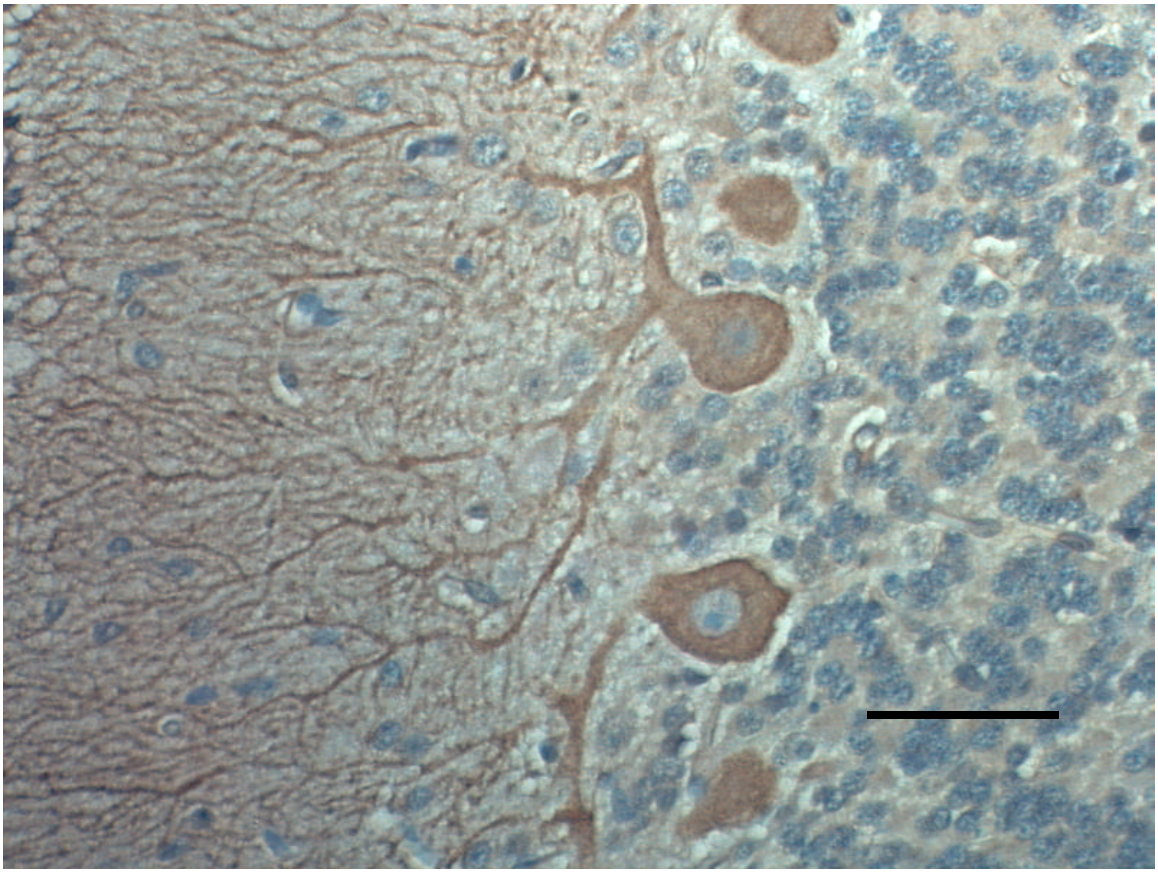

FIGURE 4.5B

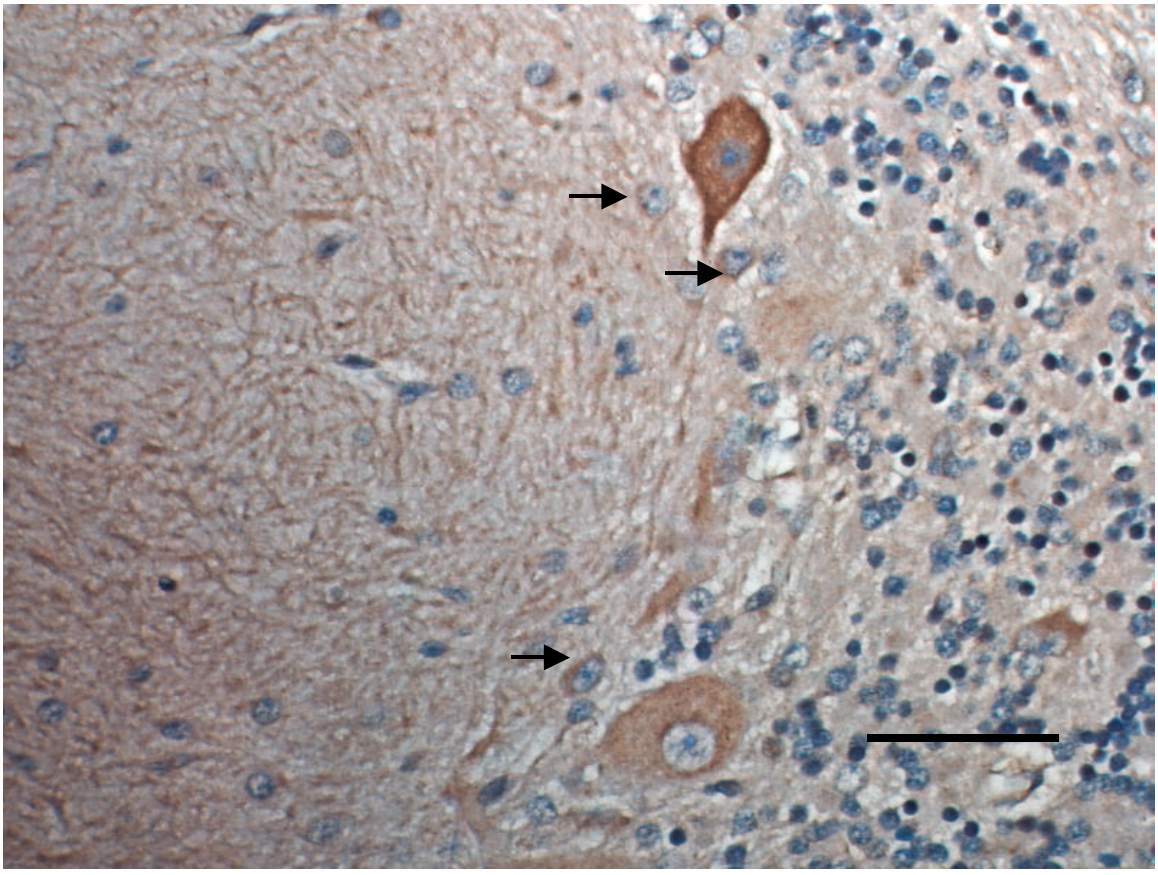

TABLE 4.1 Comparison of NGF-immunoreactivity on cerebella of ataxic JRT and normal dogs. Immunolabeled cells were subjectively compared based on the overall staining characteristics. In the ataxic dogs, stained cells located in the molecular layer were virtually less than those of the normal dogs. Purkinje cells and Golgi cells had relatively similar staining patterns between ataxic JRT and the normal dogs. Individual cases from three age groups tested were presented.

TABLE 4.1

	PC staining	Molecular layer cells	Golgi cells
Ataxic			
6 weeks	+++	+	++
	+++	+	+++
	+++	+	+++
	+++	+	+++
	+++	+	+++
7 weeks	+++	+	++
	+++	++	+++
	++	+	++
	++	+	++
	+++	++	+++
8 weeks	+++	+	+++
	+++	+	+++
	++	++	+++
	+++	+	+++
	+++	+	+++
Normal			
6 weeks	++	++	++
	+++	+++	++
	++	+++	+
7 weeks	+++	++	+++
	+++	++	+
8 weeks	+++	++	+++
	+++	+++	+++
	+++	+++	++
	+++	+++	+++
	++	+++	+++

+++; greater than 75% of cells were immunolabelled

++; 25-75% of cells were immunolabelled

+; less than 25% of cells were immunolabelled

CHAPTER 5

CONCLUSION

Cerebellar cortical degeneration is commonly recognized in domestic animals, though with higher frequency in dogs. Numerous case reports as well as studies on well-characterized disorders, such as Canine Inherited Ataxia of Gordon Setters, filled the literature (Björk et al., 1957; de Lahunta and Averill, 1976; Cork et al., 1981; Steinberg et al., 1981; Troncoso et al., 1985). Recently, a cerebellar cortical disorder was reported in Jack Russell Terriers (JRT) (Carmichael et al., 2002). This disorder differs from other cases in that there is degeneration of granule cells, without the apparent involvement of Purkinje cells. To the best of our knowledge there are only few case reports describing similar findings though with some differences (Hartley et al., 1978; Tago et al., 1993; Tatalick et al., 1993; Sandy et al., 2002). Certain murine mutants are known to show primary granule cell degeneration (Migheli et al., 1995; Norman et al., 1995). However, microscopic findings significantly differ from the current case in JRT. In several of these cases the type of cell death was reported to be apoptosis (Wüllner et al., 1995). Knowing that apoptosis plays a significant role during the normal development of the cerebellum, studies have been conducted on the role of neurotrophins that regulate neural survival. These studies have provided some knowledge of the pathophysiology of cerebellar degeneration. Such investigations into canine cerebellar degeneration are virtually absent. Most reports in dogs describe only macroscopic and light microscopic changes that occur in these disorders without explaining the underlying molecular changes. The overall goal

of this dissertation was to characterize a naturally occurring animal model of canine cerebellar degeneration and to use this model to study the molecular events leading to granule cell death. Specifically, I wanted to investigate the type of cell death that occurs during the course of the disorder, and to study possible mechanisms that might cause or regulate granule cell death.

We were able to successfully determine the type of cell death that took place in the granule cells of cerebellum from the ataxic JRT. Utilizing the TUNEL assay and electron microscopy, apoptosis was shown to be responsible for granule cell death in the current disorder. The ability of the TUNEL assay to detect true apoptotic cells has been debated extensively, elsewhere. In our case it was shown that the TUNEL assay confirms electron microscopic findings, and therefore it can still be used to provide considerable information about the dying cells. The assay was especially important in our case to make a quantitative investigation of the apoptotic cells. Using a series of different ages of ataxic dogs, we were partially able to course the level of cell death that occurred as the ataxic dogs got older. Based on the available ages of dogs used in the assay, we determined that after eight weeks of age the number of dying granule cells in ataxic JRT gradually decrease. Apoptotic cell death is apparently largely concluded by 6 months of age. This finding is in accordance with the slowly progressive clinical nature of the disorder. We have observed that after 6-8 months of age the ataxic gait does not progress, and the ataxic dogs learn how to compensate.

Presence of different levels of granule cell degeneration in individual dogs from the same age groups was significant. This finding might partially explain the reason for why ataxic siblings from the same litter occasionally show the clinical signs of different

severity. One important finding from the TUNEL assay was the presence of a few stained cells in the cerebellum of non-ataxic dogs. Apoptosis is known to play an essential role during the normal development of cerebellum by eliminating granule cells that fail to migrate from the EGL. However, we only detected very few cells in the EGL in both ataxic and non-ataxic dogs. Presence of only two or three cell layers in the EGL of cerebella in the dogs in this study might explain the very low numbers of TUNEL-stained cells in the EGL. However, a significant proportion of TUNEL-stained cells in the normal dogs was detected in the IGL. This finding is important since it is generally assumed that apoptotic cell death only takes place in the EGL and in lesser extent in the molecular layer during the development of healthy dogs. However, apoptosis-like cell degeneration has also been reported to occur, albeit in very low numbers, in the IGL and considered a normal part of development (Wood et al., 1993; Muller et al., 1995). Our findings confirm the presence of extremely low numbers of dying cells in the IGL of non-ataxic dogs. However, our finding contradicts the previous results in which the programmed cell death occurring in the IGL did not involve DNA fragmentation. Although granule cell death has been described in the IGL of healthy individuals, this is the first report describing, to the best of my knowledge that this type of cell death is due to apoptosis.

We did not recognize any changes in the pattern of staining with synaptophysin and Calbindin D-28K between the ataxic and non-ataxic dogs. These findings suggest that the granule cell death is not due to degeneration and loss of Purkinje cells. Synaptic arborization of neurons in the cerebellar cortex was also preserved in the ataxic dogs

suggesting that the death of granule cells is not due to loss of contact with other cells in the cerebellar cortex.

After establishing the type of cell death as apoptosis in the degenerating cerebellar granule cells in the ataxic JRT, immunohistochemical studies with Bcl-2 and Bax provided important findings. First, Bax is overexpressed in the IGL granule cells of ataxic dogs and is only barely detectable in the cerebellum of non-ataxic dogs. Second, Bcl-2-immunoreactivity can be detected in every type of cerebellar cortical neurons of both non-ataxic and ataxic dogs with little to no differences between the two groups. This suggests that apoptotic cell death in ataxic JRT is possibly Bcl-2 independent and that Bax overexpression may be the result of decreased trophic support to the granule cells.

Bcl-2 and Bax are two of the important regulators of apoptosis. While Bcl-2 acts in an anti-apoptotic fashion, the action of Bax is pro-apoptotic. Though this generalized functions, and their behaviors during the apoptosis or cell survival is quite complicated. Bax is shown to be overexpressed in apoptotic cell death (Vekrellis et al., 1997; Chao and Korsmeyer, 1998). It has been therefore proposed that the relative abundance of Bax to anti-apoptotic Bcl-2 family of proteins might be the cause for the initiation of apoptotic cell death (Stewart, 1994). We also showed increased Bax expression in the granule cells of cerebellum in the ataxic JRT. The number of Bax-positive cells in ataxic dogs was significantly higher than that of non-ataxic dogs. In the non-ataxic dogs, almost all of the granule cells were unreactive for Bax. It was reported that Bax is downregulated after the period of developmental cell death in the rat cerebellum and its level decreases about 140 fold (Vekrellis et al., 1997). The same report showed that though there were decreases in the overall expression of Bax in the cerebellar cortex, its expression level did not change

in Purkinje cells. Immunohistochemical labeling of cerebella of non-ataxic dogs also showed no expression of Bax in the granule cells of the IGL. However, we did not observe prominent expression of Bax in Purkinje cells. There were only occasional Purkinje cells that had very faint immunostaining in the cytoplasm in both ataxic and non-ataxic dogs. Therefore, our results suggest that there are differences in the canine and murine expression of Bax in the cerebellum. Our results, however show similar absence or downregulation of Bax expression in the cerebellar granule cells located in the IGL in non-ataxic dogs.

Presence of very low numbers of Bax-reactive cells in the cerebellum of non-ataxic dogs was an interesting finding in this study. In these dogs, similar numbers of apoptotic cells were also detected in the TUNEL-assay. These apoptotic cells were also located in the IGL. Therefore, these results show that Bax is normally expressed in the IGL of non-ataxic animals, albeit at much lower levels. Our results appear to indicate that in the canine cerebellum, Bax is involved in apoptotic granule cell death.

Other workers have shown that elimination of Bax expression during the development of the cerebellum in mice causes increased Purkinje cell, but not granule cell, numbers (Fan et al., 2001). Therefore, it was proposed that Bax is not involved in the elimination of granule cells in the EGL during the morphogenesis of the cerebellum. We also did not detect any cells with Bax-immunoreactivity in the EGL of non-ataxic dogs. On the other hand there are reports describing that Bax is overexpressed during the physiological death of cells in the EGL (Vekrellis et al., 1997). Therefore, these contradictory results require more investigation in the involvement of Bax during the development of the cerebellum.

In the current study, Bcl-2 immunohistochemistry showed that Bcl-2 is expressed in every type of cerebellar cortical neurons in both ataxic and non-ataxic dogs. There were no detectable differences in both the number and the distribution of Bcl-2 immunoreactive cells between the ataxic JRT and non-ataxic dogs. Presence of prominent Bcl-2 immunoreactivity in the cerebellar cortical cells strongly suggests survival of these cells might be controlled by Bcl-2 activity. High Bcl-2 expression in granule cells has been previously reported (Merry et al., 1994). In the same report, the authors also showed Bcl-2 expression in the Purkinje cell precursors. Our findings also confirm the presence of high Bcl-2 expression in the granule cells located in all layers of the cerebellar cortex. We were also able to show high Bcl-2 immunoreactivity in the Purkinje cells in both ataxic and non-ataxic dogs. However, there was somewhat differential staining on these cells, while some had strong immunoreactivity others did not have immunostaining or stained minimally. This might suggest that survival of Purkinje cells might not depend on the expression of Bcl-2. Another explanation for this immunolabeling pattern might be probable abundance of other anti-apoptotic molecules that might compensate for Bcl-2.

We also investigated expression of neurotrophins, NGF and NT-3, and the common neurotrophin receptor $p75^{NTR}$. Immunohistochemical labeling of cerebellar sections showed that $p75^{NTR}$ was expressed in every cell type in the cerebellar cortex. However, some granule cells with $p75^{NTR}$ overexpression were recognized. Since these receptors are potent death receptors, such an overexpression in these cells might explain the death of granule cells. Interestingly, $p75^{NTR}$ expression was recognized in granule cells located in the IGL and molecular layer in both ataxic and non-ataxic dogs. This might suggest that this receptor might act as a cell survival receptor in healthy animals.

Upon overexpression, changing pattern of functions might be mediated by $p75^{NTR}$. This receptor was shown to act as a regulator for Trk receptors (Benedetti et al., 1993; Bibel et al., 1999). $p75^{NTR}$ could also limit the trophic support by binding the available neurotrophins. Since $p75^{NTR}$ can bind all neurotrophins, its action could be broad in the nervous system.

Decreased immunoreactivity of NGF in the granule cells located in the molecular layer detected in the ataxic JRT might explain a different but may be related granule cell death pathway. During the migration of these cells, while they are still in the molecular layer, a change in the trophic support would certainly destruct the state of well being. Recently, it has been shown that NGF binding to $p75^{NTR}$ would provide anti-apoptotic signals through activation of nuclear factor kappa B in schwannoma cells (Gentry et al., 2000). These cells do not have TrkA receptor. Similarly, cerebellar granule cells do not express TrkA receptor (Ernfors et al., 1992) and we showed the presence of $p75^{NTR}$ in these neurons. Therefore, expression of $p75^{NTR}$ in the absence of TrkA might mediate NGF dependent survival of granule cells during the migration. Reduced expression of NGF in the migrating granule cells would directly be the reason for the initiation of apoptotic cell death or this decrease would disturb the homeostasis which then might cause these cells to change the expression of other genes that would initiate the apoptotic cell death. One certain change might be overexpression of $p75^{NTR}$ and Bax in these cells. Bax would certainly initiate apoptotic pathways while overexpression of $p75^{NTR}$, as suggested before (Dowling et al., 1999; Chang et al., 2000), might be only a result of cell being under stress but not the cause of cell death. Complex functions of $p75^{NTR}$ certainly require more research to clarify the role of this receptor during apoptosis and health.

Increased number of immunoreactive Golgi cells in the ataxic dogs was also an interesting finding. This increase might correlate with the trophic factor binding ability of these cells, therefore providing less survival signals to granule cells.

NT-3 is known to have important functions in the cerebellum such as, increasing the fassiculation of neurons and promoting the survival of Purkinje cells (Lindholm et al., 1993a; Segal et al., 1995). We did not detect any prominent changes both in the number and the pattern of staining for NT-3 between the ataxic JRT and non-ataxic puppies. This finding suggests that NT-3 is not involved in the degeneration of granule cells. Preservation of Purkinje cell number with unchanged expression of NT-3, might confirm the survival action of this neurotrophin on Purkinje cells, and might suggest that NT-3 might be involved in the common cerebellar cortical degeneration characterized by loss of Purkinje cells primarily.

In this study we were only able to investigate a limited number of factors that might be involved in the apoptotic cell death of granule cells. One other neurotrophin, BDNF, is also worthy of investigation in the survival of granule cells. This neurotrophin is shown to be widely expressed in the cerebellar granule cells and provide survival and axonal outgrowth of these cells (Lindholm et al., 1993b). However, BDNF was found to be not effective in protecting dying granule cells in the *weaver* mice (Gao et al., 1995). Therefore, the role of BDNF in the granule cell survival requires more research. In addition, Trk receptor expression might change in the course of granule cell degeneration. Among these receptors, TrkA and TrkB seem to be the best targets for investigation because of the binding capacity of TrkB to BDNF, and of TrkA to NGF. We detected decreased NGF immunoreactivity in the migrating granule cells. Changes in the

expression of Trk receptors in response to deprivation of neurotrophins and/or as a result of initiation of apoptotic cell death are among the subjects needed to be investigated in the future. In addition, the action of other anti- and pro-apoptotic molecules in the apoptotic granule cell death might be investigated. For example, expression of Bcl-x_L might be studied. Although we did not find any changes in the Bcl-2 immunoreactivity, decreases in the other anti-apoptotic molecules might weaken these cells' resistance against slight changes in the trophic support.

Collectively, the studies presented in this dissertation revealed that primary granule cell degeneration in Jack Russell Terriers is mediated by apoptosis. Apoptotic cell death of granule cells is due to overexpression of Bax. Decreased NGF level in the migrating granule cells of cerebellar molecular layer in the ataxic JRT might start degrading homeostasis of these cells. However, this initial change is probably not strong enough to cause cell death in this layer. On the other hand, in those cells that the initial change occur early in the development cell death can take place within the molecular layer as shown with the *in situ* TUNEL assay. Since a time lapse occurs from the initial change to the cell death, most of these dying cells are located in the deeper portions of the molecular layer. As the cells with the initial change continue to migrate into the IGL they possibily change the expression of $p75^{NTR}$. Though $p75^{NTR}$ is normally expressed in the granule cells of IGL, its function might be to regulate the specificity of Trk receptors. With the increased $p75^{NTR}$ level, these receptors might act as death receptors and cause the death of granule cells. Almost complete absence of high $p75^{NTR}$ immunoreactivity in granule cells of IGL in the non-ataxic dogs supports this hypothesis. Overall changes in the granule cells located in the IGL might easily affect other cell types located in this

cerebellar layer and cause changed expression of cell surface receptors and neurotrophins.

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