TEMPORAL EXPRESSION OF NONAPOPTOTIC CASPASE 3-POSITIVE CELLS IN THE DEVELOPING RAT CEREBELLUM

by

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ABSTRACT

Apoptosis, or programmed cell death (PCD), is a normal physiological event whereby unnecessary cells are eliminated from the body. Caspase 3 has been extensively studied as a central mediator of apoptosis. Previous research in our lab eluded to a nonapoptotic function, and possibly even a developmental role for active caspase 3 in the external granule layer (EGL) of the rat cerebellum. In the developing rat cerebellum, the folia (or lobules) express different rates of maturation for the formation of the Purkinje cells (Altman, 1985) and for the internal granule cell layer (IGL) (Altman, 1972). Although active caspase 3 has been associated with various developmental events, to date the expression of active caspase 3 in relation to cerebellar folial development has not been examined. The expression of active caspase 3 in relation to Bergmann glial cells also has never been examined.

The purpose of this study is twofold. The first hypothesis is that the expression of active caspase 3-positive cells in the vicinity of the Purkinje cell layer (PCL) is related to the development of cerebellar folia, as well as being developmentally related with respect to age and region of the cerebellum. The second hypothesis is that active caspase 3 has a nonapoptotic role in the cells surrounding the Purkinje cells (PCs) in the developing rat cerebellum.

The present study incorporates immunohistochemistry, using a confocal laser scanning microscope to determine the abundance of caspase 3-positive cells within each cerebellar folia of rats at postnatal days 9, 21, and 30. It also employs TUNEL and Annexin V to determine apoptosis, or lack thereof. We have observed the presence of active caspase 3-positive cells in close proximity to the Purkinje cells. Caspase 3-positive cells are minimal at 9 days of age. At 21 days and 30 days, caspase 3-positive cells appear to be prevalent in all lobules. The caspase 3-positive cells did not colocalize with TUNEL or Annexin V.

These findings suggest that the expression of active caspase 3-positive cells in the vicinity of the Purkinje cell layer correlates with development in the rat cerebellum with

respect to age and region, but not with folial development. They also suggest that active caspase 3 has a nonapoptotic role in the cells that are in the proximity of the Purkinje cells in the developing rat cerebellum. These cell were identified in coincidental studies in our lab as being Bergmann glial cells. The nature and functions of nonapoptotic caspase 3 in Bergmann glial cells are still being investigated by our lab, although it is believed to play a role in the differentiation of the cells. The significance of this study is that it is the first that we are aware of to examine active caspase 3 in the Bergmann glial cells. It is also the first that we are aware of to examine a nonapoptotic role for caspase 3 in the developing cerebellum.

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CHAPTER I
INTRODUCTION

Cerebellum

The cerebellum is a highly corrugated structure, located at the base of the brain. It has many infoldings, called folia or lobules. Due to the extensive folding that occurs in the cerebellum, 85% of its surface area is obscured (Barr et al., 1993). There generally are ten lobules, each with its own name, as follows: lobule I is the lingula, lobule II is the centralis (ventral), lobule III is the centralis (dorsal), lobule IV is the culmen (ventral), lobule V is the culmen (dorsal), lobule VI is the declive, lobule VII is the tuber, lobule VIII is the pyramis, lobule IX is the uvula, and lobule X is the nodulus (Fig. 1). Lobule VI is generally divided into VIa and VIb, which technically makes eleven lobules. The names of the lobules have no functional significance (Barr et al., 1993). At times, as the animal develops and ages lobules I, II, and X are lost as they diverge into other brain structures. The cerebellum is also divided into a vermis region, which is on the midline and controls spinal cord movements, and the two hemisphere regions on each side of the vermis that controls the voluntary movements of the arms and legs (Barr et al., 1993; Ganong, 2001).

Four distinct layers characterize the cerebellum (Fig. 2). The outer most layer is the external granule cell layer (EGL). It consists of two different developmental regions: the proliferative and premigratory zones. The proliferative region contains cells that are completing mitosis. The premigratory region contains cells that have already completed mitosis and are ready to migrate to their final destinations. These latter cells will eventually become the stellate cells, basket cells, and granule cells.

The second layer is the molecular layer (ML), which is the migratory region. Cells migrate from the external granule cell layer through the molecular layer to their final destinations. This layer is composed mostly of inhibitory neurons known as basket cells and stellate cells (Ganong, 2001). Stellate cells are star shaped and located in the

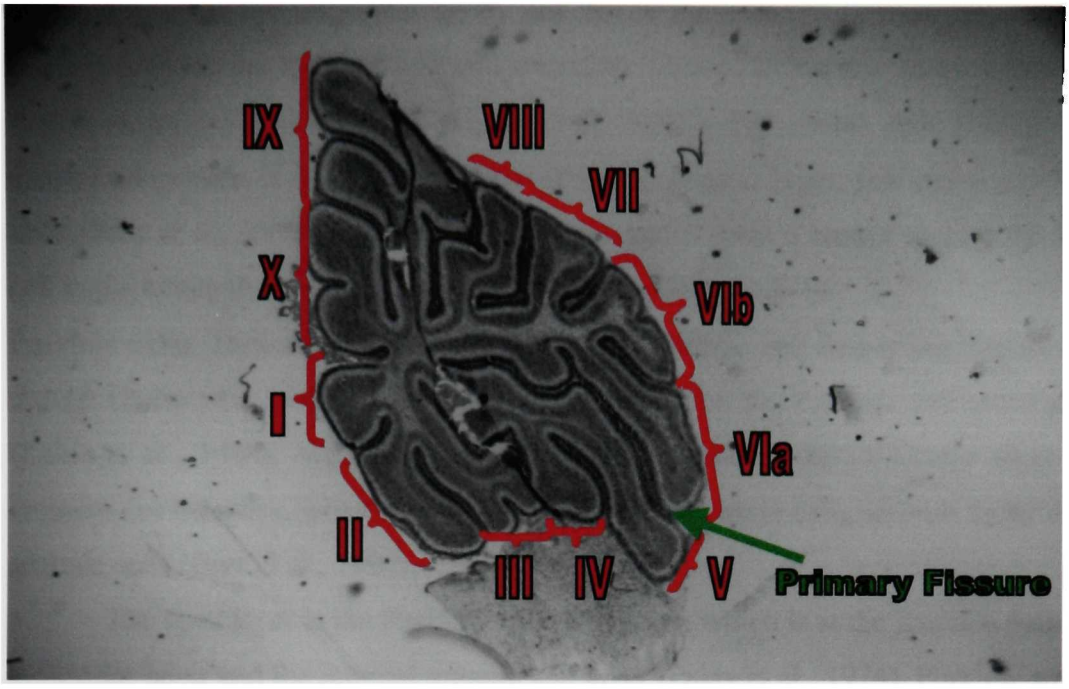

Fig. 1 Toluidine stained saggital section of the cerebellum, showing the eleven lobules. Lobule I = lingula; lobule II = centralis (ventral); lobule III = centralis (dorsal); lobule IV = culmen (ventral); lobule V = culmen (dorsal); lobule VIa and VIb = declive; lobule VII = tuber; lobule VIII = pyramis; lobule IX = uvula; lobule X = nodulus.

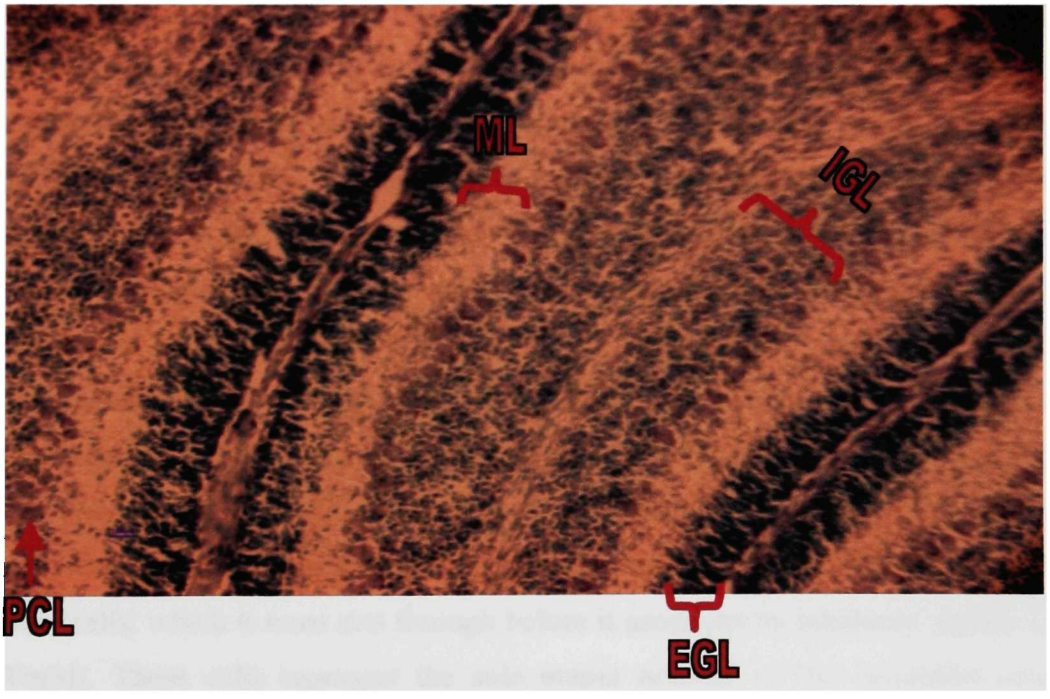

Fig. 2 Toluidine stained, 60X image showing the four distinct layers of the cerebellum. EGL = external granule cell layer; ML = molecular layer; PCL = Purkinje cell layer; IGL = internal granule cell layer.

upper portion of the molecular layer, just below the external granule cell layer. Their axons terminate on the Purkinje cell dendrites (Palay, 1974) and exert a finely tuned inhibition on the Purkinje cells (Laine et al., 1998). The basket cells are horizontally shaped and reside in the lower portion of the molecular layer, just above the Purkinje cells (Barr et al., 1993; Ganong, 2001). Their axons form a basket around the Purkinje cell body, hence their name, and may come in contact with 8-10
Purkinje cells. They usually do not contact the Purkinje cell that is the closest to them though (Palay et al., 1974). They exert an inhibition on the Purkinje cell soma and axon (Laine et al., 1998). Migrating granule cells are found in the molecular layer. It also contains the dendritic spines of the Golgi cells and Purkinje cells, as well as the axons of granule cells (Barr et al., 1993).

The third layer is the Purkinje cell (PC) layer, which is at the junction between the molecular layer and the internal granule cell layer (Palay et al., 1974). Purkinje cells are a single row of cells that outline each lobule of the cerebellum (Palay et al., 1974; Ganong, 2001). There are about 15 million Purkinje cells in the human cerebellum (Palay et al., 1974; Barr et al., 1993), but these are not the most numerous cells in the cerebellum. Purkinje cells look like little trees. Their cell body is teardrop shaped and they send their highly branched dendrites into the molecular layer to synapse with the parallel fibers (Barr et al., 1993). The Purkinje cells are spaced farther apart at the depths of each folia than they are at the crests of each folia (Palay et al., 1974). Climbing fibers originate from the inferior olivary cells in the brainstem, enter the molecular layer and twist themselves around the Purkinje cells soma and dendrites, making several synaptic contacts with each cell (Barr et al., 1993). Each climbing fiber exerts an excitation on a single Purkinje cell via glutamate neurotransmitters (Palay et al., 1974; Ganong, 2001). This excitation is known as a "complex spike." Through the mossy fibers, parallel fibers, and climbing fibers, a single Purkinje cell receives about 200,000 excitatory inputs from other cells, which it must sort through before it sends out its inhibitory signals (Purkinje World). These cells represent the sole output neurons of the cerebellar cortex. The

Purkinje cells send their axons through the granule cell layer to terminate on the deep cerebellar nuclei located in the white matter
(Barr et al., 1993; Palay et al., 1974). Neurons originating from these nuclei project to various brainstem sites. Purkinje cells are the only neuron whose axon reaches past the grey matter (Palay et al., 1974).

The Purkinje cell layer also consists of Lugaro cells and Bergmann glial cells. The Lugaro cells are neurons located at the interface between the Purkinje cells and the internal granule cell layer, at the level of the Purkinje cell soma. They are smaller than Purkinje cells, and have a fusiform cell body with long dendrites that stretch from opposite poles (Laine et al., 1992). The Lugaro cells exist in a ratio of 1:7.7 with Purkinje cells (Laine et al., 2002) They target basket cells and stellate cells in the molecular layer (Laine et al., 1998). They can also be found, to a lesser extent, interspersed in the depths of the internal granule cell layer, where they exhibit a more triangular shape (Sahin et al., 1990). Their axons run parallel to the parallel fibers. They receive inhibition from Purkinje cell axons, and excitation from granule cell axons (Laine et al., 2002). Bergmann glial cells, also known as Golgi epithelial cells, are a special kind of radial glial cell or radial glial astrocyte (Hanke et al., 1987). They are found in the cerebellum of all vertebrates from fish to humans (Hanke et al., 1987). They lie around the soma of Purkinje cells, and their fibers extend through the molecular layer out to the pial surface (Yamada et al., 2002). They are round or oval shaped, and smaller than Purkinje cells, but larger than granule cells (Shiga et al., 1983). The Bergmann glial cells thoroughly surround the somata, dendrites, and synapses of the Purkinje cells (Yamada et al., 2002). There are roughly 8.1 Bergmann cells per Purkinje cell (Yamada et al., 2002). These cells are thought to insulate, maintain, and regulate Purkinje cell structure and function in the adult brain, while their fibers (or processes) are thought to be involved with the glial guided neuronal migration of granule cells during the development of the brain (Yamada et al., 2002).

The fourth layer is the internal granule cell layer (IGL), containing the granule cells and Golgi cells. Granule cells are the smallest and most numerous neurons in the

cerebellum (Palay et al., 1974). There are about 10^{10}-10^{11} granule cells in the entire human cerebellum. There are 3000-9000 granule cells for every Purkinje cell. In the rat, there are about 250 granule cells for every Purkinje cell (Palay et al., 1974). They occupy about one-third of the entire cerebellum. Granule cells receive inputs through mossy fibers from the spinal cord and brainstem. Mossy fibers can contact an average of 28 granule cells. They send their axons up into the molecular layer, where they bifurcate to give rise to parallel fibers, which run parallel to the cerebellum (Palay et al., 1974; Barr et al., 1993). The parallel fibers synapse with the dendrites of the Purkinje cells. Each granule cell can traverse the dendritic tree of approximately 450 Purkinje cells (Barr et al., 1993). They can also excite basket and stellate cells via their parallel fibers (Ganong, 2001).

Golgi cells are dispersed throughout the entire internal granule cell layer. They are larger and more densely populated in the upper portion of the internal granule cell layer, and smaller and more sparsely populated in the lower portion of the internal granule cell layer (Palay et al., 1974). There is only one Golgi cell to 5700 granule cells (Palay et al., 1974). They establish inhibitory synapses with the granule cells on the mossy fibers. The Golgi cells receive inputs from the parallel fibers through their axons or dendrites, which then send the axonal inputs through the mossy fibers to suppress the excitation of granule cells (Ganong, 2001). Thus, the granule cells provide a negative feedback loop onto themselves. Golgi cells also receive inputs from mossy fibers, climbing fibers, and Purkinje cell collaterals, and provide a feed forward inhibition on granule cells.

Generally, in regions of the central nervous system, where cells are stacked in layers, as they are in the cerebellum, the layers are composed of postmitotic cells that originate in a ventricular to pial fashion (inside to outside). The exception to this is the formation of the granular layers, which form in an outside to inside fashion. As the cerebellum matures, cells migrate from the external granule cell layer, through the molecular layer, along Bergmann glial fibers, into the internal granule cell layer (Brown et al., 2001). The external granule cell layer begins to diminish over time, and eventually

disappears altogether, between 21 days and 30 days in the rat (Altman, 1972). The internal granule cell layer is not present at birth. It begins to appear at approximately 9 days in the rat, and around 21 days it is at its maximum thickness.

The cerebellum is vital for most motor functions, which include: maintenance of balance and erect posture, voluntary movements of the arms and legs, and motor learning when acquiring skilled orchestrated movements (Barr et al., 1993). Everyday activities require the use of the cerebellum, such as: walking, dancing, typing, riding a bicycle, skating, eating, sewing, and skiing. The cerebellum compares external messages with internal messages, and makes the necessary adjustments so that the planned movement becomes an actual, real movement that remains continuous until such time that the cerebellum receives inputs to stop the movement (Vander et al., 1994). It does not initiate voluntary movements (Vander et al., 1994), but it helps to plan, organize, and smooth the voluntary movements (Ganong, 2001).

It is also believed that the cerebellum is important for some forms of cognitive learning and sensory filter involvement. Recent findings have found that the cerebellum may play important roles in short term memory, attention, impulse control, emotion, higher cognition, the ability to plan and schedule tasks, and possibly even in conditions such as schizophrenia and autism (Bower et al., 2003). Removing the cerebellum could result in lack of motor coordination (Bower et al., 2003). Damage to the cerebellum has resulted in inaccurate judgement of duration of sounds, errors in performing certain verbal tasks, and errors in detecting speed and direction of moving objects (Bower et al., 2003). Neurodegenerative diseases that shrinks the cerebellum has resulted in inaccuracies in distinguishing sound of different tones or errors in discriminating between similar sounding words (Bower et al., 2003).

Lesions to the vermis result in spasticity of the body. Lesions of the hemispheres result in ataxia during movements. Damage to the cerebellum can also result in slurred speech, overcompensation in movements, past pointing, and intention tremors (Ganong, 2001).

Apoptosis

For every cell, there is a time to live and a time to die. Cells can die by apoptosis or necrosis. Apoptosis is termed "programmed cell death" (PCD) because the steps involved in the apoptotic process are programmed by genes. In this sense, cells "know" when to die. Apoptosis is required for normal development of the brain. The brain produces more cells than it needs in order to make the proper necessary synaptic connections between neurons. The surplus cells are eliminated through the process of apoptosis (D'Mello et al., 2000; Brown et al., 2001).

Apoptosis is necessary to destroy cells that threaten the integrity of the organism (D'Mello et al., 2000), such as cells infected with a virus, or cancer cells. Programmed cell death has been implicated in Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, and HIV. Apoptosis occurs in healthy adults. As cells continuously proliferate, the unwanted cells are destroyed via programmed cell death.

There are even some normal physiological events that occur via apoptosis. These include: the reabsorption of a tadpole's tail at the time of metamorphosis into a frog, the removal of the webbed tissue between the fingers and toes of a fetus, and the sloughing off of the inner lining of the uterus at the beginning of menstruation (Ultranet).

Characteristics of apoptosis include: condensation of the nucleus and the cytoplasm within the cell, cell shrinkage, development of bubble-like blebs on the cell membrane, DNA degradation within the nucleus, cellular decomposition into small fragments known as apoptotic bodies, engulfment by macrophages, and elimination by phagocytosis (Brown et al., 2001; Beer et al., 2000). Apoptosis is a "friendly" method of cell death, as opposed to necrosis, because it does not interfere with the function of other cells in its vicinity. With necrosis, a cell swells and bursts, which leads to inflammation of the surrounding tissue, and may cause other neighboring cells to die. Apoptosis does not elicit an inflammatory response, and it produces no ill effects (Beer et al., 2000). It is a silent, self-contained form of cell suicide.

Mechanics of Apoptosis

Apoptosis occurs by way of internal or external signals (Ultranet). In healthy cells the mitochondria expresses Bcl-2 on its surface, which binds to APAF-1 (Apoptosis Activation Factor-1) proteins located in the cytoplasm (Geocities). This binding keeps cytochrome C from escaping the mitochondria. When a cell begins to die, the Bcl-2 protein unbinds from the APAF-1 protein, which allows cytochrome C to leak out of the mitochondria through unknown mechanisms. Cytochrome C binds to the APAF-1 protein and to pro-caspase 9 (Kuan et al., 2000; Slee et al., 2001). This three-way binding forms a complex known as the "apoptosome," which aggregates in the cytosol of the cell (Ultranet). Caspase 9 is cleaved, which cleaves and activates other downstream effector caspases, like caspase 3, which in turn activates other proteins and enzymes (Kuan et al., 2000). This sequential activation of caspases and proteins is known as the caspase cascade, which ultimately leads to cell death. Cells also have "death" receptors on their cell membrane surface, such as FAS receptors and TNF (tissue necrosis factor) receptors. When "death" activators such as FAS and TNF bind to their appropriate receptors, it transmits a signal into the cytoplasm of the cell that leads to the activation of caspase 8, using external signals (Ultranet). This can also stimulate the caspase cascade.

Caspases

Caspases are a group of cysteine proteases that have a cysteine residue in their active site that cleave after aspartic acid residues (Slee et al., 2001). There are 14 known caspases to date that are divided into two main classes: initiators or activators, and effectors or executioners (Slee et al., 2001). The initiators are upstream activators of other caspases, generally reserved for caspases 8 and 9; while effectors are downstream executioners, generally 2, 3, 6, 7, and 10, which cleave other proteins that actually induce apoptosis (Geocities). Caspases exist as immature pro-caspases that must be cleaved after their aspartic acid residue in order to become active (Slee et al., 2001). Upon activation, caspases are believed to translocate to the nucleus where they trigger substrate

proteolysis, chromatin condensation, margination of chromatin to the periphery of the nucleus, and fragmentation of DNA (Slee et al., 2001).

Once active, caspases can: cleave other caspases to make them active, activate endonucleases which fragment and destroy DNA, cleave PARP which normally repairs DNA damage, activate destructive enzymes such as DNAses, promote cytochrome C release via proteins such as BID, degrade structural and regulatory proteins within a cell, and cleave other important enzymes (Kuan et al., 2000; Slee et al., 2001). The caspase cascade, mentioned earlier, is typically thought to occur in a stepwise fashion as follows: 9 - 3 - 7 - 2 - 6 - 8 -10 (Slee et al., 2001). As mentioned above, caspase 8 can sometimes initiate the caspase cascade.

Caspase 3

Caspase 3 is generally considered as an effector caspase principally because it is not the very first caspase encountered in the caspase cascade. Caspase 3 can be, and should be, considered as an initiator/effector. It is an initiator because it activates caspase 2 and 6, which are downstream from caspase 3 (Geocities). However, it is considered as the primary executioner caspase because it cleaves other important proteins that are necessary to induce apoptosis. Caspase 3 induces apoptosis by: cleaving ICAD (Inhibitor of CAD), which then allows CAD (Caspase Activated Deoxyribonuclease) to cleave DNA into fragments; cleaves nuclear lamins, which allows the nucleus to condense; and it cleaves PAK-2, which plays a role in membrane blebbing (Slee et al., 2001). As mentioned earlier, DNA fragmentation, nuclear condensation, and membrane blebbing are all hallmarks of apoptosis. Caspase 3 can also go back and cleave more caspase 9, which in turn cleaves even more caspase 3 (positive feedback loop) (Geocities). This ensures that the caspase cascade leads to irreversible cell suicide.

Caspase 3 has been shown to be either cytoplasmic or nuclear in cells of the central nervous system (Beer et al., 2000). Activated caspase 3 was shown to be expressed in neurons, astrocytes, and oligodendrocytes (Beer et al., 2000). It has also been shown that caspase 3 was not necessary for low potassium induced granule cell

death, but that it was vital for chromatin condensation and DNA fragmentation to occur (D'Mello et al., 2000). It was also found that DNA fragmentation was not necessary for cell death to occur (D'Mello et al., 2000). It was demonstrated that active caspase 3 is present in the brain during the early stages of development, but very limited in postmitotic neurons in later developmental stages (Urase et al., 1998).

Nonapoptotic Functions of Caspases

It has long been understood that caspases are essential proteins for the advent of apoptosis. Recent studies in the past few years have shown that there are dual roles for some of the caspases. Caspases have been implicated in a number of nonapoptotic roles such as regulation of cell survival pathways, cell motility, regulation of cell cycle, proliferation, and differentiation. One study proved that caspases were required for terminal erythroid differentiation (Zermati et al., 2001). The caspase activation was associated with the morphological changes that occur during erythroid maturation (Zermati et al., 2001). The fact that this caspase activation was only transiently expressed suggested that there was some regulatory mechanism in place that prevented the caspases from causing cell death (Zermati et al., 2001).

In another study, it was demonstrated that lens fiber differentiation involves part of the processes of apoptosis (Ishizaki et al., 1998). Caspase 3 is required for this differentiation process to occur (Ishizaki et al., 1998). DNA fragmentation occurs as was shown with positive TUNEL results (Ishizaki et al., 1998). PARP was cleaved during the differentiation process (Ishizaki et al., 1998). This cleavage of PARP was inhibited by caspase inhibitors, which also inhibited the formation of anucleated lens cells (Ishizaki et al., 1998). This expression of caspase 3 was also transient because the lens cells differentiate into lens fibers, which essentially last a lifetime. The cell did not shrink, fragment, and become phagocytized as it would with apoptosis. Only portions of the apoptotic machinery are utilized during the differentiation process.

It has been suggested that the activation patterns of apoptosis and differentiation follow the same signal cascade. This is particularly evident in the differentiation of

skeletal muscles. For example, actin fiber disassembly/reorganization and increased matrix metaloproteinases essential for membrane fusion are features of both skeletal muscle differentiation and apoptosis (Fernando et al., 2002). Inhibition of caspase 3 drastically prolonged myoblast fusion and impaired myotube formation (Fernando et al., 2002). It was suggested that elevated caspase 3 activity was required for effective differentiation during the early stages of skeletal myogenesis (Fernando et al., 2002).

If there are dual roles for caspases, it appears to be a common phenomenon of caspase 3 to play an essential part in cellular differentiation. The present study will represent the first that we are aware of that looks at the possibility of active caspase 3 serving a nonapoptotic function in the cerebellum.

Scope of this Study

Apoptosis and caspase 3 are hot topics for current research projects. Most research focuses on the role of caspase 3 in apoptosis, and even suggests that caspase 3 plays a vital role in neuronal apoptosis. But the reality is that the role of caspases in neurons and glia remains to be defined. Very few studies have eluded to the idea that caspase 3 is involved in a developmental phenomenon that is nonapoptotic in nature (Zermati et al., 2001; Ishizaki et al., 1998; and Fernando et al., 2002). Previous research in our lab revealed a nonapoptotic function, and possibly even a developmental role for active caspase 3 in the developing rat cerebellum.

As stated earlier, the folia in the developing rat cerebellum express different rates of maturation. It is known that lobules I, II, IX, and X are all early developing for the internal granule cell layer; whereas lobules V, VI, and VII are late developing (Altman, 1972). It has been shown that lobules I-V are early developing, lobules VIa and VIII-X are intermediate, and lobules VIb and VII are late developing for the Bergmann glial cells (Shiga et al., 1983). Although active caspase 3 has been associated with various developmental events (Zermati et al., 2001; Fernando et al., 2002), to date the expression of caspase 3 in relation to cerebellar folial development has not been examined. Our lab observed the presence of active caspase 3-positive cells in the vicinity of the Purkinje cell

layer (PCL). Based on their location, it was conceivable to think that these cells could have been migrating granule cells, Bergmann glial cells, or Lugaro cell. For now, these cells will be considered as active caspase 3-positive cells. Based on the appearance of these cells, they were believed to be nonapoptotic in nature.

This study employed immunohistochemical techniques and laser scanning confocal microscopy to map out the abundance of active caspase 3-positive cells within each folia. This study also set out to determine whether or not the active caspase 3-positive cells are apoptotic using Annexin V and TUNEL procedures.

The **first hypothesis** is that the expression of active caspase 3-positive cells in the vicinity of the Purkinje cell layer is related to development of cerebellar folia, as well as being related to age and to cerebellar region. The **second hypothesis** is that active caspase 3 has a nonapoptotic role in the cells surrounding the Purkinje cells in the developing rat cerebellum.

To test these hypotheses, three specific aims were developed. **Specific aim #1**: to conduct immunohistochemical experiments using active caspase 3 antibody on 9-day-, 21-day-, and 30-day-old rat cerebellums in the vermal as well as the hemisphere regions. Then use a confocal laser scanning microscope to collect a series of images around every folia that was representative of the abundance of cells present. Finally, to take a representative count of the number of active caspase 3-positive cells that was present in each folia. **Specific aim #2**: to perform statistical analysis on the representative cell counts in order to determine if significant differences existed between the ages, lobules, and location. Also, to determine if the abundance of active caspase 3-positve cells followed some sort of developmental pattern. **Specific aim #3**: to conduct double labeled immunohistochemical experiments using active caspase 3 and TUNEL or Annexin V on the 9-day-, 21-day-, and 30-day-old rat cerebellums to prove that these active caspase 3-positive cells are nonapoptotic in nature.

These findings suggest that the expression of active caspase 3-positive cells in the vicinity of the Purkinje cell layer correlates with the development of the cerebellum. These findings also suggest that active caspase 3 has a nonapoptotic role in the cells that

are in the proximity of the Purkinje cells in the developing rat cerebellum. This study, along with previous research in our lab, contradicts the dogma, which states an apoptotic role for caspases in glial cells of the brain and charters a new area of research.

At this point, based on their location, their size, their abundance in relation to Purkinje cells, and their immunoreactivity for glial markers, it is believed that these active caspase 3-positive cells are Bergmann glial cells. This will be detailed further in the discussion of this paper. The nature of these cells and the nonapoptotic function of active caspase 3 in these cells are still being investigated by our lab.

CHAPTER II

MATERIALS AND METHODS

Cerebellum Slice Preparation

Sprague-Dawley (Charles River Laboratories) rats that ranged from postnatal day 1 to postnatal day 60 (P1- P60) were used for these experiments. The rats were housed in the institutional animal vivarium. They were maintained on a 12-hour light/12-hour dark cycle, and were given food and water ad libitum. The use of animals for this experiment, the method of taking the animals, and all procedures discussed in the text that follows were approved by the Texas Tech University Health Science Center Animal Care and Use Committee.

In the general discovery of the active caspase 3-positive cells in the vicinity of the Purkinje cell layer, the animals were decapitated, without any prior anesthesia, using a small animal quillotine. This was done quickly, efficiently, and with the utmost care so as to minimizes traumatization to the rat. A horizontal cut was made on the occipital bone using dissecting scissors. The occipital plate was carefully removed using dissecting forceps, which then exposed the cerebrum and cerebellum (cortex). The exposed cortex was rinsed in frozen artificial cerebrospinal fluid (aCSF) containing: 30ml NaCl (125mM), 30ml NaH_2PO_4 (1.25mM), 30ml glucose (20mM), 19ml KCl (4mM), 26ml $NaHCO_3$ (20mM), 111ml distilled H20, 30ml $CaCl_2$ (2.5mM) and 30ml $MgCl_2$ (1.15mM); pH 7.40.

The cerebellar peduncles were removed from the cerebellum by making a horizontal cut using a scalpel blade. The cerebellum was placed in a 10ml beaker filled with aCSF and oxygenated with 95% O^2 : 5% CO^2 for two minutes to remove any blood present on the tissue and to reduce any metabolism still occurring in the brain. The cerebellum was moved from the beaker to an ice-cold petri dish using a spatula. Excess fluid surrounding the cerebellum was absorbed using filter paper. The hemispheres were removed by making a parasaggital cut with a Gillette razor blade, leaving only the vermis. The vermis was then affixed to the slicing block using super glue. The slicing

block was placed in a slicing chamber and submerged into aCSF that was oxygenated with 95% O^2: 5% CO^2. The slicing chamber was secured to a Fred Haer vibrotome, model OTC-3000 or OTC-4000 (Fred Haer Inc., Brunswick, ME).

The vermis was then sliced saggitally or coronally into 400μm thick sections using a blade advance speed of 2 and a vibration speed of 7-8. The 400μm sections were removed from the slicing chamber using a fine soft artists red sable brush, and placed into a holding chamber that contained normal CSF (nCSF) maintained at room temperature and continuously oxygenated with 95% O^2 : 5% CO^2. The nCSF consisted of 48ml NaCl (125mM), 25ml KCl (4mM), 44ml $NaHCO_3$ (26mM), 50ml KH_2PO_4 (1.18mM), 28ml glucose (20mM), 215ml distilled H_2O, 50ml $MgSO_4$ (1.19mM), and 40ml $CaCl_2$ (2.5mM) The sections then were allowed to recover in the nCSF for one hour. They were fixed in formalin solution (Sigma, St. Louis, MO; catalog #HT50-1-1) overnight at 4°C, then transferred to 30% sucrose (Sigma, St. Louis, MO; catalog #S-9378) in 10X Dulbecco's phosphate buffered saline (PBS) (Sigma, St. Louis, MO; catalog #D-1283), diluted to 1X and adjusted to a pH 7.3, for at least one day at 4°C for cryoprotection.

For the purposes of mapping the expression of the active caspase 3-positive cells with the development of the folia, three 9-day-old rats, three 21-day-old rats, and three 30-day-old rats were used with cardiac perfusion being performed. These ages were chosen based on the development of the internal granule cell layer vs. the external granule cell layer. First, the rats were injected with 0.05cc, 0.1, and 0.2cc of (50mg/ml) pentobarbital (Nembutal Sodium Solution; Abbott Laboratories; N. Chicago, IL) intraperitoneally for the 9-day, 21-day, and 30-day rats, respectively. When the rats no longer exhibited righting and pain reflexes, the chest cavity was opened with dissecting scissors carefully so as to not puncture any internal organs.

A small butterfly needle was inserted into the left ventricle of the heart. The right atrium was then slit to allow blood to drain out of the body. Heparin was perfused into the heart using an infusion pump (Harvard Apparatus Compact Infusion Pump, model #975) set at a flow rat of 10cc per minute, with a minimum of 10cc being perfused, depending on the weight of the rat. Then, 4% paraformaldehyde (20gms/500ml 0.1M

PBS, pH 7.4) was allowed to perfuse into the heart with a flow rate of 7cc per minute, using a total of 60-80cc, until the animal became rigid. Subsequently, it was decapitated using a small animal guillotine. A horizontal cut was made on the occipital bone using dissecting scissors. The occipital plate was carefully removed using dissecting forceps, which then exposed the cerebrum and cerebellum (cortex). The exposed cortex then was fixed in formalin solution for one to two nights. It was transferred to 20% sucrose for one night, then to 30% sucrose for at least one night. At the time cryostat slicing was done, the cerebellar vermis and hemispheres were removed from the rest of the brain, and retained for slicing along with the brainstem attached.

For cryostat slicing, first a mounting block was made by filling a plastic mold with TBS tissue freezing medium (Fisher Scientific; catalog #1518313), laying a gold block on top of it, and placing it in a cryostat machine set at -30°C to freeze. For 400μm cerebellar slices, cerebellum sections were gently laid onto a glass microscope slide using a fine artists red sable paint brush; whereas for whole cerebellar vermal sections obtained from cardiac perfusion, the specimen was positioned onto the glass slide with the aid of the paint brush and a scalpel blade. A kim wipe was used to absorb all the excess fluid surrounding the section. The section was allowed to air dry for a few seconds while the plastic mold was removed from the frozen mounting block. TBS tissue freezing medium was poured onto the section. The slide was inverted allowing the tissue freezing medium to slowly drop onto the mounting block. The slide was slowly lowered to the mounting block, keeping it as level as possible. Once the slice had touched the mounting block, and some of the tissue freezing medium had begun to freeze, a weight was applied to keep it level and continue the freezing process.

The vermis sections were sliced into 12μm saggital or coronal slices using a Tissue Tek Cyrostat (Miles Inc., Elkhart, IN; model #4553) and affixed to a coverslip that was coated in a gelatin subbing solution (5mg gelatin/1000ml) to aid in adhering the slice to the coverslip. These were used for immunohistochemical studies. The vermis sections were also sliced into 6μm slices and affixed to microscope slides that were coated with a

gelatin subbing solution. These were stained with toluidine blue and used for histological analysis and active caspase 3-positive cell mapping.

Immunohistochemistry

The 12μm saggital or coronal vermis sections, that were affixed to the subbed coverslips, were placed in 10ml beakers and treated with PBS pH 7.4 for 3 minutes to remove the tissue freezing medium (OTC), then treated with acetone for 10 minutes at -20°C to enhance antigenicity. The sections were rinsed in PBS three times for five minutes each, then transferred to a 24-well plate. Then they were permeabilized overnight with 0.5% triton (Triton X-100; Sigma, St. Louis, MO; catalog 23,472-9) at 4°C.

The slices were incubated in 250μl of blocking solution, consisting of 1700μl PBS pH 7.4, 100μl 10% triton, and 200μl goat serum (Sigma, St. Louis, MO; catalog #G-9023) for 1 hour at room temperature. The type of serum actually used depended on what animal species the secondary antibody was made in. They were rinsed five times in PBS pH 7.4 for 5 minutes each. They were treated with 1% sodium borohydride ($NaBH_4$; Sigma, St. Louis, MO; catalog #S-9125), which has been made in PBS pH 8.0 for 30 minutes at room temperature to enhance antigenicity.

The slices were rinsed five to ten times in PBS pH 7.4 for 5 minutes each, until all bubbles disappeared. Then they were incubated in a 1:50 dilution active caspase 3 (Cleaved Caspase 3 Asp 175 Antibody; Cell Signaling Technology; catalog #9661L) primary antibody (1° ab) solution overnight at 4°C. The primary antibody solution was made using: 780μl PBST (high salt PBS (1M PBS with 0.5M NaCl) with Tween 20/Polyoxyethylene Sorbital Monolaurate; Sigma, St. Louis, MO; catalog #P-7949), 100μl goat serum, 100μl 10% triton, and 20μl active caspase 3 stock. This was accomplished by placing 50–75μl of the primary antibody solution into a coverwell incubation chamber (Research Products International Corp.; catalog #247000). A negative control was obtained by not placing one slice in the primary antibody solution that contained the active caspase 3 solution. Instead, it was placed into 50-75μl of PBST only. Then the slices were moved from the 24 well plate and gently laid, face down into

the primary antibody solution. The incubation chambers were placed in the lid of a humidified 24-well plate (consisting of dampened paper towels), aluminum foil was gently placed over the top of the slices, and they were placed the humidity controlled drawer of a refrigerator to prevent the slices from drying.

The slices were returned back to the 24-well plate and rinsed in 0.1% triton for 5 minutes, then rinsed in PBS pH 7.4 five times for 5 minutes each. Then the slices were incubated with 250µl of a 1:250 dilution of goat anti rabbit (GAR) Alexa 488 (Alexa-Fluor 488 Goat Anti Rabbit IgG Conjugate; Molecular Probes, Eugene, OR; catalog #A-11034) secondary antibody (2^{o} ab) solution or a 1:500 GAR Alexa 594 (Alexa-Fluor 594 Goat Anti Rabbit IgG Conjugate; Molecular Probes, Eugene, OR; catalog #A-11037) 2^{o} ab. This was performed for 2 hours at room temperature on a rotating rocker platform. The secondary antibody for a 1:250 dilution was made using: 852µl PBST, 102µl goat serum, 42µl 10% triton, and 4µl of the Alexa 488 stock. For the 1:500 dilution, it used 854µl PBST, 102µl goat serum, 42µl 10% triton, and 2µl of the Alexa 594 stock.

The slices were rinsed in 0.1% triton for 10 minutes, then rinsed in PBS pH 7.4 ten times for 10 minutes each. The slices were rinsed in distilled water for 5 minutes, then dehydrated in decreasing ethanol steps (40%, 70%, 95%, and 100%) for three minutes each. They were mounted to a microscope slide using Prolong Antifade mounting medium (Molecular Probes, Eugene, OR; catalog #P-7481). The sections were viewed using an MRC-1024 BioRad Laser Scanning Confocal Microscope.

TUNEL (Terminal Deoxynucleotidyl dUTP Nick End Labeling)

TACS 2 TdT-Fluor In Situ Apoptosis Detection (Trevigen Inc, Gaithersburg, MD Kit; catalog #4812-30-K) allows for the detection of DNA fragmentation, which is a late apoptotic event. All reagents mentioned in this protocol were included in the apoptosis detection kit. The 12µm saggital or coronal vermis sections, that were affixed to gelatin subbed coverslips, were placed in a 24-well plate and rehydrated with decreasing ethanol steps (100%, 95%, 80%, 70%, 40%) for 3 minutes each, then with PBS pH 7.4 for 10

minutes. The slices were then permeabilized with 50μl cytonin in the coverwell incubation chambers for 60 hours at room temperature in a dessicator.

The 60-hour permeabilization was chosen after much trial and error, beginning with the 30-minute procedure that was recommended in the standard protocol that Trevigen included with their apoptosis detection kit. When this failed to produce results, as determined by viewing the positive control with the confocal microscope, the time was increased to 1 hour, 24 hours, 48 hours, and then 60 hours. Positive results still were not obtained with 48 hours. Thus, 60 hours was chosen as the optimal permeabilization treatment time.

After permeabilization, the slices were transferred back to the 24-well plate. The slices were treated with a terminal deoxynucleotidyl transferase (TdT) labeling buffer for 5 minutes. Then the slices were incubated in the TdT labeling reaction mix in the coverwell incubation chambers for 2 hours at 37°C in a humidity chamber. The TdT labeling reaction mix consisted of 1μl TdT dNTP, 1μl Mn^{2+}, 1μl TdT enzyme, and 50μl TdT labeling buffer. A negative control was obtained by omitting the TdT enzyme from the above described labeling reaction mix, and a positive control was obtained by including 1μl TdT nuclease to the above described labeling reaction mix.

The slices were placed back into the 24-well plate and rinsed in a TdT stop buffer for 5 minutes, then rinsed in PBS pH 7.4 two times for 2 minutes each. Then the slices were incubated in 50μl strept avidin fluorescein 495 solution in the coverwell incubation chambers in the dessicator for 20 minutes at room temperature in the dark. The slices were rinsed in the 24-well plate with PBS pH 7.4 ten times for 2 minutes each, then mounted on a microscope slide with an antifade mounting medium and viewed with the laser scanning confocal microscope.

Annexin V

TACS Annexin V Biotin Kit (Trevigen Inc, Gaithersburg, MD; catalog #4835-01-K) allows for the detection of cell surface changes that occur early in the apoptotic process when phosphatidyl serine becomes exposed on the cell surface after flipping from

the inner cytoplasmic membrane to the outer membrane. After the 400μm brain sections had been obtained, as described above in the cerebellum slice preparation section, and the slices had incubated for one hour in the artificial CSF, the slices were treated then with Annexin V. This was accomplished by placing the slices in a lab fabricated holding chamber that allowed for continuous oxygenation to the slices while they were incubated in the Annexin V solution. This chamber consisted of a plastic Tupperware style square container with a tightly sealing lid. Inside the plastic container, a round glass petri style dish was placed upside down and sealed with dental wax. Then little round rings of dental wax were affixed to the glass. A small hole was poked into the lid of the plastic container to allow a small oxygenation tube to fit through it.

The slices were placed inside a wax ring. For every 10μl of Annexin V stock solution used, 100μl of warm (37°C) nCSF was added. Typically, to treat 3 cerebellar slices, 20μl of Annexin V stock solution supplied in the kit was added to 200μl of warm nCSF. The Annexin V mixture was then added around the slices with a pipette, without being dropped directly onto the slices. Then warm nCSF was poured into the plastic container to surround the glass petri dish until it was just below the little wax rings. The rubber oxygenation tube was inserted into the warm nCSF and the lid was sealed. The plastic container was then placed into a preheated (37°C) water bath to allow the slices to incubate in the Annexin V solution for 30 minutes. A negative control was obtained by placing one or two slices into a little wax ring surrounded with warm nCSF that contained no Annexin V. An additional negative control was obtained by placing one or two slices directly into paraformaldehyde without any type of Annexin V treatment.

The slices were placed in formalin solution overnight, then transferred to 30% sucrose/PBS. Vermal sections were sliced into 12μm slices and 6μm slices using a Tissue Tek Cryostat as described above in the cerebellum slice preparation section, and were used for immunohistochemistry and toluidine blue staining, respectfully.

Dual Labeling with Caspase 3 and TUNEL

To obtain dual labeling, it was important that the slices were treated with primary antibody first, followed by the TdT procedure, and finalized with a combined secondary antibody solution. This was necessary to limit the number of rinse cycles used to prevent loss of fluorescent labeling.

The 12μm saggital or coronal vermal sections, that were affixed to the gelatin subbed coverslips, were placed in a 24-well plate and rehydrated with decreasing ethanol steps (100%, 95%, 80%, 70%, 40%) for 3 minutes each, then with PBS pH 7.4 for 10 minutes. The slices were then permeabilized with 50μl cytonin in the coverwell incubation chambers for 60 hours at room temperature in a dessicator.

The slices were incubated in a 24-well plate with 250μl of blocking solution, which consisted of 1700μl PBS pH 7.4, 100μl 10% triton, and 200μl goat serum for 1 hour at room temperature. The type of serum actually used depended on what animal species the secondary antibody was made in.

Slices were rinsed five to ten times in PBS pH 7.4 for 5 minutes each, until all bubbles disappeared. Then they were incubated in a 1:50 dilution active caspase 3 primary antibody (1° ab) solution overnight at 4°C. The primary antibody solution was made using: 780μl PBST 100μl goat serum, 100μl 10% triton, and 20μl active caspase 3 stock. This was accomplished by placing 50–75μl of the primary antibody solution into a coverwell incubation chamber. A negative control was obtained by not placing one slice in the primary antibody solution that contained the caspase 3 solution. Instead, it was placed into 50-75μl of PBST only. Then the slices were moved from the 24-well plate and gently laid, face down into the primary antibody solution. The incubation chambers were placed in the lid of a humidified 24-well plate (consisting of dampened paper towels), aluminum foil was gently placed over the top of the slices, and they were placed in a humidity controlled drawer in the refrigerator.

Slices were returned back to the 24-well plate and rinsed three times with PBS pH 7.4 for 5 minutes each. Slices were treated with a terminal deoxynucleotidyl transferase (TdT) labeling buffer for 5 minutes. Then the slices were incubated in the TdT labeling

reaction mix in the coverwell incubation chambers for 2 hours at 37°C in a humidity chamber. The TdT labeling reaction mix consisted of 1μl TdT dNTP, 1μl Mn^{2+}, 1μl TdT enzyme, and 50μl TdT labeling buffer. A negative control was obtained by omitting the TdT enzyme from the above described labeling reaction mix, and a positive control was obtained by including 1μl TdT nuclease to the above described labeling reaction mix.

The slices were placed back into the 24-well plate and rinsed in a TdT stop buffer for 5 minutes, then rinsed in PBS pH 7.4 two times for 2 minutes each. The slices were then treated with 50-75μl of a combined secondary antibody solution for caspase 3 and TUNEL in the coverwell incubation chambers, laid on the lid of a humidified 24-well plate and covered with aluminum foil, for 1 hour in the dessicator. The secondary antibody solution was made with 849μl PBST, 102μl serum, 42μl 10% triton, 2μl goat anti rabbit Alexa 594 (for caspase 3), and 5μl strept fluor 495 (for TUNEL). The slices were rinsed in the 24-well plate with PBS pH 7.4 ten times for 2 minutes each, then mounted on a microscope slide with an antifade mounting medium and viewed with the laser scanning confocal microscope.

Dual Labeling with Caspase 3 and Annexin V

Label the sections with the Annexin V solution as listed above in the Annexin V section above. Saggital or coronal vermal sections (12μm), that were affixed to the gelatin subbed coverslips, were placed in 10ml beakers and treated with PBS pH 7.4 for 3 minutes to remove the tissue freezing medium, then treated with acetone for 10 minutes at -20°C to enhance antigenicity. The sections were rinsed in PBS three times for five minutes each, then transferred to a 24-well plate. Then they were permeabilized overnight with 0.5% triton at 4°C.

The slices are incubated in 250μl of blocking solution, consisting of 1700μl PBS pH 7.4, 100μl 10% triton, and 200μl goat serum for 1 hour at room temperature. The type of serum actually used depended on what animal species the secondary antibody was made in. They were rinsed five times in PBS pH 7.4 for 5 minutes each. They are treated

with 1% sodium borohydride, which had been made in PBS pH 8.0 for 30 minutes at room temperature to enhance antigenicity.

The slices were rinsed five to ten times in PBS pH 7.4 for 5 minutes each, until all bubbles disappeared. Then they were incubated in a 1:50 dilution active caspase 3 primary antibody (1^{o} ab) solution overnight at 4°C. The primary antibody solution was made using: 780µl PBST, 100µl goat serum, 100µl 10% triton, and 20µl Caspase 3 stock. This is accomplished by placing 50–75µl of the primary antibody solution into a coverwell incubation chamber. A negative control was obtained by not placing one slice in the primary antibody solution that contained the active caspase 3 solution. Instead, it was placed into 50-75µl of PBST only. Then the slices were moved from the 24-well plate and gently laid, face down into the primary antibody solution. The incubation chambers were placed in the lid of a humidified 24-well plate, aluminum foil was gently placed over the top of the slices, and they were placed in a humidity controlled drawer in the refrigerator.

The slices were returned back to the 24-well plate and rinsed in 0.1% triton for 5 minutes, then rinsed in PBS pH 7.4 five times for 5 minutes each. The slices were then treated with 250µl of a combined secondary antibody solution for caspase 3 and Annexin V at room temperature, covered with aluminum foil, for 2 hours on the rocker. The combined secondary antibody solution was made of 844µl PBST, 102µl goat serum, 42µl 10% triton, 10µl strept avidin anti biotin Alexa 488 (for Annexin V), and 2µl goat anti rabbit Alexa 594 (for caspase 3). They were rinsed in 0.1% triton for 10 minutes, then rinsed in PBS pH 7.4 ten times for 10 minutes each. The slices were rinsed in distilled water for 5 minutes, then dehydrated in decreasing ethanol steps (40%, 70%, 95%, and 100%) for three minutes each. They were mounted to a microscope slide using Prolong Antifade mounting medium. The sections were viewed using an MRC-1024 BioRad Laser Scanning Confocal Microscope.

Toluidine Blue Staining

Cerebellum sections that were 6μm thick was collected onto gelatin coated microscope slides and allowed to dry. They were soaked in PBS pH 7.4 for 2-3 minutes to remove the tissue freezing medium. The slides were then dipped into toluidine blue, which is a dye that binds to the rough endoplasmic reticulum and allows one to identify the various cellular components, for 10 seconds or less. Sections were then dehydrated in increasing concentrations of alcohol (40%, 70%, 80%, 90%, and 100%) for 1.5-2 minutes each. They were then placed in xylene, then sealed by mounting a coverslip over the slide using Cytoseal 60 Mounting Medium (Fisher Scientific, Houston, TX; catalog #23244256).

Data Acquisition

Immunohistochemistry was performed on representative slices from the vermal areas as well as the hemispheric areas. This was to help in determining if there was a difference in active caspase 3-positive cell development, and cerebellar folia development obtained from medial (vermis) and lateral (hemispheric) regions of the cerebellum.

Fluorescent images of the active caspase 3-positive cells were acquired with a 60X objective, using an MRC-1024 BioRad Laser Scanning Confocal Microscope. Representative images were collected from each outer loop of a folia (gyrus) and the preceding inner loop of that same folia (sulcus). This was done for each cerebellar folium in the vermal and hemispheric regions at the respective ages. The folia present were determined by imaging the toluidine stained sections. The number of fluorescently labeled active caspase 3-positive cells within the 60X view, as well as the number of fluorescently labeled active caspase 3-positive cells within the vicinity of the Purkinje cells, was counted for each image. This was done to assess the number of cells located in the molecular layer and the deeper internal granule cell layer as opposed to those that were located within the Purkinje cell layer. This was important to establish the relevancy of the active caspase 3 expression in the cell population being analyzed.

The TUNEL procedure was then performed on the slices immediately adjacent to those used for immunohistochemistry. Active caspase 3 was labeled with Alexa 594, which reflects a red fluorescence, and TUNEL was labeled with Alexa 495, which reflects a green fluorescence. For each section, five randomly selected outer folia loops were imaged, and five randomly selected inner folia loops were imaged. For each randomly selected folium, an image was taken for active caspase 3 independently, then for TUNEL independently, and then a combined image was taken. Apoptosis was determined by the presence of colocalization of the two Alexas, which would have appeared as yellow or orange fluorescence. The number of active caspase 3-positive cells versus the number of TUNEL positive cells were counted for each image obtained. A similar method was used to determine the presence of apoptotic cells using Annexin V.

Data Analysis and Statistics

A three-way analysis of variance (ANOVA) was used to determine whether there was a significant difference in the number of active caspase 3-positive cells present in each of the eleven cerebellar folia for each age group being tested (9, 21, and 30 days old). A three way ANOVA was also used to determine if a significant difference existed between the three ages, and between the vermis and hemisphere regions. A statistically significant level was set at $p < 0.05$. A Student-Newman-Keuls test was also performed on the data in establishing significant differences in folia, age, and region. A paired t-test was used to determine statistical significance between nonapoptotic caspase 3-positive cells vs. apoptotic caspase 3-positive cells as revealed by labeling with TUNEL or Annexin V.

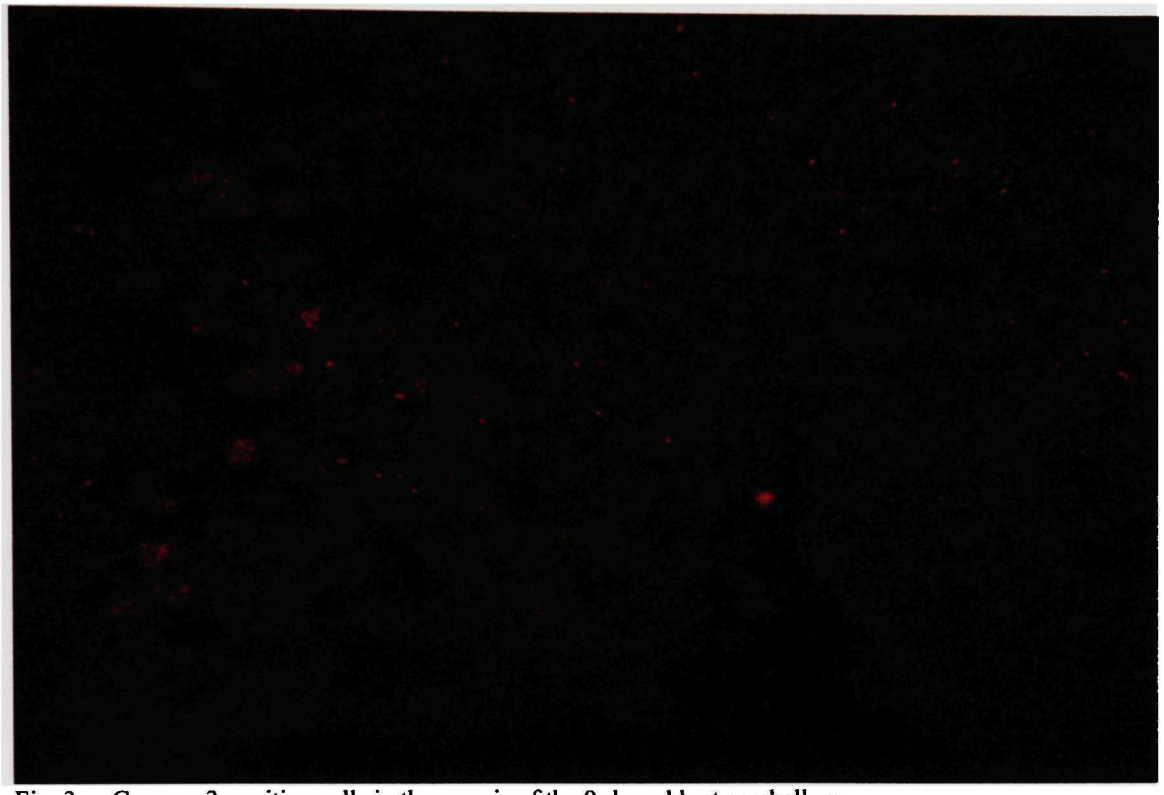

Fig. 3 Caspase 3-positive cells in the vermis of the 9-day-old rat cerebellum.

CHAPTER III

RESULTS

Nuclear Expression of Active Caspase 3 in the Rat Cerebellum

In performing some immunohistochemical experiments and confocal microscopy in our lab for a previous study, which looked at caspases in Purkinje cells, a serendipitous finding was made. Several cells surrounding the Purkinje cells were expressing strong nuclear immunofluorescence for active caspase 3. These cells became of interest to us, particularly because they morphologically did not appear to be apoptotic.

Although the core of the present study only concentrated on the 9-, 21-, and 30-day old animals, these active caspase 3-positive cells have been seen in our lab as young as 2 days old, and as late as 60 days old. We have never examined animals older than 60 days. The active caspase 3-positive cells in the younger and older animals were not found in every folia, and an evaluation of the populated folias was not performed.

Spatial and Temporal Expression of Caspase 3-Positive Cells in the Vicinity of the Purkinje Cells

9-Day-Old Animals

The active caspase 3-positive cells were not present throughout the entire cerebellum in the 9-day-old. Where they did appear, the cells were not in great abundance. Cells were fluorescently labeled at this age in the vermis and the hemisphere (Fig. 3), but they were only found in one out of three rats in the both regions. The immunohistochemistry experiments for this age were performed on four separate occassions with saggital sections from all three rats in an attempt to find caspase 3-positive cells from more than one rat for both the vermis and the hemisphere, but this proved unsuccessful. Of particular peculiarity is that the one rat that exhibited active caspase 3-positive cells in the vermis was not the same one that expressed them in the hemisphere.

Of the active caspase 3-positive cells found in the 9-day-old animal, approximately 98% of those in the vermis and 84% of those in the hemisphere were

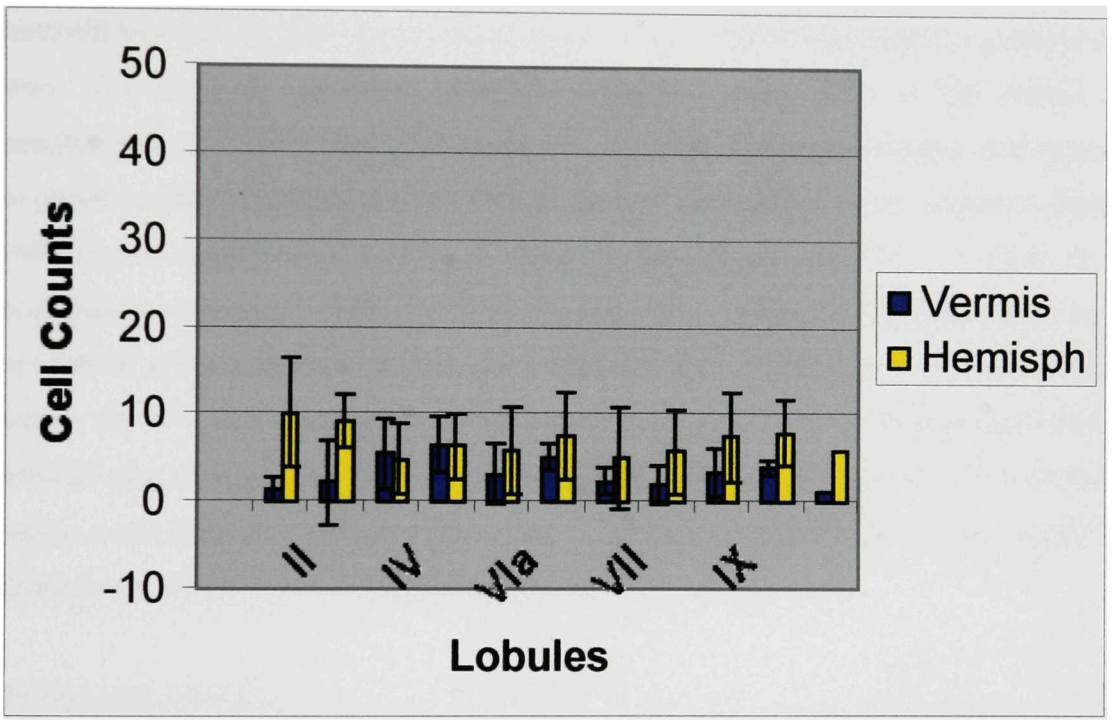

Fig. 4 Lobular distribution of active caspase 3-positive cells in the vermis and hemisphere of the 9-day-old rat cerebellum (+/- S.E., n=3).

found within the Purkinje cell layer. This was determined by the number of active caspase 3-postive cells within the 60X images vs. those that were only within the PCL (data not shown). The rest of the cells were found in the molecular layer. In most of the lobules, the hemisphere exhibited a greater abundance of cells than did the vermis (Fig. 4).

21-Day-Old Animals

An example of the active caspase-3 positive cells found in the vermis and hemisphere of the 21-day-old rat can be seen in Fig. 5. The active caspase 3-positive cells were ubiquitous in significant numbers throughout every folia in the vermis and hemispheres of every 21-day-old rat (Fig. 6). The cells were more distinct, and appeared to contain a brighter fluorescence than they did at 9 days. Of the active caspase 3-positive cells found, approximately 80% of those in the vermis and 87% of those in the hemisphere were found within the Purkinje cell layer. Again, this was determined by the number of active caspase 3-postive cells within the 60X images vs. those that were only within the PCL (data not shown). The rest were found in the molecular layer and the internal granule cell layer. There did not appear to be a pattern as to which cerebellar region contained the most numerous cells. In most but not all lobules, the vermis had greater cell counts than did the hemisphere (Fig. 6).

30-Day-Old Animals

Figure 7 shows representative images of the active caspase 3-positive cells in the vermis and the hemisphere for the 30-day-old animals. Although the cells were still ubiquitous and still quite abundant in every folia in the 30-day-old rats, the numbers in the vermis were slightly diminished from those in the 21-day-old, while most of them in the hemisphere increased in abundance over the 21-day-old (Fig. 8). Between 21 days

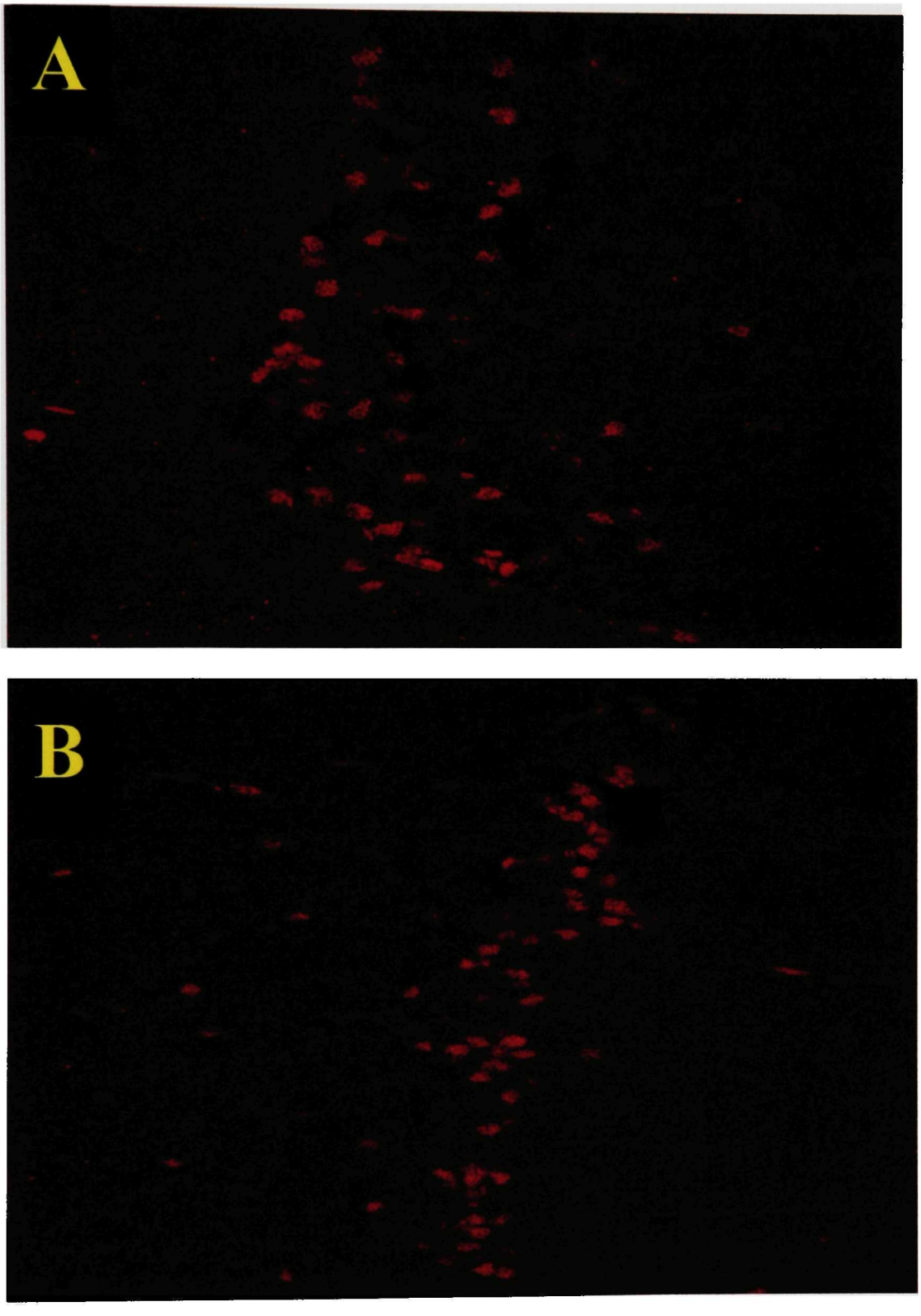

Fig. 5 Caspase 3-positive cells in the vermis (A) and hemisphere (B) of the 21-day-old rat cerebellum

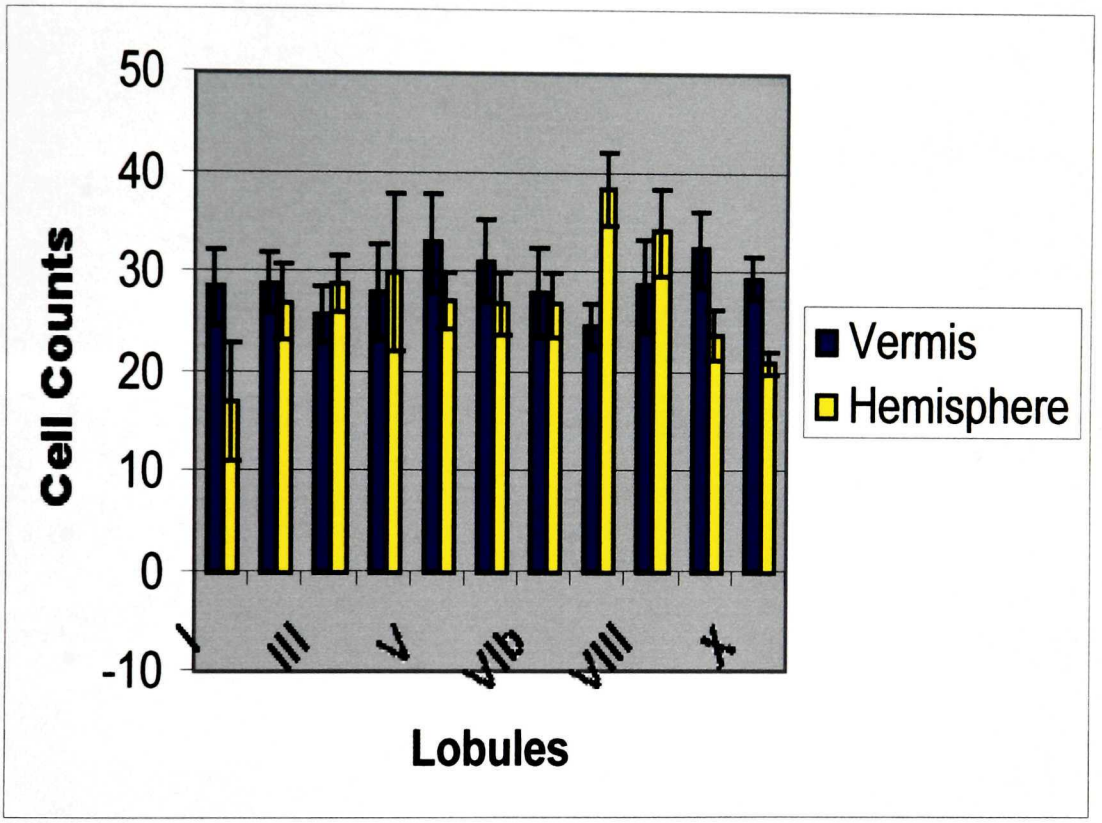

Fig. 6 Lobular distribution of active caspase 3-positive cells in the vermis and hemisphere of the 21-day-old rat cerebellum (+/- S.E., n=3).

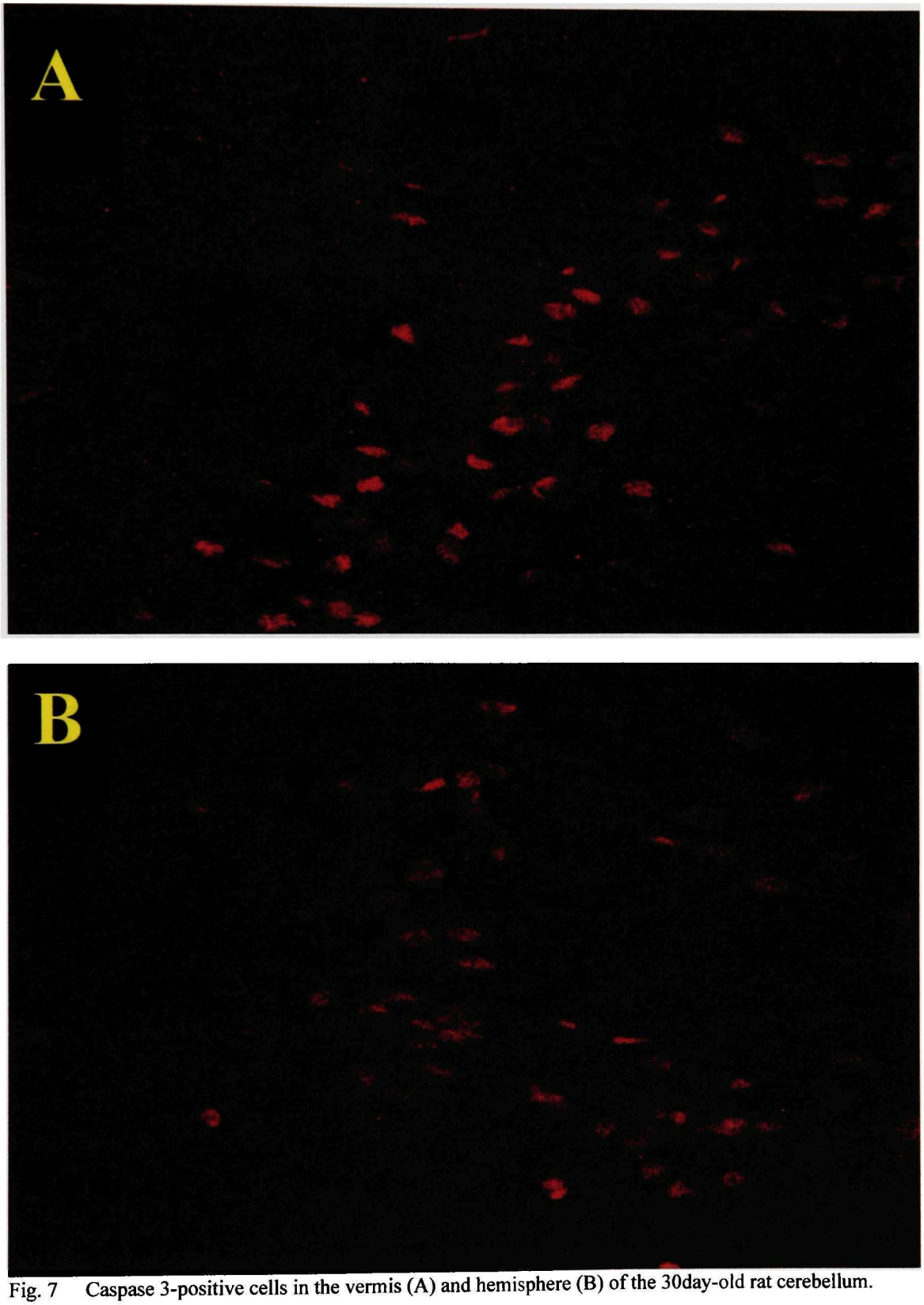

Fig. 7 Caspase 3-positive cells in the vermis (A) and hemisphere (B) of the 30day-old rat cerebellum.

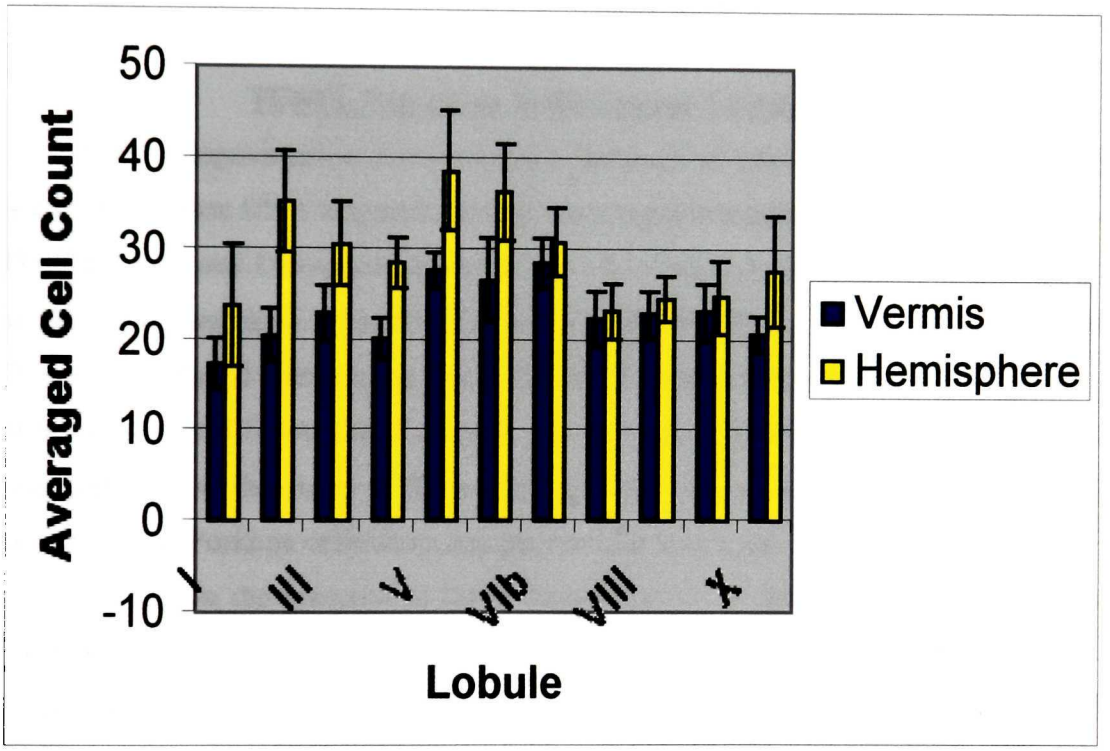

Fig. 8 Lobular distribution of active caspase 3-positive cells in the vermis and hemisphere of the 30-day-old rat cerebellum (+/- S.E., n=3).

and 30 days, more active caspase 3-positive cells within the hemisphere had come to reside in this layer.

Of the active caspase 3-positive cells found in the 30-day-old rats, approximately 79% of those in the vermis and 81% of those in the hemisphere were found in the vicinity of the Purkinje cells. This again was determined by the number of active caspase 3-postive cells within the 60X images vs. those that were only within the PCL (data not shown). The rest, as in the 21-day-old, were found in the molecular layer and the internal granule cell layer. In every lobule, the hemisphere had a greater abundance of cells than in the vermis (Fig. 8).

TUNEL Expression in the Caspase 3-Positive Cells

DNA fragmentation is considered a hallmark of late apoptotic events. Caspase 3 is known to cause DNA fragmentation by cleaving downstream substrates such as ICAD. TUNEL (Terminal Deoxynucleotidyl dUTP Nick End Labeling) allows for the detection of DNA fragmentation. The dUTP attaches to the 3'-OH end of the fragmented DNA. This is then labeled with a strept avidin fluorescent tag, which allows for the visualization of the fragmented DNA. Since caspase 3 is an important effector of apoptotic events, it was important to determine if these particular active caspase 3-positive cells within the vicinity of the Purkinje cells were also positive for apoptosis.

To assess the presence of DNA fragmentation, or lack thereof, in the caspase 3-positive cells, cerebellar slices were dually labeled with an in situ TUNEL detection assay and active caspase 3 immunohistochemistry. The dual labeling procedure was performed on various ages of animals. These results will concentrate on the three ages of interest in this particular study, the 9-day-old, 21-day-old, and the 30-day-old. Consistently, the vast majority of active caspase 3-positive cells within the Purkinje cell layer were negative for TUNEL labeling (Fig. 9). For all three ages of interest, approximately 97% (97.74%, 97.39%, and 96.97%, respectively) of the active caspase 3-positive cells were not apoptotic as indicated by TUNEL negative immunoreactivity.

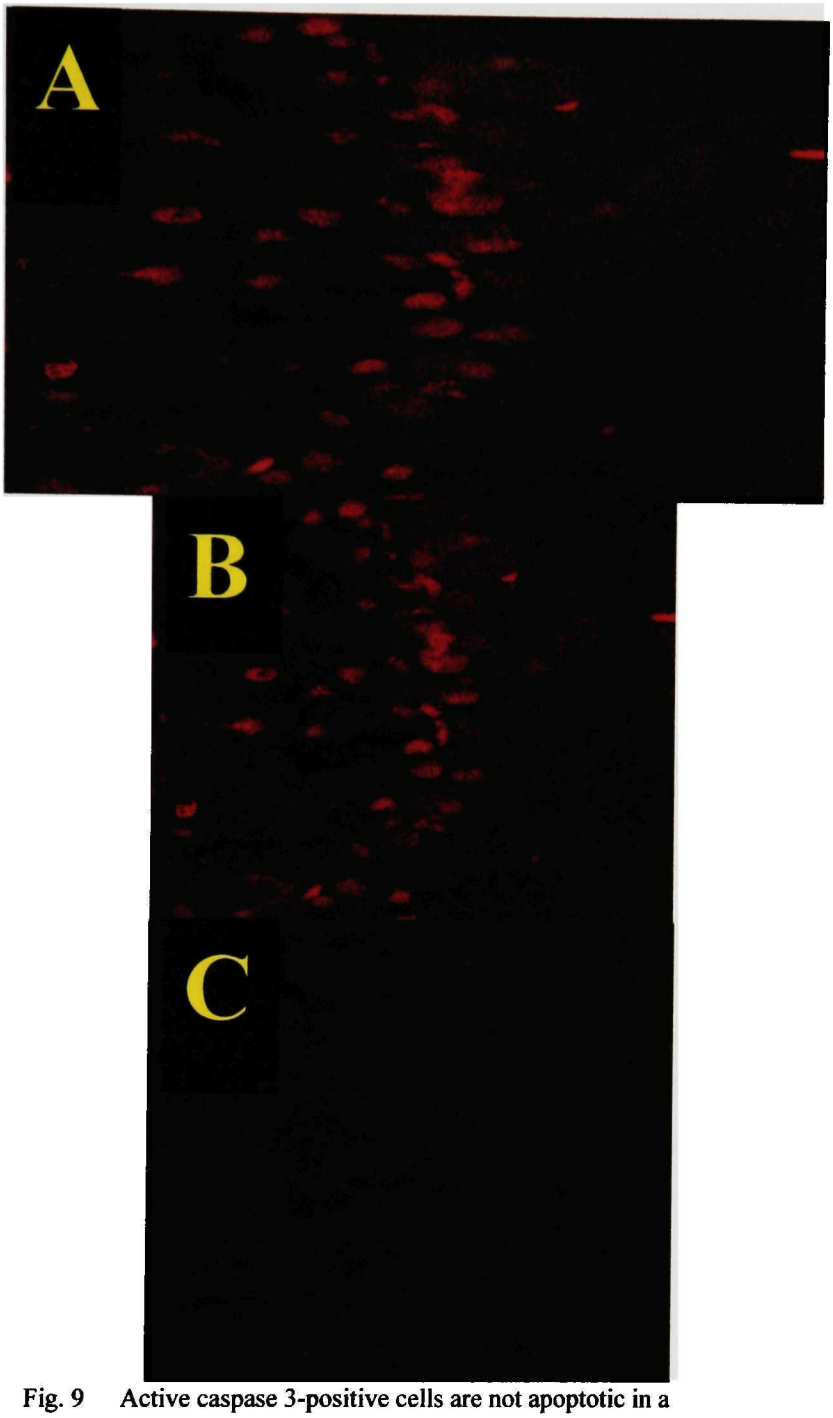

Fig. 9 Active caspase 3-positive cells are not apoptotic in a handling control slice as revealed by TUNEL. A) Colocalized active caspase 3 (red) and TUNEL (green); B) Caspase 3-positive cells (red); C) TUNEL (green) immunoreactivity shows lack of staining in the active caspase 3-positive cells shown in B.

To test the sensitivity and specificity of the TUNEL labeling, a positive control was always included. With a positive control, DNAse was introduced in the TUNEL reaction. DNAse artificially creates the necessary DNA fragments, which allows the TUNEL immunoreactivity to show positive results (Fig. 10). With the positive control, a majority of active caspase 3-positive cells were also TUNEL positive. In the handling control, or test slices, approximately 97% of the active caspase 3-positive cells were negative for TUNEL, whereas in the positive control slices, about 96% of the active caspase 3-positive cells were also positive for TUNEL. There were many other TUNEL positive cells throughout the slices that were not caspase 3-positive. In fact, of the all TUNEL positive cells, only 34.24% of the cells in the 9-day-old, 52.20% of the cells in the 21-day-old, and 36.99% of the in the 30-day-old animals exhibited caspase 3 expression (data not shown).

Annexin V Expression in the Caspase 3-Positive Cells

During the early stages of apoptosis, phosphatidyl serine is externalized from the inner leaflet of the membrane to the outer leaflet. Phosphatidyl serine is identified by the biotinylated lipid binding protein, Annexin V, which is then fluorescently tagged with strept avidin Alexa 488. Fresh, unfixed cerebellar slices of various ages were exposed to biotinylated Annexin V. After fixation, the slices were processed for active caspase 3 immunohistochemistry. It is obvious that the active caspase 3-positive cells surrounding the PC's lack the membrane labeling of phosphatidyl serine (Fig 11 and 12).

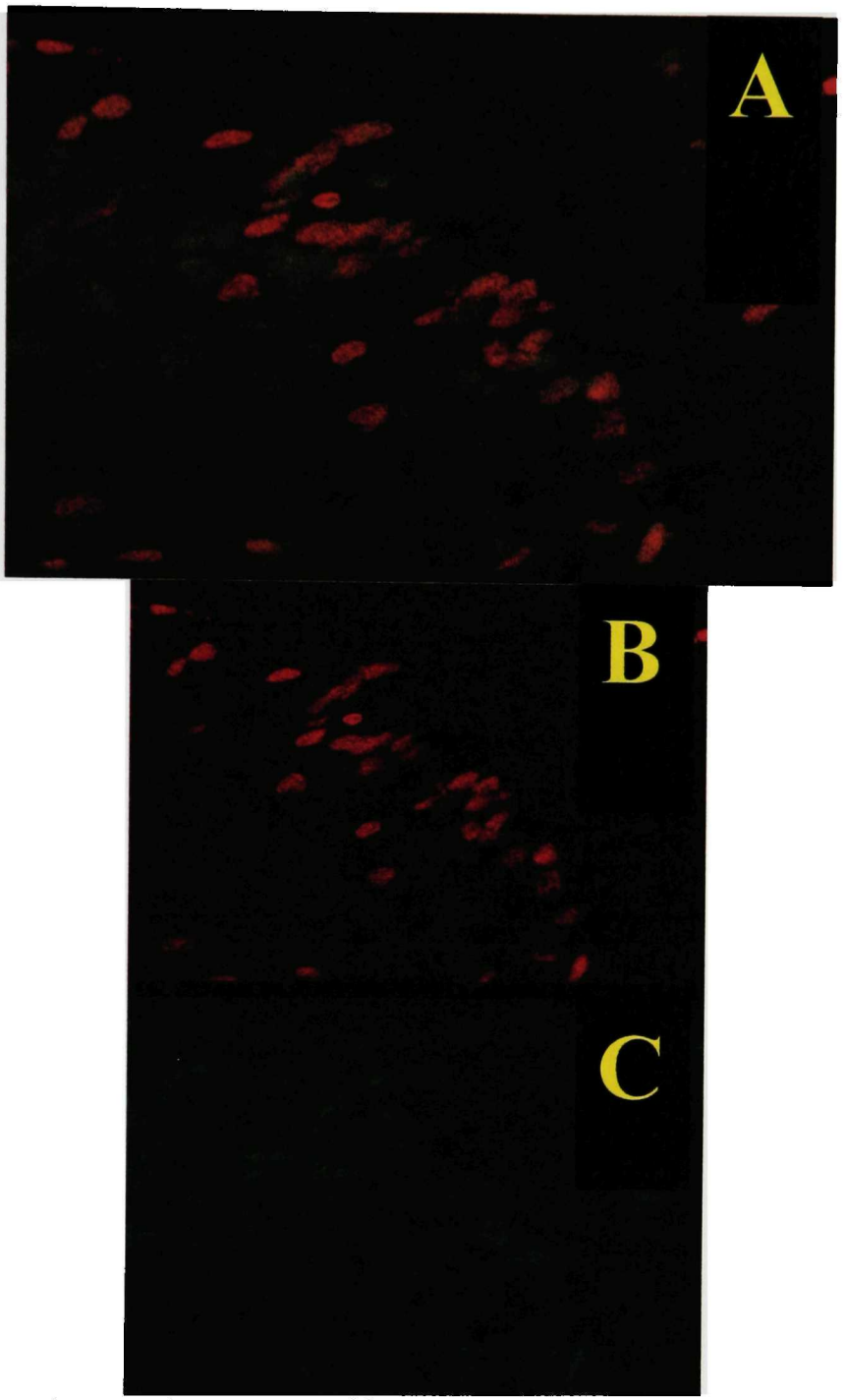

Fig. 10 Active caspase 3-positive cells are apoptotic in a positive control slice as revealed by TUNEL. A) Colocalized (orange) active caspase 3 and TUNEL; B) Caspase 3-positive cells (red); C) TUNEL (green) immunoreactivity in the active caspase 3-positive cells shown in B.

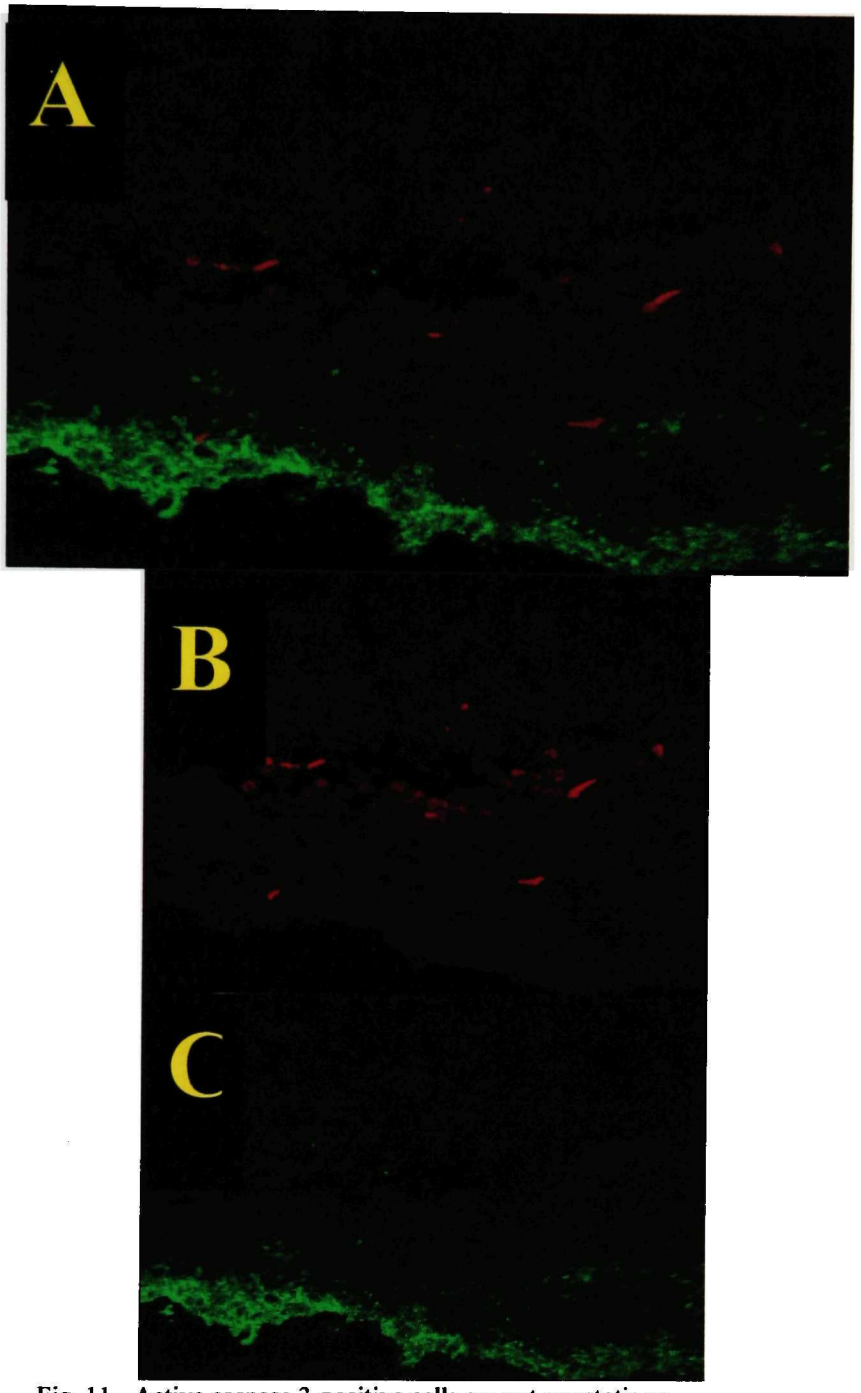

Fig. 11 Active caspase 3-positive cells are not apoptotic as revealed by Annexin V. A) Colocalized active caspase 3 (red) and Annexin V (green); B) Caspase 3-positive cells (red); C) Annexin V (green) immunoreactivity shows lack of staining in the active caspase 3-positive cells shown in B.

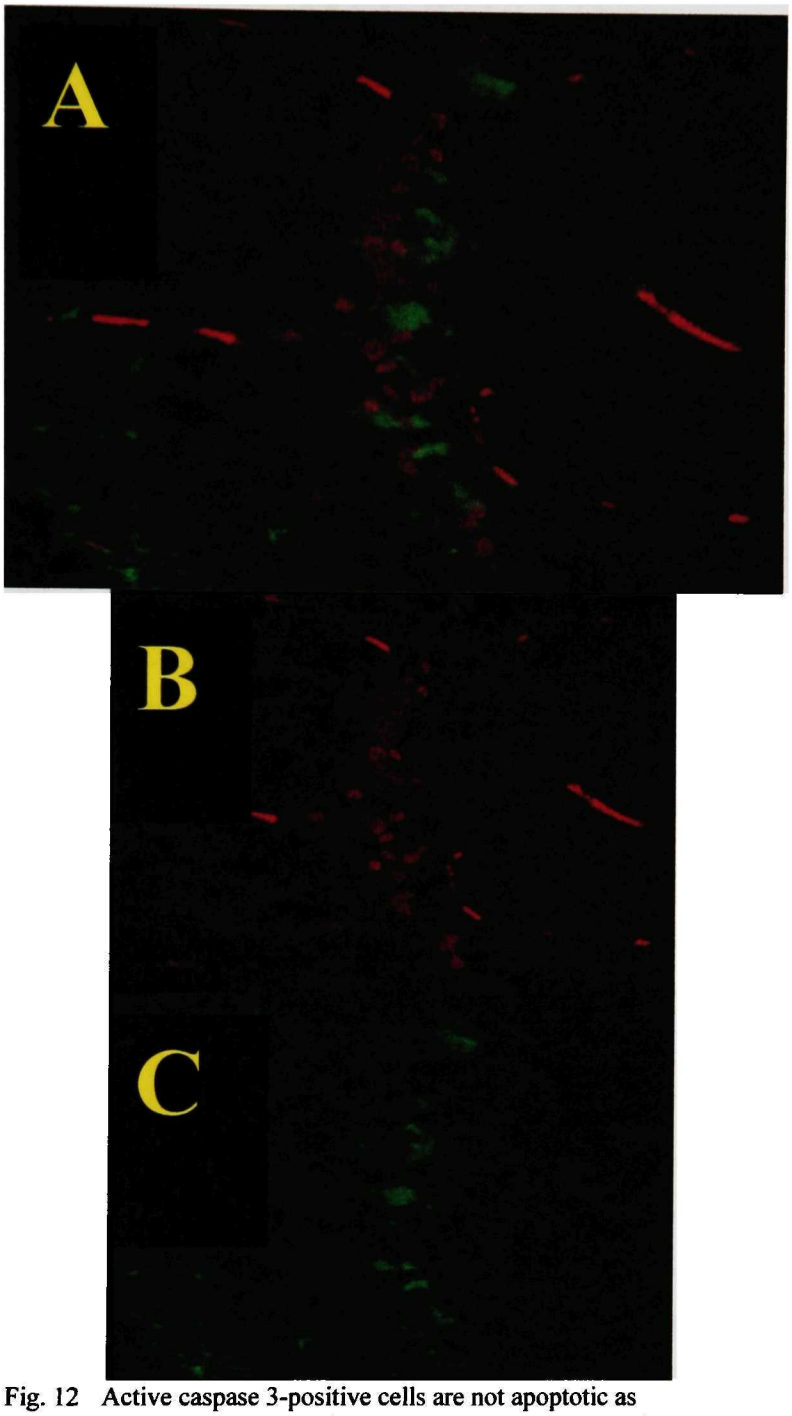

Fig. 12 Active caspase 3-positive cells are not apoptotic as revealed by Annexin V. A) Colocalized active caspase 3 (red) and Annexin V (green); B) Caspase 3-positive cells (red); C) Annexin V (green) immunoreactivity shows lack of staining in the active caspase 3-positive cells shown in B. Green Annexin V positive cells in A and C are Purkinje cells. This shows the relationship of the active caspase 3-positive cells to the Purkinje cells.

CHAPTER IV

STATISTICAL ANALYSIS

Spatial and Temporal Expression of Caspase 3-Positive Cells in the Vicinity of the Purkinje Cells

In order to perform the statistical analysis on the cell counts, some rules had to be established. Several images were taken from the sulcus and gyrus around every folia. One image from the sulcus of each folia and one image from the preceding gyrus was chosen. The images chosen from each crest and its preceding depth were those with the greatest abundance of cells. So, for each lobule, two cell counts were collected, then they were averaged, using Microsoft Excel's descriptive statistics to give us a representative cell count for each folia.

Table 1 shows the results of the ANOVA between the lobules, regions, and ages on cell counts. With an F value of 5.24 and a probability of a greater F ($Pr > F$) equal to <0.0001, the analysis verified a significant difference had occurred between the main effects (specifically, region and age, as seen in table 2) and the occurrence of active caspase 3-positive cells within the vicinity of the Purkinje cells. This also indicated that the model being used was valid for the data set obtained.

Hypothesis Testing #1: The expression of active caspase 3-positive cells in the vicinity of the Purkinje cell layer is related to development of cerebellar folia, as well as being related to age and to cerebellar region.

Table 2 summarizes the three-way ANOVA results for the different variables being tested. It looks at the cell counts for the different lobules within the vermis and hemisphere regions in the 9-day-old, 21-day-old, and 30-day-old rats. It also shows the various interactions of lobule*region, lobule*age, region*age, and the three-way combination of lobule*region*age.

The F value for lobule was 0.72 with a probability of a greater F ($Pr > F$) equal to 0.7081, indicating no significant difference in the presence of active caspase 3-positive cells between the different lobules. The F values for lobule*region, lobule*age, and

Table 1. ANOVA for Caspase 3-Positive Cells from 9-day-old, 21-day-old and 30-day-old rats.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	65	23870.86348	367.24405	5.24	<0.0001
Error	126	8838.79327	70.14915		
Corrected Total	191	32709.65675			

Table 2. ANOVA Hypothesis Testing for Cell Counts

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Lobule (L)	10	502.31354	50.23135	0.72	0.7081
Region (R)	1	400.66955	400.66955	5.71	0.0183
Age (A)	2	19922.90830	9961.45415	142.00	<0.0001
L*R	10	233.47560	23.34756	0.33	0.9708
L*A	20	670.37576	33.51879	0.48	0.9707
R*A	2	501.36696	250.68348	3.57	00.0309
L*R*A	20	880.50112	44.02506	0.63	0.8857

lobule*region*age were 0.33, 0.48, and 0.63, respectively, with probabilities of a greater F (Pr > F) equal to 0.9708, 0.9707, and 0.8857, respectively. This indicated that all of the interactions involving lobule effects also had no significant differences on the occurrence of active caspase 3-positive cells within the Purkinje cells.

On the other hand, the F value for region was 5.71 with a Pr > F of p equal to 0.0183, and an F value for age of 142.00 with a Pr > F of p equal to <0.0001 verified that significant differences for these two variables did exist. The greatest difference that affected the expression of active caspase 3 in the cells surrounding the Purkinje cells occurred between the different ages. The interaction of region*age also displayed a significant difference with an F value of 3.57 and a Pr > F of p equal to 0.0309.

The ANOVA was followed by a Student-Newman-Keuls test for difference in cell counts between the different lobules, regions and ages. As seen in table 3 there was no significant difference between the different lobules. A significant difference between the vermis and the hemisphere did exist with a mean of 18.294 vs. 21.474 as seen in table 4. Also, as table 5 demonstrates, there was a significant difference between 9 and 21 days of age (mean of 5.583 vs. 28.640) and between 9 and 30 days (mean 5.583 vs. 26.138), but not between 21 and 30 days (mean 28.640 vs. 26.138).

Hypothesis Testing #2 via TUNEL Immunoreactivity: Active caspase 3 has a nonapoptotic role in the cells surrounding the Purkinje cells in the developing rat cerebellum.

In order to determine if a significant difference existed between active caspase 3-positive cells and colocalized TUNEL positive cells in a handling control slice, a paired two-sample t-test was conducted for each age of interest. For the 9-day-old animals, with a t Stat value of 6.929023 with the Pr > T of t equal to 0.00048, a significant difference was established.

This same pattern held for the 21-day-old animals, with a t Stat of 10.85732 and the Pr > T of t equal to 0.0000181. The 30 day old animals had a t Stat value of 22.627 with the Pr > T of t equal to 0.0000945.

Table 3. Student-Newman-Keuls Test for Differences in Cell Counts Between Lobules

SNK Grouping	Mean	Lobule
A	15.357	I
A	19.475	II
A	19.694	III
A	19.917	IV
A	22.972	V
A	22.333	VIa
A	20.778	VIb
A	20.083	VII
A	20.222	VIII
A	19.333	IX
A	17.206	X

Note: Means with the same letter are not significantly different.

Table 4. Student-Newman-Keuls Test for Differences in Cell Counts Between Regions

SNK Grouping	Mean	Region
A	21.474	Hemisphere
B	18.294	Vermis

Note: Means with the same letter are not significantly different.

Table 5. Student-Newman-Keuls Test for Differences in Cell Counts Between Ages

SNK Grouping	Mean	Age
B	5.583	9
A	28.640	21
A	26.138	30

Note: Means with the same letter are not significantly different.

A t-test was also conducted to determine if significant differences exited between active caspase 3-positive cells and colocalized TUNEL positive cells on a positive control slice. A positive control slice is one in which DNAse was introduced to create DNA fragments. No significant differences were detected between the active caspase3-positive cells and the TUNEL positive cells as shown with t Stats of 0, 20745626, and 1 along with a Pr > T of t equal to 1, .051606, and 0.5 for the 9-day-old, 21-day-old, and 30-day-old, respectively. This proved that active caspase 3 is capable of labeling apoptotic cells with the techniques used in this study.

Finally a t-test was conducted to compare the TUNEL positive cells on a handling control slice to the TUNEL positive cells on a positive control slice. There was a significant difference detected between TUNEL positive cells on handling control slice and those on a positive control slice. The t Stats were –6.299753, -9.970252, and –16.97056 along with Pr > T of t equal to 0.008081, .0.000569, and 0.000446 for the 9-day-old, 21-day-old, and 30-day-old, respectively. This proved that the active caspase 3-positive cells being examined in this study were indeed nonapoptotic in nature. The significance of active caspase 3-positive cells compared to TUNEL positive cells for the handling control slices is shown in Fig. 13 and for positive control slices in Fig. 14. Figure 15 shows the significance of TUNEL positive cells on a handling control slice compared to TUNEL positive cells on a positive control slice.

Hypothesis Testing #2 via Annexin V Immunoreactivity: Active caspase 3 has a nonapoptotic role in the cells surrounding the Purkinje cells in the developing rat cerebellum.

In order to determine if a significant difference existed between active caspase 3-positive cells and colocalized Annexin V positive cells in a handling control slice, a paired two-sample t-test was conducted on all ages combined. In the developing cerebellum, a significant difference between active caspase 3-positive cells and colocalized Annexin V positive cells was established with a t Stat value of 17.81544 and a Pr > T of t equal to 4.12×10^{-17}. Figure 16 shows the significance of this difference between active caspase 3-positive cells compared to Annexin V positive cells.

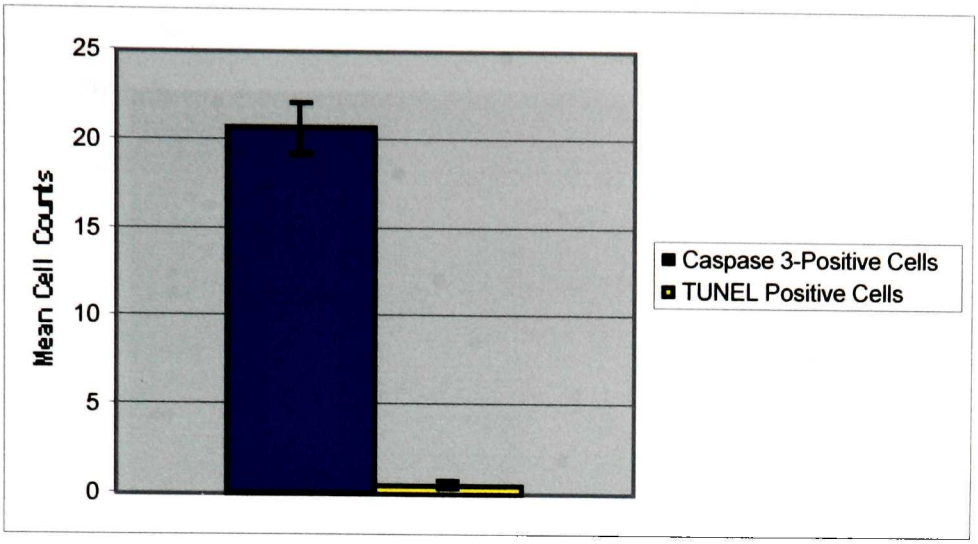

Fig. 13 Caspase 3-positive cells compared to TUNEL positive cells in handling control slices (+/- S.E).

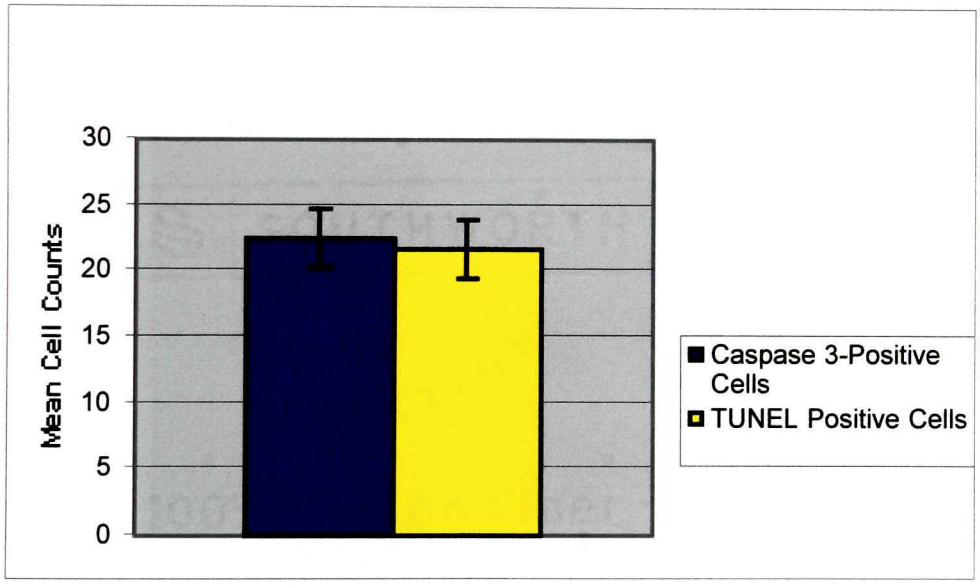

Fig. 14 Caspase 3-positive cells compared to TUNEL positive cells positive control slices (+/- S.E.).

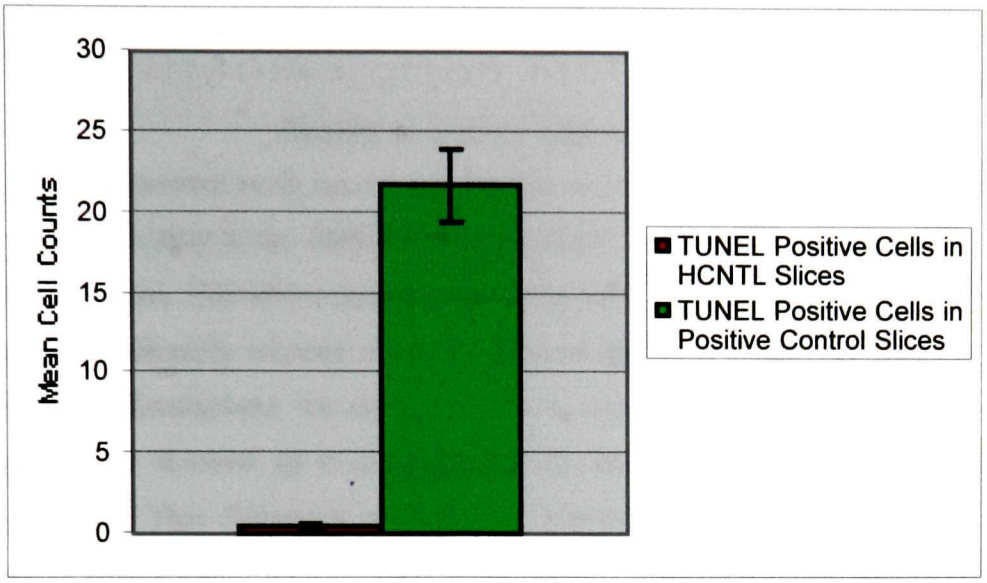

Fig. 15 TUNEL positive cells in handling control slices compared to TUNEL positive cells in positive control slices (+/- S.E.).

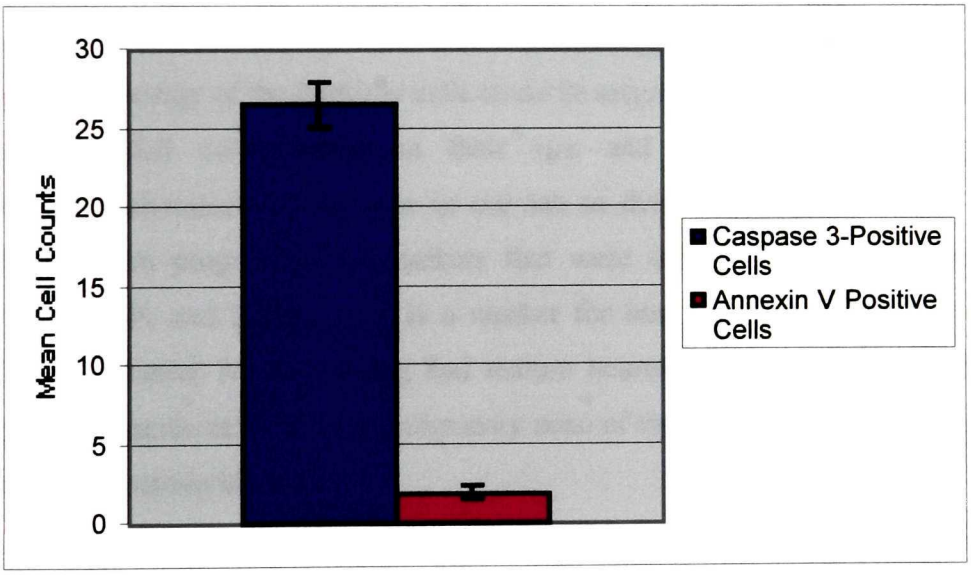

Fig. 15 Caspase 3-positive cells compared to Annexin V positive cells in handling control slices(+/- S.E.).

CHAPTER V
DISCUSSION

Identity of Active Caspase 3-Positive Cells

The present study has shown that the expression of active caspase 3-positive cells in close proximity to the Purkinje cells is closely related to the developmental patterns of the cerebellum. The active caspase 3-positive cells in the vermis are minimal at postnatal day 9, significantly increase up to 21 days of age, then diminish slightly at 30 days of age. In the hemisphere, the active caspase 3-positive cells are minimal at 9 days of age, significantly increase up to postnatal day 21, and then increase slightly more up to 30 days of age. This difference in expression between the vermis and the hemisphere could only be explained by the fact that the vermis develops faster than the hemisphere, so the caspase 3-positive cells have not reached their peak in the hemisphere at 21 days like it has in the vermis. This was supported by the significant difference obtained with the region*age interaction effect in the three-way ANOVA.

As mentioned earlier, there was a possibility that the active caspase 3-positive cells in the vicinity of the Purkinje cells could be migrating granule cells, Lugaro cells, or Bergmann glial cells, based on their size and location. We performed many immunohistochemical experiments in our lab to determine the identity of these cells (publication in progress). The markers that were used included TuJ1, NeuN, Ki67, PCNA, GFAP, and S-100. TuJ1 is a marker for immature postmitotic neurons, while NeuN is a marker for developing and mature neurons. NeuN preferentially labels the nucleus of granule cells in the premigratory zone of the EGL, ML, and the IGL. GFAP is a marker for astrocytes.

In performing double labeled immunohistochemistry experiments, it was found that our active caspase 3-positive cells were negative for TuJ1 and NeuN (results not shown, publication in progress). This indicated that these cells were not neurons, or at least not granule cells. Thus, the active caspase 3-positive cells must be glial cells. Based on these results, plus the known abundance of cells in relation to Purkinje cells, we were

able to quickly rule out the thought of these cells being migrating granule cells (250 GC's per PC) or Lugaro cells (1 LC per 7.7 PC's), both of which are neurons.

The results of another set of double labeled immunohistochemical experiments gave us GFAP labeling in the fibers of our active caspase 3-positive cells, as well as the fibers of all other astrocytes present (results not shown, publication in progress). Bergmann glial cells are one type of astrocyte present in the cerebellum. Due to the abundance of GFAP labeling, it was difficult to correlate the fibers exactly with the cells. The active caspase 3-positive cells were also positive for S-100, a glial marker, although the S-100 also labeled all glial cells present (results not shown, publication in progress). Based on the findings of GFAP positive labeling and S-100 positive labeling, in addition to the proximity of these active caspase 3-positive cells to the Purkinje cells, and the abundance of these cells in relation to Purkinje cells, we deduced that the active caspase 3-positive cells must be Bergmann glial cells (8.1 BGC's per PC).

The presence of Bergmann glial cells, and in particular, their fibers, within the cerebellum have been known about for over 100 years. This study represents the first that we are aware of that examines active caspase 3 being present in the cells.

Spatial, Temporal, and Lobular Development of Bergmann Glial Cells

Thymidine Studies of Embryonic Origin

There is much controversy over the origin and development of Bergmann glial cells. Most studies have shown the existence of Bergmann glial cells at birth (Shiga et al., 1983b; Hanke et al., 1987), believed to be derived from embryonic progenitors, but there is at least one study (Das, 1976) that believes these cells to be of EGL origin.

The presence of immature Bergmann glial cells with irregularly contoured cell bodies has been observed by some researchers (Shiga et al., 1983b) within the Purkinje cell layer at the time of birth, but they have been observed by others (Hanke et al., 1987) as early as embryonic day 20. Both studies found that during the first two postnatal weeks there is a period of extensive proliferation of these cells in the developing rat brain.

Immature Purkinje cells are also found at birth, but decrease in number during the first 4 postnatal days to align themselves in a single row (Shiga et al., 1983b). The first two weeks after birth are characterized by an increase in the thickness of the EGL, an increase in the thickness of the molecular layer, and an increase in the number of Bergmann glial cells (Shiga, 1983b et al.; Hanke et al., 1987). Also, during these first two weeks, the cells partake in a period of rapid and complex revolution with regards to the morphology of the Bergmann glial cells (Shiga et al., 1983b; Hanke et al., 1987). The contour of the cell body begins to smooth out (Shiga et al., 1983b).

There is also an increase in the number of fibers arising from Bergmann glial cells during the first two postnatal weeks, as well as an increase in the thickness of each fiber. Two types of fibers protrude from Bergmann glial cells, thick fibers and filiform fibers. Shiga et al. (1983b) found that the number of filiform fibers protruding from each Bergmann glial cell did not begin to decrease until postnatal day 15, while Hanke et al. (1987) found that they began decreasing after the first postnatal week. The cells present at embryonic day 20 have only one main process protruding from the cell body to the pial surface (Hanke et al., 1987). The cells exhibited a crude adult morphology between 15–20 postnatal days (Hanke et al., 1987). From day 16 onwards all cells established a constant number of about 5 cell processes (Hanke et al., 1987).

Shiga et al. (1983b) and Hanke et al. (1987) had a difference of opinion when it came to the maturation of the Bergmann glial cells. Shiga et al. (1983b) felt that Bergmann cells reached their full maturity by postnatal day 25, while Hanke et al. (1987) found that cell differentiation continued from day 16 onwards, even up to two years of age. It was found that the number of bushy protrusions from each process increased significantly up to 5 months of age, then to a lesser extent afterwards (Hanke et al., 1987). It was also found that the length of each process steadily increased up to two years of age as the molecular layer increased in thickness, and each process got thinner during this lengthening period (Hanke et al., 1987).

It has been studied and known that proper neuronal activity is necessary for the development of the normal structure and function of Bergmann glial cells. One study

hypothesized that neuronal activity after the first week of life was the cause of elaboration of the glial fibers (Grosche et al., 2002). The circuitry of neuronal dendrites, axons, and their synapses is set forth during the first two weeks after birth, and before the Bergmann glial cells begin the morphometric revolution of their fibers. They found that the surface area of Bergmann glial cells increased by a factor of 50 or more. This increase in surface area was due to the increasing complexity of the glial fibers.

Molecular Markers of Embryonic Origin

The above mentioned studies all used [^{3}H] thymidine to label the cells. Yamada et al. (2002) took a different approach in using neuronal and glial markers to look at different cell populations and their relationships.

GLAST was found to exclusively label all radial glial cells, although a strong expression persisted in the Bergmann glial cells into adulthood while it diminished during development in all other astrocytes (Yamada et al., 2002). GLAST is localized on the cell membrane surrounding the excitatory PC synapses, which allows for the visualization of the position of the Bergmann glial cells in relationship to Purkinje cells (Yamada et al., 2002). Calbindin is a known marker for postmitotic Purkinje cells.

Most, if not all glial cells, regardless of type, take on the form of radial glial cells during embryonic life (Yamada et al., 2002). Glial precursors are found in the form of radial glial cells during the postmitotic migration of Purkinje cells (Yamada et al., 2002). The radial glial fibers are apposed to and in contact with the calbindin positive PCs (Yamada et al., 2002). Radial glial cells function as neuronal precursors before the exclusive generation of specific types of astrocytes, thus functioning as neural stem cells (Yamada et al., 2002). Thus it is conceivable to think that perhaps Bergmann glial cells and Purkinje cells are derived from the same glial precursor cells, which could explain why they reside in the same locale in the postnatal rat brain.

It has been shown that the radial glial fibers in the embryonic brain provide the scaffolding to facilitate and direct neuronal migration (Parnavelas et al., 2001). This migration is thought to be aided by a number of molecular markers, including:

astrotactin, neuregulin, brain lipid binding protein (BLBP), and tenascin (Parnavelas et al., 2001). Evidence has indicated that at the end of the embryonic neuronal migration these precursors are then transformed into fibrous and/or protoplasmic astrocytes. Radial glial cells express vimentin, nestin, and RC2 (Parnavelas et al., 2001). Then as the cells are transformed into astrocytes, they lose the expression of these molecular markers and acquire the expression of GFAP (Parnavelas et al., 2001). It was shown that BLBP in radial glial cells is expressed later than RC2, which suggested that BLBP served a purpose after the initial induction of radial glial cell identity (Gregg et al., 2002). BLBP was not found to be in most proliferating cells (Gregg et al., 2002). Rather it was found to correlate with neural differentiation and migration (Gregg et al., 2002).

This idea of radial glial cells serving as neuronal precursors is a relatively new thinking, within the past 10–15 years. This is based on studies that have shown the ability of radial glial precursors to produce neurons and astrocytes in culture (Parnavelas et al., 2001). Other studies using time lapsed video microscopy have shown that radial glial precursors divide to give rise to mitotic radial glial cells and postmitotic neurons, and then the daughter neurons migrated along the fibers of the parent radial glial precursor (Parnavelas et al., 2001). This supports the idea of a triple role for radial glia in neurogenesis, gliogenesis, and neuronal migration guidance. This phenomenon is extremely possible, considering that radial glial precursor cells are the earliest cells to differentiate from the neuroepithelium in the embryonic brain, before the emergence of Purkinje cells (Gregg et al., 2002).

Other views on this subject believe there are cycling progenitor cells that appear to be of glial lineage, but can produce neurons when they are taken from their normal environments (Goldman, 2003). It is also thought that these progenitor cells comprise of two populations (Goldman, 2003). The minor population is multipotential and can produce neurons, while the major population gives rise to the astrocytes (Goldman, 2003). This is potentially true also given that Bergmann glial cells exist in a ration of 8.1:1 with Purkinje cells. The Bergmann glial cells could arise from the major population of progenitors while the Purkinje cells arise from the minor population of progenitors.

From embryonic day 16 – embryonic day 20, there is an active migration of Purkinje cells and Bergmann glial cells from the mitotic zone towards their final resting spots in the Purkinje cell layer in the postnatal developing rat cerebellum (Yamada et al., 2002). The GLAST expressing glial cells are evenly distributed among the PCs, and are always situated just beneath them (Yamada et al., 2002). The foremost migrating glial cells never exceed the front line of migrating PCs. Bergmann glial cell migration appeared to be regulated in tight correlation with Purkinje cells (Yamada et al., 2002). During this time of active migration, and on into the first postnatal week of life, Bergmann glial cells are distinguished from other astrocytes by their location, unipolar shape, association with PCs and their association with radial glial/astrocytic markers (Yamada et al., 2002).

Based on the idea above that Bergmann glial cell migration is regulated in correlation with Purkinje cells, along with that of Grosche et al. (2002) that Purkinje cells affect the complex fiber formation of Bergmann glial cells, it is apparent that intimate neuronal-glial interactions exist between these two cells types. It appears as if each cell type has a role in regulating the other cell type, a bi-directional regulation. This could possibly be another effect of both cell types being derived from the same radial glial precursors.

Using GFAP to label the astrocytic Bergmann glial fibers, and double labeling with GLAST, which was localized to the processes from the GFAP positive fibers, Yamada et al. (2002) found a transformation of the fibers during the second and third postnatal weeks (Yamada et al., 2002). This correlates exactly with the findings of Shiga et al. (1983b) and Hanke et al. (1987). So the immunohistochemical analysis matches that of the thymidine analysis. During this time, the Purkinje cell fibers are oriented parallel to and in contact with the Bergmann glial fibers (Yamada et al., 2002). This is also the period of active production and migration of granule cells.

It is also believed that the Bergmann glial fibers are involved in transporting granule cells from the EGL to the IGL. In order for Bergmann glial cells to play an active role in the guidance of granule cells from the EGL to the IGL, fibers of the Bergmann

glial cells must already be present at the time of birth. One study observed the presence of immature Bergmann fibers as early as prenatal day 17 (del Cerro et al., 1976). Although the fibers did display some degree of differentiation, they were smooth, without any processes being emitted, but they did follow a regular course up to the EGL along the columns of EGL cells (del Cerro et al., 1976).

In another study, it was observed that Bergmann glial cells come into existence during postnatal development with the majority of them being formed between 9–12 days (Das, 1976). The Bergmann glial cells were considered to be in a migratory stage from postnatal day 6–8 (Das, 1976). The cells that were in their early migratory phase were located just below the EGL, while those in more advanced stages of migration were in the lower regions of the ML (Das, 1976). During this time, they had some filiform processes that trailed behind them, reaching the pial surface of the cerebellum (Das, 1976). These filiform processes also increased in number during this time (Das, 1976).

By 10–12 days of age, Bergmann glial cells were found in the lower ML and in the Purkinje cell layer (Das, 1976). During this time, the soma underwent a change from an irregular morphology to a smoothed morphology (Das, 1976). The thick processes decreased in abundance, while the filiform processes increased in abundance and thickness, which agrees with Shiga et al. (1983b). These changes were used as an indicator that migration had ceased and the Bergmann glial cells had arrived at their destination points (Das, 1976).

Further differentiation of the filiform processes occurred from postnatal days 15–18 (Das, 1976). The cells possessed fewer processes than they did at earlier ages (Das, 1976). Some processes underwent resorption while others grew thornlike structures along their lengths and growth cones on their terminal ends (Das, 1976). The processes continue to become thicker, longer, and more uniform into adulthood resulting in fully differentiated Bergmann glial cells (Das, 1976).

The importance of Das' study was that it showed that Bergmann glial cells originated postnatally from the EGL. The Bergmann cells migrated downward from the EGL, apparently from some ameboid type of action. These cells could be distinguished

from other cell types leaving the EGL by the differentiation of filiform processes that terminated at the pial surface, and the fact that they did not possess any processes that traversed the IGL. It is thought that the Bergmann glial cell migration precedes that of the granule cells, and that the filiform processes are important in guiding the migration of the granule cells from the EGL to the IGL. Migrating GCs are tightly bound to the Bergmann glial cell fibers, with little extracellular space around them (Yamada et al., 2002). Thus, the Bergmann glial fibers displayed cellular affinities for differentiating PCs and migrating GCs.

Numerous investigations (Shiga et al., 1983b; Hanke et al., 1987; and Das, 1976) have reported an increase in the number of Bergmann glial cells during the second postnatal week. This could very likely explain why the present study showed an increase in the abundance of active caspase 3-positive cells in the vicinity of the Purkinje cells between the 9-day-old and the 21-day-old rats.

The finding that Bergmann glial cells are of prenatal origins, and migrate through the white matter to reside within the PC layer (Shiga et al., 1983b; Hanke et al., 1987; and Yamada et al., 2002) could explain the findings of the present study that some active caspase 3-positive cells are located in the deeper IGL. The findings of Das (1976) that Bergmann glial cells are of postnatal origins, and migrate from the EGL through the molecular layer to reside among Purkinje cells could be a possible explanation for the active caspase 3-positive cells occasionally found in the ML in the present study.

In order for Bergmann glial cells to continue postnatal proliferation while among the Purkinje cells, indicating that they had to already exist, which means that Bergmann glial cells must have developed prenatally. Some of them may have already been present in the PCL at birth, while others may have still been migrating through the white matter postnatally to reside among the Purkinje cells.

Of course there is the possibility that Bergmann glial cells originate from both the fourth ventricular region and from the EGL. The early cells that are already present in the PC layer at birth may have come from prenatal origins around the fourth ventricle, and were used to guide PCs to their final destinations. Then, during postnatal development, a

second population of Bergmann glial cells could derive from the EGL to assist in guiding granule cell migration. The other possibility is that the other active caspase 3-positive cells in this study that were located within the molecular layer and deeper internal granule cell layer are other types of astrocytes, and not Bergmann glial cells at all.

Taken together, it appears as if radial glial precursors are formed early during embryonic development. They have a significant involvement in the genesis, proliferation, and differentiation of Purkinje cells. Then the PCs play a role in the transformation of radial glial progenitors into the astrocytic Bergmann glial cells. Both cells migrate together in a specific spatial relationship, with the Purkinje cells apparently migrating along the radial glial fibers, such as to guide and regulate one another until they reach their final destination in the cerebellum within the Purkinje cell layer. Then Bergmann glial cells begin their initial stages of differentiation by extending their processes up towards the EGL before granule cell migration. The Bergmann glial fibers are an essential factor that guides the migration of granule cells from the EGL to the IGL. Once the granule cells reach their destinations, then the Bergmann glial fibers proceed with terminal differentiation into their adult morphology. The fact that our lab has seen active caspase 3-positive Bergmann glial cells as early as day 2 postnatally, and as late as 60 days, could mean that active caspase 3 is a necessary and vital component in the differentiation and migration of prenatal (Purkinje) as well as postnatal (granule) neuronal cells.

Lobular Formation

Takashi Shiga et al. (1983a) found that the labeling index for Bergmann glial cells reached a peak in lobules I–VIa and lobules VIII–X by postnatal days 6–7, whereas lobules VIb and VII reached their peak at ages 8–9. They classified the lobules into three categories: early, intermediate, and late based on the labeling indexes. Lobules I–V are early developing, lobules VIa and VIII–X are intermediate, and lobules VIb and VII are late developing. They found that the later the labeling index of Bergmann glial cells, the higher the ratio of late forming granule cells found. Their study verified that the

Bergmann glial cells in the rat continue mitosis after birth, with the bulk of postnatal proliferation occurring between 6–9 days of age. Lobule I was the earliest lobule to be labeled for Bergmann glial cells, while lobules VIb and VII the latest to be formed.

Bergmann glial cells have even been shown to continue mitosis postnatally when they are in the vicinity of the Purkinje cells. The Purkinje cells in the rat have been shown to mature earlier in lobules I–IV and VIII–X, which according to the study by Shiga et al. (1983), are the same lobules that had the earliest peak in labeling index for Bergmann glial cells. But they did not find any studies correlating Bergmann glial cells and Purkinje cells.

Yamada et al. (2002) found that the stage of peak glial production is different from lobule to lobule, and correlates with the temporal order of granule cell production and migration. The present study did not find this to be the case. The present study found no correlation between the presence of active caspase 3-positive Bergmann glial cells and the different lobules.

The findings of the present study were similar to those found by Ichikawa et al. (1983), which examined the postnatal proliferation of glial cells in the parietal cortex. Although, they looked at glial cells in general instead of Bergmann glial cells specifically, and they looked in the parietal cortex rather than the cerebellum, there are a few findings that could be applied to the current study. Glial cells have the potential to continue mitosis after leaving the ventricular region while neurons do not. Ichikawa found that the cortical glial cells were formed around the lateral ventricle and migrates to the neocortex through the white matter. The Bergmann glial cells are formed around the fourth ventricle. They probably migrate to the cerebellum through the white matter also. Ichikawa et al. (1983) also found that the rate of proliferation in all the laminae was high from postnatal age 3 to postnatal age 7, although they found no significant differences in the rate of proliferation among the different laminae. There was no significant differences found in the present study with regard to the presence of active caspase 3-positive cells in the various folia.

The maturation of the glial cells did not appear to be related to position in the cortex as it does with neurons. They showed that mitosis of glial cells does occur postnatally within the grey matter. It was also found that postnatal proliferation in these glial cells followed the inside out direction of maturation that is typically found in the central nervous system. This inside out direction of maturation appears to hold true for Bergmann glial cells also, as revealed by other studies.

Purkinje Cell Production and Migration

It is our belief that if Bergmann glial fibers are important in the migration and differentiation of Purkinje cells as well as granule cells, then their existence within the vicinity of the Purkinje cell layer should correlate with the lobular development of Purkinje cells and/or granule cells. It was found that in general, the Purkinje cells in the hemisphere are produced earlier than those in the vermis (Altman et al., 1985). The cytogenetic gradient of PC production appeared to follow a rostral to caudal direction in the hemisphere, while the reverse was true for the vermis (Altman et al, 1985). This pattern did not appear to affect the settling of cells into the Purkinje cell layer. The PCs in the vermis migrate to their final destinations earlier than they do in the hemispheres (Altman et al., 1985). The settling patterns in both regions followed the caudal to rostral direction (Altman et al., 1985). It was also discovered that the PCs begin settling in the lobules in the following order: IX, VIII, VII, VI, V, IV, X, III, II, and I (Altman et al., 1985). This settling occurs from embryonic day 17 to embryonic day 22 (Altman et al., 1985).

Granule Cell Migration

Shiga et al. (1983b) felt that if Bergmann glial cells do guide granule cells through the molecular layer, then their maturational and morphological events should correlate with changes in the migratory activity of granule cells. The fact that they demonstrated the presence of Bergmann glial cells in the newborn rat gives some support of the guidance of granule cells during their migration from the EGL though the ML to

the IGL. They felt that the change in thickness of the EGL and number of filiform fibers demonstrate an intimate relationship between granule cells and Bergmann glial cells.

As found by Shiga et al. (1983a) the later the labeling index of Bergmann glial cells, the higher the ratio of late forming granule cells found This suggests that an intimate relationship between Bergmann cells and granule cells must exist. It has been proposed that Bergmann glial cells play an important role in the guidance of granule cells migrating from the EGL through the molecular layer to the IGL. If this is so, then the regional and temporal differences that exist for granule cells as found by Altman (1972) should also exist for Bergmann glial cells. Lobule I was the earliest lobule to be labeled for Bergmann glial cells, which was also classified by Altman (1972) as an early lobule for granule cells. The late formation of lobules VIb and VII were also late forming for granule cells. Shiga et al. (1983b) felt that the spatiotemporal pattern of mitotic activity in these two cell types shows that there is a close correlation between them, and possibly even a common functional phenomenon.

It is the belief of the present study that if Bergmann glial fibers were involved in the migration of granule cells from the EGL to the IGL, then their existence should somewhat correlate with the lobular pattern of granule cell migration. It was found that migration from the EGL begins around postnatal day 4, and formation of the IGL begins around postnatal day 9 (Altman, 1972). The granule cells begin their journey from the EGL to the IGL in the vermis in more or less the following order: X, I, II, III, IX, IV, V, VI, VIII, and VII (Altman, 1972). This is quite different from the formation of the Purkinje cell layer.

Caspases

Caspases have been shown to be located in the plasma membrane, the mitochondria, the endoplasmic reticulum, the nucleus, and the cytoplasm of the cell (Algeciras-Schimnich et al., 2002). They have been shown to act as negative and positive regulators of survival pathways (Algeciras-Schimnich et al., 2002). Activation of most death receptors has led to two opposing pathways, induction of apoptosis through

recruitment of caspase 8, or cell survival by the activation of transcription factors (Algeciras-Schimnich et al., 2002). They have also been implicated in the control of cell movement (Algeciras-Schimnich et al., 2002). Nuclear caspases have been suggested to play a role in the regulation of cell cycle checkpoints (Algeciras-Schimnich et al., 2002). Caspases have been shown to cleave various cell cycle proteins (Algeciras-Schimnich et al., 2002). It is believed that activation of caspases during cell cycle events results in cleavage and inactivation of proteins that act as negative regulators of the cell cycle machinery (Algeciras-Schimnich et al., 2002). Caspases may play a role in the cell cycle to ensure that only healthy cells complete the cell cycle (Algeciras-Schimnich et al., 2002).

With such a diverse localization, and a vast array of potential functions, locally selective activation of caspases and selective processing of substrates could explain the apparent contradiction between nonapoptotic and apoptotic functions of caspases. It is likely that caspases, like many other proteins in cells, have multiple functions that include cell survival, cell growth, proliferation, differentiation, and cell death.

As mentioned in the introduction, caspase 3 was shown to be either cytoplasmic or nuclear (Beer et al., 2000). The present study observed a nuclear localization of active caspase 3, which indicates that upon activation, caspase 3 must get translocated from the cytoplasm where it is cleaved by caspase 9 back into the nucleus to serve its nonapoptotic role. Also, it was shown that active caspase 3 is limited in the postmitotic neurons in later developmental stages (Urase et al., 1998), but nothing was mentioned of astrocytic glial cells. The present study found active caspase 3 to be present in postmitotic astrocytic Bergmann glial cells as late as 60 days postnatally.

TUNEL

In a TUNEL assay, the enzyme terminal deoxynucleotidyl transferase (TdT) binds to 3'-OH ends of DNA single stranded breaks (Huppertz et al., 1999). The sensitivity of the assay is critical because single stranded DNA fragments are found during mitosis and throughout the whole life of a cell, as well as during apoptosis (Huppertz et al., 1999).

Low levels of DNA fragments are found in all cells and false positive identification of apoptotic cells can easily be achieved (Huppertz et al., 1999). Since the results of the present study did not reveal any significant TUNEL staining in the Bergmann glial cells, false positive identification was not a problem. Also, the use of a positive control that did give positive apoptotic Bergmann glial cells helped to substantiate the sensitivity of the TUNEL assay used in the present study.

Annexin V

The flipping of phosphatidyl serine from the inner leaflet to the outer leaflet of the plasma membrane is considered an early apoptotic event (Huppertz et al., 1999). Annexin V binds to the phosphatidyl serine. One problem with using Annexin V on tissue is that it could bind to the phosphatidyl serine on the inner membrane as well as the outer membrane, which would cause false positive identification of apoptotic cells (Huppertz et al., 1999). Since the present study did not observe any significant Annexin V staining in the Bergmann glial cells, false positive identification was not a concern.

Possible Functions of Nonapoptotic Caspase 3 in Bergmann Glial Cells

In numerous studies (Shiga et al., 1983b; Hanke et al., 1987; Das, 1976), it was shown that Bergmann glial cells continue proliferating during the first postnatal week and some even into the second postnatal week. In our lab, double labeling immunohistochemical experiments with active caspase 3 and Ki67 or PCNA were performed. Ki67 and PCNA are both markers for proliferating cells. Our active caspase 3-positive cells were negative for both proliferation markers (results not shown, publication in progress). Active caspase 3 does not appear to be involved in proliferation of Bergmann glial cells.

It appears to be a general consensus and common finding that the Bergmann glial cells are present and begin proliferating during the fetal stage, continue proliferating during the first two weeks of infancy, then enter the differentiation stage into the third week of life. The results of the present study demonstrated that active caspase 3-positive

Bergmann glial cells in the vermis are minimal at postnatal day 9, significantly increase up to 21 days of age, then diminish slightly at 30 days of age. In the hemisphere, the active caspase 3-positive cells are minimal at 9 days of age, significantly increase up to postnatal day 21, and then increase slightly more up to 30 days of age. Thus, the expression of active caspase 3 in the Bergmann glial cells does in fact correlate with the developmental changes that occur in the cerebellum as a result of age and region. This temporal relationship, along with the negative results of proliferation markers, leads to the belief that active caspase 3 is involved in the differentiation of Bergmann glial cells.

As discussed in the introduction, it appears to be a common finding of active caspase 3 playing a vital role in terminal differentiation of various cell types. The differentiation of Bergmann glial cells correlates with the temporal migration of granule cells. It is also possible that the expression of active caspase 3 in the Bergmann glial cells is necessary for the contact guidance of granule cells along the Bergmann glial fibers. Although, the fact that the present study showed the existence of active caspase 3 in the Bergmann glial cells as late as 60 days of age in the postnatal rat tend to argue against the latter, and in favor of the former.

Das likened Bergmann glial cell differentiation to metamorphosis since the immature cell did not resemble an undergrown replica of a fully differentiated cell. As mentioned earlier in the introduction of this paper, the resorption of a tadpole's tail during metamorphosis occurs via apoptosis. Caspase 3 is essential for the apoptotic degradation of cells to occur. It is therefore conceivable to believe that the presence of active caspase 3 in the cells that reside within the Purkinje cell layer is necessary for the "metamorphosis" that takes place during the differentiation of Bergmann glial cells.

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