DETERMINATION AND MECHANISMS OF TRAUMA-INDUCED GLIAL CELL DEATH IN THE DEVELOPING CHICK CENTRAL NERVOUS SYSTEM

by

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ABSTRACT

Traumatic injuries to the central nervous system can lead to debilitating and often irreversible neurological deficits, which result from a poorly understood, multifactorial degenerative response. Although the initial cellular damage incurred by the traumatic event is instantaneous, a secondary, protracted degenerative phase can greatly exacerbate tissue damage and functional impairment. A recently described feature of this secondary response is the delayed apoptotic death of white matter oligodendrocytes remote from the lesion. This highly regulated, stereotyped form of cell death is thought to be executed by the caspase family of intracellular proteases. To investigate the role of caspases in injury-induced oligodendrocyte apoptosis, I developed an i*n vivo* model of spinal cord injury in the developing chick embryo. Increased developmental apoptosis in the cervical spinal cord was observed in the cervical white matter between embryonic days 13-18, which correlated with the period of myelination of the spinal cord. Following transection during this period, a large increase in apoptotic cells was detected primarily in the ventrolateral and ventromedial white matter for several spinal cord segments caudal to the injury. Double labeling of apoptotic cells with cell specific antibodies revealed a large number of the dying cells to be oligodendrocytes but not astrocytes. *In ovo* application of caspase inhibitors significantly attenuated the transection-induced apoptotic response in the white matter remote from the lesion, but did not affect developmental apoptosis. Caspase-3-like catalytic activity was a component of the apoptotic response in this region following transection, and immunostaining for the active form of caspase-3 revealed its presence in an increased number of cells following injury. All cells expressing activated caspase-3 were apoptotic, and were distributed in the same region of spinal cord white matter as apoptotic oligodendrocytes. These results indicate oligodendrocytes undergo a period of heightened sensitivity to traumatic injury that correlates with the period of developmental oligodendrocyte apoptosis and with myelination of the spinal cord, and that caspases appear to mediate their death after trauma. The embryonic chick model of neurotrauma developed in this thesis may be used to further understand the mechanisms of oligodendrocyte death and survival in degenerative conditions characterized by oligodendrocyte apoptosis.

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ABBREVIATIONS

ACD	active cell death
Apaf-1	apoptosis protease activating factor 1
APP	amyloid precursor protein
Aβ	amyloid β peptide
BAF	benzyloxycarbonyl-Aspartate (OMe)-fluoromethyl ketone
Bcl	B cell lymphoma
CARD	caspase recruitment domain
c-FLIP	cellular FLICE/caspase-8 inhibitory protein
c-IAP	cellular inhibitor of apoptosis
CNS	central nervous system
Cyto c	cytochrome c
DED	death effector domain
DNA-PK	DNA-dependent protein kinase
FADD	Fas-associated death domain
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GGF	glial growth factor
ICAD	inhibitor of caspase-activated DNase
MBP	myelin basic protein
MS	multiple sclerosis
MSA	multiple system atrophy
NGF	nerve growth factor
NRG	neuregulin
P	postnatal/posthatching day
p75	low affinity neurotrophin receptor
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline

PCD	programmed cell death
PDGF	platelet derived growth factor
PKC	protein kinase C
PNS	peripheral nervous system
Pro-IL	pro-interleukin-1β
SCI	spinal cord injury
SREBP	sterol-regulatory element binding protein
TBI	traumatic brain injury
TfBP	transferrin binding protein
TNF	tumour necrosis factor
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated uridine triphosphate-biotin nick end-labeling
TUNEL+	TUNEL-positive
v-FLIP	viral FLICE/caspase-8 inhibitory protein
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER 1: GENERAL INTRODUCTION

Traumatic Injuries to the Central Nervous System

Traumatic injuries to the brain and spinal cord can result in severe and debilitating neurological deficits, which are in many cases irreversible. According to recent surveys by the Paralyzed Veterans and Canadian Paraplegic Associations North America, approximately 11, 000 new cases of spinal cord injury (SCI) are reported each year, and the American Brain Injury Association estimates 5.3 million Americans live with disabilities caused by traumatic brain injuries (TBI). The socio-economic impact of these injuries to individuals, their families, friends, and to society is tremendous and well understood; however, many of the pathophysiological mechanisms underlying central nervous system (CNS) trauma remain to be elucidated. Although the initial cellular damage incurred by the traumatic event is instantaneous, and therefore not amenable to treatment, a significant amount of secondary degeneration occurs more slowly over a period of hours and days. This indirect destruction of neural tissue can extend and exacerbate neurological deficits, making it an important target for therapeutic intervention. The protracted nature of secondary degeneration provides a window of opportunity for potential treatments aimed at attenuating the progression of tissue damage in hopes of improving neurological outcome following CNS trauma. This rationale was first substantiated by the results of the Second National Acute Spinal Cord Injury Study, which demonstrated some therapeutic potential for the anti-inflammatory steroid methylprednisilone in reducing secondary damage when administered in high doses within 8 hours after SCI (Bracken et al 1990). However, the modest benefits of methylprednisolone and the subsequently employed ganglioside, GM-1 (Geisler *et al.*, 1991) have prompted the search for alternative therapeutic targets and combinatorial treatment strategies.

Traumatic injury to the CNS results from mechanical damage and leads to a myriad of pathophysiological and biochemical responses that develop over the subsequent hours and days. The primary insult causes mechanical damage to neural cell membranes, nerve fibres and blood vessels, leading to necrosis, apoptosis and interrupted axonal conduction at the site of the injury (Blight, 1993; Dusart and Schwab, 1994; Schwab and Bartholdi, 1996). Subsequently, ischemia, excitotoxicity, inflammation, deregulation of ionic homeostasis, free radical toxicity, and pathological activation of cellular proteases (e.g.,

caspases and calpains) have all been implicated in the multifaceted response that mitigates the secondary degradation or injury which spreads from the lesion epicentre (Dusart and Schwab, 1994; Ito *et al.*, 1997; Kao and Chang, 1977; McBride *et al.*, 1999; Schumacher *et al.*, 1999; Schwab and Bartholdi, 1996; Tator, 1995; Tator and Fehlings, 1991; Zhang *et al.*, 1997b). These processes result in further loss of tissue and increased neurologic deficits. The secondary cell loss has been shown to include neurons, astrocytes, microglia and oligodendrocytes (e.g., Yong et al., 1998). Other pathological responses include astrogliosis and formation of an astrocytic scar (Fitch and Silver, 1999; Schwab and Bartholdi, 1996), invasion and activation of microglia, macrophages, neutrophils and polymorphonucleocytes, and demyelination (Blight, 1985; Blight, 1992; Dusart and Schwab, 1994; Schwab and Bartholdi, 1996). The precise role and consequences of these reactive cellular responses in the secondary injury response is unclear, but appear, in a general sense, to function in wound healing.

Forms of Cell Death

Neural cell death during secondary degeneration forms an important component of the CNS response to trauma. This cell death has been categorized into two principal forms, necrosis or active cell death (ACD, including apoptosis or programmed cell death). In general terms, necrotic cell death is a passive, unregulated process initiated by a severe, often mechanical insult that results in cell lysis and involves an inflammatory response (Buja *et al.*, 1993; Kerr *et al.*, 1995). Active forms of cell death, on the other hand, are highly regulated. In these forms of "cellular suicide," the cell actively participates in its own demise, a process often requiring *de novo* gene and/or protein expression/activation (e.g., Milligan et al., 1994; Oppenheim et al., 1990). Unlike necrosis, ACD is a discrete process in which a single cell can be removed from a system without eliciting an immune response. Active cell death has often been referred to as programmed either because it occurs at particular stages during development (Cowan *et al.*, 1984; Oppenheim, 1991) or because it relies on a genetic program for its execution (Jacobson *et al.*, 1997; Pettmann and Henderson, 1998). The terms programmed cell death (PCD) and apoptosis have often been used interchangeably. However, although PCD can be apoptotic in nature, it can assume other morphologies (described below). For the purpose of discussion in this thesis, I will ref

er to all forms of non-necrotic cell death as ACD, and only refer to specific forms of ACD when appropriate. Such clarification of terminology is important as the molecular machinery driving different ACD morphologies may vary; thus, the development of therapeutic approaches to arrest cell loss may depend on the precise form of ACD in a given condition.

Necrosis is characterized by massive and immediate cellular disruption. Early in the necrotic process, cell membranes rupture and organelles, including mitochondria, swell, whereas nuclear changes are not exceptional and occur late in the process (Buja *et al.*, 1993; Kerr *et al.*, 1995). As a result, cellular components, including lysosymes, are released into the extracellular space, eliciting an inflammatory response, which, in the CNS, does not necessarily lead to wound repair. On the contrary, inflammation in the CNS can initiate a process of cavitation that leads to substantial tissue loss and corresponding loss of neurologic function (Dusart and Schwab, 1994; Ito et al., 1997; Kao and Chang, 1977; Schwab and Bartholdi, 1996; Taoka and Okajima, 1998; Tator, 1995; Tator and Fehlings, 1991; Zhang et al., 1997).

The morphology of active forms of cell death varies greatly, and differs from that of necrosis. ACD can be classified into three main types based on their ultrastructural phenotypes (Clarke, 1990). Type 1 or apoptotic cell death is characterized by early nuclear events including chromatin condensation (pyknosis) and oligonucleosomal DNA fragmentation. Cellular membranes and organelles stay intact until the latest stages of degradation. This includes mitochondria, which appear normal throughout the apoptotic process. The normal appearance of the mitochondrion is, however misleading, as it appears to play a pivotal role in the progression of apoptotic signalling in response to a variety of apoptotic stimuli (Green and Reed, 1998; Kroemer *et al.*, 1998). Other morphological changes during apoptosis include cell shrinkage and membrane blebbing into apoptotic bodies. The relocation of phosphotidylserine from the inner membrane to extracellular membrane surface is another feature of cells undergoing apoptosis, a process that allows for the recognition and elimination of apoptotic cells by phagocytic cells *in vivo* (Koopman *et al.*, 1994; Zhang *et al.*, 1997a). Type 2 or autophagic cell death can involve pyknosis and membrane blebbing, but is distinguished by the presence of autophagic vacuoles. Organelles are sometimes dilated. Type 3 or cytoplasmic cell death

is characterized by predominant cytoplasmic alterations that resemble necrosis. Nuclear changes occur late in the degenerative process. Unlike necrotic cells, cells undergoing Type 3 cell death round up and are eliminated by heterophagic mechanisms.

The three forms of ACD are not mutually exclusive within a given neural population. For example, both Type 1 and Type 3 cell death are detected in avian spinal motor neurons undergoing developmental ACD (often referred to as PCD), and Types 1 and 2 are seen during the period of PCD in the developing chick isthmo-optic nucleus. Furthermore, many observations have been made in which individual cells display a mixture of morphological phenotypes from not only the three forms of ACD but necrosis as well (Clarke, 1990; Portera-Cailliau *et al.*, 1997). Thus, the dichotomous division of cell death into necrosis and apoptosis, as is often found in the literature, or even into necrosis and ACD, may not accurately reflect cellular processes leading to cell death. It may be more accurate to consider the variation in cell death morphologies as a continuum with apoptosis at one extreme and necrosis at the other (Lemaire *et al.*, 1998; Portera-Cailliau *et al.*, 1997). The cell death morphology expressed by a given cell likely depends on several factors including the cell type and the nature or severity of the death-inducing stimulus. Unfortunately, the molecular events underlying the different forms of cell death are unknown. However, the activation of specific cell death proteases such as the caspases may be important in determining the morphological outcome of a given death stimulus in a certain cell population.

Apoptotic Pathways: The Central Role of Caspases

Most nucleated mammalian (and likely avian) cells constituitively express the proteins required for ACD, indicating that ACD may represent the default cellular fate in the absence of survival factors that antagonize it (Ishizaki *et al.*, 1995; Raff *et al.*, 1993). The regulation of apoptosis is complex and varies between both cell types and apoptotic stimuli. With few exceptions, apoptotic pathways converge onto a unique family of cell death proteases, the caspases; thus, caspases are critical components of the apoptotic pathway, and as such, are attractive therapeutic targets in degenerative conditions involving inappropriate apoptosis.

Caspases (cysteine aspartate proteases) were first identified in mutational studies using *Caenorhabditis elegans* which identified the ced-3 gene as necessary for the normal

expression of programmed cell death during development (Yuan *et al.*, 1993). Subsequently, an entire family of mammalian homologues that share sequence, structure, and recognition sequence similarities has been discovered (Cohen, 1997; Thornberry and Lazebnik, 1998). In general, these caspases can be classified as mediators of inflammatory response (Group I; caspase-1. –4, -5, -11), effectors of apoptosis (Group II; caspase-3, -7, -2) or initiators of apoptotic caspase cascades (Group III; caspase-6, -8, -9, -10). Alternatively, they can be classified on the basis of their N-terminal prodomains. Class I caspases are initiators and possess long prodomains (including caspase-2, -8, -9, -10), whereas class II caspases are effectors and have a short or non existent prodomain (including caspase-3. –6, -7; reviewed in Kumar and Colussi, 1999). The properties and nomenclature of the known human caspases are listed in Table 1-1.

All caspases contain the sequence QACXG, in which C is the active site cysteine. Three-dimensional crystal structures of active caspases-1 and -3 indicate these enzymes utilize a typical cysteine protease mechanism involving a catalytic dyad of cysteine and histidine (Rotonda *et al.*, 1996; Walker *et al.*, 1994; Wilson *et al.*, 1994). However, caspases are unique in that they have a strict requirement for aspartate in the P1 position of their enzymatic targets, and require at least four amino acids N-terminal to the cleavage site (Thornberry and Lazebnik, 1998). Caspases are synthesized as inactive proenzymes (procaspases) with a prodomain of varying length and a large (p17-20) and small (p10-12) subunit. Upon activation, the prodomain and two subunits are cleaved from each other at caspase consensus sequences (indicating autoprocessing) and a heterodimer is formed through association of the large and small subunits. Two heterodimers then combine to form the active tetrameric complex, with two independent catalytic sites spanning the large and small subunits (reviewed in Cohen, 1997; Thornberry and Lazebnik, 1998).

In general, caspases can be activated in two ways (Figure 1-1). Ligand binding of death receptors ("death by murder") leads to the rapid induction of initiator caspases (e.g., caspase-8), which then activate effector caspases (e.g., caspase-3), the mediators of the morphological changes associated with apoptosis (Cohen, 1997; Janicke *et al.*, 1998; Stennicke *et al.*, 1998; Thornberry and Lazebnik, 1998). This method of caspase activation is a common mechanism of apoptosis induction in immune responses, and is

Figure 1-1. A wide variety of apoptotic signals converge upon effector caspases such as caspase-3, which execute the morphological changes characteristic of apoptosis. In general stimulation of death receptors such as Fas and TNF receptor, or mild cytotoxic stimuli such as growth factor withdrawal, irradiation, and oxidants activate initiator caspases, which then activate the effector caspases. Several viral and endogenous inhibitors prevent the activation of effector caspases either directly or indirectly through inhibition of initiator caspase activity. Therapeutic targets exist at every level of the pathway. Abbreviations: FADD (Fas-associated death domain), Apaf-1 (apoptosis activating factor 1), cyto c (cytochrome c) FLIP (FLICE/caspase-8 inhibitory protein), XIAP (X-linked inhibitor of apoptosis protein), c-IAP1 and 2 (cellular inhibitor of apoptosis protein 1 and 2). (Adapted from McBride *et al*, 1999)

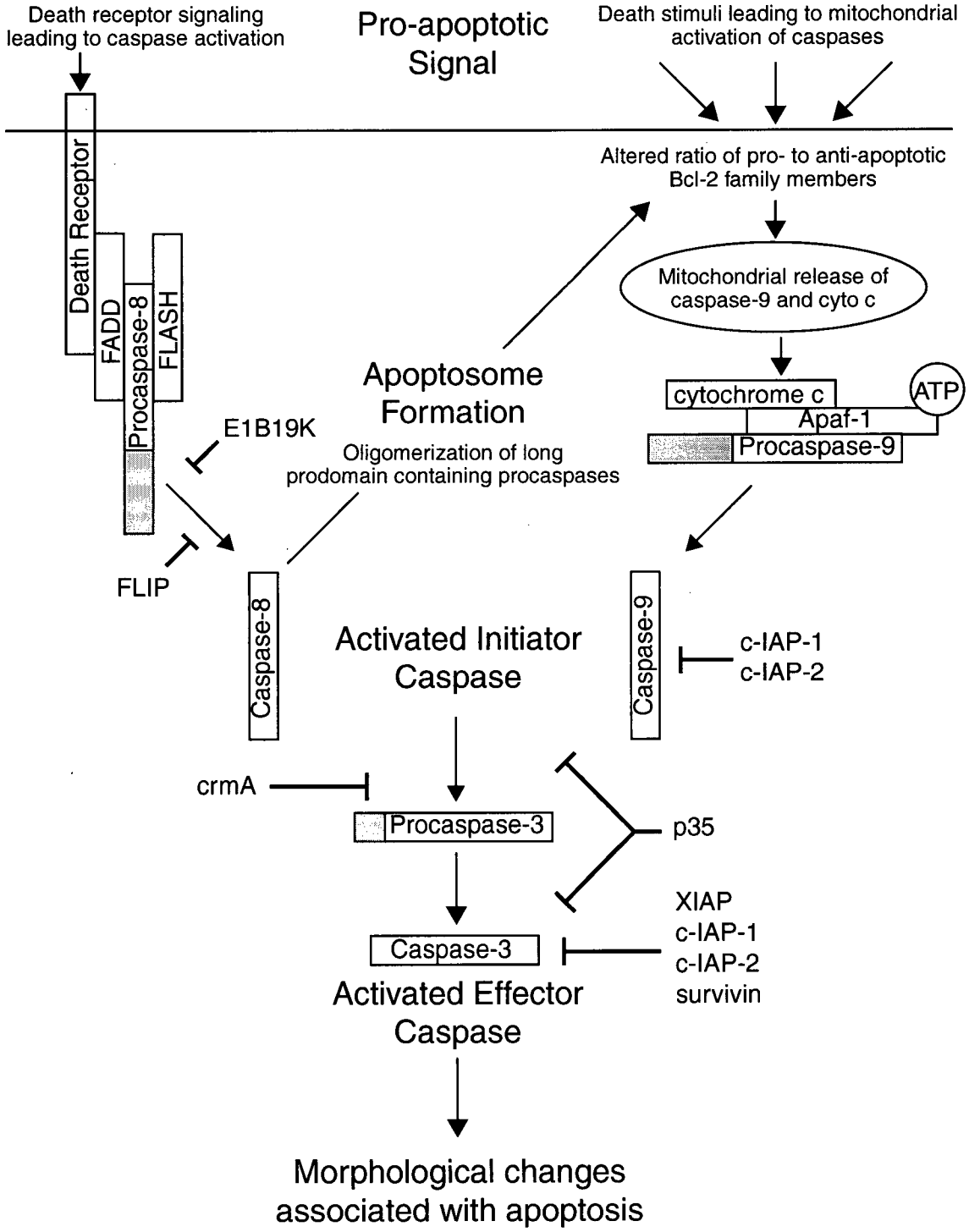

Death receptor signaling leading to caspase activation
Pro-apoptotic Signal
Death stimuli leading to mitochondrial activation of caspases
Death Receptor
FADD
Procaspase-8
FLASH
Altered ratio of pro- to anti-apoptotic Bcl-2 family members
Mitochondrial release of caspase-9 and cyto c
Apoptosome Formation
cytochrome c
ATP
Apaf-1
Procaspase-9
E1B19K
Oligomerization of long prodomain containing procaspases
FLIP
Caspase-8
Caspase-9
Activated Initiator Caspase
c-IAP-1
c-IAP-2
crmA
Procaspase-3
p35
XIAP
c-IAP-1
c-IAP-2
survivin
Caspase-3
Activated Effector Caspase
Morphological changes associated with apoptosis

Table 1-1. Properties of known human caspases (adapted from McBride et al, 1999).

Group (Function)	Caspase	Former Name	Optimal Substrate Sequence	Endogenous/ Viral Inhibitors	Examples of Cellular Substrates
I (Inflammation)	**Caspase-1**	ICE	WEHD	crmA, p35	Pro-IL-1, Pro-IL-18
	Caspase-4	ICE_{rel}-II, TX, ICH-2	(W/L)EHD	p35	
	Caspase-5	ICE_{rel}-III, TY	(W/L)EHD		
II (Effectors)	**Caspase-3**	CPP32/Yama/ Apopain	DEVD	p35, XIAP, cIAP1 & 2, survivin	PARP, ICAD, DNA-PK, fodrin, lamins, FAK, PKC, Bcl-2, Bcl-X_L, huntingtin, APP, presenilins Aβ
	Caspase-7	Mch3, ICE-LAP3, CMH-1	DEVD	XIAP, cIAP1 & 2, survivin	SREBPs
	Caspase-2*	ICH-1	DEHD	p35	
III (Initiators)	**Caspase-6****	Mch2	VEHD		Lamin B, PARP, APP (Swedish mutation)
	Caspase-8	MACH, FLICE, Mch5	LETD	crmA, c-FLIP	Procaspase-3 Bid
	Caspase-9	ICE-LAP6, Mch6	LEHD	XIAP, cIAP1 & 2	Procaspase-3
	Caspase-10*	Mch4		c-FLIP	

*some isoforms can inhibit apoptosis (Seol and Billiar, 1999; Srinivasula *et al.*, 1999).

** may be an effector as it cleaves lamin (Orth *et al.*, 1996; Takahashi *et al.*, 1998).

Abbreviations: c- FLIP (cellular FLICE/caspase-8 inhibitory protein), XIAP (X-linked inhibitor of apoptosis protein), c-IAP1 and 2 (cellular inhibitor of apoptosis protein 1 and 2), Pro-IL-1 and -18 (pro-interleukin-1β-1 and -18), PARP (poly(ADP-ribose) polymerase, ICAD (inhibitor of caspase-activated DNase), DNA-PK (DNA-dependent protein kinase), FAK (focal adhesion kinase), PKC (protein kinase C), APP (amyloid precursor protein), Aβ (amyloid β peptide), SREBPs (sterol-regulatory element binding proteins).

regulated by molecules such as FLIP (FLICE/caspase-8 inhibitory proteins (Bertin *et al.*, 1997; Hu *et al.*, 1997; Thome *et al.*, 1997). Alternatively, mild cytotoxic stimuli or withdrawal of trophic support ("death by neglect") can activate caspases in a more regulated and protracted manner in which mitochondria play a central role (Green and Reed, 1998). In this mode of caspase induction, factors such as cytochrome c and apoptosis activating factor (Apaf) are released from mitochondria that promote the activation of initiator caspases (e.g., caspase-9), which in turn activate the effector caspases (Adams and Cory, 1998; Deshmukh and Johnson, 1998; Gorman *et al.*, 1999; Kluck *et al.*, 1997a; Kluck *et al.*, 1997b; Kroemer, 1997; Li *et al.*, 1997; Martinou *et al.*, 1999; Reed, 1997a; Reed, 1997b; Slee *et al.*, 1999; Zou *et al.*, 1997). Several molecules upstream of mitochondria regulate entry into the caspase activation cascade, the most prominent being members of the Bcl-2 family. Members of this family are either pro- or anti-apoptotic and it appears that the ratio of these molecules regulates entry into the apoptotic pathway at the level of the mitochondrion (Adams and Cory, 1998; Farrow and Brown, 1996; Kluck *et al.*, 1997a; Kroemer, 1997; Reed, 1997b). Other cellular inhibitors such as the IAPs (inhibitors of apoptosis proteins) act directly at the level of effector caspase activation (Deveraux *et al.*, 1997b; Roy *et al.*, 1997).

In both pathways, the general scheme is for initiator caspases to be activated via oligomerization in complexes termed apoptosomes. The activated initiator caspases then activate effector caspases. Effector caspases execute the final stages of apoptosis through cleavage of distinct intracellular targets, including endogenous apoptosis inhibitors, structural proteins, repair enzymes, and endonucleases (Cohen, 1997; Porter *et al.*, 1997; Stroh and Schulze-Osthoff, 1998). Cleavage of these substrates serves to mitigate and facilitate the stereotyped morphological changes of apoptotic cell death. For example, caspase-3 cleavage of nuclear lamins contributes to chromatin condensation (Porter *et al.*, 1997; Stroh and Schulze-Osthoff, 1998), and disinhibition of caspase-activated Dnase (CAD) through cleavage of its inhibitor, ICAD, results in the characteristic DNA fragmentation associated with apoptosis (Enari *et al.*, 1998; Liu *et al.*, 1997a; Sakahira *et al.*, 1998).

Whether the activation of caspases represents the "point of no return" in the fate of the cell is unclear. Inhibiting caspase activity does lead to the rescue of cells in many

models of death. However, how long they survive is unclear, as most studies have examined only short-term effects (usually 2 weeks at the most). It is also unclear as to whether caspase inhibition following release of mitochondrial death-related signals will be a viable treatment, as the mitochondria may be irreversibly damaged by this timepoint (rendering the cell metabolically inactive). Thus, caspase inhibition may only delay or prevent the morphology of apoptosis but not the eventual death of the cell itself (McCarthy *et al.*, 1997; Vanderluit *et al.*, 1999). Nevertheless, caspase inhibition can, in some cases, reverse mitochondrial changes (Martinou *et al.*, 1999). In addition, evidence from models of stroke/ischemia and traumatic brain injury, argue there may be therapeutic potential for caspase inhibition in the rescue of damaged cells and improvement of neurological function (Chaudhary *et al.*, 1999; Chen *et al.*, 1998; Endres *et al.*, 1998; Kermer *et al.*, 1998; Ma *et al.*, 1998; Schulz *et al.*, 1998; Schulz *et al.*, 1999; Yakovlev *et al.*, 1997).

Apoptosis and SCI

It has been accepted for some time that necrosis plays an important role in CNS pathology following traumatic injury (Dusart and Schwab, 1994; Ito *et al.*, 1997; Kao and Chang, 1977; Schwab and Bartholdi, 1996; Zhang *et al.*, 1997b). It is only recently, however, that the relevance of apoptosis to SCI (Crowe *et al.*, 1997; Emery *et al.*, 1998; Katoh *et al.*, 1996; Li *et al.*, 1996; Liu *et al.*, 1997b; Lou *et al.*, 1998; Shuman *et al.*, 1997; Yong *et al.*, 1998) and TBI (Conti *et al.*, 1998; Rink *et al.*, 1995; Yakovlev *et al.*, 1997) has been appreciated. Apoptosis is first detected in the spinal cord near the site of injury 4-6 hours after contusion injury (Crowe *et al.*, 1997; Li *et al.*, 1996; Liu *et al.*, 1997b; Lou *et al.*, 1998). An increase in apoptotic cells at the lesion epicenter is observed over the following 24 hours, after which there is a decline. This apoptotic response consists of a variety of cell types including neurons and glia (Li *et al.*, 1996; Liu *et al.*, 1997b; Yong *et al.*, 1998). Surprisingly, a second phase of apoptosis is seen within regions remote to the lesion. Crowe et al (Crowe *et al.*, 1997) were the first to report the appearance of apoptotic cells in the spinal white matter of both rat and monkey several segments rostral and caudal to the site of injury. At this time, I detected a similar phenomenon in transected spinal cords of developing chick embryos (McBride *et al.*, 1996). Others have subsequently confirmed this finding (Emery *et al.*, 1998; Liu *et al.*,

1997b; Shuman *et al.*, 1997). Many of the apoptotic cells in regions remote from the injury have been identified as oligodendrocytes (Crowe *et al.*, 1997; Emery *et al.*, 1998; Liu *et al.*, 1997b; Shuman *et al.*, 1997). Such oligodendrocyte cell death and the concomitant loss of myelin may have important implications in the neurological outcome after injury (discussed below in *The Oligodendrocyte Response to SCI).*

Oligodendrocyte Description and Function

Oligodendrocytes are the myelinating cells of the central nervous system (c.f., Bunge, 1968). The myelin they produce forms an essential component of the CNS, allowing for efficient conduction of most large and medium diameter axons. Pathological demyelination, as occurs in multiple sclerosis (MS) and SCI, leads to functional conduction block that manifests as neurological deficits (Blight, 1983; Blight, 1993; Bunge *et al.*, 1993; Gledhill and McDonald, 1977; Martin *et al.*, 1992; Shi *et al.*, 1997; Waxman, 1992). Oligodendrocyte-derived myelin may also function in the "hard wiring" of the intricate neuronal patterns formed during development (Colello *et al.*, 1994; Keirstead and Steeves, 1998). A consequence of myelin's stabilization of the CNS pattern appears to be the inhibition of axonal regeneration after traumatic injury to the CNS (reviewed in Schwab, 1992; Schwab and Bartholdi, 1996; Schwab et al., 1993; Steeves et al., 1993; Steeves et al., 1994; Steeves and Tetzlaff, 1998; Keirstead, 1998). The inhibitory effects of myelin on regenerating CNS neurons have been described in several species, including chick (reviewed in Schwab and Bartholdi, 1996). Work done in this laboratory has demonstrated that the change from a permissive to a restrictive period for axonal regeneration of transected chick brainstem-spinal neurons (Hasan *et al.*, 1993) correlates with the appearance of myelin in the developing chick spinal cord (Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Keirstead *et al.*, 1997). Furthermore, experimental complement-mediated suppression of myelin formation or removal can facilitate regeneration of injured CNS neurons (Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Keirstead *et al.*, 1998a; Keirstead *et al.*, 1997).

Major components of the myelin membrane include proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP). MBPs comprise of family of related proteins derived by alternative spicing of a single gene (de Ferra *et al.*, 1985; Kimura *et al.*, 1985; Takahashi

et al., 1985). MBPs' highly positively charge causes them to avidly bind the negatively charged lipids of membranes. For this reason, it has been proposed that MBP mRNA is translocated to the loci of insertion in order that the MBPs do not insert into the membranes of intracellular organelles (Staugaitis *et al.*, 1996). The high reactivity of oligodendrocytes with membranes contributes to the compaction of myelin lamellae and the formation of the major dense line. However, not all MBP isoforms are located in the myelin sheath. Many oligodendrocytes express MBP in the soma and nucleus, suggesting a regulatory role in myelination (Hardy *et al.*, 1996). The differential distribution of MBP in oligodendrocytes also suggests phenotypic heterogeneity. Electron microscopic analysis of oligodendrocytes indicates three main types based on morphology (Mori and Leblond, 1970). The light and medium phenotypes are believed to have higher metabolic activity and be involved in myelin production and maintenance (Cho and Lucas, 1995; Sturrock, 1974).

Oligodendrocyte Development

Oligodendrocytes are of neuroepithelial origin and differentiate from focally induced precursor cells located in the ventral ventricular zone of the developing spinal cord (Ono *et al.*, 1995; Richardson *et al.*, 1997). From there, they migrate as dividing but committed cells to their final location in the mature spinal cord (Miller, 1996; Ono *et al.*, 1995). A2B5 is the earliest characterized cell surface antigen, in oligodendrocyte precursors (Eisenbarth *et al.*, 1979). In response to growth factors such as fibroblast growth factor (FGF) and platelet derived growth factor (PDGF), the A2B5 precursors proliferate (McKinnon *et al.*, 1990; Miller, 1996; Noble *et al.*, 1988). These precursors have been termed oligodendrocyte-type-2-astrocyte (O-2A) progenitor cells as *in vitro*, they are bipotential, possessing the ability to form type-2 astrocytes or oligodendrocytes depending on the trophic environment (Raff *et al.*, 1983). The relevance of this developmental plasticity *in vivo* is unknown. As the precursors mature, they begin to express cell surface antigens detected by the monoclonal antibody, O4 (Sommer and Schachner, 1981). Subsequently, they begin to express Ranscht monoclonal antibody (R-mAb/O1) immunoreactivity and the major myelin glycolipid, galactocerebroside (GalC) (Raff *et al.*, 1978; Ranscht *et al.*, 1982). The expression of GalC correlates with an abrupt decline in oligodendrocyte precursor proliferation and the onset of their differentiation

into mature oligodendrocytes (Fok-Seang and Miller, 1994; Hart *et al.*, 1989a; McKinnon *et al.*, 1990). As the differentiating oligodendrocyte matures it begins to express other myelin proteins such as MBP (Reynolds and Wilkin, 1991).

In the chick spinal cord, the O4-positive precursors are first detected on E6 (Ono *et al.*, 1995). At this stage, the O4-positive cells are localized to the ventral ventricular area and ventral mantle layer. By E11, intensely stained O4-positive cells are observed throughout the spinal white matter and scattered throughout the grey matter. It is at this time that oligodendrocyte precursors begin their final differentiation, and shortly after, begin to express myelin associated proteins such as MBP, which appears on approximately E12-13 (Keirstead *et al.*, 1992; Macklin and Weill, 1985; Richardson *et al.*, 1997; State *et al.*, 1977). MBP is initially expressed in the cytosol and nucleus of premyelinating and promyelinating (cells forming initial myelin wraps around axons) oligodendrocytes, and subsequently in the lamellar extensions of the myelin sheath (Butt *et al.*, 1997; Hardy *et al.*, 1996).

Oligodendrocyte precursors proliferate in response to platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), neurotrophin-3 (NT-3), and basic FGF (reviewed in Barres and Raff, 1994; Miller, 1996; Richardson et al., 1997). *In vivo*, proliferation appears to be dependent on electrical activity in axons (Barres and Raff, 1993). Tetrodotoxin-induced blockade of electrical activity in the rat optic nerve reduces oligodendrocyte precursor proliferation by 80%. This reduction can be reversed by administration of PDGF to the optic nerve, indicating electrical activity induces the release of oligodendrocyte precursor mitogens. A likely source of these mitogens is astrocytes, which make and release PDGF, IGF-1, and NT-3 (Barres and Raff, 1994). However, PDGF only promotes the survival of newly formed oligodendrocytes: as oligodendrocytes mature, they lose their PDGF receptors and are no longer receptive to PDGF (Hart *et al.*, 1989b; McKinnon *et al.*, 1990).

Oligodendrocyte precursors do not divide indefinitely: they possess an intrinsic clock that stops proliferation and initiates differentiation after approximately eight divisions *in vitro* (Temple and Raff, 1986). The clock appears to be made of two components, one that counts time or cell division and one that stops proliferation (Barres and Raff, 1994). The effector component consists of intracellular receptor activation by steroids and

retinoic acid (Barres *et al.*, 1994). Thyroid hormone plays an important role in halting proliferation and promoting myelination of differentiating oligodendrocytes (Barres and Raff, 1994). There is evidence to suggest limited reserves of oligodendrocyte precursors are present in adult spinal cord and may differentiate to remyelinate demyelinated regions (Keirstead *et al.*, 1998b). Mature oligodendrocytes are generally considered to be postmitotic (Gard and Pfeiffer, 1989; Hardy and Reynolds, 1991; Keirstead and Blakemore, 1997). However, exposure of cultured mature oligodendrocytes to FGF-2 or the neuregulin, GGF can result in their dedifferentiation into early stage oligodendrocyte precursors capable of cell division (Canoll *et al.*, 1999; Grinspan *et al.*, 1996b). The relevance of these findings remains to be determined *in vivo;* however, oligodendrocyte dedifferentiation is thought to occur following optic nerve transection in P21 rats (Butt and Colquhoun, 1996).

Developmental Oligodendrocyte Cell Death

It has become accepted that significant neuronal cell death occurs during CNS development (Cowan *et al.*, 1984; Oppenheim, 1991; Pettmann and Henderson, 1998). In populations such as chick spinal motor neurons and the isthmo-optic nucleus, cell death claims 50-60% of the developing cells. It is believed excess neurons are eliminated through competition for limiting amounts of trophic support from neuronal targets and afferent inputs (Cowan *et al.*, 1984; Oppenheim, 1991). The initial excess of neurons is thought to provide a developmental safety factor, ensuring the appropriate number and targeting of connections within the nervous system, a process that can occur in an epigenetic manner. The recent discovery of developmental oligodendrocyte death in the rat optic nerve indicates a process that may be analogous to developing neuronal populations. Approximately 50% of oligodendrocytes die during development of the optic nerve (Barres *et al.*, 1992b). It has been suggested that, analogous to neuronal cell death, this death occurs as a result of competition for a limited amount of trophic support required by newly formed oligodendrocytes for their survival and ensures appropriate myelination of CNS axons (Barres *et al.*, 1992a; Barres *et al.*, 1993a; Barres and Raff, 1994; Barres *et al.*, 1993b).

The source of trophic factors required for oligodendrocyte survival is unknown. However, the presence of axons is critical for the survival of newly formed

oligodendrocytes. If the rat optic nerve is transected on P12, axons degenerate and 90% of the oligodendrocytes die over a 10 day period (Barres *et al.*, 1993a). By contrast, transection of the C56B1/6-WLD mutant mouse optic nerve did not induce an increase in oligodendrocyte apoptosis (Barres *et al.*, 1993a). This mouse contains a genetic defect that decreases the rate of degeneration of the distal stump of the transected nerve (Perry *et al.*, 1991). Thus, the mere presence of axons, which need not necessarily have continuity with a cell body, may be sufficient to promote oligodendrocyte survival. This idea is supported by *in vitro* experiments demonstrating oligodendrocyte survival in cultures containing axons but not in axon conditioned medium alone, suggesting a trophic mechanism dependent on contact rather than a secreted factor. Further evidence for the role of axons in oligodendrocyte survival is provided in a study using a bcl-2 transgenic mouse in which the number of axons in the optic nerve is increased as a result of decreased developmental cell death of retinal ganglion cells (Burne *et al.*, 1996). In this model, the number of oligodendrocytes, astrocytes and microglia is also increased proportionately to the number of axons. Interestingly, the neuron specific enolase promoter-driven bcl-2 transgene was detected in all oligodendrocytes; however, bcl-2 was unable to protect oligodendrocytes from trophic factor deprivation-induced PCD, indicating that the transgene was not directly responsible for the increase in oligodendrocytes. Furthermore, transection of the transgenic optic nerve on P18 resulted in oligodendrocyte death that is proportionately greater than that in wild type mice. Similar axon dependence has been reported for myelinating Schwann cells in the developing sciatic nerve, and in the development of *Drosophila* midline glia (Sonnenfeld and Jacobs, 1995).

The precise identity of axon-derived oligodendrocyte survival factors remains elusive. However, despite the inability of axon conditioned medium to promote oligodendrocyte survival, exogenous cilliary neurotrophic factor (CNTF) and insulin-like growth factor-1 (IGF-1) can reduce oligodendrocyte death after optic nerve transection (Barres *et al.*, 1993a), and axonally-derived neuregulin can influence oligodendrocyte responses through the Her4 receptor (Vartanian *et al.*, 1997). The mechanisms by which oligodendrocytes die is similarly unclear, but likely involves caspase activation (Emery *et al.*, 1998; Gu *et al.*, 1999). Whether or not axons promote oligodendrocyte survival

directly or indirectly is unclear. In vitro, purified neuronal cultures can promote oligodendrocyte survival; however, the possibility that *in vivo* astrocytes contribute to the promotion of oligodendrocyte survival can not be ruled out. Astrocytes upregulate CNTF after optic nerve transection, although it is not clear as to whether it is secreted *in vivo* (Barres *et al.*, 1993a). Astrocytes can also influence oligodendrocyte maturation. For example, oligodendrocyte contact with astrocytes in culture results in the expression of MBP isoforms involved in membrane compaction (Sakurai *et al.*, 1998).

The Oligodendrocyte Response to SCI

The oligodendrocyte response to traumatic injury remains largely enigmatic. Although they are not believed to subserve a reactive role in the primary or secondary injury response, trauma-induced oligodendrocyte pathology may have important neurological implications. Oligodendrocytes have been observed undergoing apoptosis in the perilesion region and in the white matter remote to the site of primary injury (Crowe *et al.*, 1997; Emery *et al.*, 1998; Liu *et al.*, 1997b; Shuman *et al.*, 1997). Apoptotic oligodendrocytes in the white matter remote to the lesion appear to be associated with degenerating axons (Crowe *et al.*, 1997; Liu *et al.*, 1997b; Shuman *et al.*, 1997). As a single oligodendrocyte can myelinate multiple axons, degeneration of an unknown proportion of axons associated with an oligodendrocyte may induce its death, a phenomenon that may explain the appearance of demyelinated regions of the injured spinal cord. Observations of trauma-induced demyelination and interference of conduction of ascending and descending axons dates back to the turn of the 20th century (Holmes, 1906). The occurrence of this aspect of the secondary injury response has since been substantiated in both experimental SCI animal models and human SCI patients (Blight, 1983; Blight, 1985; Blight, 1993; Bunge *et al.*, 1993; Gledhill and McDonald, 1977; Shi *et al.*, 1997). The appearance of demyelinated regions in injured spinal cords correlates with the peak of the distal apoptotic response of white matter oligodendrocytes. The observed demyelination is not, however, restricted to degenerating axons as intact axons also become demyelinated (Blight, 1992; Blight, 1993; Blight and Decrescito, 1986; Blight *et al.*, 1991; Bunge *et al.*, 1993; Shi *et al.*, 1997; Waxman, 1992). Such demyelination results in functional conduction block in axons due to the low density of sodium channels and the unmasking of potassium channels in the internodal axonal

membrane, which serve to fix the membrane close to the potassium equilibrium potential (Waxman, 1992). This can be partially overcome through administration of the potassium channel blocker, 4-aminopyridine (Blight *et al.*, 1991; Shi and Blight, 1997), which effectively delays repolarization of the membrane, thereby increasing the membrane length constant.

Oligodendrocytes appear to be particularly susceptible to many cell death stimuli, including those that may exist in the setting of neurotrauma and secondary injury. Cultured oligodendrocytes are vulnerable to elevated glutamate (Oka *et al.*, 1993) via AMPA/kainate receptor-mediated glutamate excitoxicity (Rosenberg *et al.*, 1999), ceramide (Casaccia-Bonnefil *et al.*, 1996a), TNF-α (Akassoglou *et al.*, 1998; D'Souza *et al.*, 1995; Louis *et al.*, 1993; Selmaj *et al.*, 1991), Fas (D'Souza *et al.*, 1996b), oxidative stress (Back *et al.*, 1998), FGF-2 (Muir and Compston, 1996), and interleukin-2 (Eizenberg *et al.*, 1995). It should be noted that although microglia express many of these cytotoxins, they themselves are susceptible to apoptosis induced by the same agents. Thus, microglia may induce oligodendrocyte apoptosis after injury but may also undergo apoptotic death as well. Such a scenario has been observed in the spinal white matter of the rat following spinal contusion injury (Shuman *et al.*, 1997). Astrocytes are another potential source of cytotoxic agents such as TNF-α and may contribute to oligodendrocyte pathology following trauma (Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995). Astrocytes appear to be far more resistant to the above mentioned cytotoxins and do not readily undergo apoptosis after injury, except in the immediate vicinity of the injury (Yong *et al.*, 1998).

Developmental Model of CNS Injury

The primary goals of this thesis were to determine the nature of SCI-induced apoptosis in the spinal cord distal to the lesion and to determine the role of apoptotic proteases (caspases) in this death. In order to accomplish the latter goal, it was necessary to develop an *in vivo* model of trauma-induced apoptosis in which sufficient levels of cells would undergo apoptosis in a condensed timeframe to enable biochemical analysis of protease activity in injured tissue. Such a model could also be used to determine the efficacy of specific protease inhibitors to inhibit the death. Current models of SCI typically involve adult rodents. These models effectively mimic the human condition and

have provided great insight into the responses and mechanisms of CNS trauma. Of particular relevance to my work is the finding of oligodendrocyte apoptosis in the distal spinal white matter of rats and monkeys that received spinal contusion injuries (Crowe *et al.*, 1997; Shuman *et al.*, 1997). This death occurred over a protracted timecourse with only a small but significant number of apoptotic cells detected at any given time; therefore, adult rodent models are unattractive for quantitative biochemical analysis of the apoptotic response remote from the injury.

Developmental models have proven invaluable for understanding many aspects of neural cell death and axonal regeneration following injury (Henderson, 1996; Oppenheim, 1991; Snider *et al.*, 1992; Steeves *et al.*, 1993; Steeves *et al.*, 1994; Tetzlaff *et al.*, 1994). An important advantage of developmental models is the rapid onset of reactions and the large scale of the cellular responses. For example, axotomy at certain points during development results in almost total loss of many neuronal populations (Clarke, 1992; Cowan *et al.*, 1984; Oppenheim, 1991; Snider *et al.*, 1998). The neuronal death occurs rapidly after injury, generating large numbers of dying cells during this time. Similar injuries in adult animals usually result in only a small amount of neuronal death that occurs over a prolonged time course. Similarly, Schwann cells in the developing sciatic nerve and oligodendrocytes in the developing optic nerve are more sensitive to axonal perturbations during development than in the mature animal (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996; Butt *et al.*, 1997; Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Ludwin, 1990; Ludwin, 1992; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996). Such models have provided important information regarding the trophic requirements of the injured cells and agents that may attenuate their death. It is believed that the mechanisms underlying developmental cell death and regeneration may provide insight into the mechanisms of injury, degeneration and regeneration not only in mature animal models, but also after human neurotrauma.

Why are neural populations more sensitive to injury-induced death during development? This is a question that remains to be answered sufficiently; however, several theories exist. The fundamental explanation may revolve around the neurotrophic theory, which states that developing neurons must compete for a limited supply of target-derived trophic factor, and those not making appropriate connections will be eliminated.

This would allow for the precise formation of the nervous system connections and patterning through epigenetic influences. A similar theory has been proposed for oligodendrocyte development in which an appropriate number of oligodendrocytes must be formed to match the number of neurons requiring myelination (Barres *et al.*, 1993a; Barres and Raff, 1994). By producing an initial excess of neurons or oligodendrocytes, the nervous system has a built in, epigenetic safety mechanism to ensure sufficient connections are made. It has been proposed that all nucleated cells constituitively express the cellular death machinery and that ACD is the default program that must be suppressed by appropriate trophic factors (Ishizaki *et al.*, 1995; Raff *et al.*, 1993). During development, levels of these death-related molecules are often elevated compared with adult levels. Thus, in developing systems, cells are "primed" to die and perturbations that further restrict the levels of essential trophic support can accentuate the number of dying cells.

It is unclear why mature neurons and glia are more resistant to the same types of injury that cause massive ACD during development. In addition to down-regulating expression of death inducing proteins, it has been postulated that neurons and glia switch their trophic dependencies once functional connections have been made, and may initiate autocrine survival loops to maintain their own survival (Cheng and Patterson, 1997; Kobayashi *et al.*, 1996; Yang *et al.*, 1998c). Thus, adult neurons and glia, although capable of undergoing apoptosis, may not be as primed to do so.

Embryonic Chick Model of CNS Trauma

I chose the embryonic chick as a model in which to examine the distal apoptotic response to cervical SCI. The embryonic chick has been an important model for understanding CNS development, neuronal cell death, and axonal regeneration. Early work by Hamburger and Levi-Montalcini in the chick nervous system lead to the discovery of nerve growth factor (NGF) and development of the neurotrophic theory of cell survival (Levi-Montalcini, 1987). The work of Oppenheim on spinal motor neuron death indicated the extent and mechanisms of neuronal death and survival in the developing CNS (Oppenheim, 1991), and Clarke's studies of the cell death in the developing isthmo-optic nucleus revealed the different ultrastructural morphologies dying cells express (Clarke, 1990). Work on the developing chick CNS in the laboratory of

John Steeves has shed important light on the inhibitory role of myelin on regeneration within the CNS (Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Keirstead *et al.*, 1997; Steeves *et al.*, 1994; Steeves and Tetzlaff, 1998).

One reason the embryonic chick has been used so extensively in studies of CNS development is its experimental accessibility. Surgical and pharmacological manipulations can be made with a greater degree of simplicity and efficiency than in similar developmental staged mammalian embryos. This fact also reduces the cost of investigation. It is important to note that the CNS of the chick is very similar to that of mammalian species (c.f., Steeves et al., 1993; Steeves et al., 1994). However, developmental events appear to be accelerated in the chick (a precocial vertebrate), another desirable feature for investigations (c.f., Steeves et al., 1993; Steeves et al., 1994).

Development of the embryonic chick occurs over approximately 21 days. The first functional synapses in the spinal cord from brainstem-spinal neurons are detected on embryonic day (E) 7, and by E11, the mature pattern of supraspinal projections is formed (Glover, 1993; Okado and Oppenheim, 1985; Sholomenko and O'Donovan, 1995). It is shortly after this stage that myelination of the chick spinal cords begins. MBP immunoreactivity appears on E12-E13 (Keirstead *et al.*, 1992; Macklin and Weill, 1985; Richardson *et al.*, 1997), and it is at this time that the chick spinal cord undergoes a transition from a permissive environment for regeneration of axotomized brainstem-spinal neurons to one that is restrictive (Hasan *et al.*, 1993). This transition is in part due to the appearance of myelin (Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Keirstead *et al.*, 1997; Steeves *et al.*, 1994). The blood brain barrier also begins to form at this time (Wakai and Hirokawa, 1978). Unfortunately, less is known about the development of ascending pathways in the chick spinal cord, although the pattern of spinocerebellar projections is complete by approximately E14 (Okado *et al.*, 1987) and longitudinal projection patterns of lumbar primary sensory afferents develop by E10 (Eide and Glover, 1995)

Hypotheses and Relevance

The impetus for embarking on the present project was based on the preliminary observation of the appearance of apoptotic cells in the sciatic nerve following transection

of this nerve on E12. To develop the TUNEL technique in embryonic chick nervous tissue, I used embryonic sciatic nerve transection to induce apoptosis in dorsal root ganglion (DRG) cells. I was able to successfully label large numbers of apoptotic DRG cells using TUNEL. Surprisingly, during the analysis of my ability to use this technique, I also observed increased numbers of TUNEL+ cells in the dorsal root of the axotomized nerve compared to the uninjured contralateral nerve. Soon after making this observation, it was reported that transection of the neonatal mouse sciatic nerve induced Schwann cell apoptosis (Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996), the likely cellular identity of the increased apoptotic response in the chick sciatic nerve. It was because of this, that I postulated a similar phenomenon would occur in oligodendrocytes after spinal cord transection, a speculation supported by the large-scale, injury-induced oligodendrocyte apoptosis in the developing rat optic nerve concomitant with the onset of myelination (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne and Raff, 1997). The emerging understanding of the central role of caspases in apoptosis suggested these pro-apoptotic proteases could mediate such a response in the injured spinal cord. Thus, **I hypothesized oligodendrocytes would be vulnerable to trauma-induced apoptosis in the developing chick spinal cord, particularly during the time of myelination of the spinal white matter tracts, and that this apoptotic response would be dependent upon caspase activation.**

In order to test these hypotheses, I examined the apoptotic response to transection of the cervical spinal cord at different times during development. I focussed on the reaction remote from the injury in order to avoid the complex and heterogeneous nature of the focal injury response. The amount of apoptosis was quantified distal to the lesion at various timepoints during development and after injury. The anatomical distribution of apoptotic cells was assessed, and the cell types determined. This initial study revealed a sensitive period for oligodendrocyte apoptosis in the spinal white matter several segments from the injury site during the active, developmental period of myelination. These results indicated that spinal transection of the embryonic chick was a viable model in which to study and perhaps quantify the mechanisms underlying the apoptotic process in the distal spinal white matter. Specifically, I examined the expression and activity of caspases in this response, and the potential for using caspase inhibitors to attenuate it. I observed

preferential activation of caspase-3-like activity over caspase-1-like activity, and *in ovo* application of synthetic peptide inhibitors attenuated the apoptotic response in the white matter remote from the site of injury.

These results provide insight into the mechanisms of trauma-induced glial apoptosis in the CNS, and suggest a potential for caspase inhibitors to attenuate this response. This may be important in light of the oligodendrocyte apoptosis recently found in humans victims of SCI (Emery *et al.*, 1998). In addition, the findings of this project could be of relevance to the understanding of the regulation of neuropathological diseases involving oligodendrocyte death such as MS (Dowling *et al.*, 1997; Oshako and Elkon, 1999; Tsunoda *et al.*, 1997), multiple system atrophy (Probst-Cousin *et al.*, 1998), and cognitive and spastic motor deficits observed in premature infants (Noble and Mayerproschel, 1996). The embryonic chick transection model developed here may also prove useful in elucidating the stimuli for injury-induced glial apoptosis and testing putative trophic factors that may promote their survival. This will be a critical development as caspase inhibition may only extend the therapeutic window to allow application of survival promoting factors that will promote the long term survival of rescued cells.

CHAPTER 2: DEVELOPMENTAL OLIGODENDROCYTE APOPTOSIS

INTRODUCTION

Developmental Glial ACD

It has been appreciated for some time that neuronal cell death is a prominent feature of nervous system development (Cowan *et al.*, 1984; Oppenheim, 1991). It is believed that much of this death results from a failure to successfully compete for a limited supply of trophic support, and that the initial excess of cells ensures the appropriate number and pattern of connection is made (Cowan *et al.*, 1984; Oppenheim, 1991). Recently, the phenomenon of glial cell death during development of the CNS and PNS has gained attention. The mechanisms governing the survival of developing oligodendrocytes and Schwann cells are believed to be similar to those in neurons, in that differentiating oligodendrocytes and Schwann cells compete for a limited supply of trophic support. Those that fail to successfully compete for these survival factors are eliminated in a controlled, unobtrusive manner. The initial excess of these cells likely ensures appropriate myelination of axonal pathways.

The precise nature of oligodendrocyte survival cues and their source or sources is unclear, as are the mechanisms underlying their death. However, axons appear to be essential for their survival. In the developing rat optic nerve, approximately 50% of the newly generated oligodendrocytes die (Barres *et al.*, 1993a; Barres and Raff, 1994). This death increases dramatically when the optic nerve is severed and decreases when the number of axons in the optic nerve is increased via bcl-2 transgene expression in retinal ganglion cells (Burne *et al.*, 1996). Similarly, damage to the developing peripheral nerve results in increased Schwann cell apoptosis (Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Trachtenberg and Thompson, 1996). Glial growth factor (GGF) may also promote survival as this axonal neuregulin influences oligodendrocyte development through the HER4 receptor (Vartanian *et al.*, 1997). However, it is unclear whether axons directly affect oligodendrocyte survival or indirectly through cells such as astrocytes. Although the presence of purified neuronal cultures was sufficient for oligodendrocyte survival, the role of astrocytes *in vivo* can not be excluded. IGF-1, NT-3, CNTF, and PDGF-AA, have also been shown to regulate oligodendrocyte survival (Barres *et al.*, 1992a; Barres *et al.*, 1993a; Barres *et al.*, 1993b). Astrocytes are known to produce many of the trophic factors

that promote oligodendrocyte survival (Barres *et al.*, 1993a). Therefore, it is possible that intact axons trigger the astrocytic release of these factors.

The extent to which mature oligodendrocytes depend on the trophic factors required during development is also unknown. However, evidence suggests they are not as heavily reliant on axons. Transection of mature optic nerve results in only a small loss of oligodendrocytes, and surviving oligodendrocytes are observed in Wallerian degenerated regions for long periods after the injury (Butt *et al.*, 1997). Similarly, Schwann cells appear to tolerate sciatic nerve transection in adult rodents (Meier *et al.*, 1999). Nevertheless, recent studies have shown the involvement of trauma-induced oligodendrocyte apoptosis in adult rodent and primate spinal cords (Crowe *et al.*, 1997; Liu *et al.*, 1997b; Shuman *et al.*, 1997). Moreover, the dying cells are often associated with degenerating axons. It is unclear what triggers the death of these cells, and whether the axonal degeneration is directly responsible for it. Demyelination is observed in regions of intact axons following SCI, indicating that other factors may initiate the apoptotic response in oligodendrocytes after injury. (Blight, 1992; Blight, 1993; Blight and Decrescito, 1986; Blight *et al.*, 1991; Bunge *et al.*, 1993; Shi *et al.*, 1997; Waxman, 1992)

The trauma-induced oligodendrocyte apoptosis observed in the adult rat and monkey was observed both at the site of primary injury and in regions remote from the lesion (Crowe *et al.*, 1997; Liu *et al.*, 1997b; Shuman *et al.*, 1997). The functional consequence of this phenomenon is unknown, but may relate to functional deficits resulting from demyelination-induced conduction block after SCI (Blight, 1992; Blight, 1993; Blight and Decrescito, 1986; Blight *et al.*, 1991; Bunge *et al.*, 1993; Shi *et al.*, 1997; Waxman, 1992). In short, little is known about the mechanisms underlying SCI-induced oligodendrocyte apoptosis. The protracted nature of the SCI-induced oligodendrocyte apoptosis and the small number of dying oligodendrocytes observed at any given post-injury timepoint in these models makes it difficult to determine biochemical causes. Thus, an alternative *in vivo* model in which large numbers of apoptotic oligodendrocytes can be induced to die over a short time is desirable for investigating the cellular and molecular regulation of their death. The heightened susceptibility of developing neurons and glia to injury has proven developmental models useful in understanding the

mechanisms of active neuronal cell death and survival. Findings from developmental cell death models have provided insight into the mechanisms mediating unscheduled ACD in several neurodegenerative disorders (Takahashi *et al.*, 1985).

Embryonic Chick Model of CNS Trauma

I chose the embryonic chick as a model in which to examine the distal apoptotic response to cervical SCI. The embryonic chick has been an important model for understanding CNS development, neuronal cell death, and axonal regeneration. Early work by Hamburger and Levi-Montalcini in the chick nervous system lead to the discovery of nerve growth factor (NGF) and development of the neurotrophic hypothesis (c.f., Levi-Montalcini, 1987). Analysis of embryonic chick spinal motor neuron death indicated the extent and mechanisms of neuronal death and survival in the developing CNS (Oppenheim, 1991). The embryonic chick has been used extensively in the Steeves laboratory to shed important light on the inhibitory role of myelin on regeneration within the CNS (Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Keirstead *et al.*, 1997). The CNS of the chick is very similar to that of mammalian species (Steeves *et al.*, 1993). However, since chickens are oviparous, surgical and pharmacological manipulations can be made with a greater degree of simplicity and efficiency than in similar developmental staged mammalian embryos. In addition, the chick's neurodevelopmental events appear to be accelerated and compressed in time, another desirable feature for investigations of ACD.

Chicken embryo development occurs over a 21 day period. Functional synapses from brainstem-spinal neurons are detected in the spinal cord on E7, and by E11, the pattern of supraspinal projections is fully formed (Glover, 1993; Okado and Oppenheim, 1985; Sholomenko and O'Donovan, 1995). Shortly after this stage, myelination of the chick spinal cords begins (Keirstead *et al.*, 1992; Macklin and Weill, 1985; Richardson *et al.*, 1997; State *et al.*, 1977), and the blood brain barrier forms (Wakai and Hirokawa, 1978). With respect to ascending pathways in the chick spinal cord, the pattern of spinocerebellar projections is complete by approximately E14 (Okado *et al.*, 1987) and longitudinal projection patterns of lumbar primary sensory afferents are complete by E10 (Eide and Glover, 1995).

As mentioned, myelination of the chick spinal cord begins on approximately E13. Myelination of the cervical spinal white matter proceeds in a rostrocaudal and

dorsoventral direction, and myelination of the entire cord is complete by hatching (State *et al.*, 1977). Myelin basic proteins (MBPs) are first detected at all levels of the spinal cord on E13 (Macklin and Weill, 1985). MBP exists as one of several isoforms derived from a single gene via alternative splicing of exons 2, 5, or 6 (Takahashi *et al.*, 1985). The chick, unlike several mammals, does not express the 21.5- or 20.2 kD isoforms; instead, the prominent isoforms in chick are 18.5- and 17.3 kD (Kerlero de Rosbo *et al.*, 1991). The concentration of these isoforms rises in the chick spinal cord from E13 to post hatching day (P) 5. The 18.5 kD isoform has been shown to localize to the nucleus in rat oligodendrocytes and is not highly reactive with membranes (Allinquant *et al.*, 1991). This indicates it may not be involved in myelin compaction and may somehow regulate myelinogenesis. Proteolipid protein is detected approximately one day later than MBP and CNPase activity follows MBP detection by 3-4 days. Overall, the period of myelination in the chick is shorter and begins earlier than in some mammals, such as the rat (Hartman *et al.*, 1979; Macklin and Weill, 1985), a feature that may relate to the precocial development of a hatchling chick that is capable of independent life upon hatching.

Detection of Apoptosis

In the studies undertaken in this thesis, I examined the apoptotic response to traumatic CNS injury. Apoptosis is the most commonly investigated form of ACD. This may be due in part to its prevalence in many different tissues and cell lines, and to the relative ease of its detection. To date, electron microscopic analysis of cellular ultrastructure is the only definitive means by which to differentiate the different forms of ACD. Although electron microscopy remains the 'gold standard" in determining apoptotic death, other more readily accessible methods have been developed to rapidly screen for the presence and extent apoptosis in cells and tissue. These methods take advantage of hallmark features of apoptosis, including DNA fragmentation into oligonucleosomal fragments, chromatin condensation, and changes in cell surface markers (Kerr *et al.*, 1995). The stereotyped fragmentation of DNA in cells undergoing apoptosis is the most commonly exploited feature used in the detection. The characteristic oligonucleosomal laddering of DNA following electrophoresis on agarose gels was one of the first of these techniques used to identify apoptosis (Eastman, 1995).

However, it is not always sensitive enough to detect apoptosis in tissue containing heterogeneous cell populations with different sensitivities to apoptotic stimuli (such as found in the CNS). When a small but significant number of cells are undergoing apoptosis within a given population, or when apoptosis of a given population is asynchronous and occurs over a protracted period, electrophoretic analysis of apoptotic DNA ladering may not be sensitive enough to detect apoptosis. Furthermore, DNA laddering techniques cannot give precise information as to the histology of the dying cells.

To circumvent the shortcomings of electrophoretic methods for analyzing DNA fragmentation, histochemical techniques that identify apoptotic DNA fragments through the addition of labeled nucleotides have proven useful in determining the location, cellular phenotype, and extent of apoptosis in tissue section or *in vitro* cultures (Willingham, 1999). The most commonly used techniques are *in situ* End Labeling (ISEL) (Wijsman *et al.*, 1993; Wood *et al.*, 1993), *in situ* nick translation (ISNT) (Willingham, 1999), and terminal deoxynucleotidyl transferase (TdT)-mediated uridine triphosphate-biotin nick end-labeling (TUNEL) (Gavrieli *et al.*, 1992). All of these techniques utilize an enzymatic activity that adds biotin- or fluorochrome-conjugated nucleotides to the 3'-hydroxy group of the apoptotic DNA fragments. Unfortunately, DNA fragmentation is a relatively late event in the apoptotic pathway. Cells at this stage of degeneration are rapidly phagocytized and removed from the tissue, and, thus, are not always detected by end labeling techniques. For example, axotomized neonatal rat facial motor neurons remain detectable by TUNEL for only four hours (Rossiter *et al.*, 1996), and apoptotic oligodendrocytes may be cleared within one hour in the developing optic nerve (Barres *et al.*, 1992b). Thus, the rapid clearance of apoptotic cells likely leads to an underestimation of the actual amount of apoptosis occurring in any given system.

Although end-labeling techniques are highly selective for apoptotic cells, caution must be taken in verifying the apoptotic nature of the positively labeled cells as necrotic cells can also be labeled by these techniques (Charriaut-Marlangue and Ben-Ari, 1995). Direct visualization of nuclear morphology can be a practical means by which to confirm the presence of apoptosis. Nuclear stains such as bisbenzamide (e.g., Hoechst 33258) have been successfully used to demonstrate the patterns of chromatin condensation

typical of apoptotic cell death. Although Hoechst labels all nuclei, the rounded shape containing intensely labeled clumps of condensed and partitioned chromatin readily distinguishes the nuclei of apoptotic cells. Their appearance is similar to that of pyknotic cells labeled by cresyl violet, another counterstain that can verify apoptosis in peroxidase-based TUNEL-labeled cells. Hoechst staining is easily performed in end-labeled tissue and provides assurance of apoptotic morphology in cells positively labeled for DNA fragmentation.

Methods that utilize antigen translocation such as immunostaining for annexin V are not amenable to analysis in tissue sections. Annexin V immunostaining only works in cultured cells as it detects the translocation of phosphatidyl serine from the intracellular to extracellular membrane (Koopman *et al.*, 1994; Zhang *et al.*, 1997a), both of which become exposed when tissue is sectioned. More recently, detection of the activation of specific proteases has been used as an indicator of apoptosis. In particular, caspase activation and cleavage products of their cellular substrates may be useful and important indicators of apoptosis (Jeon *et al.*, 1999; Siman *et al.*, 1999; Srinivasan *et al.*, 1998; Yang *et al.*, 1998a). However, the extent to which this and the other apoptosis detection techniques label other forms of ACD is unknown. Importantly, because none of the techniques are absolutely specific for apoptosis, it is important to use at least two of them to substantiate findings of apoptosis.

In this study, I used TUNEL/Hoechst staining to examine the amount of apoptosis in the embryonic chick cervical spinal cord before, during, and after the period of myelination of this region. The apoptotic response to SCI in remote regions of the spinal cord caudal to the injury was subsequently assessed. I hypothesized that during the period of spinal cord myelination, oligodendrocytes would undergo developmental apoptosis, and that they would be more susceptible to injury-induced death during this time. The findings of this study support this hypothesis. Increased TUNEL labeling of apoptotic cells in the cervical spinal cord was observed during the period of myelination of this region. In addition, transection of the cervical spinal cord at this time resulted in a massive increase in TUNEL labelling. This response was observed for several segments distal to the injury and was highly localized to cells of the ventrolateral and ventromedial white matter. The anatomical localization to the white matter combined with double

fluorescence and peroxidase labeling of oligodendrocyte specific and apoptotic markers indicated many of the dying cells to be of oligodendrocyte origin. These results support findings that axonal injury can lead to apoptotic death of axon-associated glia at sites remote from the injury (Crowe *et al.*, 1997; Liu *et al.*, 1997b; Shuman *et al.*, 1997). The impressive magnitude of this response in the present study indicates this model will be useful for furthering the acquisition of knowledge regarding the mechanisms of oligodendrocyte apoptosis in order to direct treatment strategies aimed at arresting degenerative disorders involving the apoptotic death of these cells.

METHODS

Animals

Fertilized white leghorn chicken (*Gallus gallus domesticus*; Coastline Chicks, Abbotsford, BC) eggs were incubated in a rotating, incubator maintained at 38°C and a relative humidity of approximately 60%. Hatchling chicks were maintained in a commercial brooder with food and water provided *ad libitum*. For analysis of apoptosis in the developing chick cervical spinal cord, embryos were sacrificed on various days between E10-E20. Treament of animals conformed to university animal care guidelines.

Tissue Processing

All embryonic and hatchling chicks were sacrificed by intracardial perfusion with phosphate buffered saline (PBS), pH 7.4, followed by perfusion fixation with 4% paraformaldehyde in PBS. Prior to perfusion, older embryos (E17 and older) and hatchlings were anesthetized with a lethal dose of Somnotol (sodium pentobarbitol; approximately 75 mg/kg). The spinal column was then removed and post-fixed overnight in fresh 4% paraformaldehyde. Spinal cords were then dissected out from the spinal column, cryoprotected in 18% sucrose, and frozen in OCT (Tissue Tek; JB EM Services; Dorval, Que, #JBF 172) compound with liquid nitrogen. Frozen spinal cords were stored at –70°C until cryosectioned at –21°C on a Zeiss cryostat. Cryosectioned tissue was subsequently air dried and stored at –70°C until stained.

Embryonic Surgery

Prior to spinal cord transection, the developmental stage of embryos was determined using the criteria established by Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were operated on E10-E14. Due to the size and the ossification of the vertebral column, consistent spinal transection in embryos older than E14 was not possible, and thus, were not included in the study. A small window was made in the shell using a single-edged razor blade and the inner shell membrane was removed with #5 forceps. Using Dumont 5/45 forceps (Fine Science Tools, North Vancouver, BC), a small hole was made in the underlying chorioallantoic membrane. Special care was taken to minimize disruption of the blood vessels of this highly vascularized membrane. A "hooked" glass probe was then inserted through the hole in the chorioallantoic membrane to secure the neck for transection of the cervical segment (C) 3-C5 spinal cord with 5/45 forceps. The transection was performed by inserting the forceps tips on either side of the dorsal neck midline and closing them entirely. This procedure has been used extensively in this laboratory and reliably transects the spinal cord completely (Hasan *et al.*, 1993; Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Steeves *et al.*, 1993). Sham operations were also performed in which the forceps were inserted into the neck but were not closed. Following transection, the window was covered with filament tape and sealed with paraffin wax. Eggs were then returned to the incubator (but not rotated) until sacrificed as described above. E10 embryos were sacrificed 24 and 96 hours after transection. Embryos transected on E13 were sacrificed at various times between 12 hours and 7 days after transection.

Cervical transections were performed for three main reasons. First, the cervical spinal cord contains the largest number of descending axons from the brainstem; thus, a greater response may be expected from injuries in this region than from those at more caudal levels. Second, this region is surgically the most accessible and results in much more consistent surgical outcomes, namely complete transection. Third, this is the first region of the spinal cord to become myelinated, allowing studies to be performed at earlier times in development, which makes the operation easier and more consistent with respect to the completeness of the transection. The operation is well tolerated by the embryos with typical survival rates of approximately 75%.

The efficacy of transections was determined upon dissection of the fixed spinal cord and in cresyl violet stained sections of the injured spinal cords. Only those cords exhibiting complete transections were included in the study.

Hatchling Surgery

On postnatal day (P) 2 hatchling chicks were anesthetized with a mixture of Rompum and ketamine. Due to the poor survival of chicks receiving complete cervical transections, complete transections were performed at the thoracic level, an operation well tolerated by hatchling chicks. An incision was made above the region corresponding to thoracic level 4 of the spinal cord. The spinal cord was exposed and a corneal blade was passed through the spinal cord. Chicks receiving sham operations were prepared similarly except the spinal cord was not transected. Following transection, the incision was closed with #5 surgical silk and the chicks placed in a post-operative recovery brooder where they were assisted with feeding and drinking until they could function independently.

Apoptosis Detection

Apoptosis was determined using a modified TUNEL technique (Gavrieli *et al.*, 1992) to label fragmented DNA *in situ* and with the nuclear bisbenzamide stain, Hoechst 33258 (Sigma, St. Louis, MO). TUNEL staining was performed on 10 μm transverse and longitudinal spinal cord sections. In both techniques, spinal cord sections were initially washed three times in PBS (5 minutes per wash), then subjected to proteinase K (2 μg/ml in PBS: Promega; Madison, WI, #V3021) digestion for 5 minutes at room temperature. Sections were again washed three times in PBS before continuing with the TUNEL procedure. Two variations of the TUNEL procedure were used, one intended for light and one for fluorescent microscopic analysis.

For peroxidase-based TUNEL intended for light microscopy, endogenous peroxidase activity was quenched by immersing sections in 0.6% hydrogen peroxide in methanol for 20 minutes at room temperature. Sections were washed twice in PBS before being equilibrated in terminal deoxynucleotidal transferase (TdT) buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate and 1 mM cobalt chloride). The TUNEL reaction was performed at 37°C for 1 hour using 0.15 U/μl TdT (Boehringer Mannheim; Laval, Que.,

#220 582) and 2 nM biotin-16-dUTP (Boehringer Mannheim; #1093070) in TdT buffer. The reaction was stopped by immersing sections for 15 minutes at room temperature in buffer containing 300 mM sodium chloride and 30 mM sodium citrate. Sections were then immersed in 2% bovine serum albumin (BSA) fraction V (Sigma; St. Louis, MO, #A-8551) in PBS for 20 minutes at room temperature. Following 3 x 5 minute rinses in PBS, sections were incubated with ABC solution (ABC Elite standard kit, Vector; Burlingame, CA, #PK-6100) for 30 minutes at 37 °C. Subsequently, sections were rinsed twice in PBS, then in Tris-HCl (pH 7.6) before the reaction was visualized with diaminobenzidine (DAB) peroxidase substrate kit, with nickel enhancement (Vector, #SK-4100) or with VIP peroxidase substrate kit (Vector, #SK-4600). Sections were then rinsed with water and dehydrated through a series of ascending alcohols (2 minutes each in 70%, 95%, 100%, and 100% ethanol). After clearing sections in two rinses in xylene, sections were mounted in Permount (Fisher Scientific). In negative controls, TdT was omitted from the reaction mixture.

For TUNEL intended for fluorescent analysis, sections were incubated with 1% normal goat serum (NGS: Jackson ImmunoResearch Laboratories; West Grove, PA, #017-000-127) in PBS for 20 minutes at room temperature. Sections were then rinsed two times in PBS, equilibrated in TdT buffer for 15 minutes at room temperature, and incubated with the TUNEL reaction mixture as described for peroxidase-based TUNEL. The reaction was terminated in the stop buffer as above. Sections were then incubated for 30 minutes at 37 °C in PBS containing 1% NGS, 0.1% Triton X-100 (BDH; Toronto, Ont, #R06433), and Cy3-conjugated ExtrAvidin (Sigma; #E-4142) diluted 1:100. After 3 five minute rinses in PBS, sections were stained in 1 μg/ml Hoechst 33258 (Sigma, #B 2883) in PBS for 2 minutes to verify the apoptotic morphology of TUNEL-positive (TUNEL+) cells. Sections were rinsed in PBS and mounted in PBS glycerol (Sigma Mounting Medium: Sigma, #1000-4). Nail polish was used to seal the coverslip to the slide. In negative controls, TdT was omitted from the reaction mixture.

Immunohistochemistry and TUNEL Double Labeling

To determine the cell type(s) undergoing apoptosis in the developing and transected chick spinal cord, indirect immunofluorescence or immunoperoxidase staining with cell-specific antibodies was used in combination with TUNEL and/or Hoechst 33258. Table

2-1 lists the antibodies and Table 2-2 lists the secondary antibodies, stains, and kits used in this study, respectively.

Table 2-1. Antibodies used in this study.

Antibody	Cell Type/ Antigen Detected	Host	Concen-tration	Source	Catalog No.
Anti-human ferritin	Oligodendrocyte cell bodies	Rabbit	1:2	Sigma (St. Louis, MO)	F-5012
Anti-MBP	Oligodendrocyte cell body and processes	Rabbit	1:400-1:800	Chemicon (Temecula, CA)	AB980
Monoclonal anti-GFAP	Astrocytes	Mouse	1:500	Sigma (St. Louis, MO)	G-3893
O4	Oligodendrocytes	Mouse	10 µg/ml	Chemicon (Temecula, CA)	MAB345
Polyclonal anti-GFAP	Astrocytes	Rabbit	1:400-1:600	Dako (Carpinteria, CA)	Z334
Rip - supernatant	Oligodendrocyte cell bodies	Mouse	1:5	Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA)	Rip
Anti-chick TfBP	Oligodendrocyte cell bodies	Rabbit	1:800-1:1200	Dr. John J. Lucas, State University of New York at Syracuse	Gift

Abbreviations: MBP (myelin basic protein), GFAP (glial fibrillary acidic protein), GalC (galactocerebroside) TfBP (transferrin binding protein)

Table 2-2. Secondary Antibodies, Stains, and Kits.

Secondary Antibody/Stain	*Concentration*	Company Source	Catalog No.
ABC Elite Kit		Vector	PK-6100
Alexa 488 goat anti-rabbit conjugate	1:200	Molecular Probes (Eugene, OR)	A-11034
DAB peroxidase kit		Vector	SK-4100
Donkey anti-mouse biotin conjugate	1:200	Jackson ImmunoResearch	715-065-150
Donkey anti-mouse Cy3 conjugate	1:200	Jackson ImmunoResearch	715-165-150
Donkey anti-rabbit biotin conjugate	1:200	Jackson ImmunoResearch	711-065-152
Donkey anti-rabbit Cy3 conjugate	1:200	Jackson ImmunoResearch	711-165-152
ExtrAvidin-Cy3 conjugate	1:100	Sigma	E-4142
Goat anti-mouse rhodol green conjugate	1:200	Molecular Probes (Eugene, OR)	R-6412
Goat anti-rabbit rhodol green conjugate	1:200	Molecular Probes (Eugene, OR)	R-6470
Hoechst 33258	1 µg/ml	Sigma	B-2883
VIP peroxidase kit		Vector	SK-4600

In general, the TUNEL procedure was performed first, as described above. Following the TUNEL DAB or VIP reaction, residual peroxidase activity was quenched by immersing sections in 0.6% hydrogen peroxide in methanol for 20 minutes at room temperature. Sections were then blocked in PBS containing 0.01%-1% Triton X-100 and 5% normal donkey serum (NDS: Jackson ImmunoResearch, #165-000-171) or NGS. Sections were then incubated overnight at 4 °C with the primary antibody diluted in PBS containing 1% NDS or NGS and Triton X-100. Sections were then rinsed 3 times in PBS and incubated for one hour at room temperature with an appropriate biotinylated secondary antibody (see Table 2 for details) diluted 1:200 in PBS containing 1% NDS or NGS and Triton. Following 3 PBS rinses, sections were incubated with ABC solution for 1 hour at room temperature before being rinsed twice in PBS then once in Tris-HCl. DAB with or without nickel enhancement or VIP was used to visualize the reaction. Sections were passed through an ascending series of ethanol and cleared in xylene before being mounted with Permount. In control sections, the TdT and/or the primary antibody was omitted and following immunoperoxidase staining, sections were counterstained in cresyl violet before being dehydrated, cleared and mounted in Permount.

Following fluorescent TUNEL, sections were blocked in 0.01%-1% Triton X-100 and 5% NDS or NGS. Sections were then incubated overnight at 4 °C with the primary antibody diluted in PBS containing 1% NDS or NGS and Triton X-100. After 3 rinses in PBS, sections were incubated for one hour at room temperature with an appropriate Cy3-, Rhodol Green-, or Alexa488-conjugated secondary antibody (see Table 2 for details). Sections were then rinsed 3 times in PBS, stained in 1 μg/ml Hoechst 33258, rinsed in PBS, and mounted in Sigma Mounting Medium. In negative controls, either TdT and/or the primary antibody was omitted.

Cell Counts and Imaging

To assess the amount of apoptosis, TUNEL+ cells were counted in every 20th 10 μm transverse section through 4-6 mm (depending on developmental stage) of cervical spinal cord (see Figure 2-1). In transected embryos, the 4-6 mm region beginning 1 mm caudal to the most caudal extent of the lesion site was analysed (see Figure 1-1). This region avoids the perilesion region, which contains large numbers of apoptotic as well as necrotic cells that could also be labeled by TUNEL. An equivalent region of untreated or

sham controls was analysed. Counts were performed blind with respect to treatment and developmental age. The amount of apoptosis was expressed as the mean number of TUNEL+ cells per 10 μm transverse section. Similarly, the number of cells double-labeled for specific cell types and markers of apoptosis was determined in this region. However, in double-labeling experiments, 5 random sections were examined for each animal. Computer lucida was performed using Adobe Photoshop (versions 4.0-5.0: Adobe; San Jose, CA) on images captured on a Spot CCD (Diagnostic Instruments; Sterling Heights, MI) attached to a Zeiss Axiophot Microscope (Carl Zeiss; Thornwood, NY). This was done to analyze the spatial distribution of TUNEL+ cells in the spinal cord. Data were recorded in Microsoft Excel and statistical analysis was performed using StatView (SAS Institute; Cary, NC). Data was compared using Fisher post hoc analysis of variance (ANOVA). Differences with a p-value <0.05 were considered significant.

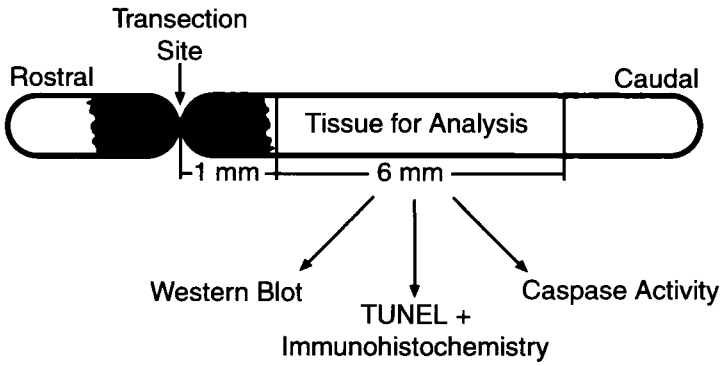
RESULTS

Developmental Apoptosis in the Cervical Spinal of the Developing Chick

TUNEL+ cells were detected in the cervical spinal cord of all embryonic and hatchling chicks; however, their number and distribution differed at different developmental stages. As shown in Figure 2-2, the greatest number of TUNEL+ cells was observed between E13-E15, the period when myelination of the chick spinal cord begins (Keirstead *et al.*, 1992; Macklin and Weill, 1985; Richardson *et al.*, 1997; State *et al.*, 1977). Prior to this time (E10-E12), a smaller number of apoptotic cells are observed. By E18, the number of TUNEL+ cells decreased, and only a small number were detected in hatchlings. The decrease in apoptotic activity in the cervical spinal cord correlates with the cessation of myelination of this region, and the number of TUNEL+ cells at this time was the same as just before the onset of myelination (E11 and E12).

The distribution of TUNEL+ cells within the cervical spinal cord also changes over development. Figure 2-2(b) shows representative computer lucida images of developmental apoptosis during the development of the cervical chick spinal cord. Prior to E12, apoptotic cells are present with a roughly equal distribution in grey and emerging white matter. By E13, the TUNEL+ cells are confined almost entirely to the white matter.

Figure 2-1. Schematic representation of the region of cervical spinal cord analysed in this thesis. Six mm of spinal cord was dissected 1-7 mm caudal to the transection site. The region within 1 mm of the transection site (in red) was characterized by a massive apoptotic and necrotic response in both grey and white matter. Caudal to this zone, large numbers of apoptotic cells were confined to the ventrolateral and ventromedial white matter. Tissue from comparable levels of uninjured and sham transected control spinal cord was harvested. The harvested tissue was analysed for DNA fragmentation (TUNEL), nuclear morphology (Hoechst 33258), immunohistochemistry, western blot, or caspase activity. In E13-E18 embryos, one spinal cord segment is approximately 2 mm long.


Transection
Site
Rostral
Caudal
Tissue for Analysis
1 mm
6 mm
Western Blot
TUNEL +
Immunohistochemistry
Caspase Activity

Although the number of TUNEL+ cells decreases as the chicks mature, their distribution remains almost exclusively confined to the white matter. Occasionally, what appeared to be TUNEL+ motor neurons were observed in embryos prior to E13. As expected, many TUNEL+ cells were also detected in the dorsal root ganglia (DRG) of E10 and E11 chicks.

Hoechst staining revealed a near 1:1 ratio of TUNEL+ cells with apoptotic nuclei (e.g., Figure 2-7c, bottom right panel), indicating TUNEL selectively labels apoptotic cells in this region of the spinal cord. TUNEL labeling was absent in negative control sections in which TdT was omitted from the reaction mixture. Thus, TUNEL was an accurate and reliable method for apoptosis detection in the developing chick spinal cord.

Sensitive Period for Transection-Induced Apoptosis in the Spinal White Matter Correlates with the Period of Myelination and Developmental Glial Apoptosis

Cervical transection of the embryonic and hatchling spinal cord resulted in a massive apoptotic response within approximately 1 mm of the lesion site (Figure 2-3). This response was observed in both the grey and white matter. In many cases, grey matter was much more heavily stained than the surrounding white matter. Hoechst staining of cell nuclei within this region indicated a large number of TUNEL+ cells to have non-apoptotic, possibly necrotic appearing nuclei, indicating false-positive TUNEL labeling of apoptotic cells may have been prevalent in this region. Beyond 1mm from the lesion, the apoptotic response became highly confined to the white matter. In addition, TUNEL+ cells in this region all displayed apoptotic nuclear morphologies. Analysis of the anatomical distribution of TUNEL+ cells in transverse sections several segments caudal from the injury showed the apoptotic cells to be concentrated in the ventromedial and ventrolateral spinal cord. This corresponded to the location of descending supraspinal pathways in the chick.

As shown in Figure 2-4, transection prior to the period of myelination of the cervical chick spinal cord (before E13) results in an increase in apoptosis caudal to the lesion compared to sham or unoperated controls. Twenty-four hours after cervical transection on E10 the number of TUNEL+ cells increased (Figure 2-4a). The TUNEL+ cells were distributed equally between grey and developing white matter regions of the cord (Figure 2-4). Four days after E10 transection (during the phase of increased myelination and

developmental apoptosis in the developing spinal white matter), the number of TUNEL+ cells increased compared to control animals, and the apoptotic cells became localized almost exclusively to the white matter tracts. Approximately 91-94% of the TUNEL+ cells were observed in the white matter 4 days after injury compared with only 56-61% 24 hours after injury. The number of TUNEL+ cells in the grey matter did not increase compared with sham injured controls (typically 1-4 per section 4 days after injury).

Complete transection of the E13 cervical spinal cord resulted in a massive and rapid apoptotic response that was localized almost exclusively to the white matter (Figure 2-5). Statistically significant increases in the number of TUNEL+ labeled cells was observed 24 hours after transection, and reached a maximum between 24 and 72 hours after transection. The number declined by 6 days post injury but remained greater than control animals. Of the total number of TUNEL+ cells counted per section, the percentage of these cells in the white matter was similar at all postoperative times (approximately 88-94%). Longer post-operative times were not possible due to the inability of the embryos to survive hatching.

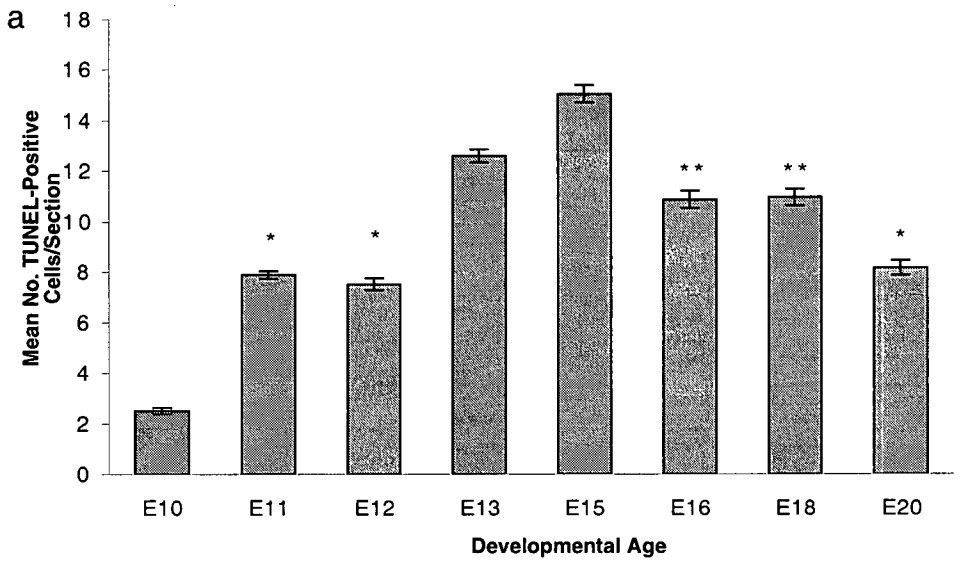
Complete transection of the thoracic spinal cord (T4) of P2 hatchlings resulted in a small but significant increase in TUNEL+ cells (Figure 2-6a) that were, again, localized to the white matter caudal to the lesion. Thus, the period of increased developmental apoptosis in the spinal white matter correlates with the process of myelination and an increased sensitivity to axonal perturbation.

As in the unoperated spinal cord, almost all TUNEL+ cells in the transected cervical cord at sites remote from the injury displayed apoptotic nuclei, as revealed by Hoechst. At the lesion epicentre and the region immediately adjacent to it, however, a wide variety of nuclear morphologies was observed, many which appeared to be necrotic.

Identity of Apoptotic Cells in the Developing and Injured White Matter Tracts

The strict localization of TUNEL+ cells to the white matter of E13 and older chick cervical spinal cords indicated the dying cells are likely of glial origin, as very few neuronal cell bodies are present in these regions. Furthermore, the temporal correlation between increased apoptosis and the onset of myelination suggested oligodendrocytes may have been a principal cellular component of the observed apoptosis. To investigate this possibility, sections were double-labeled for apoptotic DNA fragmentation (TUNEL)

Figure 2-2. Analysis of TUNEL labeling in the cervical spinal cord of the developing chick embryo. (a) Histogram of the mean number of TUNEL-positive cells per 10 μm section +/- SEM of cervical spinal cords from different staged embryos (n=5/timepoint). A significant increase in the amount of apoptosis was observed between E12 and E15 ($p<0.001$), which was followed by a decline through E20 ($p<0.001$). (b) Computer lucida representations of typical distribution of spinal cords at different stages of development. Note the distribution of TUNEL+ cells is equally distributed in E11 and E12 embryos but is confined mostly to the white matter in older embryos. * indicates no significant differences in the number of TUNEL+ cells in E11, E12, and E20 ($p>0.05$). ** indicates no difference between E16 and E18 ($p>0.05$). Scale=1000 μm.


a
Mean No. TUNEL-Positive Cells/Section
0
2
4
6
8
10
12
14
16
18
*
*
**
**
*
E10
E11
E12
E13
E15
E16
E18
E20
Developmental Age

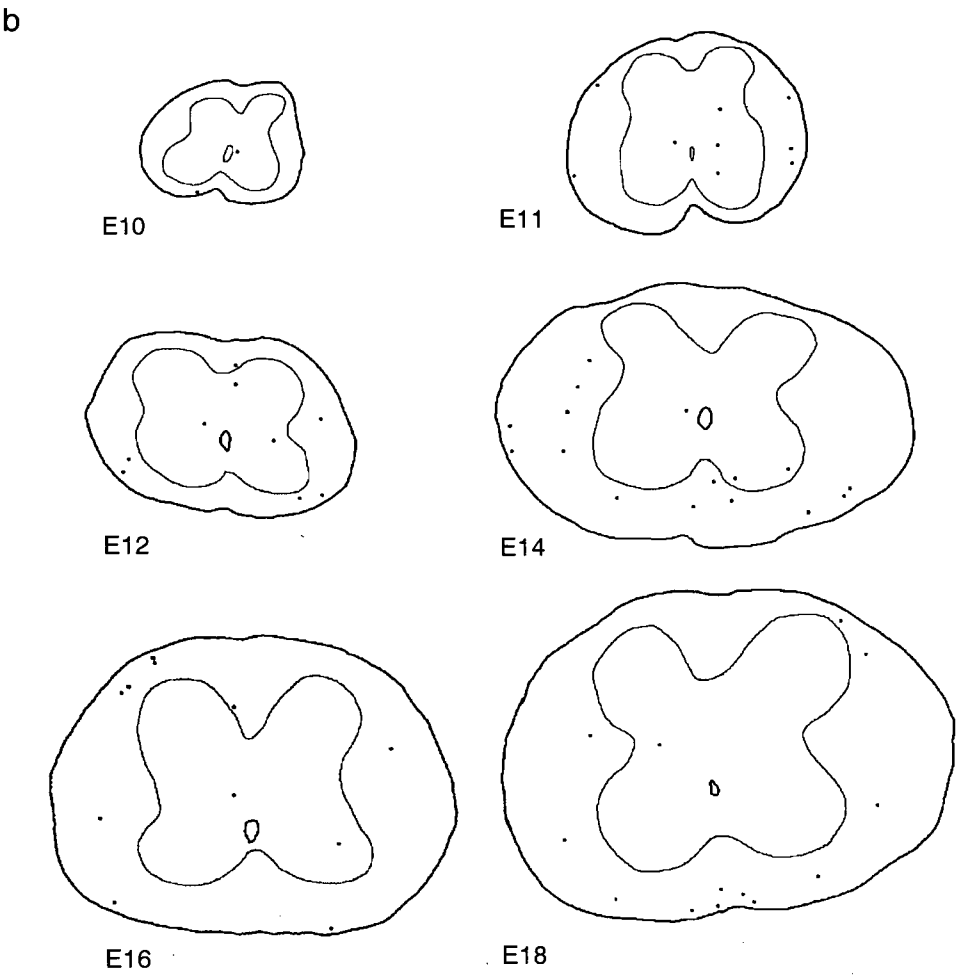

b
E10
E11
E12
E14
E16
E18

Figure 2-3. Twenty-four hours after transection on E13, TUNEL labeling of sections at the site of injury reveals a massive apoptotic response in both grey and white matter (a) 0.3 mm and (b) 0.6 mm caudal to the lesion. (c) One mm beyond this region, TUNEL+ cells are confined to the white matter. (d) Representative longitudinal section demonstrates the progression of TUNEL+ cells from the grey and white matter within the region immediately adjacent to the lesion (as seen in top of section) to the white matter caudal to the lesion. Scale bar = 500 μm.

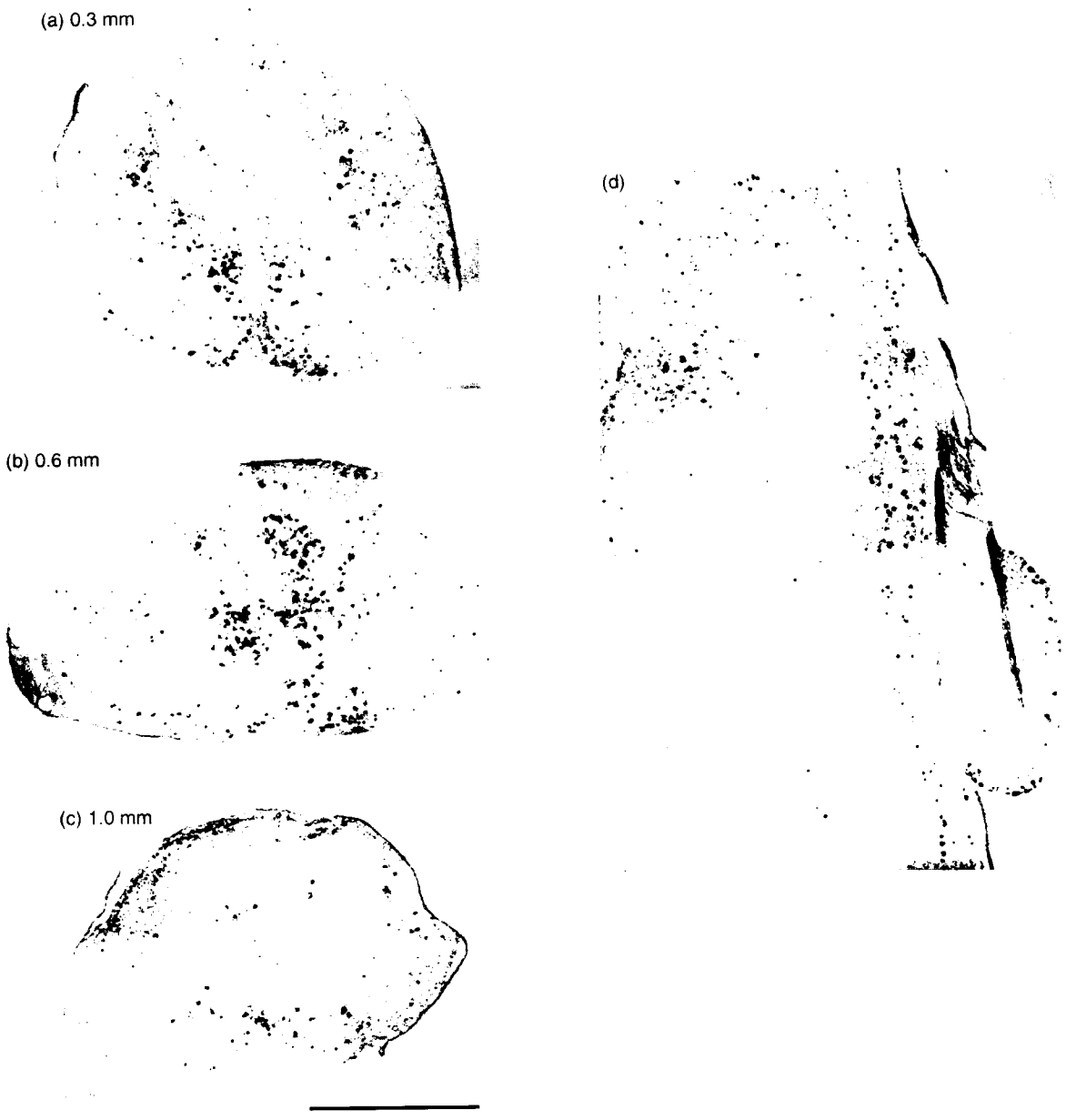

(a) 0.3 mm
(b) 0.6 mm
(c) 1.0 mm
(d)

Figure 2-4. Analysis of TUNEL labeling in the cervical spinal cord following transection on E10. (a) Compared to sham operates, transected spinal cords showed a significant increase in TUNEL labeling 1-7 mm caudal to the lesion at 24 and 96 hours after injury (*$p<0.001$; n=4/treatment). The increase in 96 hour transects was also significant when compared with 24 hour transects ($p<0.001$). Computer lucida shows the differential distribution of TUNEL+ cells 24 hours (c) and 96 (e) hours after injury.(b) and (c) represent 24 hour and 96 hour sham controls, respectively. Scale = 500 μm.

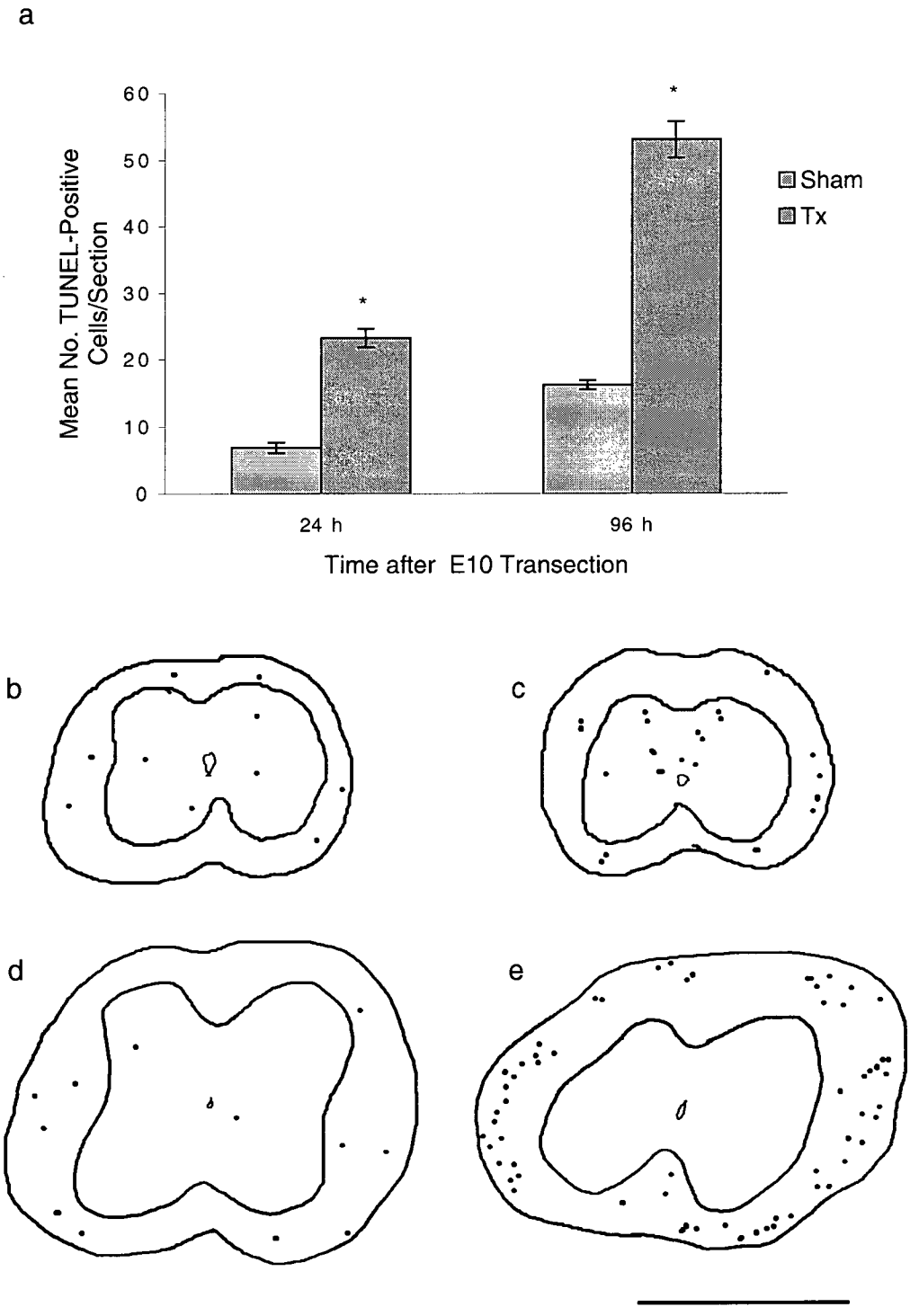

a
60
50
40
30
20
10
0
Mean No. TUNEL-Positive Cells/Section
*
*
Sham
Tx
24 h
96 h
Time after E10 Transection
b
c
d
e

Figure 2-5. Analysis of TUNEL labeling in the cervical spinal cord following transection on E13. (a) Transection lead to a significant increase in apoptosis compared to sham controls at all time points examined (*$p<0.001$; n=5/treatment). The apoptotic response 6 days after injury was significantly smaller than that at 24-72 hours (**$p<0.001$). Computer lucida of transected spinal cords 24 (b) hour and 6 days (e) hour transects reveals a large increase in TUNEL staining compared to sham controls (b and d, respectively) that is confined to the white matter. The distribution appears to shift from a predominantly ventral to a predominantly lateral position. Scale bar = 500 μm.

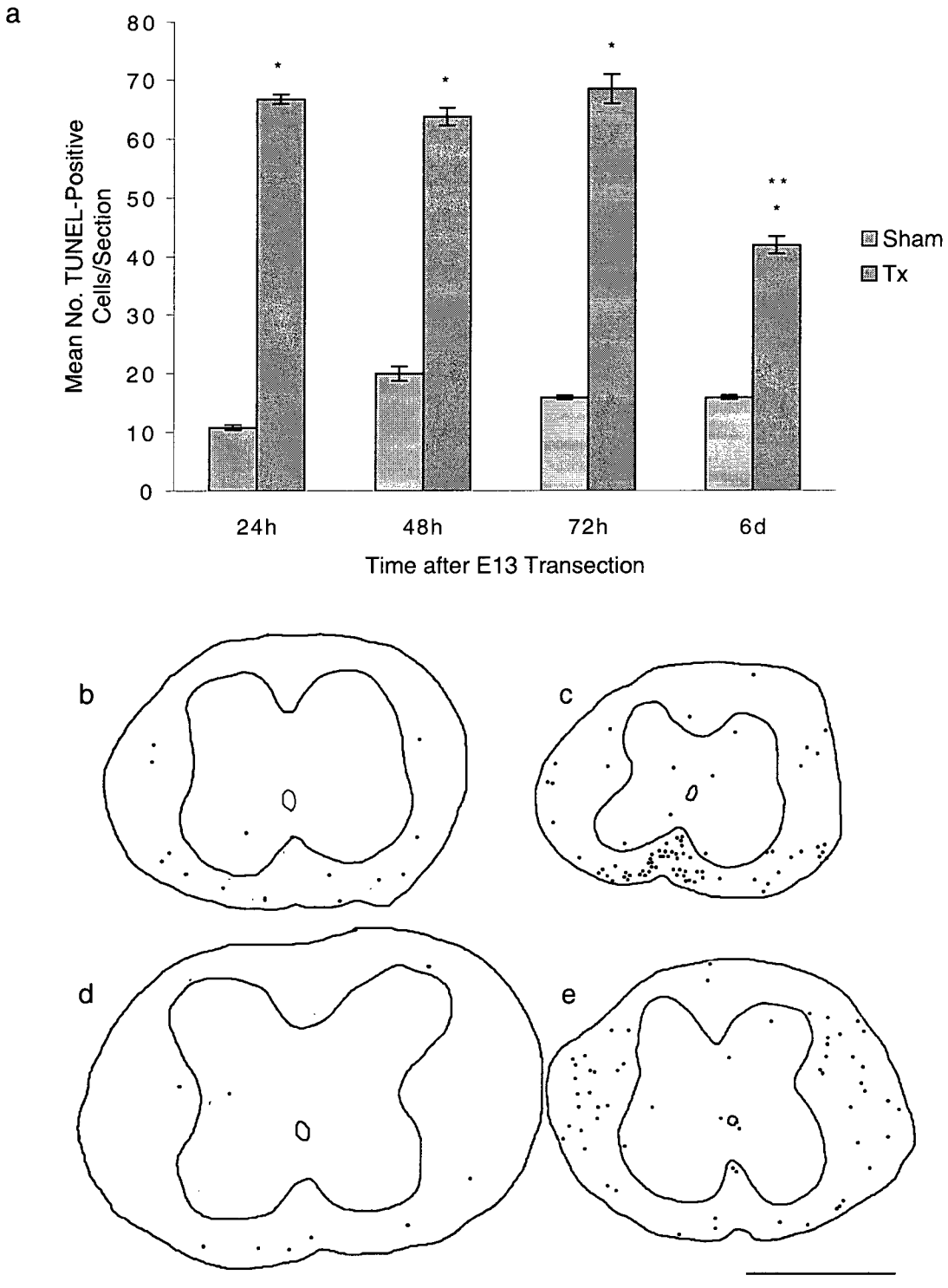
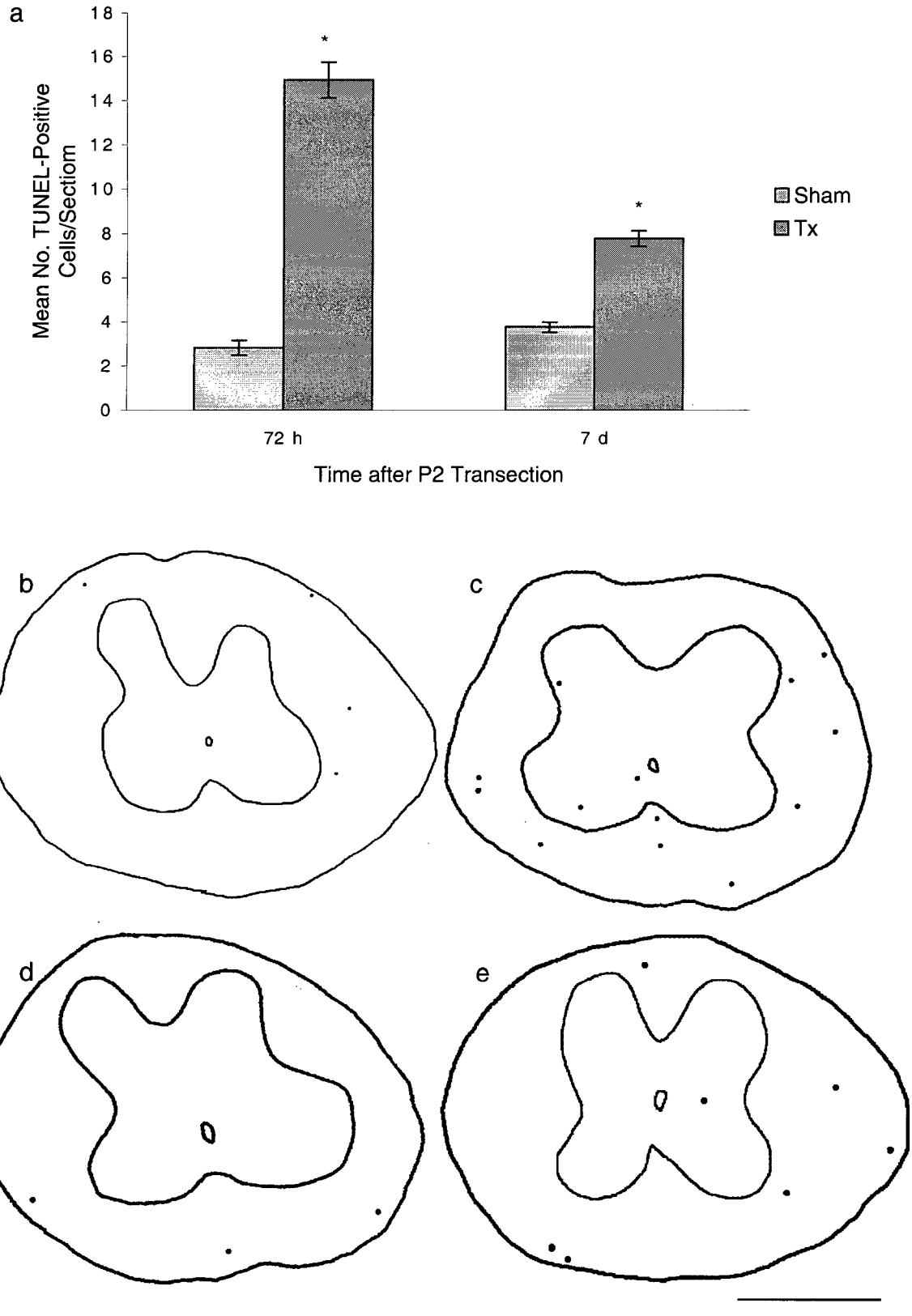

a
80
70
60
50
40
30
20
10
0
Mean No. TUNEL-Positive Cells/Section
24h
48h
72h
6d
Time after E13 Transection
Sham
Tx
b
c
d
e

Figure 2-6. Analysis of TUNEL labeling in the thoracic spinal cord following transection on P2. (a) A significant rise in apoptosis was detected 1-7 mm caudal to the injury 72 hours and 7 days after injury compared with sham controls (* $p<0.001$, n=3/treatment), however, the response after 7 days was diminished compared to that at 72 hours. Computer lucida reveals a similar distribution of TUNEL+ cells in the ventral and lateral white matter 24 hours after sham (b) or transection (c), and 7 days following sham (d) or transection (e). Scale=1mm.


a
Mean No. TUNEL-Positive Cells/Section
18
16
14
12
10
8
6
4
2
0
*
*
Sham
Tx
72 h
7 d
Time after P2 Transection
b
c
d
e

and various immunohistochemical markers for oligodendrocytes or astrocytes (see Table 2-2), the two main non-axonal components of spinal white matter. Microglia were not examined due to the lack of cross reactivity of microglial markers such as OX42 and ED-1 with chick tissue.

Double fluorescence and peroxidase labeling indicated oligodendrocytes but not astrocytes were the predominant cell type undergoing apoptosis in the developing spinal cord. TUNEL+ cells immunoreactive for the oligodendrocyte markers MBP, Rip, ferritin and TfBP were seen in all sections; whereas, GFAP-positive TUNEL-labeled cells were rarely observed (Figure 2-7). TfBP provided the most easily distinguished colocalization of an oligodendrocyte marker with TUNEL, and was, therefore, used for quantitative analysis of the number of oligodendrocytes undergoing apoptosis. TfBP has been identified as heat shock protein 108, and specifically labels oligodendrocytes in chick but not mammalian CNS (Cho and Hyndman, 1991; Cho and Lucas, 1995; Cho *et al.*, 1999; Cho *et al.*, 1997). Approximately 27-38% and 24-31% of TUNEL+ cells displayed TfBP immunoreactivity 24 and 48 hours after E13 transection, respectively, compared with 26-33% and 11-19% in respective sham controls. However, many TUNEL+ cells did not colocalize with oligodendrocyte-specific markers. This may indicate oligodendrocytes lose their phenotype as they undergo apoptosis, as is the case for Rip immunoreactivity in oligodendrocytes undergoing apoptosis in the developing optic nerve (Barres *et al.*, 1992a). Alternatively, it may indicate the involvement of other cell types or oligodendrocyte precursors not yet expressing these mature oligodendrocyte antigens.

The onset of MBP immunoreactivity was detected on E12-E13, which is in agreement with previous studies on MBP expression in the developing chick (Keirstead *et al.*, 1992; Macklin and Weill, 1985; Richardson *et al.*, 1997). The developmental onset of TfBP immunoreactivity had not been examined at early timepoints. Therefore, I examined the developmental profile of TfBP immunoreactivity before, during, and after myelination of the cervical spinal cord. TfBP immunoreactive cells were first detected on E11 (Figure 2-8). These cells appeared bipolar and radiating out toward the white matter. By E13, TfBP-positive cells lay predominantly within the white matter and had a round appearance with occasional processes emanating from the soma, a morphology consistent with staining of adult chick oligodendrocytes (Cho and Hyndman, 1991; Cho and Lucas,

1995; Cho *et al.*, 1999; Cho *et al.*, 1997). The number of TFBP-positive cells in the white matter increased from E13-E19. Unfortunately, the O4 antibody used in this study under the conditions necessary for detection of apoptosis did not yield satisfactory results. Thus, potential detection of apoptotic O4-positive precursors was not possible.

DISCUSSION

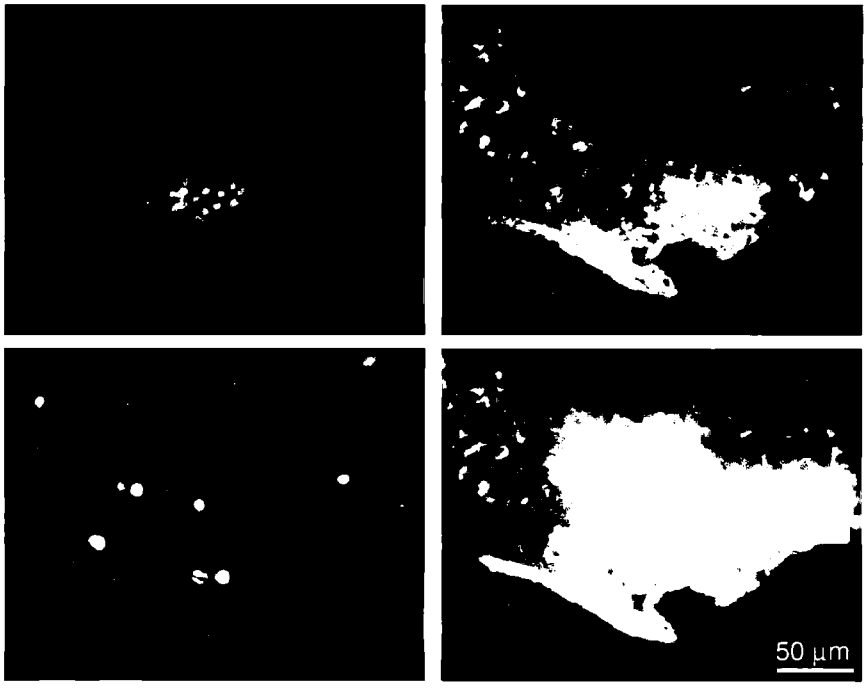
A recently discovered component of the secondary response to SCI is oligodendrocyte apoptosis in the white matter remote from the injury site (Crowe *et al.*, 1997; Liu *et al.*, 1997b; Shuman *et al.*, 1997). Unfortunately, the small numbers of apoptotic cells detected at any one time in these models of SCI may preclude their use in determining the mechanisms underlying this reaction. Thus, I developed an *in vivo* (*in ovo* to be precise) model of trauma-induced oligodendrocyte apoptosis in the chick embryo.

Period of Increased Susceptibility of White Matter Glia

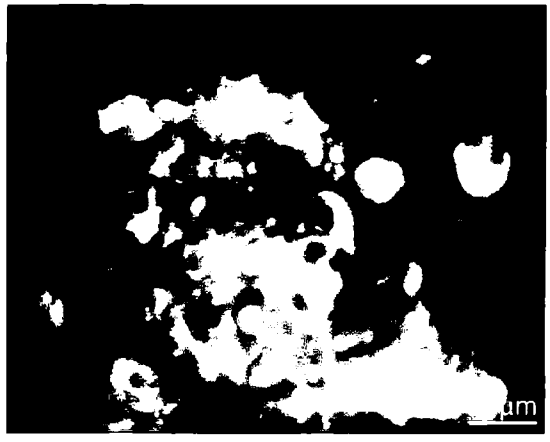
Results of this study demonstrated an increase in developmental glial apoptosis in the cervical spinal white matter during the previously defined period of myelination (Keirstead *et al.*, 1992; Macklin and Weill, 1985; Richardson *et al.*, 1997; State *et al.*, 1977). In addition, white matter cells at this time are at a heightened state of vulnerability to cell death by means of spinal cord transection. This increased susceptibility to transection-induced death corresponds with the increase in developmental apoptosis within the white matter. Prior to and subsequent to the completion of myelination, only small numbers of apoptotic cells were seen distributed throughout the cord, and injury induced only a moderate amount of apoptosis. These findings are consistent with the increased vulnerability of oligodendrocytes to transection in neonatal rodent optic nerve (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996) and of Schwann cells following sciatic nerve injury in neonatal rodent and embryonic chick (Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996). Schwann cells become increasingly resistant to sciatic nerve damage as animals mature (Butt *et al.*, 1997; Ludwin, 1990; Ludwin, 1992).

Figure 2-7. Double and triple immunofluorescent and immunoperoxidase staining of white matter glia in E13 transected spinal cords distal from the lesion. (a) Hoechst 33258 (blue), TUNEL (red), and TfBP (green) triple-labeling reveals a number of apoptotic oligodendrocytes. (b) High magnification of the same section reveals the colocalization of these markers. (c) Immunolabeling for MBP, and Rip reveal apoptotic oligodendrocytes (arrows). GFAP staining does not colocalize with apoptotic nuclei - note the apoptotic nuclear morphology as detected with Hoechst 33258 (blue) in TUNEL+ cells (red). Arrows in (d) indicate TUNEL+ cells that are not immunoreactive for GFAP (i.e., are not astrocytes).

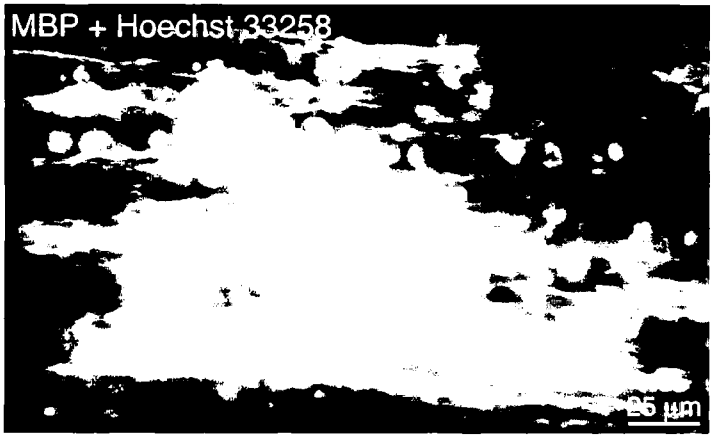
a

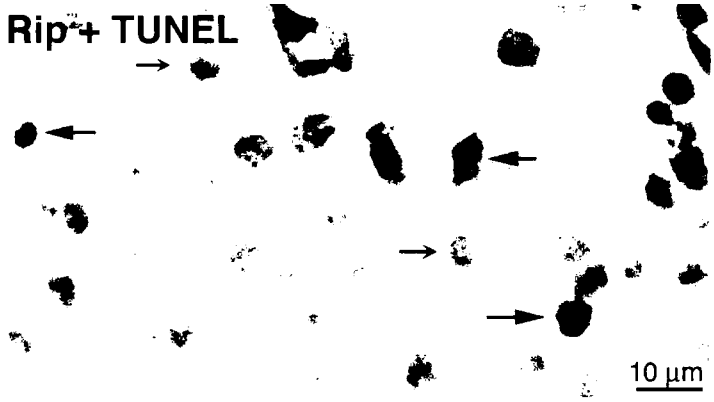

50 µm

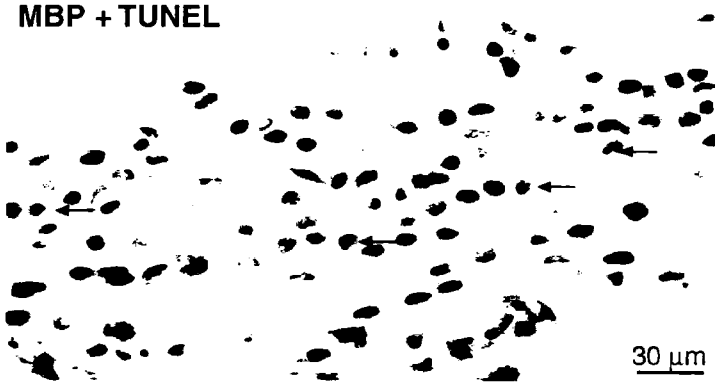
b


µm

c


MBP + Hoechst 33258
25 µm


Rip+ TUNEL
10 µm


MBP + TUNEL
30 µm

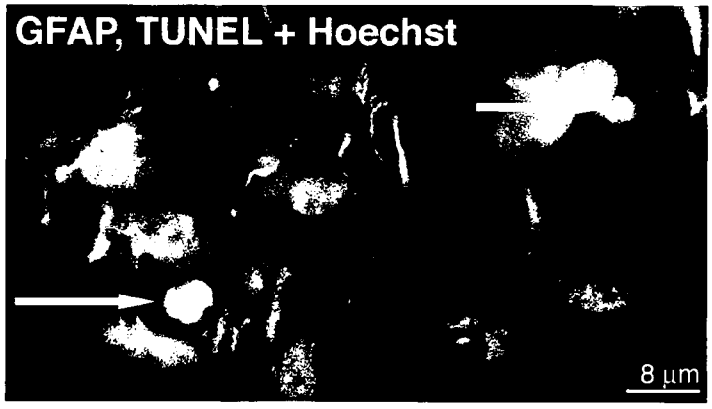
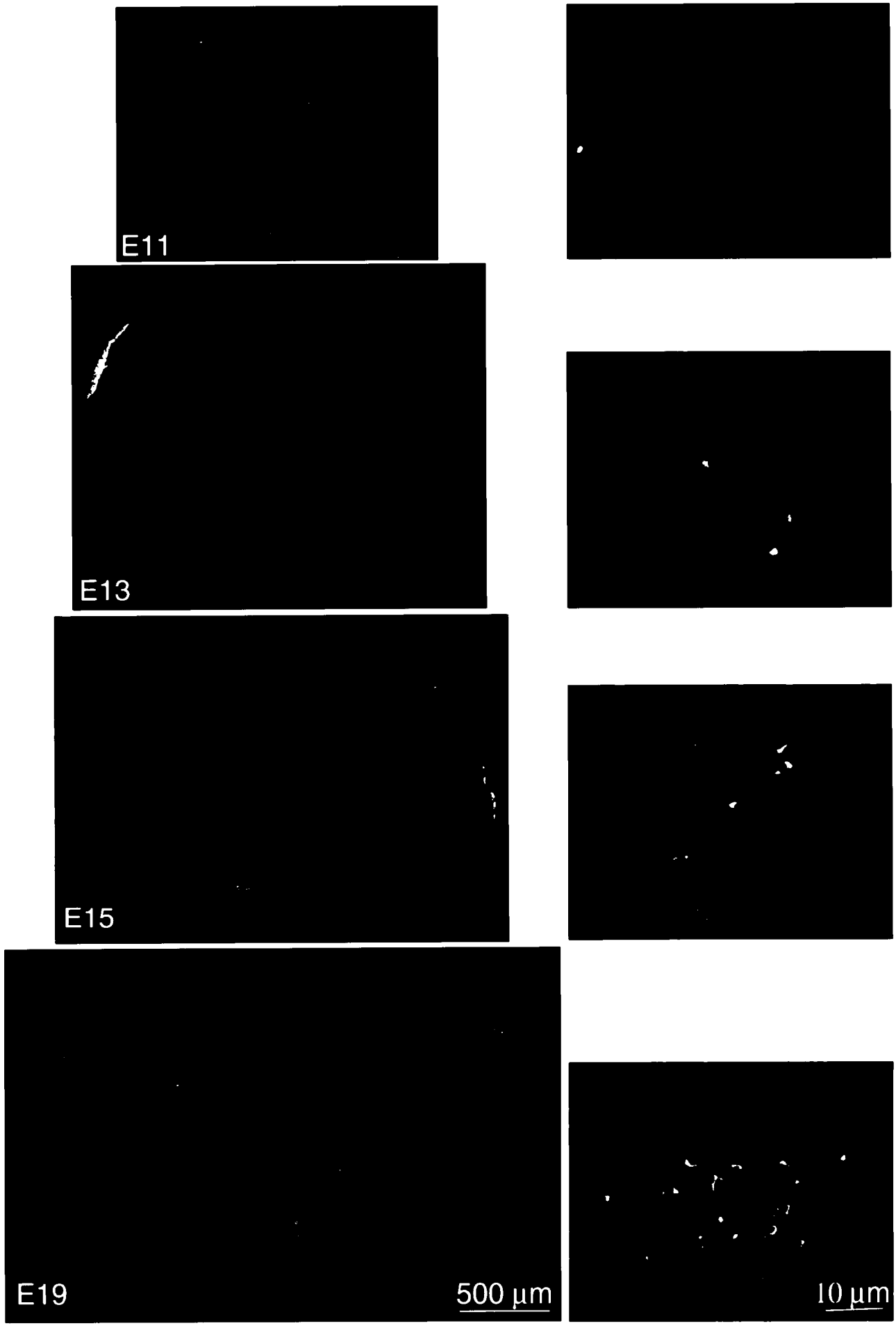

GFAP, TUNEL + Hoechst
8 µm

Figure 2-8. Analysis of TfBP development in the cervical chick spinal cord. Immunoreactivity is detected as early as E11. These cells appear bipolar and to be radiating from central regions of the cord (corresponding high magnification image on the right). A small number of TfBP+ cells are observed in the E13 spinal white matter. These cells appear to have a more mature phenotype, as observed in E15, E18, and hatchling (not shown) chicks. By E15, substantial numbers of TfBP+ cells are found in the white matter, and by E18, an adult-like pattern is formed.


E11
E13
E15
E19
500 µm
10 µm

This also appears to be the case for white matter glia in the mature chick spinal cord as hatchling transection initiated a only a moderate increase in apoptosis in regions remote from the site of injury. This response is similar to findings in the contused, adult rat spinal cord (Crowe *et al.*, 1997; Shuman *et al.*, 1997).

Transection of the cervical spinal cord at all developmental stages examined elicited a massive apoptotic response at and immediately adjacent to the site of injury. This response occured rapidly after injury, persisted over several days, and affected both white and grey matter. Equivalent responses have been seen in rodent models of spinal cord injury (Crowe *et al.*, 1997; Emery *et al.*, 1998; Katoh *et al.*, 1996; Li *et al.*, 1996; Liu *et al.*, 1997b; Shuman *et al.*, 1997). As in these models, necrosis was a prevalent component of the injury response. Double fluorescent labeling of TUNEL labeled sections with Hoechst 33258 indicated that many TUNEL+ cells had nuclear morphologies consistent with a necrotic form of death. Although TUNEL is highly selective for apoptosis, it is not specific and can label cells undergoing necrosis (Charriaut-Marlangue and Ben-Ari, 1995). Within the perilesion area, high concentrations of TUNEL+ cells are observed in the grey matter, perhaps indicating an increased susceptibility of grey matter neurons to the degenerative milieu of the secondary injury response immediately surrounding the lesion site. However, beyond 1 mm caudal to the lesion site, TUNEL+ cells became highly localized to the white matter tracts. At this point, TUNEL specifically labeled apoptotic cells; however, cells with apoptotic nuclei, as determined by Hoechst 33258, were occasionally observed without TUNEL labeling, perhaps indicating a difference in the onset of pyknosis and DNA fragmentation in the apoptotic pathway.

It has been estimated that dying oligodendrocytes in the developing optic nerve are removed within one hour of becoming pyknotic (Barres *et al.*, 1992b). Thus, the number of dying cells detected by TUNEL represented only a small proportion of the total cellular loss. The large numbers of cells observed in the white matter distal to the lesion indicates the magnitude of the glial cell loss during development, and particularly, following developmental injury.

Identity of the TUNEL+ Cells

The strict localization of TUNEL+ cells to the white matter in both developing and transected spinal cords, and the correlation of the peak of TUNEL+ cells during

development and their heightened sensitivity to injury with the period of spinal cord myelination strongly suggested the apoptotic cells were of oligodendrocyte origin. This is supported by similar findings in developing rodent optic nerve (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996), and for Schwann cells in the developing sciatic nerve (Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996).

Direct identification of apoptotic oligodendrocytes is inherently difficult due to the small cytoplasmic volume of their cell bodies and the paucity of antibodies that bind selectively to constitutively expressed antigens in this region of the cell. Compounding the difficulty is the loss of cytoplasmic volume that occurs during apoptosis (Clarke, 1990; Kerr *et al.*, 1995). However, TfBP, which specifically labels oligodendrocytes in avian CNS (Cho and Hyndman, 1991; Cho and Lucas, 1995; Cho *et al.*, 1999; Cho *et al.*, 1997) revealed a large number of the dying cells in the injured chick spinal white matter to be oligodendrocytes. In addition, MBP and Rip also identified dying cells as oligodendrocytes in TUNEL and or Hoechst stained sections.

The death of these cells may reflect their dependence on intact axonal signalling for their survival. This suggestion is supported by the observation of increased apoptosis caudal to the injury in the ventrolateral and ventromedial white matter regions containing descending but not ascending projections (i.e., degeneration of distal axonal segments contributed to the apoptotic response). Similar patterns of degeneration-associated oligodendrocyte apoptosis have been identified in contused rat spinal cord, albeit over a different timecourse (Crowe *et al.*, 1997; Shuman *et al.*, 1997). This may reflect a loss of axon-derived trophic support required by white matter glia as has been proposed for oligodendrocyte apoptosis in the transected rodent optic nerve (Barres *et al.*, 1993a). In this sense, the apoptotic response is likely a secondary reaction to axonal injury. This idea is supported by the similar increase and distribution of TUNEL+ cells observed 24 hours after transection on E13 and 4 days after transection on E10. In the latter case, the absence of axons may reduce trophic support for newly differentiating or premyelinating oligodendrocytes, resulting in their death.

Recent studies of oligodendrocyte cell death in the developing rat brain and spinal cord indicate it is the pre- or promyelinating differentiated oligodendrocytes that undergo

developmental apoptosis (Calver *et al.*, 1998; Trapp *et al.*, 1997). This is consistent with findings of Schwann cell apoptosis in the developing sciatic nerve of rat and chick, in which the apoptotic Schwann cells also appear to be at premyelinating stages in their differentiation (Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a). These studies indicate that the peak of oligodendrocyte and Schwann cell apoptosis occurs prior to or during the onset of myelination.

In the present study, the peak of apoptosis in the developing cervical white matter occurs well after the onset of myelination. However, the asynchronous nature of oligodendrocyte differentiation and myelination combined with the condensed timecourse of myelination in the chick spinal cord (Macklin and Weill, 1985) may result in the presence of greater numbers of premyelinating oligodendrocytes during later stages of myelination than may be found in other models. Importantly, studies that identified premyelinating oligodendrocytes as the predominant cell type undergoing developmental apoptosis did not examine the injury-induced apoptotic responses in newly myelinated fibres.

In light of the large transection-induced apoptotic response observed well into the period of myelination of the embryonic chick spinal cord, it seems likely that both premyelinating and myelinating oligodendrocytes undergo apoptosis. In the present study, however, I do not make the distinction between these developmental stages. The antibodies used in this study to identify apoptotic oligodendrocytes label both premyelinating and myelinating oligodendrocytes. MBP immunoreactivity in the cell bodies of premyelinating oligodendrocytes has been previously reported (Trapp *et al.*, 1997), and Rip labels oligodendrocytes from early points in their differentiation (Daston and Ratner, 1994). The appearance of TfBP immunoreactivity in the spinal white matter on E11 indicates this antibody also detects premyelinating oligodendrocytes. The bipolar morphology of E11 TfBP-positive cells is consistent with the morphology of early stage oligodendrocytes found in the developing chick CNS (Ono *et al.*, 1995; Ono *et al.*, 1997). TfBP has been identified as heat shock protein 108, and has been suggested to be involved in iron metabolism (Cho and Lucas, 1995; Cho *et al.*, 1997). Oligodendrocytes are the predominant iron-containing cells in the CNS, in which iron is a crucial component for myelin formation (Connor and Menzies, 1996). It is likely, therefore that

TfBP initially labels premyelinating oligodendrocytes preparing for myelination of the spinal cord.

Although large numbers of apoptotic cells exhibited oligodendrocyte specific immunoreactivity, many dying cells did not. This may indicate the loss of oligodendrocyte specific immunoreactivity during apoptosis. Apoptotic oligodendrocytes in the developing optic nerve rapidly lose Rip immunoreactivity (Barres *et al.*, 1992a). This may explain why fewer Rip-positive apoptotic cells were detected than MBP-positive cells. MBP and TfBP immunoreactivity appear to be retained further into the apoptotic process, but may be lost during latter stages. In the jimpy mouse, MBP is not lost from the cytoplasm of oligodendrocytes undergoing apoptosis, but is redistributed (Vela *et al.*, 1996). Loss of phenotypic markers could also occur if injured cells de-differentiate before dying. Oligodendrocyte de-differentiation is thought to occur following optic nerve transection in P21 rats (Butt and Colquhoun, 1996). It is also conceivable that some apoptotic cells represent immature oligodendrocytes or their precursors, which may not yet express the phenotypes examined in this study. Additionally, heterogeneity among oligodendrocytes may contribute to the absence of TfBP immunoreactivity in many apoptotic cells. TfBP is found primarily in the highly metabolic light to medium oligodendrocytes (Cho and Lucas, 1995). Thus, dark oligodendrocytes undergoing apoptosis would not have been detected with the TfBP antibody.

Alternatively, the absence of oligodendrocyte phenotypic markers in TUNEL+ cells may indicate the involvement of other cell types in the apoptotic response. Although astrocytes mature during the same period as oligodendrocytes in the chick spinal cord (McGraw, 1999), the absence of GFAP-positive apoptotic cells indicates astrocytes do not undergo developmental or transection-induced apoptosis in the spinal white matter remote from the lesion. This is consistent with the lack of astrocyte cell death observed in the developing optic nerve (Barres *et al.*, 1992a; Burne *et al.*, 1996) and in injured adult rat spinal cord (Li *et al.*, 1996). The failure to label apoptotic astrocytes is not due to the loss of phenotype as GFAP immunoreactivity is retained during apoptosis (Burne *et al.*, 1996). It is possible that microglia are represented in the apoptotic response in the present model. Apoptotic microglia have been observed in regions of oligodendrocyte apoptosis

in the developing spinal cord of the jimpy myelin mutant mouse and in the white matter of contused rat spinal cords. (Knapp *et al.*, 1986; Shuman *et al.*, 1997). Whereas it is possible that labeling of apoptotic nuclei in microglia actually represents phagocytosis of apoptotic oligodendrocytes, evidence suggest that at least some microglia actually undergo apoptosis themselves (Shuman *et al.*, 1997; Vela *et al.*, 1996). The significance of microglial apoptosis is unclear, but may represent a means by which to remove themselves from the CNS once their primary function had been fulfilled. Alternatively, their death may be an indirect consequence of an ongoing microglial reaction to degenerating cells. Microglia possess many pro-apoptotic signals used to eliminate unwanted cells from the CNS, but which could conceivably result in their own removal (discussed below). Unfortunately, the microglial response could not be examined in this model due to the lack of reliable avian markers of these cells.

Developmental neuronal death is not likely to have played a significant role in the developmental or transection-induced apoptosis in this model, as TUNEL+ cells were confined to the white matter, and different phases of neuronal ACD are detected in the developing chick spinal cord prior to E12 (Burek and Oppenheim, 1996; Homma *et al.*, 1994; Okado and Oppenheim, 1984; Oppenheim, 1984; Oppenheim, 1991; Oppenheim *et al.*, 1988). This raises the possibility that neuronal death contributes to the TUNEL labeling observed in the cervical spinal cord of E10 and E11 embryos. The more widespread distribution of apoptotic cells in these earlier staged embryos may reflect a neuronal component. Alternatively, it may represent the death of immature and migrating oligodendrocytes. Interneurons do not undergo developmental ACD (McKay and Oppenheim, 1991) and, therefore, did not likely contribute to the apoptosis detected in the developing cord in the present study.

What is the Death Stimulus

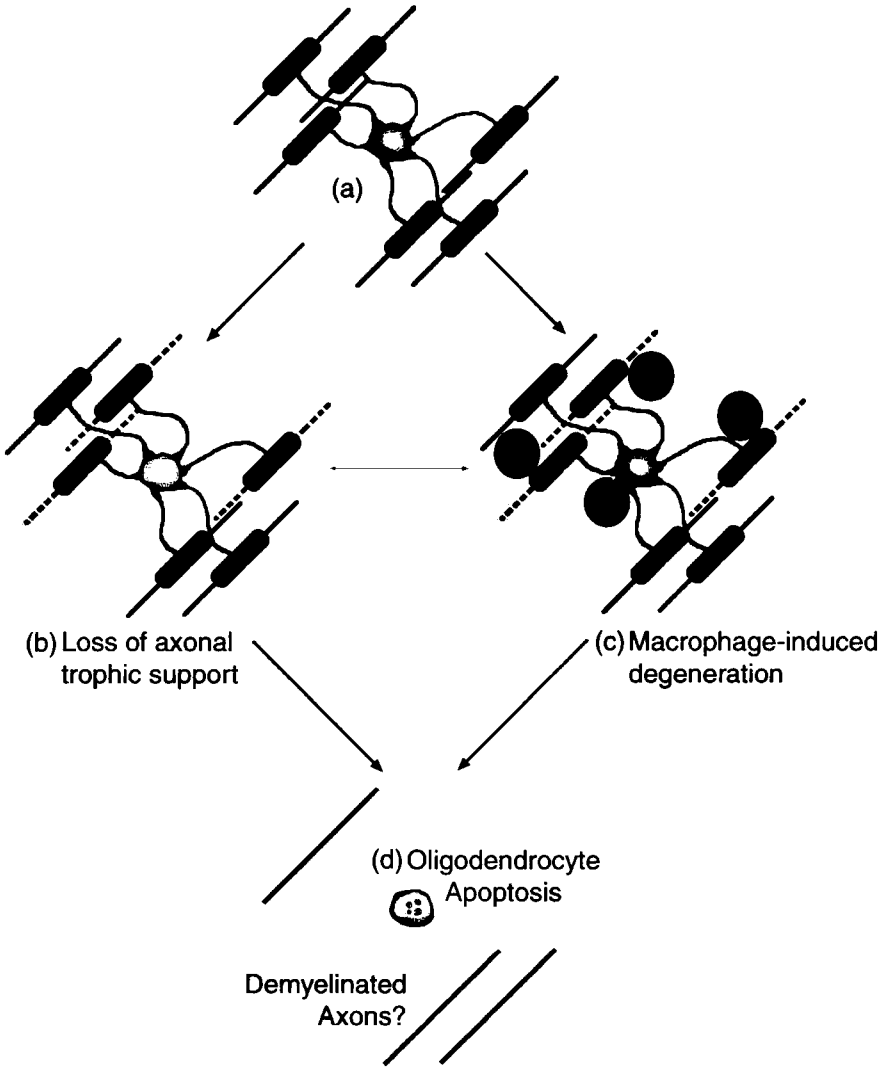
The stimuli responsible for initiating the apoptotic program in oligodendrocytes following SCI remain unknown; however, two potential mechanisms have been proposed (Figure 2-9). The first mechanism, "death by neglect," involves the loss of axon-derived trophic support (Barres *et al.*, 1993a; Barres and Raff, 1994; Raff *et al.*, 1993). The findings of the present study appear consistent with this proposal, as the apoptotic response in the white matter is largely associated with the distal axons of descending

supraspinal pathways (discontinuous with the neuronal cell body) in the ventrolateral and ventromedial cord, and not with proximal fibres of ascending projections in the dorsal columns (continuous with neuronal soma). This concurs with the pattern of injury-induced oligodendrocyte apoptosis in the developing rat optic nerve (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996) and the contused spinal cord of the adult rat (Crowe *et al.*, 1997; Shuman *et al.*, 1997). The precise nature of the putative axon-derived trophic factor remains elusive. However, possible candidates include IGF-1, NT-3, and PDGF-AA, all of which regulate oligodendrocyte survival (Barres *et al.*, 1992a; Barres *et al.*, 1993a; Barres *et al.*, 1993b). Glial growth factor (GGF) may also promote survival as this axonal neuregulin influences oligodendrocyte development through the HER4 receptor (Vartanian *et al.*, 1997).

The second mechanism proposed to induce trauma-induced oligodendrocyte apoptosis involves cytotoxic activities of immune cells such as microglia or macrophages (Shuman *et al.*, 1997; Vela *et al.*, 1996), and has been termed "death by murder." In spinal cords of both the myelin mutant jimpy mouse (Vela *et al.*, 1996) and contused rat spinal cord (Shuman *et al.*, 1997), most apoptotic oligodendrocytes are contacted by microglia. The temporal sequence of microglia activation and oligodendrocyte apoptosis in the distal white matter remote from the epicenter of spinal contusion led Shuman et al (1997) to postulate that microglia have a role that extends beyond phagocytosing apoptotic cells and that they may exert direct death-inducing influence on oligodendrocytes. This could explain the presence of demyelinated regions of intact axons after SCI (Blight, 1983; Blight, 1985; Blight, 1993; Bunge *et al.*, 1993; Gledhill and McDonald, 1977; Shi *et al.*, 1997). Indeed, the temporal sequence of microglial activation and the appearance of demyelination are consistent with this hypothesis.

Recently, Fas has been shown to be involved in neuronal cell death in the developing rat cortex (Cheema *et al.*, 1999). This death receptor mediated death may also occur in the spinal cord, and may involve oligodendrocytes. Oligodendrocytes express Fas receptor (D'Souza *et al.*, 1996b), and are sensitive to other death ligands activating apoptosis through the tumor necrosis factor (TNF) receptor, p55 (D'Souza *et al.*, 1995; D'Souza *et al.*, 1996a) and the low affinity neurotrophin receptor, p75 (Gu *et al.*, 1999).

Figure 2-9. Two proposed stimuli for transection-induced oligodendrocyte apoptosis. (a) A healthy oligodendrocyte myelinates (blue) multiple axons (green). Trauma induces degeneration of some axons (red dashed) associated with an oligodendrocyte. (b) This could induce oligodendrocyte apoptosis through the loss of axon-derived trophic support (death by neglect). (c) Alternatively, microglia/macrophages (red circles) recruited to the area of degenerating axons could actively induce apoptosis of oligodendrocytes (death by murder). The grey arrow indicates the two mechanisms need not be mutually exclusive. (d) In either scenario, the apoptotic death of oligodendrocytes could lead to the demyelination of intact axons, resulting in deficits due to functional conduction block.


(a)
(b) Loss of axonal trophic support
(c) Macrophage-induced degeneration
(d) Oligodendrocyte Apoptosis
Demyelinated Axons?

Factors such as TNF are produced by microglia and increase after SCI (Klusman and Schwab, 1997; Pan *et al.*, 1997; Wang *et al.*, 1996), suggesting a possible role in the induction of oligodendrocytes apoptosis following SCI. It is uncertain, however, what initiates the microglial response in regions remote from the lesion. It may be degenerative responses such as trophic factor deprivation-induced apoptosis of oligodendrocytes and/or Wallerian degeneration attract microglia to clean up the debris. As a consequence of this microglial activation, surviving oligodendrocytes may inadvertently be induced to undergo apoptosis by cytotoxic agents released by the microglia to which oligodendrocytes are particularly susceptible.

The mechanisms of death by neglect or murder need not be mutually exclusive. However, in developing fibre tracts such as those found in the spinal cord or the optic nerve, the former hypothesis may be more relevant due to the increased reliance of oligodendrocytes and Schwann cells on axon-derived trophic support. As these cells mature, however, they may establish autocrine or juxtacrine/paracrine survival loops, which would reflect their diminished reliance on axons for their survival. Such developmental events have been reported for neurons (Cheng and Patterson, 1997; Kobayashi *et al.*, 1996; Yang *et al.*, 1998c) and Schwann cells (Cheng *et al.*, 1998a; Meier *et al.*, 1999; Syroid *et al.*, 1999). Interestingly, cultured neonatal oligodendrocytes may also regulate their development through a neuregulin autocrine or juxtacrine (cell-to-cell) pathway (Raabe *et al.*, 1997). It would appear from the paucity of astrocyte apoptosis in this and other studies, that they are not dependent on axons for their survival to the degree that oligodendrocytes might be, or that astrocytes are not susceptible to cytotoxic microglial factors. It is unclear as to why microglia would effect the removal of oligodendrocytes from spared, uninjured axons: it may be an inadvertent consequence of the microglial response to degenerating axons and the oligodendrocytes associated with them.

Conclusions

Results of this study indicate the embryonic chick model of SCI produces an apoptotic glial response similar to other developmental models of axotomy-induced oligodendrocyte ACD (e.g., Barres *et al.*, 1993a; Barres and Raff, 1994), and mimics trauma-induced degenerative responses in adult mammalian systems (e.g., Crowe *et al.*,

1997; Emery *et al.*, 1998; Liu *et al.*, 1997b; Shuman *et al.*, 1997). The greater magnitude of the glial apoptotic response in the distal white matter in this developing chick model as compared to that in adult rodents make it a useful model with which to investigate the molecular mechanisms mediating oligodendrocyte cell death and survival. Information obtained from this model may not only further our knowledge of mechanisms underlying trauma-induced oligodendrocyte apoptosis, but will be of importance in understanding and managing other degenerative conditions involving oligodendrocyte apoptosis such as MS and SMA. Furthermore, evidence suggests oligodendrocyte precursors are capable of remyelinating demyelinated regions (Keirstead *et al.*, 1998b). However, there is a limited supply of these cells. It will, therefore, be important to prolong oligodendrocyte survival in order to maintain functional myelination of axons in remyelination events.

CHAPTER 3: CASPASE INVOLVEMENT IN TRANSECTION-INDUCED GLIAL APOPTOSIS

INTRODUCTION

Traumatic injury to the brain and spinal cord is characterized by two separate pathophysiological components (Schwab and Bartholdi, 1996; Taoka and Okajima, 1998; Tator and Fehlings, 1991). The first comprises the immediate mechanical damage (primary injury), whereas the second involves delayed secondary degeneration. The primary response is mediated by physical disruption of tissue and cellular membranes (Schwab and Bartholdi, 1996; Taoka and Okajima, 1998; Tator and Fehlings, 1991). This passive process differs dramatically from the delayed secondary injury that evolves over the subsequent hours and days. The secondary response is an active process mediated by a myriad and complex set of factors including ischemia, excitotoxicity, inflammation, deregulation of ionic homeostasis, free radicals, and pathological activation of cellular proteases, such as calpains and caspases (Dusart and Schwab, 1994; Ito *et al.*, 1997; Kao and Chang, 1977; Schumacher *et al.*, 1999; Schwab and Bartholdi, 1996; Tator, 1995; Tator and Fehlings, 1991; Zhang *et al.*, 1997b). Although extraordinarily complex, the delayed and protracted nature of the secondary response may make it amenable to therapeutic intervention. Such intervention is a critical goal in SCI as many injuries are initially incomplete. Because as little as 10% sparing of axons can dramatically improve neurological function, any preservation of neural tissue may have significant benefits for those who suffer a CNS injury (Eidelberg *et al.*, 1997; Fehlings and Tator, 1995).

SCI & Oligodendrocyte Apoptosis

Apoptosis has been shown to be a component of the secondary response to neurotrauma (Crowe *et al.*, 1997; Emery *et al.*, 1998; Katoh *et al.*, 1996; Li *et al.*, 1996; Liu *et al.*, 1997b; Lou *et al.*, 1998; Shuman *et al.*, 1997; Yong *et al.*, 1998). In particular, oligodendrocytes undergo a biphasic apoptotic response following SCI. Initially oligodendrocytes in the immediate region of the injury undergo apoptosis within 24 hours of the injury (Crowe *et al.*, 1997; Liu *et al.*, 1997b; Yong *et al.*, 1998). This is followed by a delayed response in the distal white matter several days after the primary injury (Crowe *et al.*, 1997; Emery *et al.*, 1998; Liu *et al.*, 1997b; Shuman *et al.*, 1997). These apoptotic oligodendrocytes are associated with degenerating axons in rat, monkey and human spinal cord following traumatic injury (Crowe *et al.*, 1997; Emery *et al.*, 1998;

Liu *et al.*, 1997b; Shuman *et al.*, 1997). The results of the study outlined in Chapter 2 demonstrate that the embryonic chick model of SCI effectively mimics this response except that magnitude of the apoptotic reaction is much larger and occurs over a condensed time frame.

Very little evidence exists for the mechanisms underlying oligodendrocyte apoptosis *in vivo*. However, following SCI, de novo protein synthesis is a requirement for the death of these cells as treatment of the contused rat spinal cord with cyclohexamide reduced tissue loss and improved neurological outcome (Liu *et al.*, 1997b). As discussed in the previous chapter, SCI-induced glial apoptosis may represent death by "neglect" or by "murder". Irrespective of the precise nature of the death-inducing stimulus, the apoptotic morphology of glial cell death in spinal cord injured rodents and humans suggests involvement of caspases. Indeed, presumptive oligodendrocytes in the injured human spinal white matter exhibit immunoreactivity for antibodies raised against activated caspase-3 (Emery *et al.*, 1998), the central effector of the apoptotic pathway in the CNS (Cohen, 1997; Porter and Janicke, 1999; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998).

Caspases

Caspases are regarded as the final common executioners of apoptotic pathways (Cohen, 1997; Porter and Janicke, 1999; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). These cysteine proteases contain the amino acid sequence QACXG, in which C is the active site cysteine. Three-dimensional crystal structures of active caspases-1 and -3 indicate these enzymes utilize a typical cysteine protease mechanism involving a catalytic dyad of cysteine and histidine; however, caspases are unique in that they have an absolute requirement for aspartate in the P1 position of their substrate (Garcia-Calvo *et al.*, 1998; Talanian *et al.*, 1997; Thornberry *et al.*, 1992; Thornberry *et al.*, 1997). Caspases show the greatest specificity at the P4 position as the P4 side chains occupy defined pockets that vary amongst different members of the caspases (Garcia-Calvo *et al.*, 1998; Talanian *et al.*, 1997). Caspases are synthesized as inactive proenzymes (procaspases) with a prodomain of varying length and a large (p17-20) and small (p10-12) subunit. Upon activation, the prodomain and two subunits are cleaved from each other at caspase consensus sequences (indicating autoprocessing) and a

heterodimer is formed through association of the large and small subunits. Two heterodimers then combine to form the active tetrameric complex, with two independent catalytic sites spanning the large and small subunits (Cohen, 1997; Thornberry and Lazebnik, 1998).

Caspases can be classified as mediators of inflammatory response (Group I; caspase-1 –4, -5, -11), effectors of apoptosis (Group II; caspase-3, -7, -2) or initiators of apoptotic caspase cascades (Group III; caspase-6, -8, -9, -10) (McBride *et al.*, 1999; Thornberry and Lazebnik, 1998). In general, caspases can be activated in two ways. Ligand binding of death receptors ("death by murder") leads to the rapid induction of initiator caspases, which then activate effector caspases, the mediators of the morphological changes associated with apoptosis (Cohen, 1997; Janicke *et al.*, 1998; Stennicke *et al.*, 1998; Thornberry and Lazebnik, 1998). This method of caspase activation is a key mechanism of apoptosis induction in immune responses, and is regulated by molecules such as FLIP (FLICE/caspase-8 inhibitory proteins (Bertin *et al.*, 1997; Hu *et al.*, 1997; Thome *et al.*, 1997). Alternatively, mild cytotoxic stimuli or withdrawal of trophic support ("death by neglect") activate caspases in a more regulated and protracted manner in which mitochondria play a central role (Green and Reed, 1998). In this mode of caspase induction, factors are released from mitochondria that promote the activation of initiator caspases, which, in turn, activate the effector caspases. Several molecules upstream of mitochondria regulate entry into the caspase activation cascade, the most prominent being members of the Bcl-2 family. Members of this family are either pro- or anti-apoptotic and it appears that the ratio of these molecules regulates entry into the apoptotic pathway at the level of the mitochondrion (Farrow and Brown, 1996; Kroemer, 1997; Reed, 1997b). Other cellular inhibitors such as the IAPs (inhibitors of apoptosis proteins) act directly at the level of effector caspase activation (Deveraux *et al.*, 1997b; Roy *et al.*, 1997).

Caspase-3 has been shown to be a critical effector caspase in numerous models of apoptosis (Cohen, 1997; Janicke *et al.*, 1998; Porter and Janicke, 1999; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998; Woo *et al.*, 1998; Zheng *et al.*, 1998). It appears to be of particular importance in CNS development as deletion of the caspase-3 gene causes gross anatomical abnormalities within the CNS characterized by enlarged brain regions due to decreased cell death (Kuida *et al.*, 1996). Other *in vivo* studies

support a role for caspase-3 in developmental apoptosis within the CNS (Kuida *et al.*, 1996; Li *et al.*, 1998b; Urase *et al.*, 1998; Woo *et al.*, 1998). Both neuronal (Barnes *et al.*, 1998; Chen *et al.*, 1998; Deshmukh *et al.*, 1996; Dodel *et al.*, 1999; Dodel *et al.*, 1998; Eldadah *et al.*, 1997; Gorman *et al.*, 1999; Hisahara *et al.*, 1997; Jeon *et al.*, 1999; Keane *et al.*, 1997; Kouroku *et al.*, 1998; Krajewska *et al.*, 1997; Li *et al.*, 1998b; Marks *et al.*, 1998; Miller *et al.*, 1997; Namura *et al.*, 1998; Porter and Janicke, 1999; Schulz *et al.*, 1998; Siman *et al.*, 1999; Srinivasan *et al.*, 1998; Tamatani *et al.*, 1998; Tanabe *et al.*, 1998; Urase *et al.*, 1998; Vanderluit *et al.*, 1999; Yakovlev *et al.*, 1997) and oligodendrocyte (Emery *et al.*, 1998; Gu *et al.*, 1999; Miura *et al.*, 1999) apoptosis have been shown to involve caspase-3 activation. However, it is clear that other caspases are important to the death of these cells and that different stimuli may activate different caspases (e.g., (Gu *et al.*, 1999; Hakem *et al.*, 1998; Li *et al.*, 1998b; Park *et al.*, 1998; Stefanis *et al.*, 1998; Troy *et al.*, 1997; Troy *et al.*, 1996). Neonatal rat cortical oligodendrocytes express caspases-1, -2, -3, and -8 (Gu *et al.*, 1999). However, in response to NGF signaling through the p75 neurotrophin receptor, only caspase-1, -2 , and -3 become activated. Caspase-8 can become activated in these cells in response to staurosporine but not radiation or p75 activation (Gu *et al.*, 1999).

Caspase Inhibition

Synthetic peptide-based caspase inhibitors have been developed utilizing the substrate specificity of the different caspases(Garcia-Calvo *et al.*, 1998; Talanian *et al.*, 1997; Thornberry *et al.*, 1997) and can be either reversible or irreversible (Livingston, 1997). Reversible inhibitors include aldehydes, nitriles, and ketones. These inhibitors undergo nucleophilic addition of the catalytic cysteine to form thiomiactetals, thiomidates and thioemiketals, respectively (Thornberry and Molineaux, 1995). The similarity of these compounds to the tetrahedral and acyl-enzyme intermediates formed during hydrolysis of the substrate accounts for the potency of these inhibitors. Irreversible inhibitors include diazomethyl, acylomethyl and halomethyl ketones, which function through the formation of a thiomethylketone with the active cysteine via expulsion of the leaving group (Thornberry and Molineaux, 1995). Inactivation appears to proceed via formation of a thioemiketal.

The most extensively utilized caspase inhibitors are the reversible aldehyde (CHO) peptides and the irreversible halomethyl ketones. The fluoromethyl ketones (fmk) exhibit less reactivity than chloromethyl ketones (cmk) and are, therefore, more specific. The estrification of aspartate residues in fmk inhibitors neutralizes the charge and increases cell permeability (Garcia-Calvo *et al.*, 1998; Livingston, 1997). In general, YVAD peptide inhibitors inhibit caspase-1-like caspases (caspase-1, -4, -5), whereas DEVD inhibits caspase-3-like caspases (caspase-3, -7) (Garcia-Calvo *et al.*, 1998; Talanian *et al.*, 1997; Thornberry *et al.*, 1997). Recently, a pan-specific caspase inhibitor, BAF (boc-aspartyl(OMe)-fluoromethylketone) has been successfully used to inhibit caspase activity and apoptosis *in vivo* (Cheng *et al.*, 1998b).

The first report of caspase inhibition *in vivo* demonstrated the attenuation of developmental neuronal cell death of spinal motor neurons and of other cell types in the developing chick embryo (Milligan *et al.*, 1995). Recent studies have demonstrated the involvement of caspases in secondary injury in rodent models of TBI (Yakovlev *et al.*, 1997) and ischemia (Chen *et al.*, 1998; Cheng *et al.*, 1998b; Hara *et al.*, 1997; Kaushal *et al.*, 1998; Namura *et al.*, 1998; Schulz *et al.*, 1999) and SCI in humans (Emery *et al.*, 1998). Caspase inhibition has been shown to attenuate tissue loss and improve neurological outcome in several of these rodent models of neurotrauma (Chaudhary *et al.*, 1999; Chen *et al.*, 1998; Cheng *et al.*, 1998b; Kermer *et al.*, 1998; Ma *et al.*, 1998; Schulz *et al.*, 1998; Schulz *et al.*, 1999; Yakovlev *et al.*, 1997).

The overall involvement of caspases in SCI-induced glial cell death in the distal spinal white matter and the ability of caspase inhibitors to rescue these cells *in vivo* is unknown. However, the apoptotic nature of oligodendrocyte death in this region suggests caspases are involved. Observations of activated caspase-3 in human spinal white matter supports this suggestion (Emery *et al.*, 1998). Thus, based on the central role of caspases in apoptosis and my previous findings of transection-induced oligodendrocyte apoptosis in the developing chick spinal cord, I hypothesized that caspases would play a crucial role in mediating this apoptotic response. Using the model developed in Chapter 2, I examined the contribution of caspases in this response to trauma. Administration of caspase inhibitors attenuated the apoptotic response after transection of the cervical spinal cord but did not affect developmental apoptosis. The degree of inhibition varied

with the different inhibitors and suggested caspase-3-like caspases to be more involved in the apoptotic response in the spinal white matter remote from the lesion. This was corroborated by an increase of caspase-3-like catalytic activity compared to caspase-1-like activity in this region of the transected spinal cord. Furthermore, activated caspase-3 immunoreactivity was detected in this region. These data indicate a central role for caspase-3-like catalytic activity in the injury-induced apoptosis in the spinal white matter remote from the lesion, and indicate a potential avenue for therapeutic intervention to limit the secondary response to SCI. The results may also be relevant to other degenerative conditions involving oligodendrocyte apoptosis such as some forms of multiple sclerosis (Dowling *et al.*, 1997; Oshako and Elkon, 1999; Tsunoda *et al.*, 1997), multiple system atrophy (Probst-Cousin *et al.*, 1998), and in the cognitive and spastic motor deficits of premature infants (Noble and Mayerproschel, 1996).

A note on terminology

Due to the promiscuity of caspases with respect to substrate specificities, the currently employed tetrapeptide inhibitors are not specific for a single caspase (Garcia-Calvo *et al.*, 1998; Thornberry *et al.*, 1997). However, substrate preferences for particular caspase subgroups show a high degree of selectivity. Therefore, it is more accurate when using substrates such as YVAD and DEVD to refer to the associated caspase activity in terms of the subgroup most specific for the substrate. The two caspase subgroups investigated in the present study are the Group I, caspase-1-like caspases, caspase-1, -4, and –5, and the Group II, caspase-3-like caspases, caspase-3 and –7.

MATERIALS AND METHODS

Animals

As described in Chapter 2, fertilized white leghorn chicken (*Gallus gallus domesticus*; Coastline Chicks, Abbotsford, BC) eggs were incubated in a rotating, incubator maintained at 38°C and a relative humidity of approximately 60%. Hatchling chicks were maintained in a commercial brooder with food and water provided *ad libitum*.

Embryonic Surgery

Surgery was performed as described in Chapter 2. Prior to spinal cord transection, the developmental stage of embryos was determined using the criteria established by Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were operated on E13. E13 was selected as it is the day on which myelin appears and SCI induces large numbers of TUNEL-positive cells in the remote spinal white matter. In addition, E13 embryos were found to tolerate SCI better than earlier stage embryos. A small window was made in the shell using a single-edged razor blade and the inner shell membrane was removed with #5 forceps. Using Dumont 5/45 forceps (Fine Science Tools, North Vancouver, BC), a small hole was made in the underlying chorioallantoic membrane: special care was taken to minimize disruption of the blood vessels of this highly vascularized membrane. A "hooked" glass probe was then inserted through the hole in the chorioallantoic membrane to secure the neck for transection of the cervical segment (C) 3-C5 spinal cord with 5/45 forceps. The transection was performed by inserting the forceps tips on either side of the dorsal neck midline and closing the forceps entirely. This procedure has been used extensively in this laboratory and reliably transects the spinal cord completely (Hasan *et al.*, 1993; Keirstead *et al.*, 1995; Keirstead *et al.*, 1992; Steeves *et al.*, 1993). Sham operations were also performed in which the forceps were inserted into the neck but were not closed. Following transection, the window was covered with filament tape and sealed with paraffin wax. Eggs were then returned to the incubator (but not rotated). Embryos were sacrificed at various times between 12 hours and 7 days after transection.

Cervical transections were performed for three main reasons. Firstly, the cervical spinal cord contains the largest number of descending axons from the brainstem; thus, a greater response may be expected from injuries in this region than from those at more caudal levels. Second, this region is surgically the most accessible and results in much more consistent surgical outcomes, namely complete transection. Third, this is the first region of the spinal cord to become myelinated, allowing studies to be performed at earlier times in development, which makes the operation easier and more consistent with respect to the completeness of the transection. The operation is well tolerated by the embryos with typical survival rates of approximately 75%.

The efficacy of transections was determined upon dissection of the fixed spinal cord and in cresyl violet stained sections of the injured spinal cords. Only those cords exhibiting complete transections were included in the study.

Inhibition of Caspase Catalytic Activity In Ovo

Cervical spinal cord transections in E13 chick embryos were performed as described above. Caspase inhibitors were applied directly onto the chorioallantoic membrane in a volume of 50-75 µl, 12 and 22 hours following the transection, or at equivalent times for sham and unoperated controls. Inhibitor control embryos received vehicle alone. Inhibitor application began 12 hours post injury due to the 20-30% post surgery mortality rate and the cost of inhibitors. Mortality is unpredictable but if it occurs, it typically happens within the first 12 hours after transection. Thus, little mortality is expected in embryos after 12 hours and is not likely to occur during the inhibitor treatment. Embryos were sacrificed 8-10 hours following the final application of inhibitor (30-32 hours after transection). The tetrapeptide aldehyde inhibitor, acetyl (Ac)-YVAD-cho (Bachem; King of Prussia, PA, #H-8410) was dissolved in distilled water and diluted in 0.9% saline. The fluoromethyl ketone (fmk) inhibitors, Ac-DEVD-fmk, and benzyloxycarbonyl-Asp(OMe)-fmk (BAF) (Enzyme Systems Products; Dublin, CA, #FK-010 and –011, respectively) were dissolved in DMSO and subsequently diluted in saline with a final DMSO concentration of 2%. Each animal received 250 µg YVAD, 100 µg DEVD, 100 µg BAF, or vehicle control per application.

Apoptosis Detection

To quantify the number of apoptotic cells in the embryonic cervical spinal cord (see Figure 2-1), embryonic chicks were sacrificed by intracardial perfusion with phosphate buffered saline (PBS), pH7.4 followed by perfusion fixation with 4% paraformaldehyde in PBS. The spinal column was then removed and post-fixed overnight in fresh 4% paraformaldehyde. Spinal cords were then dissected out from the spinal column, cryoprotected in 18% sucrose, and frozen in OCT (Tissue Tek; JB EM Services; Dorval, Que, #JBF 172) compound with liquid nitrogen. Frozen spinal cords were stored at –70°C until cryosectioned at –21°C on a Zeiss cryostat. Cryosectioned tissue was subsequently air dried and stored at –70°C until stained.

Apoptosis was determined using a modified TUNEL technique (Gavrieli *et al.*, 1992) to label fragmented DNA *in situ* and with the nuclear bisbenzamide stain, Hoechst 33258 (Sigma, St. Louis, MO). Both peroxidase and fluorescent TUNEL staining was performed on 10 μm transverse and longitudinal spinal cord sections. In both techniques, spinal cord sections were initially washed three times in PBS (5 minutes per wash), then subjected to proteinase K (2 μg/ml in PBS: Promega; Madison, WI, #V3021) digestion for 5 minutes at room temperature. Sections were again washed three times in PBS before continuing with the TUNEL procedure. Two variations of the TUNEL procedure were used, one intended for light and one for fluorescent microscopic analysis.

For peroxidase-based TUNEL intended for light microscopy, endogenous peroxidase activity was quenched by immersing sections in 0.6% hydrogen peroxide in methanol for 20 minutes at room temperature. Sections were washed twice in PBS before being equilibrated in terminal deoxynucleotidal transferase (TdT) buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate and 1 mM cobalt chloride). The TUNEL reaction was performed at 37°C for 1 hour using 0.15 U/μl TdT (Boehringer Mannheim; Laval, Que., #220 582) and 2 nM/10 μl biotin-16-dUTP (Boehringer Mannheim; #1093070) in TdT buffer. The reaction was stopped by immersing sections for 15 minutes at room temperature in buffer containing 300 mM sodium chloride and 30 mM sodium citrate. Sections were then immersed in 2% bovine serum albumin (BSA) fraction V (Sigma; St. Louis, MO, #A-8551) in PBS for 20 minutes at room temperature. Following 3 5 minute rinses in PBS, sections were incubated with ABC solution (ABC Elite standard kit, Vector; Burlingame, CA, #PK-6100) for 30 minutes at 37 °C. Subsequently, sections were rinsed twice in PBS, then in Tris-HCl (pH 7.6) before the reaction was visualized with diaminobenzidine (DAB) peroxidase substrate kit, with nickel enhancement (Vector, #SK-4100) or with VIP peroxidase substrate kit (Vector, #SK-4600). Sections were then rinsed with water and dehydrated through a series of ascending alcohols (2 minutes each in 70%, 95%, 100%, and 100% ethanol). After clearing sections in two rinses in xylene, sections were mounted in Permount (Fisher Scientific). In negative controls, TdT was omitted from the reaction mixture.

Double immunofluorescent staining with fluorescent TUNEL and Hoechst 33258 was performed on selected sections to verify the apoptotic nature of the TUNEL+ cells.

For TUNEL intended for fluorescent analysis, sections were incubated with 1% normal goat serum (NGS: Jackson ImmunoResearch Laboratories; West Grove, PA, #017-000-127) in PBS for 20 minutes at room temperature. Sections were then rinsed two times in PBS, equilibrated in TdT buffer for 15 minutes at room temperature, and incubated with the TUNEL reaction mixture as described for peroxidase-based TUNEL. The reaction was terminated in the stop buffer as above. Sections were then incubated for 30 minutes at 37 °C in PBS containing 1% NGS, 0.1% Triton X-100 (BDH; Toronto, Ont, #R06433), and Cy3-conjugated ExtrAvidin (Sigma; #E-4142) diluted 1:100. After 3 five minute rinses in PBS, sections were stained in 1 μg/ml Hoechst 33258 (Sigma, #B 2883) in PBS for 2 minutes to verify the apoptotic morphology of TUNEL-positive (TUNEL+) cells. Sections were rinsed in PBS and mounted in PBS glycerol (Sigma Mounting Medium: Sigma, #1000-4). Nail polish was used to seal the coverslip to the slide. In negative controls, TdT was omitted from the reaction mixture.

Caspase Activity Assays

Caspase catalytic activity was measured in spinal cord extracts taken from transected and sham operated controls at various times post operation, and in transected and sham embryos receiving treatment with the pan-specific caspase inhibitor BAF, as described above. Embryos were sacrificed by decapitation. Six millimeters of spinal cord was removed from the cervical spinal cord 1-7 mm caudal to the lesion or the corresponding region in sham operates (see Figure 2-1). The dissected segments from 4-5 embryos were frozen on dry ice and stored at -70 °C. Protein extracts from dissected spinal cords were prepared by homogenization on ice in lysis buffer (25 mM HEPES, pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM $MgCl_2$, 5 mM DTT, and 10 μg/ml each of pepstatin A, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) followed by centrifugation at 14, 000 g for 12 minutes at 4°C. The supernatant was collected and protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford IL, #23225). Caspase catalytic activity in spinal cord lysates was determined by adding 200 μM of colourimetric (p-nitroaniline, pNA) caspase-1-like substrate (Ac-YVAD-pNA; Biomol, Plymouth Meeting, PA, #P-408) or caspase-3-like substrate (Ac-DEVD-pNA; Biomol, #P-412) to 50 μg of extracted proteins in assay reaction buffer (25 mM HEPES,

pH7.5, 0.1% CHAPS, 5 mM DTT, 5 mM EDTA, and 10% sucrose). Reactions were performed at 37°C in microwell plates and enzyme-catalyzed release of pNA was measured at 405 nm in a microplate reader (Bio-Rad). Measurements were taken at 20 minute intervals over a 2 hour period and data was plotted as absorbance versus time for each sample. Reactions were performed in triplicate. Slopes of the line fitted to the linear portion of the data were calculated and averaged for replicate data. The activity of the samples was expressed as pmol substrate per minute using a conversion factor determined by the concentration and absorbance of substrate standard in assay buffer. The sample activity was then calculated as average slope (optical density/minute) x conversion factor (μM/optical density) x assay volume. The specific activity (pmol/minute/μg protein) of the samples was then calculated as the activity divided by the amount of protein in the reaction.

Western Blot Analysis

Tissue was harvested, and prepared for SDS-PAGE as for the caspase activity assay, except that the spinal cords were homogenized in lysis buffer containing 10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM MgCl2, 1mM PMSF, 0.1 mM EDTA/EGTA, 1mM DTT, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.1% CHAPS. Fifty micrograms of protein from the supernatant and 6 μl biotinylated molecular weight marker (Phototope-Star Western Blot Detection Kit: anti-rabbit IgG; New England Biolabs, #7051) was loaded into 15% SDS gels for electrophoretic separation using Bio-Rad Mini Protean II protein gel apparatus (Bio-Rad, #165-2944). Protein was then transferred onto Immobilon-P PVDF transfer membranes (Millipore, #P-15552). Transfer membranes were wetted in methanol, rinsed in distilled water and equilibrated in TBST. Membranes were subsequently blocked in 5% milk in TBST for 40 minutes at room temperature before incubating with rabbit anti-caspase-3 primary antibody (R280, diluted 1:20,000, gift of Dr. George Robertson, Merck Frosst) for one hour at room temperature in 1% blocking solution. The Phototope-Star Western Blot Detection Kit: anti-rabbit IgG was used to detect the protein bands recognized by the primary antibody. Membranes were rinsed in TBST, and incubated with anti-rabbit secondary antibody (diluted 1:5000

in 1% blocking solution) for 1 hour at room temperature. Alkaline phosphatase-conjugated anti-biotin (1:1000 dilution) was included in the incubation solution to detect the molecular weight marker. Following rinses in TBST and Wash Buffer (1X), membranes were incubated in CDP-Star substrate (1:250 dilution in 1X Assay Buffer) for 5 minutes. The membrane was drained of excess developing solution, wrapped in plastic wrap, and exposed to Biomax ML x-ray film (Kodak, #8194540).

Immunohistochemisrty

Detection of active caspase-3 in sections of cervical spinal cord corresponding to the region analysed for caspase activity (i.e., 1-7 mm caudal to the lesion, Figure 2-1) was performed using the polyclonal antibody, MF-397, raised against the conformation of the active caspase-3 tetramer, (Xu *et al.*, 1999). Spinal cord tissue was fixed and prepared as described above for TUNEL labeling. A 1:200 dilution of the MF-397 antibody was diluted in PBS containing 0.01% Tween-20 and 2 % NDS, and applied to 10 μm sections of transected or sham operated spinal cords. Sections were incubated overnight at 4°C, rinsed in PBS and incubated in Cy3-conjugated donkey-anti-rabbit secondary antibody diluted 1:200 in PBS containing 0.01% Tween-20 and 2 % NDS. Sections were rinsed in PBS containing 1 μg/ml Hoechst 33258 before coverslipping in PBS-glycerol. Comparison of the number of MF-397 immunoreactive cells in transected versus sham sections of spinal cord was compared using an unpaired Student's t-test. Ten randomly selected sections from 3 animals in each group were analysed.

RESULTS

Caspase Inhibitors Attenuate Transection-Induced Glial Apoptosis in the Developing Spinal Cord

The previous study demonstrated massive glial apoptosis in the spinal white matter of the developing chick spinal cord during the period of myelination. To test the potential involvement of caspases in mediating this response, specific and non-specific, synthetic peptide caspase inhibitors were administered *in vivo* following cervical transection on E13. Two doses of the tetrapeptide caspase-1-like inhibitor, Ac-YVAD-cho; the tetrapeptide caspase-3-like inhibitor, Ac-DEVD-fmk; the pan-specific caspase inhibitor

BAF; or vehicle control were applied at 12 and 22 hours after injury. Consistent with results from the previous study, transection of the E13 cervical spinal cord lead to an increase in the number of TUNEL+ cells in the spinal cord 1-7 mm caudal to the caudal-most extent of the lesion in saline treated controls 32 hours after injury. The distribution of the apoptotic cells was also similar, occurring primarily in the ventolateral and ventromedial white matter. Hoechst labeling of selected sections stained with fluorescent TUNEL indicated the TUNEL+ cells had apoptotic nuclear morphologies.

Administration of all three inhibitors significantly attenuated the apoptotic response compared to saline treated controls (Figure 3-1). However, the reduction by YVAD was modest compared to DEVD and BAF, indicating a relatively minor contribution of caspase-1-like caspases in transection-induced apoptosis in the spinal white matter remote from the injury. Treatment with both DEVD and BAF lead to a more than 2-fold reduction in the number of TUNEL-positive cells. Although significant ($p<0.01$), the reduction of apoptosis by BAF was not that much greater than for DEVD, suggesting BAF lead to a reduction in DEVD-specific activity (i.e., caspase-3-like activities). The reduction of TUNEL+ cells occurred primarily in the ventromedial and ventrolateral white matter, the region of greatest apoptotic induction following cervical transection in the chick (see chapter 2, Figure 2-4).

Interestingly, BAF did not have a significant effect on the amount of developmental apoptosis in sham or untreated embryos when compared to vehicle treated controls. Once again, the number and distribution of TUNEL+ cells in the sham transected controls was consistent with findings in the previous study (Chapter 2).

Transection-Induced Increased Caspase Catalytic Activity

The caspase-1-like (YVAD) and caspase-3-like (DEVD) catalytic activities were examined in extracts of the cervical cord corresponding to the region used to used to quantify apoptosis in the previous experiment (i.e., 1-7 mm from the caudal-most extent of the lesion, 30-32 hours after transection). Figure 3-2(a) shows a higher degree of cleavage of DEVD compared to YVAD, indicating that of these two caspase activities, caspase-3-like activity is the predominant caspase involved in the apoptotic response in this region of the cord following transection. Consistent with the BAF-mediated attenuation of transection-induced apoptosis, BAF treatment of transected spinal cords

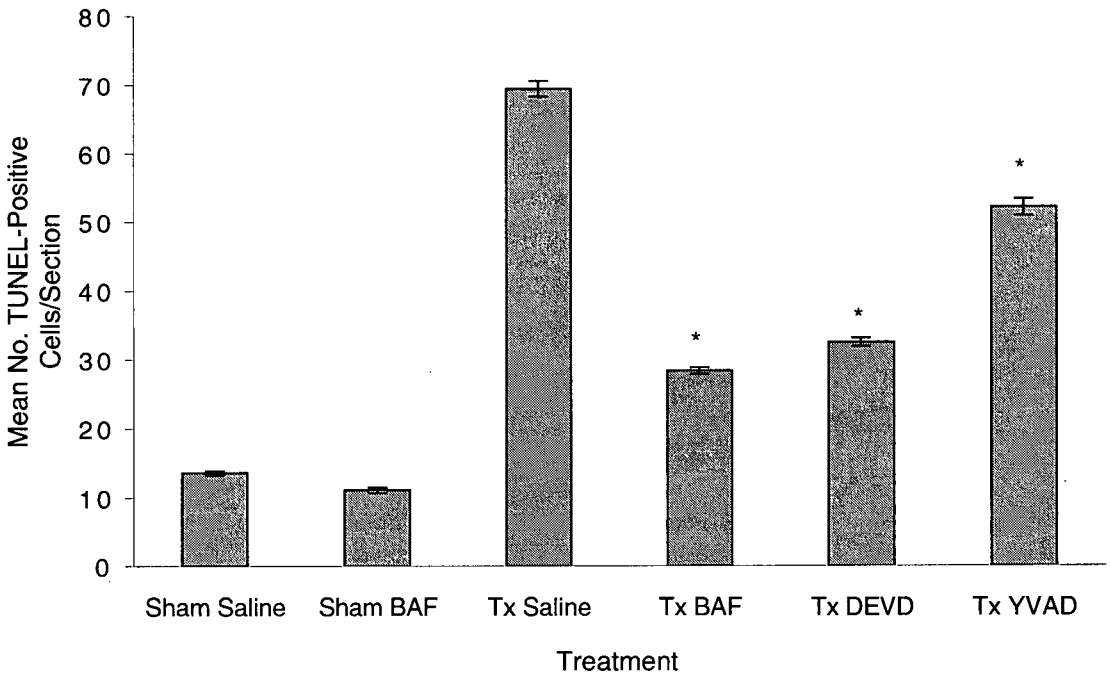
lead to a 2-fold reduction in caspase-3-like catalytic activity (Figure 3-2c). Analysis of caspase-3-like catalytic activity at different times after injury showed an increase over 48 hours followed by a gradual decline that remained elevated compared with sham transected controls (Figure 3-2b). The level of caspase-3-like activity was observed to decline in sham transected animals between E14-E16, but unexpectedly rose sharply on E17. Except for the E17 sham operates, the results of changes in caspase-3-like activity correlate with changes in the amount of apoptosis observed in the same region of the injured but untreated spinal cord and during development.

Detection of Active Caspase-3 following Transection of the Developing Spinal Cord

The results of the caspase inhibitor and catalytic activity studies suggested caspase-3-like caspases were important components of transection-induced apoptosis in the developing spinal white matter. The induction of caspase-3-like activation was verified by western blot analysis of extracts from injured and sham operated spinal cord and by immunohistochemistry with an antibody that preferentially detects the active form of caspase-3. In western blots, the antibody, R280, recognizes the 32 kD proform of caspase-3, but also detects the cleaved p17 subunit, which is indicative of caspase-3 activation. Western blot analysis using this antibody revealed the presence of both the 32 kD procaspase-3 and the 17kD cleavage fragment in all embryonic stages examined (E13-20; Figure 3-3). Although not quantified, the level of the 32 kD proform appeared similar at all stages of development examined. The 17 kD fragment appeared slightly elevated in E13 cervical spinal cords compared with the same region at E18, perhaps reflecting the onset of developmental glial apoptosis, as determined in Chapter 2.

Transection of the cervical spinal cord on E13 lead to an increase in the level of the 17 kD subunit of caspase-3 24 hours post injury compared to 48 and 72 hours after transection. Compared to sham transected controls, the level of the 17 kD fragment appeared elevated 24 hours after transection. However, at 48 and 72 hours after injury, the level of this fragment appeared to be reduced compared with sham operate cervical cords. The level of inactive procaspase-3 (32kD) remained appeared to be slightly reduced in transected cords compared to those sham transected. The unexpected

Figure 3-1. Caspase inhibitors attenuate the transection-induced apoptotic response in the cervical spinal cord caudal to the lesion 32 hours after injury. BAF and DEVD lead to a significantly greater reduction in TUNEL+ cells than did YVAD ($p<0.001$), indicating a greater role for caspase-3-like activity in this death. None of the inhibitors reduced the death to sham operated levels and BAF was unable to inhibit developmental apoptosis in this region of the spinal cord. (*$p<0.001$ compared to transected saline control, n=5/treatment).


80
70
60
50
40
30
20
10
0
Mean No. TUNEL-Positive Cells/Section
*
*
*
Sham Saline
Sham BAF
Tx Saline
Tx BAF
Tx DEVD
Tx YVAD
Treatment

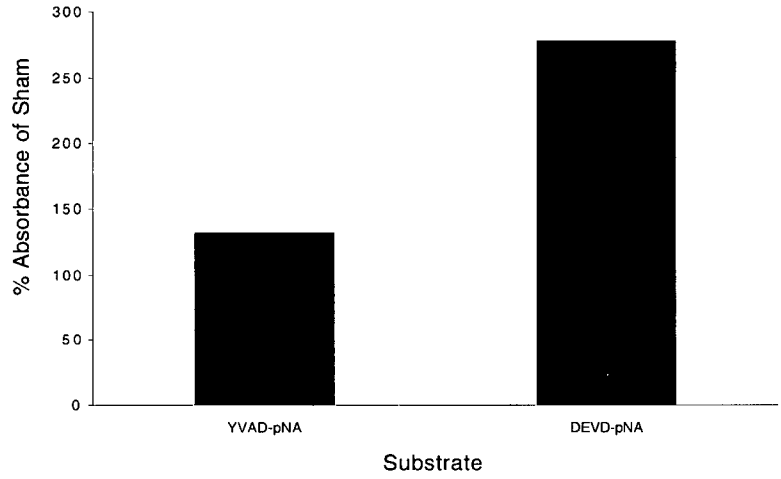
decline in the level of the caspase-3 protein appears to be consistent with changes in its gene expression. Although not shown here, reverse transcriptase PCR on similarly treated tissue indicates levels of caspase-3 mRNA and cyclophilin mRNA consistently decline 48 hours after transection, which may represent an overall decrease in cellular activity in this region of the cord.

Although the western blot data presented unexpected changes in caspase-3 levels, it, at the very least, demonstrated the presence of caspase-3 in the chick cervical spinal cord. The presence of the 17 kD cleavage fragment of caspase-3 suggested procaspase-3 processing, but did not necessarily indicate its activation. Therefore, immunofluorescent staining of sectioned cervical spinal cord in the region used in the western blot and caspase activity analyses was performed using the MF-397 antibody raised against the active caspase-3 tetramer (Xu *et al.*, 1999). A small number of MF-397-positive cells was detected in sham controls. These cells were also predominantly confined to the white matter (Figure 3-4a). Thirty hours after transection, the number of MF-397 immunoreactive cells in distal sections caudal to the injury increased dramatically. The MF-397 immunoreactivity was localized to the ventrolateral and ventromedial white (Figure 3-4b). Hoechst 33258 double labeling of MF-397 stained sections revealed that the nuclei of all MF-397 immunoreactive cells in sham and transected spinal cord sections were apoptotic (Figure 3-4e). The immunoreactivity was for the most part confined to the cytoplasm, and only occasionally were apoptotic nuclei observed in cells devoid of MF-397 immunoreactivity.

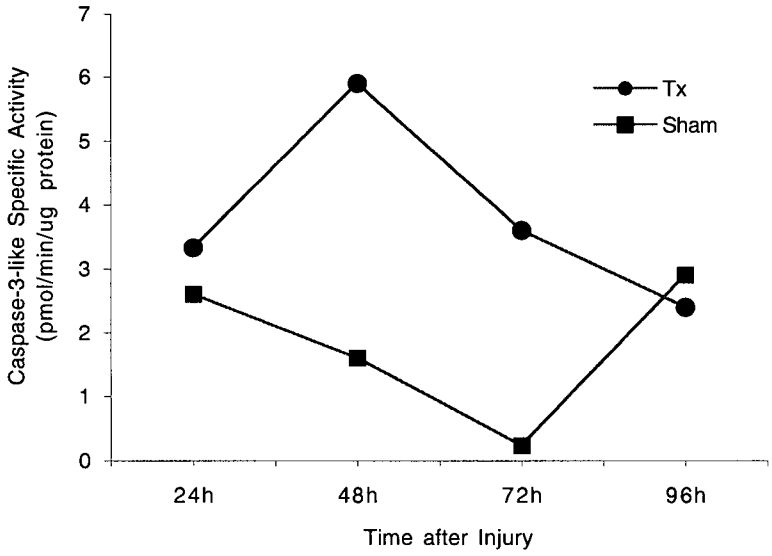
Thus, the pattern of activated caspase-3 immunoreactivity corresponds to the apoptotic response observed in the intact and transected developing cervical spinal cord. Based on the colocalization of apoptotic cells and oligodendrocyte specific markers observed in Chapter 2, it is likely that much of the activation of caspase-3 occurs in oligodendrocytes undergoing apoptosis. Unfortunately, double labeling of active caspase-3 and oligodendrocytes markers, such as TfBP could not be performed as both were rabbit polyclonal antibodies and monoclonal antibodies with similar specificity and cross reactivity were unavailable.

Figure 3-2. Caspase catalytic activity was analysed in the transected spinal cord 1-7 mm caudal to the lesion. (a) Compared with sham operation, transection induced a greater increase in DEVD-pNA cleavage than YVAD-pNA 32 hours after injury. Values are expressed as percent change in absorbance in transected versus sham operated spinal cord extracts. (b) Following transection, caspase-3-like (DEVD-pNA) activity increased over 48 hours then dropped to control levels. In sham operated animals the specific activity of caspase-3-like proteases declined between E14-E16, but increased to E14 levels on E17. (c) *In ovo* application of BAF after transection reduces caspase-3-like catalytic specific activity (DEVD-pNA) compared to transected saline-treated control, but not to the level of sham operated, untreated controls.

a


% Absorbance of Sham
300
250
200
150
100
50
0
YVAD-pNA
DEVD-pNA
Substrate

b


Caspase-3-like Specific Activity
(pmol/min/ug protein)
7
6
5
4
3
2
1
0
Tx
Sham
24h
48h
72h
96h
Time after Injury

c

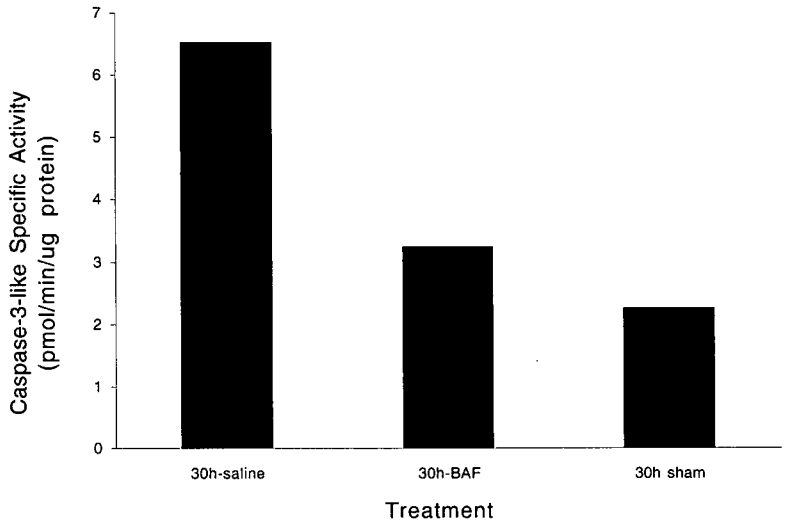

Caspase-3-like Specific Activity
(pmol/min/ug protein)
7
6
5
4
3
2
1
0
30h-saline
30h-BAF
30h sham
Treatment

Figure 3-3. Western blots using the R280 antibody raised against the caspase-3 proenzyme (32 kD) reveal the presence of procaspase-3 within the developing and injured chick cervical spinal cord. In addition, the 17 kD cleavage fragment of caspase-3, indicative of procaspase-3 processing by initiator caspases, is detected. (a) Levels of procaspase-3 are relatively constant through development. Expression of the 17 kD fragment is slightly higher at E13 compared with later embryonic stages. (b) Twenty-four hours after transection, the level of the p17 fragment of caspase-3 increases transiently, then declines. Unexpectedly, levels of this fragment are lower in the transected cord (Tx) compared with sham operated controls (Sh). Each lane represents the extracts of 3-5 cervical spinal cords harvested 1-7 mm caudal to the lesion.

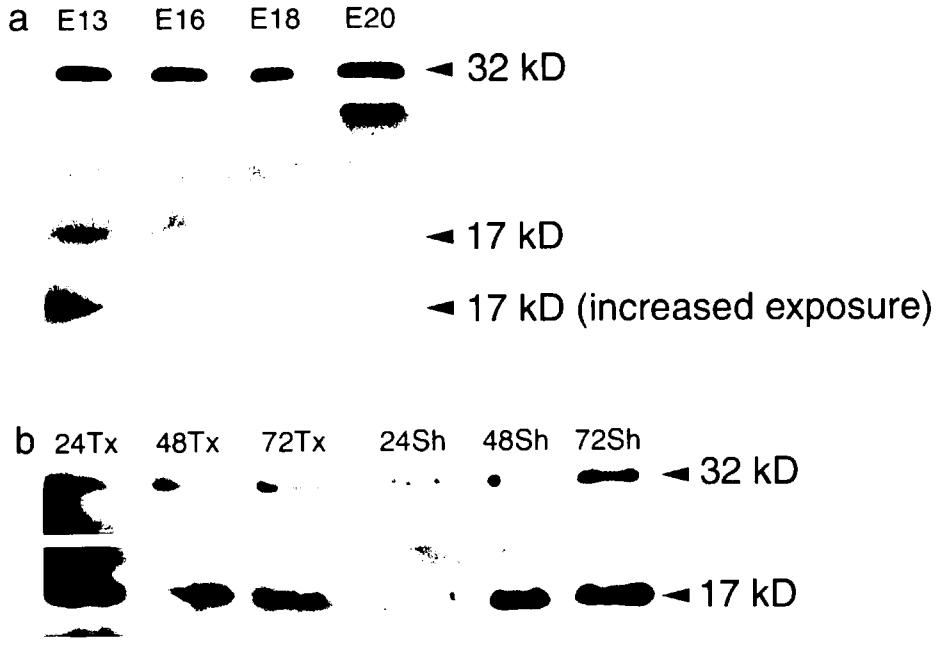
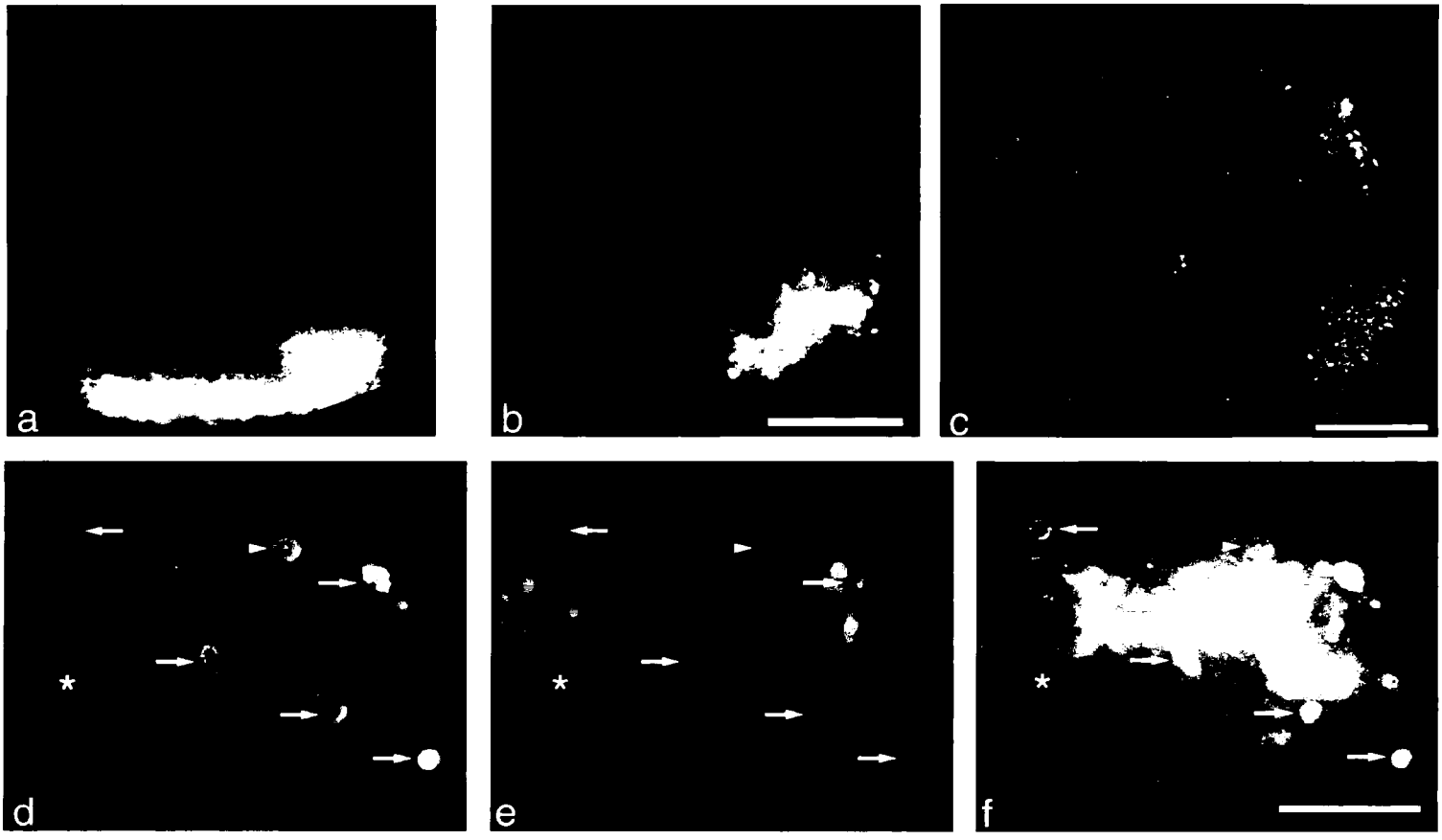

a
E13
E16
E18
E20
32 kD
17 kD
17 kD (increased exposure)
b
24Tx
48Tx
72Tx
24Sh
48Sh
72Sh
32 kD
17 kD

Figure 3-4. Immunoreactivity to active caspase-3 increases in the spinal white matter 1-7 mm caudal to the lesion following cervical transection on E13. (a) Active caspase-3 is observed in a small number of cells in the ventrolateral and ventromedial white matter of sham transected controls. (b) In contrast, transected spinal white matter displays a marked increase in the number of cells in this region expressing the active form of caspase-3 30 hours after injury. (c) Distribution of active caspase-3 immunoreactivity in the transected spinal white matter reveals a similar staining pattern as TUNEL as immunoreactive cells are located primarily in the ventrolateral and ventromedial white matter. (d-f) Higher magnification of the transected white matter reveals the predominantly cytoplasmic distribution of the active caspase-3 (d). Double fluorescence labeling with Hoechst 33258 (e) reveals the colocalization (arrows) of apoptotic nuclear morphology with active caspase-3 immunoreactivity in these cells (f). Occasionally, apoptotic cells were observed without active caspase-3 immunoreactivity (asterisk). The arrowhead indicates an active caspase-3 immunoreactive cell with a nucleus in the early stages of chromatin condensation and nuclear segregation. Scale bars = 20 μm (a, b), 250 μm (c) and 75 μm (d-f).


a
b
c
d
e
f

DISCUSSION

The finding that systemic administration of all three inhibitors of caspase catalytic activity used in this study caused a significant reduction in apoptosis indicates it was an effective delivery route in this model. This is supported by the ability of BAF to reduce caspase-3-like catalytic activity *in ovo* following transection. The efficacy of systemic treatment with caspase inhibitors may reflect the immature nature of the blood brain barrier, which develops on approximately E13 (Wakai and Hirokawa, 1978). However, the degree of cellular penetration by the different inhibitors may have differed, which could partially explain the differences in their ability to attenuate the apoptotic response. In this respect, the YVAD used in this study may have been less effective than DEVD because of its aldehyde group, which makes it a reversible inhibitor and less cell permeable than the fluoromethyl ketone group of DEVD and BAF. However, the reduced ability of YVAD to inhibit transection-induced apoptosis in the distal white matter was not due entirely to these properties as caspase activity assays revealed YVAD-specific catalytic activity was minor compared to DEVD-specific activity. BAF's small size and fmk group may also improve penetration of cells within the CNS. To date, BAF is the only caspase inhibitor shown to reduce trauma-induced apoptosis via systemic administration (Cheng *et al.*, 1998b).

The greater ability of BAF to reduce injury-induced apoptosis may also reflect its ability to inhibit several different caspases operating at different levels of the apoptotic cascade. Thus, BAF could inhibit caspase-3 and the Group III (initiator) caspases that activate them. Alternatively, BAF could inhibit caspase-3 in addition to other caspases that activate cell death through caspase-3 independent pathways. However, although BAF-mediated attenuation of transection-induced apoptosis was significantly higher than DEVD, the difference between these two inhibitors was not large, suggesting they may inhibit similar caspases, including, perhaps, caspase-3. The BAF-induced reduction of caspase-3-like activity *in ovo* following transection is consistent with the attenuation of the apoptotic response and the hypothesis of caspase-3 involvement in the trauma-induced oligodendrocyte apoptosis.

The results of the caspase activity assay also support the predominant role of caspase-3-like activity compared to caspase-1-like activity. Although inhibitors of caspase-1-like caspases have been shown to decrease neuronal ACD (Gagliardini *et al.*, 1994; Milligan *et al.*, 1995), caspase-1 is primarily involved in inflammatory reactions (Black *et al.*, 1989; Dinarello, 1998; Kostura *et al.*, 1989; Thornberry and Lazebnik, 1998; Wang *et al.*, 1998). In fact, caspase-1 and the recently identified caspase-11 (Wang *et al.*, 1998) appear to be the only known caspases involved in inflammation. Thus, it seems likely that inflammatory responses do not mediate the glial apoptotic response in regions remote to the lesion in the embryonic chick. Other caspases were not directly examined in this study; however, with the increasing availability of specific caspase inhibitors, substrates, and antibodies, the present model may be a useful tool for investigating the roles of other caspase family members in trauma-induced secondary degeneration in the spinal white matter.

Changes in caspase-3-like catalytic activity during development, as seen in sham transected controls, were consistent with the decline in developmental apoptosis in the cervical spinal cord. However, the sudden rise of this activity in E17 embryos was unexpected as the amount of apoptosis at this stage continues to decline. This may reflect an increase in nonapoptotic functions of caspases. For example, caspases have been suggested to play a role in synaptic plasticity (Chan and Mattson, 1999). The increased caspase activity could, therefore, represent a period of synaptic remodeling in the cervical spinal cord.

The presence of caspase-3 in cervical chick spinal cord was verified by western blot analysis. Both procaspase-3 and its 17 kD cleavage fragment were detected during development and in both sham and transected spinal cord extracts. The reduction in levels of the 17 kD fragment was unexpected and the explanation for it is unclear, particularly in light of the activity assay data that show increased catalytic activity of caspase-3-like activity. It should be noted that the presence of the 17 kD caspase-3 fragment indicates cleavage of the caspase-3 zymogen, but does not necessarily indicate caspase-3 activation. Endogenous caspase inhibitors such as XIAP and c-IAP-1 and –2 can act to prevent caspase-3 activity (Deveraux *et al.*, 1997b; Roy *et al.*, 1997). In addition, changes in the level of caspase-3 protein may not, on its own, be the sole determinant of changes

in its level of activity. The ratio of pro- and antiapoptotic proteins is critical to the progression of the apoptotic pathway (Adams and Cory, 1998; Farrow and Brown, 1996; Kluck *et al.*, 1997a; Kroemer, 1997; Reed, 1997b). Thus, antiapoptotic regulators of caspase activation may be expressed at higher levels in shams, and undergo a relatively greater decline than caspase-3 following transection, resulting in increased caspase-3 catalytic activity. It will, therefore, be of interest to examine changes in the levels of antiapoptotic proteins such as Bcl-2, Bcl-X, and the IAPs. It is also possible that caspase-7, which has a similar substrate specificity to caspase-3 (Thornberry *et al.*, 1997), could account for the changes in caspase-3-like activity. However, caspase-7 has not been detected in the normal mammalian CNS (Juan *et al.*, 1997). Nevertheless, caspase-7 expression has not been examined in conditions of CNS trauma, and, therefore, cannot be ruled out. Unfortunately, caspase-7 antibodies that cross-react with chick caspase-7 were not available at the time the present study was conducted.

Neonatal rat cortical oligodendrocytes have been shown to express caspases-1, -2, -3, and -8 (Gu *et al.*, 1999). However, in response to NGF treatment, only caspase-1, -2, and -3 become activated, a response mediated by the p75 neurotrophin receptor (Gu *et al.*, 1999). In this *in vitro* model of oligodendrocyte apoptosis, NGF preferentially activates caspase-1 and to a lesser extent caspase-3. In the present study, caspase-1-like activity increased only modestly in comparison with caspase-3-like activity. The minor contribution of caspase-1 activity in transected embryonic chick spinal cord in regions remote to the lesion indicates this caspase and possibly inflammation may not be critical mediators of this glial apoptotic response to injury. This does not however, rule out the possibility of immune-related cytokines initiating glial apoptosis through related death receptors such as the p55 TNF receptor or Fas (Ashkenazi and Dixit, 1998; Cohen, 1997; Muzio *et al.*, 1996; Nagata and Golstein, 1995; Salvesen and Dixit, 1997). For example, proinflammatory conditions inducing Fas-mediated apoptosis of rheumatoid arthritis synovial cells involves activation of caspase-3 but not caspase-1 (Sekine *et al.*, 1996). Fas and TNF-α initiate caspase activation and the apoptotic pathway through caspase-8 (Ashkenazi and Dixit, 1998; Bertin *et al.*, 1997; Cohen, 1997; Muzio *et al.*, 1996; Muzio *et al.*, 1998; Salvesen and Dixit, 1997; Yang *et al.*, 1998b). Caspase-8 is an initiator caspase that leads directly to caspase-3 activation (Stennicke *et al.*, 1998), and can

become activated in neonatal rat oligodendrocytes in response to staurosporine, but not radiation or p75 stimulation (Gu *et al.*, 1999). It should be noted that p75 mediated apoptosis has only been demonstrated in cultured neonatal oligodendrocytes (Casaccia-Bonnefil *et al.*, 1996b; Gu *et al.*, 1999) but not in cultured mature oligodendrocytes (Ladiwala *et al.*, 1998). The relevance of p75 apoptotic signalling after SCI is unknown; however, p75 expression in oligodendrocytes has been reported in MS lesions. As microglia produce NGF, they could be a source of this putative death ligand for p75-mediated apoptosis (Elkabes *et al.*, 1996; Frade and Barde, 1998; Heese *et al.*, 1998).

Although the p75 neurotrophin receptor stimulation doesn't result in caspase-8 expression in oligodendrocytes (Gu *et al.*, 1999), other members of the TNF receptor superfamily such as the p55 TNF receptor and Fas may be capable of activating this protease. Both TNFα and Fas can induce oligodendrocyte apoptosis (Akassoglou *et al.*, 1998; D'Souza *et al.*, 1995; D'Souza *et al.*, 1996b; Hisahara *et al.*, 1997). Microglia constituitively express Fas ligand (Spanaus *et al.*, 1998) and oligoendrocytes express Fas receptor (D'Souza *et al.*, 1996b). Therefore it is plausible that microglia trigger oligodendrocyte apoptosis through Fas activation of caspases. Similarly, microglia and other invading immune cells produce TNF-α (Stollg and Jander, 1999), and TNFα increases in the spinal cord following traumatic injury (Klusman and Schwab, 1997; Wang *et al.*, 1996). Thus, these pro-inflammatory cytokines have the potential to mediate oligodendrocyte apoptosis in the distal white matter after injury. However, the extent to which these responses occur in regions remote from the injury is unknown.

The caspase activity assays and positive MF-397 immunostaining indicated the presence of caspase-3 activity in both the developing and transected cervical spinal cord. Why then did caspase inhibitors fail to reduce developmental cell death in this study? It may be that certain cells in this region die via caspase independent events or that caspase inhibition does not prevent the ultimate death of the cell. The DNA fragmentation could have occurred through AIF (apoptosis inducing factor) activity, which can signal mitochondrial driven apoptosis in a caspase independent manner, (Lorenzo *et al.*, 1999). Apparent caspase-independent forms of cell death have been reported in the CNS. For example, not all forms of developmental and injury-induced ACD in the chick are

prevented by caspase inhibitors (Milligan *et al.*, 1995), although pan-specific caspases were not tested.

In this study, I do not examine the long-term efficacy of caspase inhibition. The dynamic nature of the developing system, the potential for the constant production of newly differentiating oligodendrocyte precursors following injury, and the fact that few transected embryos survive the hatching process combine to make long term assessment of survival problematic. However, the ability to promote long term survival is an important issue to consider: it may be that caspase inhibition merely delays rather than prevents cell death. Several examples exist for this phenomenon in neuronal populations. The role of caspases may then be to mediate the rapid and efficient cellular degeneration and removal of unwanted or compromised cells (Borner and Monney, 1999; Thornberry and Lazebnik, 1998). In the absence of caspase activity, however, other death-promoting proteolytic activity, may be unmasked. For example, calpains may be an important component of an alternate death pathway (Patel *et al.*, 1996). Calpain increases within the spinal cord after injury and may participate in the loss of myelin (Banik *et al.*, 1997a; Banik *et al.*, 1997b; Banik *et al.*, 1998; Ray *et al.*, 1999; Schumacher *et al.*, 1999; Springer *et al.*, 1997). As mentioned, another mediator of caspase-independent death may be the phylogenetically ancient mitochondrial apoptosis inducing factor (AIF; (Lorenzo *et al.*, 1999). Models such as the one developed in this thesis will be useful for examining the role of these factors in oligodendrocyte apoptosis *in vivo*.

Conclusions

This initial study on the role of caspases in the distal trauma-induced apoptotic response indicates that caspases do indeed play an important function in executing this response. The prevalence of caspase-3 compared to caspase-1 is consistent with its role as the central effector of the apoptotic degradation pathway in the CNS. This study also demonstrates that the embryonic chick is a good model in which to study the mechanisms of cell death and conversely, cell survival. The staining pattern of active caspsae-3 following transection was very similar to that of TUNEL. Results of the previous study demonstrate the presence of apoptotic oligodendrocytes in these regions. However, until more specific and complimentary reagents are available, the specific role of caspase-3 in trauma-induced oligodendrocyte apoptosis in this model cannot be tested directly.

However, the spatial and temporal correlation of caspase-3 activation in regions containing apoptotic oligodendrocytes strongly suggest that caspase-3 is indeed involved in mediating this response in oligodendrocytes, as has been shown recently in cases of human spinal cord injury (Emery *et al.*, 1998).

CHAPTER 4 GENERAL DISCUSSION

Introduction

Results of the studies outlined in this thesis demonstrate (1) a period of increased cell death in the cervical spinal white matter that is coincident with the developmental period for spinal cord myelination; (2) during this stage of development white matter cells exhibit an increased susceptibility to SCI-induced apoptosis, even in regions remote from the site of injury; (3) oligodendrocyte apoptosis is an identified cellular constituent of the observed apoptotic response; (4) caspases, particularly caspase-3, play a role in executing the transection-induced apoptosis within the spinal white matter caudal to the lesion; (5) inhibition of caspase-3-like activity attenuates this injury-induced apoptotic response; and (6) the embryonic chick model of SCI mimics adult mammalian models, and because of the high level of injury-induced cell death during the sensitive developmental period, may prove useful in furthering our understanding of the molecular and biochemical mechanisms of glial death and survival.

Comparison with Models of Glial ACD during Development

The results of my initial studies on the occurrence of developmental and transection-induced apoptosis in the developing chick spinal cord are consistent with mammalian models of glial and Schwann cell death during development (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996; Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996). The apparent axon dependence of glial survival in these mammalian models argues for an analogous means of oligodendrocyte survival and death in the chick spinal cord. However, in these mammalian models, oligodendrocytes and Schwann cells undergo apoptosis at the onset of myelination and appear to be at a premyelinating stage of development. The oligodendrocyte apoptosis in the developing chick spinal white matter, on the other hand, occurs well into the period of myelination. Thus, it seems likely that at least some of these dying cells have begun the myelinating process. More studies are needed to determine the kinetics of oligodendrocyte myelination and death in this model.

It also seems likely that, similar to glial cell death in developing mammalian models, axonal signals mediated oligodendrocyte survival during development. To further substantiate this hypothesis it will be important to demonstrate an association between

dying oligodendrocytes and degenerating axons. The present model may be used to examine the survival promoting potential of putative oligodendrocyte trophic factors. Factors such as PDGF, IGF-1, NT-3, and CNTF have been implicated in oligodendrocyte survival signalling (reviewed in Barres and Raff, 1994; Miller, 1996; Richardson *et al.*, 1997), as have the neuregulins (Vartanian *et al.*, 1997). It will be interesting to see if application of these factors, either individually or in combination, will inhibit the apoptotic pathway in oligodendrocytes after neurotrauma. Conversely, if they are required for axon-dependent survival, inhibiting their signalling (e.g., with function blocking antibodies) in oligodendrocytes would be expected to exacerbate their apoptotic response to similar injury.

Why are White Matter Glia More Susceptible to Transection-Induced Apoptosis at Certain Times During Development?

It has been proposed that all nucleated mammalian cells constituitively express cell death proteins, and that in the absence of survival factors, ACD is the default pathway (Ishizaki *et al.*, 1995; Raff *et al.*, 1993). During particular stages of nervous system development, neurons and glia express elevated levels of pro-apoptotic proteins such as Bax and caspase-3 (Madison and Pfeiffer, 1996; Vanderluit *et al.*, 1999; Vanderluit *et al.*, 1997). This elevation typically coincides with the period of developmental ACD, and may serve to facilitate the removal of superfluous cells that were unsuccessful in competing for limited supplies of trophic support. In this sense, cells are primed to die. As illustrated by the gross morphological abnormalities in the CNS due to decreased cell death and the limited lifespan of caspase-3 gene deletion mice, whose viability is greatly compromised, cell death in the developing nervous system must occur for appropriate formation and proper function of the CNS (Kuida *et al.*, 1996). The effect of caspase-3 gene deletion on glial ACD has not been examined, but would be expected to increase the number of surviving oligodendrocytes.

As the nervous system matures, cells typically down-regulate the expression of death-related proteins. This down-regulation coincides with apparent changes in trophic dependencies. For example, developing but not adult motor neurons are highly dependent on target derived trophic support, and axotomy of the facial or sciatic nerve in the neonate results in almost total loss of motor neurons; whereas, a similar injury to adult

nerves results in only modest motor neuron cell death (Oppenheim, 1986; Snider *et al.*, 1992). An analogous response is seen in Schwann cells. Schwann cells are dependent on axonal survival signals during development, and undergo apoptosis in response to axonal degeneration during development but not in the mature animal (Ciutat *et al.*, 1996; Grinspan *et al.*, 1996a; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996).

A similar scenario may exist for oligodendrocytes. Alterations of axon number in developing rat optic nerve greatly affect the number of surviving oligodendrocytes, presumably through altered levels of trophic support (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996). The results presented in this study are consistent with this concept. White matter glia, including oligodendrocytes, were much more susceptible to injury-induced apoptosis during the developmental period of oligodendrocyte maturation, myelination, and maximal developmental apoptosis. Transection of the hatchling spinal cord resulted in a diminished apoptotic response within the white matter, which, in sham operated controls, displayed only a small amount of naturally occurring apoptosis. These findings are corroborated by studies of axotomy-induced oligodendrocyte death in the developing versus adult optic nerve. As mentioned, transection of the neonatal optic nerve leads to a large apoptotic response in oligodendrocytes (Barres *et al.*, 1993a; Barres and Raff, 1994; Burne *et al.*, 1996). However, in the mature optic nerve, transection leads only to minimal cell loss (Butt *et al.*, 1997; Ludwin, 1990; Ludwin, 1992). This may indicate a switch in trophic dependency from an axon-derived factor to something else. In addition, decreased levels of pro-apoptotic proteins may play a role in the decreased trophic dependencies of mature cells.

The identity and source of trophic factors in mature neurons and glia is unknown. Increasingly, evidence is pointing to the development of autocrine trophic signaling in neurons and glia. Autocrine survival loops have been observed in neurons (Cheng and Patterson, 1997; Kohn *et al.*, 1999), and may reflect a transition from target dependence. Similarly, Schwann cells and oligodendrocytes may switch their trophic dependence from axon-derived signals to autocrine and/or paracrine signals (Meier *et al.*, 1999; Syroid *et al.*, 1999). A putative autocrine circuit for Schwann cell survival consists of IGF-1, NT-3, and PDGF-BB (Meier *et al.*, 1999; Syroid *et al.*, 1999). Although IGF-1, NT-3, and PDGF-AA regulate oligodendrocyte survival (Barres *et al.*, 1992a; Barres *et al.*, 1993a;

Barres *et al.*, 1993b) it is unclear as to whether they function as autocrine factors. It may be these factors work in a paracrine manner, released from astrocytes and neurons, and that the difference between the modes of survival in these two myelinating cell populations may reflect differing evolutionary pressures (Meier *et al.*, 1999). The greater risk but less serious consequence of PNS trauma may have dictated the evolution of self-dependency, a strategy more likely to promote survival than relying on support from other cells. The low probability of an organism surviving CNS trauma may not have necessitated a similar evolutionary development for oligodendrocytes.

Nonetheless, large numbers of oligodendrocytes survived complete transection of the cervical embryonic chick spinal cord. This indicates axons are likely not the sole source of oligodendrocyte trophic support. Neuregulins have been suggested to be a component of oligodendrocyte autocrine signalling at different stages of their development (Raabe *et al.*, 1997; Vartanian *et al.*, 1999). Whether autocrine or paracrine survival pathways regulate their survival remains to be elucidated.

Alternative Death Stimuli

Loss of axon-derived trophic support may not be the only stimulus for the induction of the apoptotic pathway in oligodendrocytes following trauma. As discussed in Chapter 2, microglia and/or macrophages recruited to the region of degenerating axons may play an active role in inducing oligodendrocyte apoptosis (see Figure 2-9). Such a mechanism has been proposed by Shuman et al (1997), who observed microglia contacting dying oligodendrocytes in regions of axonal degeneration. Activated microglia and macrophages could induce oligodendrocyte apoptosis through release of death factors such as Fas ligand and TNFα, which could activate death receptors present on oligodendrocytes (Akassoglou et al., 1998; D'Souza et al., 1995; D'Souza et al., 1996; Hisahara et al., 1997; Spanaus et al., 1998; Stollg and Jander, 1999). These two mechanisms need not be mutually exclusive, however. It is conceivable that oligodendrocyte apoptosis resulting from loss of axonal trophic support could also recruit microglia and/or macrophages, which could subsequently directly induce the apoptotic death of neighbouring oligodendrocytes. However, it will be a difficult task to determine the temporal and causal events in the induction of oligodendrocyte apoptosis following injury. Application of putative trophic molecules such as IGF-1, NT-3, PDGF-AA, and

GGF (Barres et al., 1992; Barres et al., 1993; Barres et al., 1993; Vartanian et al., 1997) could be used to determine the importance of survival factors in regulating oligodendrocyte death after spinal cord trauma. Rescue of oligodendrocytes with such factors would indicate that the loss of trophic support after injury is an important death stimulus for the cells.

Axonal degeneration following traumatic CNS injury may underlie another potential oligodendrocyte death stimulus. The degenerating axons could release cytotoxic factors, such as proteases, free radicals, and excitotoxins that could induce the apoptotic death of neighbouring oligodendrocytes. Cultured oligodendrocytes have been shown to be particularly vulnerable to many of these stimuli (e.g., Oka et al., 1993; ; Casaccia-Bonnefil et al., 1996; Back et al., 1998). However, the asynchronous nature of oligodendrocyte apoptosis in the spinal white matter remote from the lesion may argue against this mechanism of apoptosis induction. More concentrated or clustered TUNEL labeling might be expected if degenerating axons were killing oligodendrocytes through the passive release of cytotoxins, as appears to be the case near the lesion site. Nevertheless, this possibility cannot be ruled out. Future studies addressing the nature of the transection-induced death stimuli will, therefore, need to examine the levels of cytotoxic factors in the spinal white matter.

The role of ischemia may also need to be investigated as the transection procedure invariably interrupts blood flow. Ischemia has been reported to induce apoptotic death in mammalian spinal cord (Kato *et al.*, 1997; Mackey *et al.*, 1997). However, although ischemia likely plays a role at the site of injury, it is unclear how much of a role it has in tissue remote from the lesion. It will be very difficult to isolate the effects of ischemia in the developing chick embryo. The small size of the embryonic spinal cord combined with its somewhat gelatinous form may prove problematic for the surgical manipulations required to interrupt blood flow without damaging nerve fibres.

Caspases and Glial Apoptosis

Although the effect of inhibiting all the caspases was investigated, I only examined the specific contribution of caspase-1-like and caspase-3-like caspases in the transection-induced apoptotic response within the developing chick spinal cord caudal to the injury. Certainly, other caspases may subserve important regulatory and effector functions in this

model. Cultured immature oligodendrocytes express multiple caspases including caspase-1, -2, -3, and -8 (Gu *et al.*, 1999). It is likely that caspase-9 is also expressed. It is, however, unlikely that caspase-7 is involved in the present model as it is not expressed at appreciable levels in the mammalian CNS (Juan *et al.*, 1997). Understanding which caspases are involved the apoptotic pathway may provide important clues as to the nature of the stimulus inducing it. For example, caspase-9 is released from the mitochondria during apoptotic events initiated by mild cytotoxic stimuli or withdrawal of trophic support (Cohen, 1997; Green and Reed, 1998; Thornberry and Lazebnik, 1998). In combination with other mitochondrial released, proapoptotic factors such as Apaf-1 and cytochrome c, caspase-9 becomes active, and in turn, activates caspase-3 (Slee *et al.*, 1999). Caspase-8, on the other hand, is activated in response to death receptor stimulation (Cohen, 1997; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Once activated, it too activates caspase-3 (Stennicke *et al.*, 1998). Thus, differential caspase activation may provide insight into whether the oligodendrocyte death in the present model is stimulated by neglect (caspase-9) or by murder (caspase-8). A caveat to this proposition is the potential cross talk between death receptor and mitochondria-driven caspase signalling cascades. Both caspase-8 and -3 can cleave Bcl-2 family members, resulting in increased proapoptotic activity related to mitochondrial activation of caspases (Cheng *et al.*, 1997; Clem *et al.*, 1998; Grandgirard *et al.*, 1998; Li *et al.*, 1998a). This likely serves as an amplification step to expedite the apoptotic process, but may blur the distinction of the pathway initially activated in response to the original apoptotic stimulus.

Nevertheless, it will be import to understand the precise nature of the caspase signalling cascade in order to target upstream regulators that may be used to alter cellular fate in traumatic or degenerative conditions characterized by unscheduled apoptosis. Potential targets for the upstream regulation of apoptosis include Bcl-2 family members and Akt, both of which regulate the apoptotic pathway prior to mitochondrial involvement (Adams and Cory, 1998; Brunet *et al.*, 1999; Cardone *et al.*, 1998; Crowder and Freeman, 1998; Kennedy *et al.*, 1999; Reed, 1997b). Arresting the apoptotic pathway prior to caspase activation may be a desirable goal as the ultimate death of the cell may be secured once caspases become activated.

It is unclear as to whether caspase inhibition can provide long term survival and "health" of the cell. Many examples illustrate how caspase inhibition can transiently rescue cells, but ultimately fails to prevent their death (e.g., McCarthy et al., 1997; Vanderluit et al., 1999). Thus, the delay in death effected by caspase inhibitors may reflect the importance of caspases in the efficient cell removal. However, caspase inhibition can, in some cases, reverse mitochondrial changes thought to be important in the apoptotic pathway (Martinou *et al.*, 1999). In addition, evidence from models of stroke/ischemia and traumatic brain injury, argue for the therapeutic potential of caspase inhibition in the rescue of damaged cells and improvement of neurological outcome (Chaudhary *et al.*, 1999; Chen *et al.*, 1998; Endres *et al.*, 1998; Kermer *et al.*, 1998; Ma *et al.*, 1998; Schulz *et al.*, 1998; Schulz *et al.*, 1999; Yakovlev *et al.*, 1997). Another class of cellular apoptosis inhibitors, IAPs, have also been shown to inhibit caspase activation and rescue cells from apoptosis (Clem and Duckett, 1997; Crocker *et al.*, 1997; Deveraux and Reed, 1999; Deveraux *et al.*, 1997a; Liston *et al.*, 1996; Roy *et al.*, 1997). These too may be targets for regulating unscheduled apoptosis.

Relevance of Oligodendrocyte Apoptosis

Knowledge concerning the mechanisms of oligodendrocyte apoptosis will be important for determining strategies aimed at arresting this process and promoting cell survival. Promoting oligodendrocyte survival may be an important step in arresting the demyelinating pathology of degenerative conditions such as those found in degenerative disorders and after traumatic injury. The significance of oligodendrocyte apoptosis in the white matter remote from the injury is unclear, but may be a component of the demyelination process that occurs after SCI (Blight, 1992; Blight, 1993; Blight and Decrescito, 1986; Blight *et al.*, 1991; Bunge *et al.*, 1993; Shi *et al.*, 1997; Waxman, 1992). Similarly, the apoptotic oligodendrocyte death in some forms and models of MS indicate a role in the progressive demyelination characterized by the disease (Dowling *et al.*, 1997; Oshako and Elkon, 1999; Tsunoda *et al.*, 1997). Other pathological conditions also involve oligodendrocyte apoptosis, such as multiple system atrophy and (Probst-Cousin *et al.*, 1998), and cognitive and spastic motor deficits in premature infants (Noble and Mayerproschel, 1996). Thus, prevention of oligodendrocyte apoptosis is an important goal in arresting the progression of these conditions.

Understanding the mechanisms governing the death and survival of differentiated oligodendrocytes may also be of relevance to another aspect of adult human pathology. Recent findings suggest that following demyelination, oligodendrocyte precursors differentiate and remyelinate the affected region (Keirstead *et al.*, 1998b). However, the supply of these precursors is limited. Therefore, it may be important to promote the survival of these cells in conditions such as MS, in which multiple demyelination events can occur and in some cases there is a progressive loss in the ability to remyelinate. Developmental models of oligodendrocyte death, such as the embryonic chick spinal cord, may be useful in understanding the death and survival pathways, in order to achieve maximum benefit from a limited supply of these cells.

Considerations Regarding Inhibition of Oligodendrocyte Apoptosis

Will treatments aimed at inhibiting trauma-induced oligodendrocyte death promote their long-term survival? Clearly the answer to this question remains equivocal. As discussed above, treatments that inhibit caspase activity may only delay the death of the cell. Thus, approaches that employ such a strategy must consider complimentary and combinatorial approaches that take into account the myriad of cellular responses to neurotrauma. For example, the combination of caspase inhibition and inhibition of glutamate toxicity acts synergistically to prevent cell loss and tissue damage, and to improve neurological function after ischemia (Ma *et al.*, 1998; Schulz *et al.*, 1998). Caspase inhibition may temporarily arrest the death process, thereby extending the therapeutic window for agents designed to promote cell survival. For example, caspase inhibition can block the death of trophic deprived sympathetic neurons, and subsequent application of NGF can then reverse apoptotic mitochondrial changes, thereby promoting the health of rescued cells (Martinou *et al.*, 1999).

Whether or not it is desirable to promote oligodendrocyte apoptosis after SCI is also an important question to consider. In cases of myelin damage, mature oligodendrocytes are only capable of poor and incomplete myelination (Keirstead and Blakemore, 1997). It may, therefore, be advantageous to remove injured oligodendrocytes to allow oligodendrocyte progenitors to differentiate and remyelinate the demyelinated region (Keirstead *et al.*, 1998b). Nevertheless, the chronic demyelination observed after SCI (Blight, 1992; Blight, 1993; Blight and Decrescito, 1986; Blight *et al.*, 1991; Bunge *et*

al., 1993; Shi *et al.*, 1997; Waxman, 1992) suggests this may not always be a tenable approach and that initially preventing oligodendrocyte loss may also be desirable. Any sparing of functional tissue after SCI is of vital importance for as little as 10% sparing of axons can dramatically improve neurological function (Eidelberg *et al.*, 1997; Fehlings and Tator, 1995).

It is important to note that the current generation of caspase inhibitors will have little therapeutic benefit in humans due to their peptide nature. However the knowledge gained from their inhibitory properties and capabilities may facilitate the development of small, molecule, nonpeptide-based drugs. In the case of acute neurotrauma, it will be desirable to develop oral treatments that quickly penetrate the CNS. However long term caspase inhibition will need to target specific cells as deregulated apoptosis could lead to tumor development.

Future Directions

This thesis addresses the occurrence and cellular identity of trauma-induced apoptosis in the cervical spinal cord remote from the injury, and the role of specific caspases in this process. It does not, however, directly address the nature of the stimulus inducing the apoptotic response. The embryonic chick model of spinal cord trauma developed in this thesis could prove useful in understanding the mechanisms responsible for initiating transection-induced oligodendrocyte apoptosis. As discussed above, it will be important to establish the relationship between degenerating axons and dying oligodendrocytes. Electron microscopy will be a valuable tool for analyzing this relationship. The efficacy of putative trophic factor application for reducing oligodendrocyte apoptosis after injury will also be necessary to demonstrate the importance of the loss of trophic support in this process. The present model may be advantageous for such a study as the trophic factors could likely be administered directly onto the highly vascularized chorioallantoic membrane, thereby reducing further perturbations to the spinal cord.

The development of antibodies that detect chick microglia/macrophages will allow investigation of the relationship between these cells and the dying oligodendrocytes. In addition, analysis of changes in expression of death ligands such as Fas and TNFα, and of the death receptors could indicate the role of these inducers of oligodendrocyte

apoptosis in the transected chick spinal white matter. The large amount of death induced in the present model may allow for quantitative biochemical analysis of such changes, and the changes in the death response by function blocking antibodies to these factors could be assessed. For example, the role of p75 could also be examined. As p75 has been shown to be involved in some models of oligodendrocyte death (Casaccia-Bonnefil *et al.*, 1996b; Gu *et al.*, 1999), it will be of interest to examine changes in the expression of this death/neurotrophin receptor on oligodendrocytes. Microglial NGF could be a source for p75 death signalling in oligodendrocytes (Elkabes *et al.*, 1996; Frade and Barde, 1998; Heese *et al.*, 1998).

As mentioned, the amount of glial apoptosis in the spinal white matter remote from the lesion is sufficient to allow for biochemical analysis and changes in protein expression. Thus, the embryonic chick model of traumatic spinal cord injury developed here could be useful for examining the roles of other apoptosis-related molecules. The changes in expression and activity of other caspases could be determined. In particular, initiator caspases such as caspase-8 and –9 could be examined. As discussed in Chapter 3, the relative changes of these two proteases could provide insight into the nature of the cell death stimulus following transection.

The convergence upon caspases of apoptotic signalling pathways induced by diverse and often poorly characterized death stimuli makes these central executioners of apoptosis good initial targets for regulating cellular survival. However, learning more about the specific stimuli and the pathways they initiate will provide insight into specific caspase involvement, which may allow manipulation of individual caspases. In addition, it will improve understanding of regulatory molecules upstream of caspase activation, such as Bax or Akt that could serve as future targets for altering cell fate. Arresting the cell death pathway prior to caspase activation and changes in mitochondrial membrane potential may be advantageous as cells may be maintained in a healthier state that is more responsive to survival promoting factors.

Concluding Remarks

The findings presented in this thesis demonstrate a model of injury-induced glial apoptosis in the embryonic chick CNS that is analogous to developmental and adult mammalian models of injury induced oligodendrocyte apoptosis. The accessibility of this

model to experimental manipulations and the relative ease of animal care make it an attractive alternative to mammalian models. Importantly, the large number of cells induced to undergo apoptosis following injury facilitate quantitative and biochemical analysis of this process. The findings of caspase involvement in trauma-induced apoptosis of oligodendrocytes in the developing chick spinal cord provide a small step forward in the understanding of the mechanisms regulating the death and survival of these cells. However, the model developed can serve as an important tool in furthering our knowledge of the signals and pathways that determine their fate. It will also be a convenient model in which to study the efficacy of new compounds designed to alter cellular fate. It is acknowledged that the availability of reagents and transgenic animals is limited for chick. It will, therefore be interesting to see if this model will be mirrored in developing rodents. The use of transgenic mice expressing lac z from the MBP promoter is of particular interest as the often difficult task of identifying oligodendrocytes would be greatly eased. Crossing this mouse with transgenic mice with altered caspase, bcl-2, or iap expression could yield potentially exciting results about their regulation of the apoptotic pathway in oligodendrocytes. However, many fundamental questions concerning the factors regulating glial cell death remain to be answered in the chick model. What is the precise role of axons in the development of spinal cord oligodendrocytes? What triggers the apoptotic pathway in newly formed oligodendrocytes and why are they more resistant to this death as they mature? Is the apoptotic response death by murder or neglect? The model developed in this thesis may provide the answers to these and other unanswered questions, and their lessons could be important to the understanding of pathology of human neurotrauma and ways to manage it. The embryonic chick has provided much insight into the events governing nervous system development and the pathogenesis of neurodegenerative conditions and will continue to do so in the future.

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