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Astrocytes Attenuate Oligodendrocyte Death *In Vitro*: Involvement of Growth Factors and Intra-cellular Kinases

by

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Abstract

In a number of central nervous system (CNS) pathologies, such as Multiple Sclerosis (MS) and CNS trauma, oligodendrocytes undergo cell death. Using mouse cell cultures in which oligodendrocytes undergo spontaneous death, we examined whether astrocytes could promote oligodendrocyte survival. Co-culture with astrocytes significantly reduced oligodendrocyte death; astrocyte protection was cell-type specific. Astrocyte protection required oligodendrocyte-astrocyte contact. Function blocking antibodies to ciliary neurotrophic factor (CNTF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF) and neurotrophin-3 (NT-3) (5μg/ml each, 20μg/ml combined) did not affect astrocyte protection of oligodendrocytes. Inhibitors of src kinases (5μM PP2) and phosphatidylinositol 3-kinase (PI3-K) (50nM Wortmannin, 50μM LY294002), components of integrin signaling, negated astrocyte protection of oligodendrocytes. We conclude that astrocyte protection of oligodendrocytes does not involve growth factors but may involve the integrin family of cell adhesion molecules.

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ACM = astrocyte-conditioned-medium
AMPA = α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid
bFGF = basic FGF
Bisindolylmaleimide =
(2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide)
C = Celsius
CAD = caspase-activated DNase
CAM = cell adhesion molecule
CG4 = central glia 4
CNP = 2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS = central nervous system
CNTF = ciliary neurotrophic factor
CRADD = caspase and RIP adapter with death domain
d = day(s)
DNA = deoxyribonucleic acid
DNA-PK = deoxyribonucleic acid dependent-protein kinase
dNTP-FITC = deoxynucleotidyl triphosphate-fluorescein isothiocyanate
DRG = dorsal root ganglia
e = embryonic
EAE = experimental autoimmune encephalomyelitis
ECM = extra-cellular matrix
EDTA = ethylene diamine tetra acetic acid
EGF = epidermal growth factors
ER = endoplasmic reticulum
FADD = Fas associated death domain
FAK = focal adhesion kinase
fasL = Fas ligand.
FCS = fetal calf serum
FGF = fibroblast growth factors
FT3 = Farnesyltransferase inhibitor III
GalC = galactocerebroside
GDNF = glial derived neurotrophic factor
GFAP = glial fibrillary acidic protein
GluR = glutamate receptor
h = hour(s)
H_2O_2 = hydrogen peroxide
HBSS = Hanks Balanced Salt Solution
hfA = human fetal astrocytes
HGF = hepatocyte growth factor
Iκ-B = inhibitor of κ-B
ICAM = inter-cellular adhesion molecule
IFN-γ = interferon-γ
IGF = insulin-like growth factor
IKK = inhibitor of κ-B kinase

IL = interleukin
inhibitor ICAD = inhibitor of CAD
JAK/STAT = janus kinase-signal transducer and activator of transcription
JNK = jun kinase
JNKK = jun kinase kinase
MAG = myelin associated glycoprotein
MAPK/ERK = mitogen activated protein kinase/extra-cellular regulated kinase
MBP = myelin basic protein
meF = mouse embryonic fibroblasts
MEKK = mitogen activated protein kinase/extra-cellular regulated kinase kinase kinase
MEM = minimum essential medium
mM = millimolar
μM = micromolar
μm = micrometer
μg/ml = microgram/milliliter
min = minutes
mm^2 = $millimeter^2$
MOG = myelin/oligodendrocyte glycoprotein
MS = Multiple Sclerosis
NAC = N-acetyl-L-cysteine
NBQX = 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione
N-cadherin = neural-cadherin
NCAM = neural cell adhesion molecule
NDF = neu differentiation factor
NFκ-B = nuclear factor κ-B
NGF = nerve growth factor
NIK = nuclear factor κ-B inducing kinase
nmA = neonatal mouse astrocytes
NO = nitric oxide
NT-3 = neurotrophin-3
NT-4/5 = neurotrophin-4/5
O2A = oligodendrocyte type 2 astrocyte
OL = oligodendrocyte
PARP = poly ADP-ribose polymerase
PDGF = platelet-derived growth factor
PI = phosphatidylinositol
PI(3)P = phosphatidylinositol 3-phosphate
$PI(3,4)P_2$ = phosphatidylinositol 3,4-biphosphate
$PI(3,4,5)P_3$ = phosphatidylinositol 3,4,5-triphosphate
PI3-K = phosphatidylinositol 3-kinase
PKB = protein kinase B
PKC = protein kinase C
PLP = proteolipid protein
PP2 = (4-amino-5-(4-cholorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) (AG1879)
RIP = receptor-interacting protein
RPM = revolutions per minute

SNAP = S-nitroso-N-acetyl-DL-penicillamine
SOD = superoxide dismutase
TdT = terminal deoxynucleotidyl transferase
TNF = tumor necrosis factor
TNFR-1 = tumor necrosis factor receptor 1
TNFR-2 = tumor necrosis factor receptor 2
TRADD = tumor necrosis factor receptor associated death domain
TRAF-2 = tumor necrosis factor receptor protein 2
TUNEL = terminal deoxynucleotidyl transferase-mediated nick end labeling
VCAM = vascular cell adhesion molecule
wk = week

CHAPTER ONE:

INTRODUCTION

Oligodendrocytes are glial or support cells in the central nervous system (CNS). The primary function of oligodendrocytes is the myelination of CNS axons. Myelination begins as an oligodendrocyte extends its processes and contacts adjacent axons. An individual oligodendrocyte may contact and myelinate as many as 60 axons. Following contact an oligodendrocyte wraps its processes around the axons. After several turns the cytoplasm of the processes are removed and the processes become compact (Figure 1). Compact oligodendrocyte processes are referred to as myelin (McLaurin & Yong, 1995).

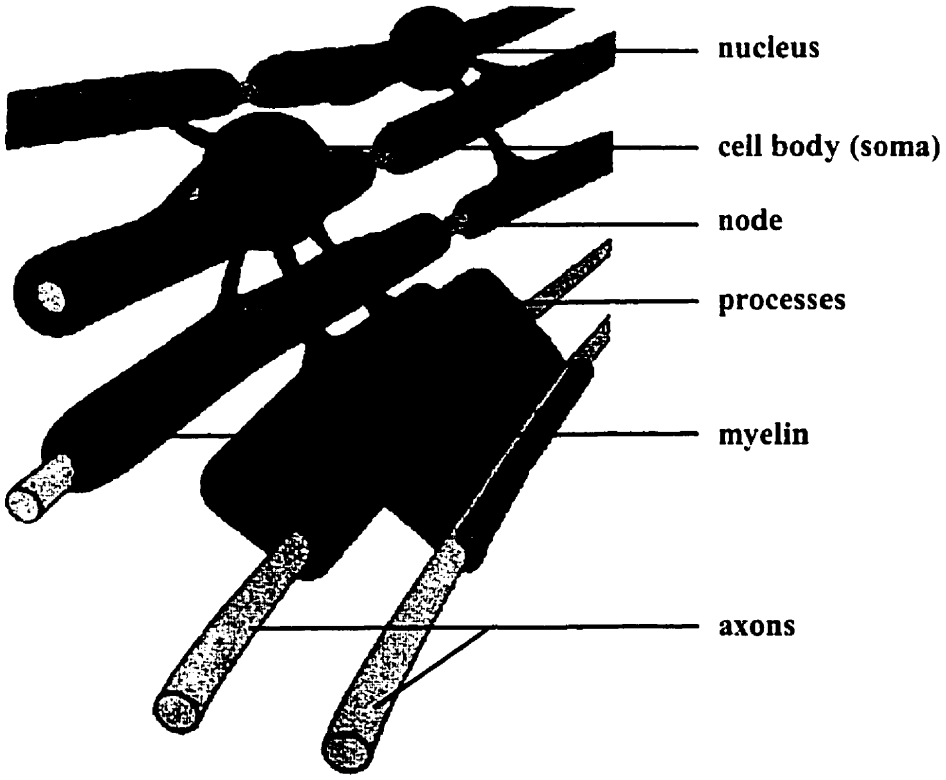

Figure 1. Oligodendrocytes and myelin
The oligodendrocytes shown have myelinated several axons. The oligodendrocyte in the foreground is in the process of myelinating two additional axons.

The composition of CNS myelin is approximately 70% lipid and 30% protein (dry weight). Myelin lipids include cholesterol, phosphatidylserine/phosphatidylcholine, cerebrosides, and ethanolamines. Cholesterol is the most abundant lipid and the most abundant component of CNS myelin. The major proteins contained in CNS myelin are lipophilin/proteolipid protein (PLP) and myelin basic protein (MBP). Other myelin proteins include Wolfgram proteins/2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), myelin associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG) (Table 1) (McLaurin & Yong, 1995).

Table 1. Composition of CNS myelin

Lipid (70% Dry Weight)	**% Total Lipids**
Cholesterol	40
Cerebrosides	20
Phosphatidylserine/phosphatidylcholine	16
Ethanolamines	13
Phosphoglycerides	5.5
Sphingomyelin	4
Gangliosides	1
Inositides	0.5
Protein (30% Dry Weight)	**% Total Proteins**
PLP/Lipophilin	30
Myelin Basic Protein (MBP)	25
Wolfgram Proteins (CNP)	4
Myelin associated glycoprotein (MAG)	1
Myelin/oligodendrocyte glycoprotein (MOG)	0.05
Others	40

Myelin is an integral component of the CNS. The CNS relies on the generation and integration of billions of electrical impulses; rapid conduction of these impulses is of

the utmost necessity. CNS myelin insulates axons and speeds impulse conduction. In this regard, myelination of axons is essential for efficient CNS function.

The manner in which axons are myelinated plays a large role in impulse conduction. A single axon is myelinated by several oligodendrocytes; each oligodendrocyte myelinates a segment of the axon. An axon becomes fully myelinated as many oligodendrocyte processes wrap and compact around its length. Oligodendrocyte processes do not overlap on the axon, instead they lie adjacent to each other and leave small unmyelinated portions or nodes between each process (Figure 1). The segmented pattern of myelination enables action potentials to 'jump' from node to node; this process is referred to as saltatory conduction. Saltatory conduction prevents action potentials from having to be regenerated continually along the axon, as a result the velocity and efficacy of propagation is greatly increased (Zigmund et al., 1999).

In a number of CNS pathologies, such as Multiple Sclerosis (MS) and CNS trauma, axons undergo a loss of myelin or demyelination. Demyelination of axons can result either from the death of oligodendrocytes or from direct damage to the myelin. In MS it is common to find numerous areas or plaques of demyelinated axons scattered throughout the CNS (Conlon et al., 1999). In focal CNS injury demyelinated axons can be found both within and around the injured area (Rosenberg et al., 1998). Demyelination of axons compromises CNS function. Demyelinated axons lose the capacity to support saltatory conduction, the propagation of action potentials along an axon is slowed and axonal conductance is impaired (McLaurin & Yong, 1995). Depending on the location of the axons, demyelination can lead to sensory, motor and cognitive deficits. Demyelination of axons in the optic nerve, for instance, can lead to

impairments in vision while demyelination of axons in the basal ganglia compromises motor function. Demyelination of temporal lobe structures is often associated with impairments in both learning and memory (Kolb & Whishaw, 1995).

Preventing demyelination and/or promoting remyelination is essential for maintaining CNS function. Demyelination and remyelination center on the oligodendrocyte. Without support from the oligodendrocyte cell body myelin degenerates and withdraws from the axon. If an oligodendrocyte dies, myelin is lost and axons become demyelinated. Oligodendrocyte death has implications for remyelination as well. Remyelination of axons depends on the production of myelin. If oligodendrocytes die, new oligodendrocytes must be generated to produce myelin. Oligodendrocytes, however, have limited regenerative capacities; dying oligodendrocytes are not readily replaced. In this regard, oligodendrocyte death severely hinders remyelination (Ludwin, 1996).

Preventing oligodendrocyte death in CNS pathology is a useful strategy for both preventing demyelination and/or promoting remyelination. Prevention of oligodendrocyte death may decrease the extent of demyelination in an injured area and prevent subsequent deficits in CNS function. It is possible that demyelination will occur regardless of oligodendrocyte survival in a pathological state. In cases where demyelination is inevitable, preventing oligodendrocyte death is a useful strategy for promoting remyelination; prevention of oligodendrocyte death maintains the adequate numbers of oligodendrocytes needed for remyelination of the demyelinated area.

Types of Cell Death

Cell death can be distinguished as either necrotic or apoptotic. During necrotic cell death the cytosolic and nuclear structures of the cell swell, the plasma membrane ruptures and the intra-cellular contents spill into the extra-cellular space generating an inflammatory reaction (Wylie et al., 1997; Granville et al., 1998). Apoptosis, also referred to as programmed cell death, is a more altruistic form of cell death; cellular contents are contained within the plasma membrane and the dying cell generates little to no inflammatory reaction. During apoptotic cell death there is a general decrease in the size of the cell as the plasma membrane blebs and contorts, the cytoplasm condenses and the cellular organelles compact. Within the nucleus of apoptotic cells the chromatin condenses and deoxyribonucleic acid (DNA) strand breaks occur between the nucleosomes (Wylie et al., 1997; Gavrieli et al., 1992). Whereas random DNA strand breaks may occur in necrotic cells, DNA strand breaks in apoptotic cells occur specifically between the nucleosomes creating multimers of 180 base pairs (Gavrieli et al., 1992). While necrotic death lacks elaborate mechanisms the apoptotic process is intricate and complex. Consequently, apoptosis occurs relatively slower than necrosis following damage to the cell (Wylie et al., 1997; Dixon et al., 1997).

Apoptosis consists of three distinct phases; initiation, execution and termination. Initiation of apoptosis occurs when intra- or extra-cellular signals activate apoptotic factors within the cell. Execution of apoptosis involves the proteolysis of structural and homeostatic proteins. Termination of the apoptotic process involves the recognition and phagocytosis of the apoptotic cell.

Events involved in the initiation of apoptosis include, but are not restricted to, damage to plasma and mitochondrial membranes, disruption of cell-cell and/or cell-extra-cellular matrix (ECM) interactions, deprivation of growth factors, DNA damage and activation of cytokine receptors (Wylie et al., 1997; Granville et al., 1998; Ruoslahti et al., 1994). Each of these events initiates a cascade of intra-cellular signaling that leads to the execution of the apoptotic process. The intra-cellular signaling involved in apoptosis is elaborate and complex; apoptotic signaling is best described by example. The activation of the tumor necrosis factor (TNF) family of cytokine receptors and the disruption of cell-cell and/or cell-ECM interactions provide excellent examples of apoptotic signaling.

The activation of the TNF family of cytokine receptors has received a great deal of attention in the area of apoptosis. The TNF receptor family includes TNF receptors 1 (TNFR-1) and 2 (TNFR-2), fas, p75, and numerous other receptors (Wang & Lenardo, 1997). Research on TNFR-1 has provided a great deal of insight into the apoptotic process. Binding of TNF to TNFR-1 activates TNFR-1 and initiates apoptosis. Following activation of TNFR-1, TNF receptor associated death domain (TRADD) binds to TNFR-1 receptor via a conserved amino acid sequence, referred to as the death domain. Binding of TRADD recruits other molecules to the TNFR-1/TRADD complex including a fas associated death domain (FADD), receptor-interacting protein (RIP), and TNF receptor protein 2 (TRAF-2) (Liu et al., 1996). Binding of FADD to the TNFR-1/TRADD complex activates caspase 8. Caspase-8 activates several caspases including caspase-3 (Granville et al., 1998, Liu et al., 1996). As we will see in the execution phase of apoptosis, activation of caspase-3 is integral to the apoptotic process.

Binding of RIP to the TNFR-1/TRADD complex recruits caspase and RIP adapter with death domain (CRADD). TRADD/RIP/CRADD activates caspases either directly or indirectly by increasing levels of intra-cellular ceramide (Granville et al., 1998). Intra-cellular ceramide has been shown to increase levels of caspase-2, -3, and –9 (Ito et al., 1999). The TRADD/RIP/CRADD complex can also induce transcription of apoptotic genes via jun kinase (JNK). TRADD/RIP/CRADD activates mitogen activated protein kinase/extra-cellular regulated kinase (MAPK/ERK) kinase kinase (MEKK); TRADD/RIP/CRADD can activate MEKK directly or through ceramide. MEKK1 activates JNK kinase (JNKK) which in turn activates JNK (Liu et al., 1996). JNK activates several transcription factors including c-jun and c-myc (Chaudhary et al., 1998). C-jun and c-myc can induce the transcription of pro-apoptotic genes (Wargnier et al., 1998; Romashkova & Makarov, 1999). Binding of TRAF-2 to the TNFR-1/TRADD complex results in an anti-apoptotic pathway. TRADD/TRAF-2 can induce transcription of anti-apoptotic genes via activation of nuclear factor κ-B (NFκ-B). TRADD/TRAF-2 activates NFκ-B inducing kinase (NIK). NIK phosphorylates inhibitor of κ-B (Iκ-B) kinase (IKK) which in turn phosphorylates Iκ-B. Iκ-B forms a complex with NFκ-B in the cytosol; the Iκ-B/NFκ-B complex prevents translocation of NFκ-B to the nucleus. Phosphorylation of Iκ-B releases NFκ-B. Upon release NFκ-B translocates to the nucleus and induces the transcription of anti-apoptotic genes (Liu et al., 1996). Anti-apoptotic genes that are transcribed by NFκ-B include A1 and anti-apoptotic inhibitor of apoptosis-2 (c-IAP2) (Granville et al., 1998).

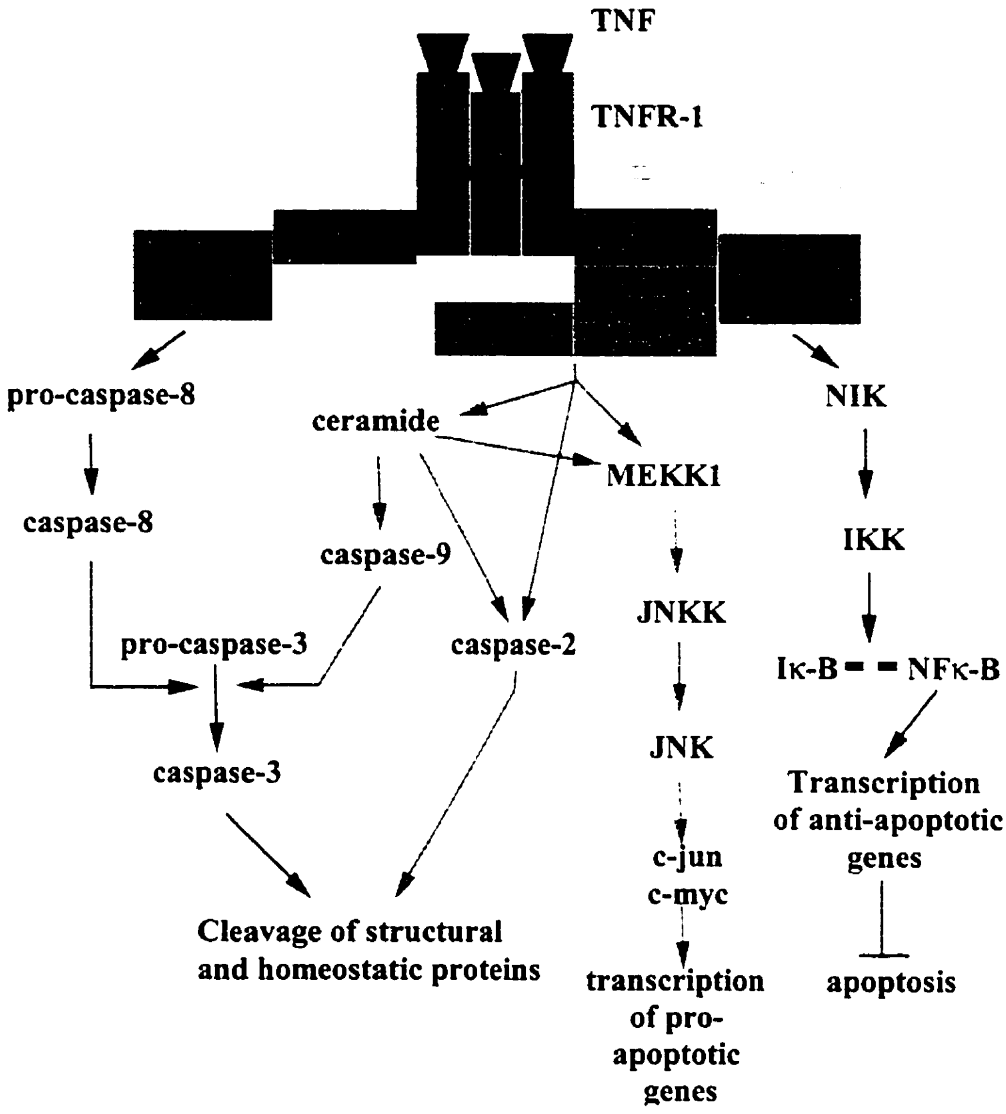

Figure 2. TNF signaling in apoptosis

The intra-cellular signaling that occurs following the disruption of cell-cell and/or cell-ECM interactions provides a second example of the complexity of apoptosis; disruption of cell-cell and/or cell-ECM interactions is of particular relevance to the research in question. Cell-cell and cell-ECM interactions are maintained largely through adhesion molecules that are present on the extra-cellular surface. Four main families of cell adhesion molecules (CAMs) have been identified at this time, integrins, cadherins,

selectins and members of the immunoglobulin superfamily (Petruzelli et al., 1999; Chothia et al. 1997; Aplin et al., 1998). Of the currently known CAMs, the integrin family has received the most attention in the area of apoptosis. Integrins can interact with proteins in the extra-cellular space and with other CAMs, such as vascular cell adhesion molecule (VCAM) and intercellular cell adhesion molecule (ICAM) (Giancotti & Ruoslahti, 1999; Boudreau & Jones, 1999). Unlike TNFR-1-induced apoptosis where death results from induction of noxious stimuli via the TNF receptor, integrin-related apoptosis results from the removal of survival stimuli that are provided by the integrin family. Integrin-ECM or integrin-CAM interactions activate integrins and initiate signaling cascades that promote cell survival; disruption of integrin-ECM or integrin-CAM interactions negates cell survival and induces apoptosis (Ruoslahti et al., 1994; Giancotti & Ruoslahti, 1999; Frisch & Ruoslahti, 1997).

The survival pathway initiated by integrin activation centers around phosphatidylinositol 3-kinase (PI3-K), a lipid kinase involved in the generation of several second messenger molecules (Leevers et al., 1999; Prescott, 1999). Integrins modulate PI3-K activity in a variety of ways. Integrin activation activates focal adhesion kinase (FAK) and Fyn kinase, a member of the src family of kinases (Giancotti & Ruoslahti, 1999; Aplin et al., 1998). FAK and Fyn kinase activate PI3-K in a number of manners. FAK can activate PI3-K directly or indirectly through src kinase family members and/or through Ras via src kinase members. Fyn kinase can activate PI3-K either directly or indirectly through activation of Ras via the Shc adaptor protein (Giancotti & Ruoslahti, 1999). Activated PI3-K phosphorylates inositol lipids at the 3′ position of the inositol ring and generates 3-phosphoinositide second messengers.

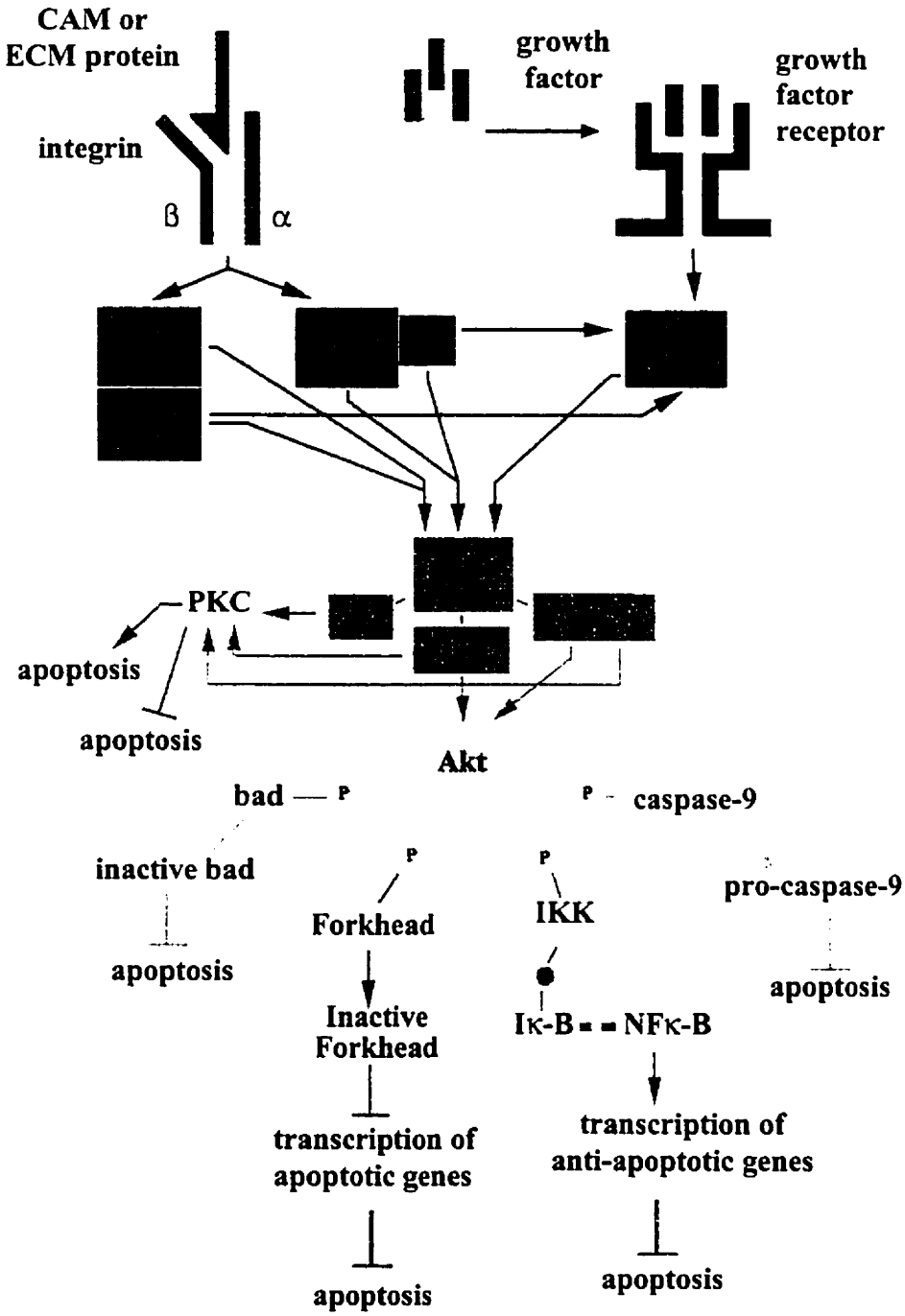

Figure 3. Integrin and growth factor signaling in cell survival

PI3-K generated phosphoinositides include phosphatidylinositol (PI) 3-phosphate (PI(3)P), PI3,4-biphosphate ($PI(3,4)P_2$) and PI3,4,5-triphosphate ($PI(3,4,5)P_3$); only $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are involved in the suppression of apoptosis. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ induce activation of the serine-threonine kinase, Akt (Leevers et al, 1999; Rameh & Cantley, 1999; Franke et al., 1997). Akt, also referred to as protein kinase B (PKB), can suppress apoptosis through four distinct mechanisms, activation of NFκ-B, phosphorylation of bad, phosphorylation of caspase-9 and phosphorylation of Forkhead transcription factors. As previously mentioned activation of NFκ-B counters apoptosis through transcription of anti-apoptotic genes. Akt activates NFκ-B indirectly through IKK and Iκ-B. Akt phosphorylates IKK; IKK, in turn, phosphorylates Iκ-B (Romashkova & Makarov, 1999). Phosphorylation of Iκ-B enables NFκ-B translocation and transcription. NFκ-B induces transcription of anti-apoptotic genes that suppress apoptosis (Granville et al., 1998). Phosphorylation of bad, caspase-9 or the Forkhead transcription factors can also lead to suppression of the apoptotic process. Bad and caspase-9 are pro-apoptotic factors that are involved in the execution of the apoptotic process. Bad is a pro-apoptotic gene while caspase-9 is a proteolytic protein. Both bad and caspase-9 are inactivated by phosphorylation (Coffer et al., 1998; Khwaja et al., 1999, Cardone et al., 1998). The Forkhead family of transcription factors induces transcription of pro-apoptotic genes such as the fas ligand (fasL) gene. Akt phosphorylates Forkhead transcription factors; phosphorylation inactivates the factors and suppresses the transcription of pro-apoptotic genes (Datta et al., 1999). PI3-K can also activate protein kinase C (PKC). PKC has been implicated in both the induction of cell death and the promotion of cell survival. It is likely that the role of PKC in cell

death/survival is dependent on the cell type in question (Lucas et al., 1995; Lavin et al., 1996).

In integrin-related apoptosis, disruption of integrin-ECM and integrin-CAM interactions prevents integrin-mediated survival. Disruption of integrin binding leads to reduced activation of FAK and Fyn kinases and a subsequent reduction in both PI3-K and Akt activity. Reduced Akt activity, in turn, leads to apoptosis either through reduced transcription of anti-apoptotic genes, increased transcription of pro-apoptotic genes or through reduced de-activation of the pro-apoptotic factors bad, caspase-9 and Forkhead transcription factors (Giancotti & Ruoslahti, 1999; Kumar, 1998, Datta et al., 1999).

The scenario in which apoptosis results from the removal of survival stimuli is not unique to the integrin family, deprivation of growth factors can induce apoptosis in a similar manner (Giancotti & Ruoslahti, 1999; Kumar, 1998). Growth factors can also promote cell survival through PI3-K. Growth factors that can activate PI3-K include insulin-like growth factors (IGF), neurotrophins, fibroblast growth factors (FGF), epidermal growth factors (EGF) and platelet-derived growth factors (PDGF) (Butler et al., 1998; Segal & Greenberg, 1996; Bikfalvi et al., 1997; Malarkey et al., 1995). Growth factors activate PI3-K via Ras (Figure 3). Growth factor deprivation decreases Ras activity and causes a subsequent decrease in the activity of PI3-K and Akt. In this regard apoptosis induced by growth factor deprivation can involve similar signaling to that of integrin-related apoptosis.

The complex signaling involved in the initiation of apoptosis ultimately leads to the execution phase of the apoptotic process. Execution of the apoptotic process involves the activation of the caspase family of cysteine proteases (Thornberry, 1997; Granville et

al., 1998; Dixon et al., 1998). Recently, the caspase family has been grouped into ICE and CED-3 subfamilies. The ICE family includes caspase-1, -4 and -5. It is currently presumed that the ICE family is involved in inflammation and not in the apoptotic process. The CED-3 family has been further grouped into effectors and executioners of the apoptotic process. The effector caspases, caspase- 6, -8, and -9 are responsible for the activation of the executioner caspases, caspase -2, -3, and –7 (Granville et al., 1998). Upon activation, executioner caspases cleave several integral cellular proteins (Casciola-Rosen et al., 1996; Thornberry, 1997). Proteins that are susceptible to caspase-induced cleavage include structural proteins such as G-actin, lamin-A and fodrin, and homeostatic proteins such as DNA-dependent protein kinase (DNA-PK) and poly ADP-ribose polymerase (PARP) (Granville et al., 1998; Casciola-Rosen et al., 1996; Thornberry, 1997). Following cleavage of DNA-PK and PARP the cell is unable to initiate DNA repair and cell death is inevitable.

Although each of the executioner caspases are capable of cleaving integral cellular proteins, caspase-3 is considered the central executioner caspase. Caspase-3 has been shown to cleave PARP and DNA-PK in various cell types during apoptosis (Granville et al., 1998). Caspase-3 is also involved in the specific DNA fragmentation that occurs in apoptotic cells. In 1998 Enari et al. demonstrated that caspase-3 can induce DNA fragmentation by activating a novel DNase. Enari et al. (1998) discovered the DNase and the inhibitor of the DNase. Enari et al. (1998) named the DNase and its inhibitor caspase-activated DNase (CAD) and inhibitor of CAD (ICAD), respectively. The inactive form of CAD was found in complex with ICAD in various types of tissues.

During apoptosis caspase-3 was shown to cleave ICAD, thereby releasing CAD in its active form and enabling it to enter the nucleus and degrade chromosomal DNA.

Factors involved in the initiation and execution phases of apoptosis are constitutively expressed in most cell types. Under normal conditions apoptotic factors are held in check by anti-apoptotic factors such as bcl-2, Akt and NFκ-B (Wylie et al., 1997, Datta et al., 1999). Found on the nuclear envelope, mitochondrial membrane and the endoplasmic reticulum (ER), bcl-2 is a pro-apoptotic gene that has been shown to block TNF and ceramide induced apoptosis and inhibit the activation of effector caspases (Brown, 1997). Akt, as mentioned, suppresses apoptosis by maintaining inactive forms of bad, caspase-9 and Forkhead transcription factors and by activating NFκ-B (Granville et al., 1998) (Coffer et al., 1998; Khwaja et al., 1999, Cardone et al., 1998) (Datta et al., 1999). NFκ-B suppresses apoptosis through transcription of the anti-apoptotic genes AI and c-IAP2 (Granville et al., 1998).

Following the initiation and execution phases of apoptosis the apoptotic process reaches termination. Termination of apoptosis involves the recognition and phagocytosis of the apoptotic cell. Apoptotic cells are recognized by changes in the plasma membrane. Membrane changes include the exposure of side chain sugars and phosphatidylserine and thrombospondin binding sites; phagocytic cells bind to these sites via lectins, phosphatidylserine receptors and integrin receptors, respectively (Savill, 1997). Phagocytosis removes the apoptotic cell and terminates the apoptotic process.

Death of Oligodendrocytes *In Vivo*

The majority of *in vivo* research on oligodendrocyte death has focussed on the occurrence of oligodendrocyte death during development and in CNS pathologies such as CNS trauma and MS.

During CNS development oligodendrocytes are produced in excess amounts and the cells must compete for limited amounts of survival factors (Raff et al., 1993). In the absence of adequate survival factors oligodendrocytes undergo cell death. During development dying oligodendrocytes exhibit DNA fragmentation and morphological characteristics of apoptosis. Dying oligodendrocytes have been detected in cortical (Trapp et al., 1997, Bertollini et al., 1997), optic nerve (Barres et al., 1992; 1993; Burne et al., 1996), and spinal cord (Calver et al., 1998) development. Addition of growth factors in the developing CNS attenuates oligodendrocyte death. In 1992 and 1993 Barres et al. demonstrated decreased oligodendrocyte death in the developing optic nerve following application of platelet-derived growth factor (PDGF) and ciliary neurotrophic factor (CNTF), respectively.

Oligodendrocyte death is a common outcome of injury to the CNS. Following injury breakdown of the blood-brain-barrier, inflammation and mechanical trauma lead to numerous changes in the CNS environment. Levels of inflammatory cytokines, free radicals, reactive oxygen species and neurotransmitters are increased in the injured area (Yakolev & Faden, 1995). Disruption of cell-cell contact can deprive cells of trophic support (Raff et al., 1993). Changes in the CNS environment impact heavily on cell survival; cell death occurs both in and around the injury site.

Dying oligodendrocytes have been detected in both brain and spinal cord models of CNS injury. In 1998 Conti et al. investigated oligodendrocyte death following a fluid percussion model of brain injury. Conti et al. detected apoptotic oligodendrocytes as early as 12 hours (h) following injury with oligodendrocyte death peaking at 24h and 1 week (wk) following injury. Similar results were reported by Bittigau et al. (1999). Bittigau et al. investigated oligodendrocyte death following a contusion model of brain injury. Apoptotic oligodendrocytes were detected as early as 6h following injury with peak levels of apoptosis occurring at 24h post-injury. Oligodendrocyte death has been well characterized following spinal cord injury. Dying oligodendrocytes have been detected in response to radiation (Li et al., 1996; 1996), compression (Crowe et al., 1997; Li et al., 1996; 1999; Liu et al., 1997; Rosenburg et al., 1999) and contusion (Shuman et al., 1997) of the spinal cord. Following radiation of the spinal cord oligodendrocyte die by apoptosis. Apoptotic oligodendrocytes have been identified as early as 4h with the peak of oligodendrocyte death occurring at 8h following radiation injury (Li et al., 1996; 1996). In compression and contusion models of spinal cord injury both necrosis and apoptosis contribute to oligodendrocyte death. Following compression and contusion dying oligodendrocytes have been identified as early as 30 minutes (min) post- injury; the peak of oligodendrocyte death occurs between 7-9 days (d) post-injury. Oligodendrocytes dying 30 min following spinal cord injury have been identified as necrotic (Shuman et al., 1997), while oligodendrocytes dying at 4h and beyond have been identified as apoptotic (Crowe et al., 1997; Li et al., 1996; 1999; Liu et al., 1997; Shuman et al., 1997). In both brain and spinal cord models of CNS injury the majority of

oligodendrocyte death occurs at one week following injury; this delay provides a therapeutic window for the attenuation of oligodendrocyte death following CNS injury.

A report by Rosenberg et al. (1998) provides evidence for the involvement of glutamate toxicity in oligodendrocyte death. Rosenburg et al. (1998) were able to attenuate acute oligodendrocyte death following spinal cord contusion through antagonism of glutamate receptor (GluR) activation on oligodendrocytes. Oligodendrocyte death was attenuated at 4h and 24h post-injury by focal micro-injection of 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX), an antagonist of the kainate and α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) subclass of GluRs. Research on mediators of oligodendrocyte death following CNS injury is scarce. To date, the majority of research has focussed on establishing that oligodendrocyte death occurs in CNS injury.

Oligodendrocyte death has been well described in MS. MS is a demyelinating disease of the CNS characterized by the presence of areas or plaques of axonal demyelination (Conlon et al., 1999, Noseworthy, 1999). Dying oligodendrocytes are commonly detected both within and at the periphery of the demyelinating plaques. Demyelination in MS is thought to occur largely from direct damage to myelin. Infiltrating macrophages and resident microglia have been shown to directly attack and phagocytize myelin in MS plaques; what causes these cells to attack myelin is still unknown (Noseworthy, 1999). Damage to myelin can result in oligodendrocyte death (Ludwin & Johnson, 1981; Rodriguez, 1985). Dying oligodendrocytes, however, have been detected prior, concomitant and subsequent to demyelination (Bruck et al., 1994). It is conceivable therefore that while oligodendrocyte death is not the sole cause of

demyelination in MS, oligodendrocyte death contributes to and exacerbates demyelination in the diseased state. In the same regard, it is probable that myelin damage is not the sole cause of oligodendrocyte death but that oligodendrocyte death occurs in response to other factors as well. Other factors that may contribute to oligodendrocyte death in MS include increased levels of inflammatory cytokines such as TNF-α and interferon-γ (IFN-γ) and increased levels of p75, fas and fas-L. As we will see in the following section TNF-α, IFN-γ and activation of p75 and fas have all been shown to induce oligodendrocyte death *in vitro*.

Dying oligodendrocytes have been detected in MS patients (post-mortem) (D'Souza et al., 1997; Bonetti et al., 1997; Dowling et al., 1999; 1996; Storch et al., 1998; Benjelloun et al, 1998; Luchinetti et al., 1996; Ozawa et al., 1994) and in animal models of MS such as experimental autoimmune encephalomyelitis (EAE) (Pender et al., 1991, Pitt et al., 2000; Smith et al., 2000). While the occurrence of oligodendrocyte death is well accepted, the nature of oligodendrocyte death in MS is still under considerable debate. It has been proposed that oligodendrocyte death occurs solely by necrosis in MS. In 1997, D'Souza et al. and Bonetti et al. examined oligodendrocyte death in the post-mortem tissue of MS patients. In both active and inactive MS plaques dying oligodendrocytes demonstrated morphological features of necrosis. While neither D'Souza et al. (1997) nor Bonetti et al. (1997) were able to detect apoptotic oligodendrocytes, both groups demonstrated the presence of apoptosis-related molecules in oligodendrocytes. In active plaques oligodendrocytes expressed increased levels of TNF receptors and fas, in silent plaques oligodendrocytes expressed increased levels of bcl-2. Other reports have also demonstrated the presence of apoptosis-related molecules

on oligodendrocytes. Dowling et al. (1996) and D'Souza et al. (1996) demonstrated increased fas expression on oligodendrocytes in MS lesions. In 1999, Dowling et al. demonstrated increased expression of p75 on dying oligodendrocytes. The presence of apoptosis-related molecules suggests an apoptotic mode of death; several publications support this view. Dowling et al. (1999; 1996), Storch et al. (1998), Benjelloun et al. (1998), Luchinetti et al. (1996), and Ozawa et al. (1994) have identified apoptotic oligodendrocytes in active and inactive plaques of MS patients. Apoptotic oligodendrocytes exhibited DNA fragmentation and morphological characteristics of apoptosis.

There is still much work to be done concerning both the factors involved in oligodendrocyte death and the nature of oligodendrocyte death in MS. Current knowledge of the area suggests that a variety of factors, including myelin damage, elevation of inflammatory cytokines, and increased expression of apoptosis-related molecules such as fas and p75, may contribute to oligodendrocyte death in the disease. With respect to the nature of oligodendrocyte death in the disease, it is likely that oligodendrocytes die by both necrosis and apoptosis in the MS plaque.

CNS pathologies such as MS and CNS injury lead to numerous changes in the CNS environment; the exact factors that induce oligodendrocyte death in these pathologies are not known. Candidate factors include altered levels of growth factors, cytokines, free-radicals, reactive oxygen species, and neurotransmitters. Many of these factors have been investigated *in vitro*.

Death of Oligodendrocytes *In Vitro*

In vitro research on oligodendrocyte death is concentrated on identifying factors that mediate oligodendrocyte death. Factors that may mediate oligodendrocyte death during development and in CNS pathology have received the greatest amount of attention.

Growth factor deprivation may contribute to oligodendrocyte death during development and CNS trauma. During development growth factors are not produced in sufficient amounts and many cells are deprived of adequate levels of growth factors. Following injury, extensive cell death can disrupt cell-cell contact and growth factor supply (Raff et al., 1993). *In vitro*, deprivation of growth factors is modeled by serum deprivation. Oligodendrocyte death induced by serum deprivation can be attenuated by a large number of growth factors including CNTF, PDGF, IGF, leukemia inhibitory factor (LIF), neurotrophin-3 (NT-3), and basic FGF (bFGF) (Barres et al., 1993; Kahn & deVellis; 1994; Barres et al., 1993; Fressinaud et al., 1996; Barres et al., 1993; Barres et al., 1993; Kahn & deVellis, 1994; Barres et al. 1993; Kumar et al. 1998; Yasuda et al., 1995).

Oligodendrocytes may undergo spontaneous death in culture. Oligodendrocytes are particularly delicate cells; dissociation and isolation of oligodendrocytes from tissue can induce a significant amount of stress on the cell. Often the stress of cell culture can induce oligodendrocyte death. In 1999 Frost et al., demonstrated that co-culture with dorsal root ganglia (DRG) protects oligodendrocytes from spontaneous death. DRG protection of oligodendrocytes was mediated by $\alpha_6\beta_1$ integrin. Growth factors can also protect oligodendrocytes from spontaneous death *in vitro*. bFGF (Grinspan et al., 1996;

Yasuda et al., 1995), IGF (Yasuda et al., 1995), PDGF (Yasuda et al., 1995), CNTF (Yasuda et al., 1995), and neu differentiation factor (NDF) (Raabe et al., 1997) can attenuate spontaneous death of oligodendrocytes.

There are instances in which growth factors are unable to attenuate oligodendrocyte death. In 1996, Vemuri & McMorris demonstrated that in the presence of PI3-K inhibitors neither IGF, PDGF, NT-3, EGF, bFGF nor CNTF could protect oligodendrocytes from cell death. Growth factors can promote cell survival by activating PI3-K and Akt. In the presence of Wortmannin and LY294002, pharmacological inhibitors of PI3-K, growth factor signaling is blocked and growth factors are unable to promote cell survival.

While a large number of growth factors prevent oligodendrocyte death, nerve growth factor (NGF) has been shown to induce apoptosis of oligodendrocytes *in vitro* (Gu et al., 1999; Yoon et al., 1998; Cassacia-Bonnefil et al., 1996). NGF-induced oligodendrocyte apoptosis is mediated by the p75 receptor, a member of the TNF receptor family. NGF-induced death of oligodendrocytes has been attenuated by both activation of TrkA, which is thought to compete with the p75 receptor for NGF binding (Yoon et al., 1998), and the inhibition of caspase-1, -2 and -3 (Gu et al., 1999). The induction of oligodendrocyte death by NGF is of particular relevance to MS, Alzheimer's Disease and Parkinson's. In MS, oligodendrocytes exhibit an increase in the expression of p75 (Dowling et al., 1999). Increased p75 expression may contribute to oligodendrocyte death, or more specifically, oligodendrocyte apoptosis in MS plaques. NGF has been proposed as a treatment for Alzheimer's Disease and Parkinson's; NGF treatment promotes neuronal survival in animal models of these diseases (Koliatsos et al.,

1996). While NGF promotes neuronal survival it is likely that NGF treatment has detrimental effects on oligodendrocytes. Nonetheless, not all reports demonstrate that NGF is toxic to oligodendrocytes. In 1998 Ladiwala et al. demonstrated that NGF did not induce the death of human oligodendrocytes.

Levels of pro- and anti-inflammatory cytokines are significantly increased in MS and CNS injury; many of these cytokines have been investigated to determine their role in oligodendrocyte death. A large number of cytokines are upregulated in MS including interleukin (IL) –1, –2, -4, -5, -6, -10 and –13, TNF-α and -β and IFN-γ (Noseworthy, 1999). Cytokines are upregulated both in and around demyelinating plaques. In CNS injury, work from our laboratory and others has demonstrated increased levels of IL-1, -2, and -6, TGF-α and β, TNF and IFN-γ (Herx et al., unpublished; Yong, 1996; Arvin et al., 1996). Of these cytokines only IFN-γ and TNF induce oligodendrocyte death *in vitro*.

IFN-γ treatment induces apoptosis of oligodendrocytes *in vitro* (Baerwald & Popko 1998; Vartanian et al., 1995); this effect can be potentiated by TNF-α (Andrews et al., 1998). Addition of LIF to oligodendrocyte cultures attenuates IFN-γ toxicity (Vartanian et al., 1995). There is evidence to suggest that IFN-γ toxicity is species-specific. While IFN-γ has been shown to induce apoptosis of rat oligodendrocytes, D'Souza et al. (1995) found that IFN-γ treatment did not induce death of cultured human oligodendrocytes.

While both TNF-α and β induce oligodendrocyte death *in vitro*, the effect of TNF-α is best documented in the literature. TNF-α induces apoptotic death of human (Ladiwala et al., 1998; D'Souza et al., 1996; 1995; 1995), bovine (Selmaj et al., 1991),

and embryonic mouse oligodendrocytes (Hisahara et al., 1997), and oligodendrocyte type 2 astrocyte (O2A) (Noble & Mayer 1996; Mayer & Noble 1994) and central glia 4 (CG4) cell lines (Claude-Louis et al., 1993). TNF-α-induced toxicity can be reversed by caspase inhibition. In Hisahara et al. (1997), inhibition of caspase-1 and –3 significantly reduced TNF-α-induced death. This observation supports the involvement of caspase activation in TNF-α-induced death and provides evidence that TNF-α signaling in oligodendrocytes is similar to that of other cell types. N-acetyl-L-cysteine (NAC) can also attenuate TNF-induced death (Noble & Mayer, 1996). NAC is a synthetic compound that replenishes intra-cellular cysteine, a precursor of the anti-oxidant glutathione (Lu et al., 1999; Gillissen et al., 1998). Addition of NAC augments glutathione levels and promotes oligodendrocyte survival. The mechanism(s) by which glutathione might attenuate TNF-α-induced death are unknown. NAC attenuates TNF-α-induced death both alone and in synergism with CNTF (Noble & Mayer, 1996). Lone CNTF also attenuates TNF-α-induced death. Three separate reports implicate CNTF in the protection of oligodendrocytes from TNF-α-induced death. Louis et al. (1993) provided the first evidence of a role for CNTF in TNF-α toxicity. Additional reports from D'Souza et al. (1996) and Noble & Mayer (1994) demonstrate the protective capacities of CNTF. While CNTF significantly attenuates TNF-α-mediated toxicity, CNTF is unable to protect oligodendrocytes from either complement-induced or T-cell mediated necrosis (D'Souza et al., 1996). Several other growth factors have been tested against TNF-α-induced toxicity. Neither BDNF, NGF, NT-3 nor neurotrophin-4/5 (NT-4/5) can protect oligodendrocytes from TNF-α-induced death (D'Souza et al. 1999). Until recently CNTF was the only growth factor which protected against TNF-α toxicity.

In 1999, Ye & D'Ercole demonstrated that IGF could also attenuate TNF-α-induced oligodendrocyte death. Given the extent of literature on TNF-α-induced oligodendrocyte death *in vitro* it is probable that TNF-α upregulation plays a significant role in oligodendrocyte death following CNS injury.

Molecules associated with the TNF signaling pathway can also induce oligodendrocyte death. The binding of TNF-α to TNFR-1 increases intra-cellular ceramide (Granville et al., 1998; Haimovitz-Friedman et al., 1997). Ceramide has been shown to induce apoptotic death in human (Ladiwala et al., 1998) and bovine oligodendrocytes (Larocca et al., 1997) and in CG4 cells (Brogi et al., 1997; Cassacia-Bonnefil et al., 1996). Ceramide production can also be stimulated by damage to the plasma membrane. Following plasma membrane damage, intra-cellular ceramide is increased through the cleavage of sphingomyelin, a membrane lipid. Sphingomyelin cleavage releases intra-cellular ceramide (Granville et al., 1998; Haimovitz-Friedman et al., 1997).

Activation of fas also induces oligodendrocyte death. The role of fas in oligodendrocyte death is controversial. Fas is a member of the TNF family of cytokine receptors. In apoptosis, fas signaling is almost identical to that of TNFR-1. Activation of fas induces apoptosis in various cell types; both fas and fas-ligand (FAS-L) are referred to as apoptosis-related molecules (Granville et al., 1998; Liu et al., 1996). Fas activation in oligodendrocytes contradicts current dogma. Following fas activation *in vitro,* oligodendrocytes exhibit morphological characteristics of necrosis (D'Souza et al., 1996). The nature of fas-induced oligodendrocyte death is of significance to MS. Fas-L and fas expression on oligodendrocytes are upregulated in MS plaques (D'Souza et al., 1996;

Bonetti et al., 1997); fas activation is likely to contribute to oligodendrocyte death in the disease. The nature of fas-induced oligodendrocyte death and the nature of oligodendrocyte death in MS continue to be a controversial area of research.

Oxidative stress may also contribute to oligodendrocyte death in MS and CNS injury. Oxidative stress can result from excessive production of free radicals. Free-radicals such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) are upregulated in MS and CNS injury (Smith et al., 1999); the effects of these free radicals has been investigated *in vitro*. Mitrovic et al. (1994; 1995) investigated the effects of S-nitroso-N-acetyl-DL-penicillamine (SNAP), a NO donor, on cultured oligodendrocytes. SNAP induced necrosis of oligodendrocytes. Work by Boullerne et al. (1999) further supported a role for both SNAP and NO in oligodendrocyte death. NO production can be attenuated and stimulated by various cytokines. Cytokines such as IL-4 and −10 and TGF-β attenuate NO production. Cytokines such as IL-1-α and -β, IFN-γ and TNF-α stimulate NO production (Smith et al., 1999). In this regard IFN-γ and TNF-α may induce oligodendrocyte directly or indirectly by increasing NO production.

Several reports indicate that H_2O_2 induces oligodendrocyte death (Vollgraf et al., 1999; Kameshwar et al., 1999; Laskiewicz et al., 1999; Uberti et al; 1999; Bhat et al., 1999; Richter-Lansdberg et al., 1998; Noble & Yong, 1995). Whereas NO induces necrosis, H_2O_2 induces apoptosis of oligodendrocytes. H_2O_2-induced death can be attenuated in part by NAC (Richter-Lansdberg et al., 1998). In 1995 our laboratory showed that oligodendrocytes were less susceptible to H_2O_2-induced death when co-cultured with astrocytes (Noble & Yong, 1995). Astrocyte protection of oligodendrocytes was thought to involve the free-radical scavenger astrocyte catalase.

Depletion of anti-oxidants such as glutathione can also lead to oxidative stress. Examination of postmortem tissue from MS patients has revealed decreased levels of glutathione in MS plaques (Smith et al., 1999). Following CNS injury the production of reactive oxygen species outweighs glutathione production; glutathione may not be present in sufficient amounts to counter oxidative stress (Azbill et al., 1997). Several papers have addressed the effect of glutathione depletion on oligodendrocytes. *In vitro* glutathione may be depleted directly by pharmacological substrates or indirectly by depletion of glutathione precursors such as intra-cellular cystine. In oligodendrocytes both cystine and glutathione depletion induce cell death (Back et al., 1998; 1998; Yonezawa et al., 1996). Cell death following cystine and glutathione depletion is apoptotic. Toxicity induced by glutathione depletion can be attenuated by antioxidants such as alpha-tocopherol and idebenone, and by addition of glutathione itself (Back et al., 1998). In 1996, Yonezawa et al. demonstrated that live astrocytes could eliminate toxicity induced by cystine depletion. Yonezawa et al. (1996) used cystine-depleted medium to induce oligodendrocyte death. Pre-incubation of cystine-depleted medium in astrocyte cultures attenuated toxicity. The mechanism by which astrocytes reduced the toxicity of the medium is not known; astrocyte-conditioned medium showed no significant changes in either cystine or cysteine concentrations.

Accumulation of neurotransmitters following CNS injury may contribute to oligodendrocyte death. Following injury extensive cell death occurs in and around the injury site. Cells dying of necrosis do not maintain membrane integrity and cellular contents, including neurotransmitters, spill into the extra-cellular space. Several papers have investigated the effect of neurotransmitters on oligodendrocyte survival. Work from

our laboratory has demonstrated that the catecholamines epinephrine and norepinephrine induce oligodendrocyte death *in vitro* (Noble & Yong, 1995). Treatment of oligodendrocyte with epinephrine- and norepinephrine was thought to induce toxicity via the production of H_2O_2. Co-culture of oligodendrocytes with live astrocytes attenuated epinephrine-, norepinephrine- and H_2O_2-mediated toxicity. As previously mentioned astrocyte protection was thought to involve the free-radical scavenger astrocyte catalase.

Excessive amounts of glutamate may also contribute to oligodendrocyte death. In MS and CNS injury infiltrating leukocytes and resident microglia release excessive amounts of glutamate (Steinman, 2000). Blockade of glutamate transmission attenuates oligodendrocyte death in models of MS (Pitt et al., 2000; Smith et al., 2000) and CNS injury (Rosenburg et al., 1999). The effect of GluR overactivation on cultured oligodendrocytes has been well described in the literature. Overactivation of GluR can induce oligodendrocyte death through two distinct pathways; excessive release of intra-cellular calcium or depletion of intra-cellular cystine. Excessive release of intra-cellular calcium induces necrotic death while depletion of intra-cellular cystine induces apoptotic death (Back et al., 1998). Glutamate and glutamate analogues kainate and AMPA induce oligodendrocyte death *in vitro* (Sanchez & Matute, 1999; Matute et al., 1997, Noble & Mayer, 1996; Mayer & Noble, 1994; McDonald et al., 1998; Yoshioka et al., 1994; 1998). Glutamate-mediated death of oligodendrocytes can be attenuated by the GluR antagonist NBQX (McDonald et al., 1998), and by both NAC (Noble & Mayer, 1996; Mayer & Noble, 1994; Yoshioka et al., 1994; 1998) and CNTF (Noble & Mayer, 1996).

In summary, *in vitro* research on oligodendrocyte death has elucidated several mediators and attenuators of oligodendrocyte death. Mediators of oligodendrocyte death *in vitro* include growth factor deprivation, cytokine toxicity, oxidative stress and neurotransmitter-mediated toxicity. Oligodendrocyte death can be attenuated *in vitro* in a variety of ways including addition of growth factors, free-radical scavengers and anti-oxidants and through antagonism of glutamate transmission. Astrocytes can also attenuate oligodendrocyte death. Astrocytes protect oligodendrocytes from oxidative stress. It is presumed that astrocyte protection involves the production of free-radical scavengers and anti-oxidants. The protective potential of astrocytes in oligodendrocyte death has been greatly overlooked in the literature.

Role of Astrocytes in Promoting Cell Survival

Astrocytes have multiple functions in the CNS including maintenance of the blood brain barrier, regulation of ionic homeostasis, release, uptake and inactivation of neurotransmitters, metabolism of energy, and production of cytokines and neurotrophic factors (Figure 4) (Yong, 1998; Norenburg, 1994; Montgomery, 1994). In CNS pathology astrocytes become activated in a process referred to as reactive astrogliosis. Reactive astrocytes are characterized by marked hypertrophy, elongation of processes, increased amounts of several organelles including mitochondria, golgi complexes, ER and lysosomes and increased amounts of neurofilamentous proteins including vimentin and glial fibrillary acidic protein (GFAP). Reactive astrogliosis is accompanied by increased production of a variety of factors including anti-oxidants, free radical scavengers, growth factors, and detoxifying proteins. Reactive astrocytes also

demonstrate increased uptake of ions and excitotoxins (Yong, 1998, Norenburg, 1994; Montgomery, 1994).

Astrocytes have an immense protective potential in the CNS. The protective potential stems from astrocyte production of free-radicals, anti-oxidants, growth factors, and detoxifying proteins and astrocyte uptake of neurotransmitters such as glutamate. Astrocyte may also promote cell survival through cell-cell and cell-ECM interactions

Astrocytes produce a variety of free-radical scavengers and antioxidants including superoxide dismutase (SOD), and catalase and glutathione and glutathione precursors (Copin et al., 1992). Astrocyte-derived anti-oxidants have been shown to protect oligodendrocytes from oxidative stress (Noble & Yong, 1995; Yonezawa et al., 1996). It is likely that the protective capacities of astrocytes extend far beyond the production of anti-oxidants.

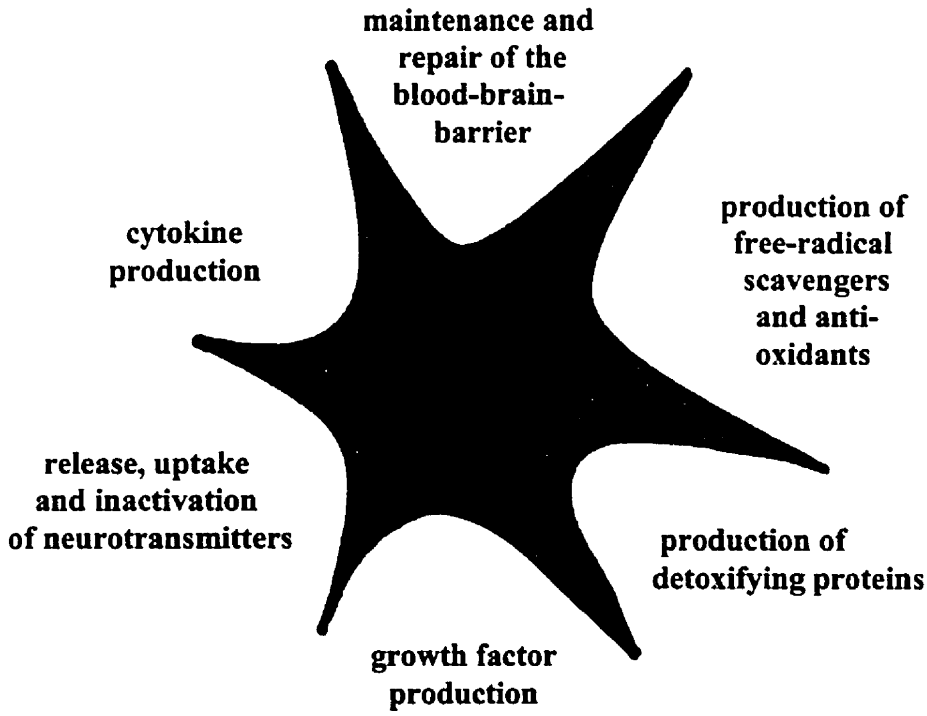

Figure 4. Some functions of astrocytes in the CNS

Astrocytes have to the potential to protect oligodendrocytes from growth factor deprivation and cytokine toxicity. Astrocytes produce a variety of growth factors including PDGF, BDNF, NGF, FGF, NT-3, IGF, CNTF, LIF and glial derived neurotrophic factor (GDNF) (Yong, 1996; Appel et al., 1997; Moretto et al., 1996; Aloisi et al., 1994). Growth factors are integral to oligodendrocyte survival. PDGF, BDNF, bFGF, NT-3, IGF, LIF, NDF and CNTF promote oligodendrocyte survival in insufficient culture conditions. CNTF, IGF and LIF also protect oligodendrocytes from cytokine toxicity *in vitro.* CNTF and PDGF can attenuate oligodendrocyte death *in vivo* during development. Growth factors have also been implicated in reducing demyelination. In 1992 Komoly et al. observed an increase in astrocyte production of IGF in a cuprizone model of demyelination. This group further demonstrated that IGF treatment reduced demyelination in EAE (Yao et al., 1995). While is it likely that astrocytes promote oligodendrocyte survival via growth factors, there is no direct evidence to support this claim. It is conceivable that growth factors secreted from other cell types such as neurons and microglia may also facilitate oligodendrocyte survival.

Astrocytes may also aid in oligodendrocyte protection through detoxification of the extra-cellular milieu. In CNS pathology astrocytes increase their uptake of ions and neurotransmitters and increase production of detoxifying proteins. Detoxifying proteins produced by astrocytes include ApoE and cathepsin G (Norenburg, 1994). ApoE is involved in lipid removal following injury. Cathepsin G is involved in the destruction of proteases and toxic cytokines. Production of Cathepsin G may enable astrocytes to attenuate cytokine-induced oligodendrocyte death.

It is likely that excessive glutamate concentrations contribute to oligodendrocyte death following injury. As previously described glutamate and glutamate analogues induce oligodendrocyte death *in vitro*. Astrocytes possess a tremendous capacity for glutamate uptake. In a paper published by Ye et al. (1998) live astrocytes were shown to reduce the concentration of glutamate in culture medium from 90 μM to 1μM within 3h. Astrocytes are the primary source of glutamate uptake in the CNS. Increased glutamate uptake by reactive astrocytes may reduce oligodendrocyte death.

Astrocyte-oligodendrocyte interactions may also promote oligodendrocyte survival. Astrocytes are often found in extremely close proximity to oligodendrocytes in the normal brain and in MS plaques; it is proposed that this proximity promotes oligodendrocyte survival and remyelination (Wu & Raine, 1992). Astrocyte promotion of oligodendrocyte survival and remyelination may be mediated by integrins. In the normal brain astrocytes express $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins (Archelos et al., 1999). When activated astrocytes also express integrin binding molecules such as inter-cellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (Lee & Benveniste, 1999). Astrocyte expression of ICAM and VCAM is upregulated in MS lesions (Lee & Benveniste, 1999). Astrocyte ECM contains a large number of integrin binding proteins including fibronectin, vitronectin, laminin, tenascin, collagen and proteoglycan (Oh et al., 1995; McKeon et al., 1995; Laywell et al., 1992; Toru-delBauffe et al., 1992; Smith et al., 1995). Astrocyte ECM proteins are upregulated following CNS injury (Brodkey et al., 1995; Pasinetti et al., 1993; Geisert et al., 1996; Stichel et al., 1994). Oligodendrocytes express a number of integrins including $\alpha_1\beta_1$, $\alpha_6\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Archelos et al., 1999). As previously mentioned,

integrins are involved in cell survival. Activation of integrins initiates a signaling cascade that counters apoptosis. Integrins can mediate oligodendrocyte survival. Frost et al. (1999) demonstrated that neurons promote oligodendrocyte survival through an integrin-dependent mechanism; it is likely that astrocytes can promote oligodendrocyte survival through a similar mechanism. Integrins can also mediate oligodendrocyte function. Work from our laboratory and others has demonstrated that integrins promote oligodendrocyte process extension. We have demonstrated that astrocyte-oligodendrocyte interactions facilitate oligodendrocyte process extension (Oh & Yong, 1996). In this work the astrocyte-oligodendrocyte interactions involved oligodendrocyte binding to astrocyte ECM. In the presence of bFGF and PKC analogues, astrocyte ECM greatly enhanced oligodendrocyte process extension. There are reports that β_1 integrin is involved in oligodendrocyte process extension (Malek-Hedayat et al., 1994; Buttery et al., 1999).

The protective capacities of astrocytes have been demonstrated in the neuronal literature. Astrocytes have been shown to protect neurons from a variety of insults. Co-culture with live astrocytes and culture with astrocyte conditioned medium (ACM) protect neurons from spontaneous death (Ohgoh et al., 1998; O'Malley et al. 1994; Kilpatrick et al. 1993) and from serum deprivation (Dringen et al. 1999; Schamlenbach et al. 1993). Protection by live astrocytes and ACM involves astrocyte-derived growth factors (Ohgoh et al., 1998; O'Malley et al., 1994; Schamlenbach et al., 1993). Astrocytes have also been shown to protect neurons from oxidative stress. In co-culture, astrocyte-derived glutathione has been shown to protect neurons from glutathione depletion (Drukarch et al 1998; Blanc et al., 1998) and from H_2O_2 toxicity (Desagher et

al., 1996). Astrocyte ACM and ECM can also protect neurons from NO-induced death (Tanaka et al., 1999). In 1998, Ye et al. demonstrated astrocyte protection of neurons following glutamate toxicity. Co-culture with astrocytes significantly reduced glutamate levels and promoted neuronal survival.

Astrocytes can also promote neuronal function. Astrocytes promote neuronal growth through cell-ECM and cell-cell interactions. The astrocyte ECM protein laminin (McKeon et al., 1995) supports neurite outgrowth *in vitro*. Co-culture with astrocytes can promote neurite outgrowth through neural (N)-cadherin, neural cell adhesion molecule (NCAM) and β_1 integrin mediated astrocyte-neuron interactions (Smith et al., 1990; Neugebaher et al., 1998; Tomaselli et al., 1988; McKeon et al., 1995). It should be mentioned that some astrocyte ECM proteins adversely affect neuronal function. Tenascin and proteoglycans inhibit neurite growth *in vitro* (Powell et al., 1999; McKeon et al, 1995; Smith et al., 1995). It is thought that astrocyte production of inhibitory ECM proteins is detrimental to CNS recovery following trauma.

In summary, astrocytes have an immense potential for promoting oligodendrocyte survival. Astrocytes uptake glutamate and produce a variety of anti-oxidants, free radical scavengers, growth factors and detoxifying proteins; many of theses factors promote oligodendrocyte survival and function. Furthermore, astrocytes protect neurons from a variety of insults and can promote neuronal function. It is likely that astrocytes can protect oligodendrocytes from other insults such as growth factor deprivation, and glutamate and cytokine toxicity. It is also possible that astrocytes support oligodendrocyte survival through integrin-mediated mechanisms

It is the purpose of this work to demonstrate that astrocytes promote oligodendrocyte survival. Using an *in vitro* system in which oligodendrocytes undergo spontaneous death, we hypothesize that co-culture with live astrocytes will attenuate oligodendrocyte death. In the event that astrocytes attenuate oligodendrocyte death we will attempt to elucidate the mechanism(s) of the astrocyte protection.

CHAPTER TWO:

METHODS

Cell Culture

Astrocyte Culture

Neonatal mouse cells were derived from the brains of post-natal day 3 CD1 mice; human fetal cells were derived from human fetal brains. Cells were dissociated using trypsin digestion and isolated as previously described (Yong & Antel, 1997). Isolated cells were plated into 100 mm^2 culture dishes at an approximate density of 50 x 10^6/dish. Astrocyte cultures were maintained in Minimum Essential Medium (MEM) containing 0.1% dextrose, 100 micrograms/millilitre (μg/ml) penicillin-streptomycin, 1mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.2 mM glutamine and 10% fetal calf serum (FCS). Mouse cultures were passaged once before use; cell passaging consisted of treatment with 0.05% trypsin to collect adherent cells followed by a replating of the cells. Human fetal cells were passaged a minimum of three times before use. Mouse cultures generated with this method were previously characterized by our laboratory (Boutros et al., 1997). Cultures were shown to contain > 90% astrocytes. The major contaminants were fibroblasts (~5%) and microglia (~1%). Human fetal cultures generated with this method have also been characterized by our laboratory (Yong & Antel, 1997). Cultures were shown to contain > 99% astrocytes with some contaminating fibroblasts and microglia. No oligodendrocytes were present in either of these astrocyte cultures at the time of oligodendrocyte-astrocyte co-culture.

Fibroblast Culture

Fibroblasts were derived from the whole embryo of embryonic day 14 C57BL/6 mice. Cells were dissociated and isolated as described in detail elsewhere (Spector, 1997). Isolated cells were plated into 100 millimeter^2 (mm^2) culture dishes at an approximate density of 50 x 10^6/dish. Cells were passaged several times before use. Fibroblasts were maintained in astrocyte medium, described above.

Oligodendrocyte Culture

Mouse oligodendrocytes were derived from whole brains of 3-4 week old CD1 mice. Cells were dissociated with trypsin digestion and isolated by Percoll gradient, as previously described by our laboratory (Yong and Antel, 1997). Isolated cells, consisting mainly of oligodendrocytes, astrocytes and microglia, were plated into 25 mm^2 flasks at an approximate density of 12 x 10^6/flask and left overnight at 37° C. In contrast to astrocytes and microglia, oligodendrocytes are poorly adherent. Following adherence of astrocytes and microglia, floating cells were collected, generating enriched oligodendrocyte cultures. Oligodendrocytes were plated onto coated (10 μg/ml poly-ornithine) 16-well chamber slides at a density of 5 x 10^4 cells /well. Oligodendrocytes were maintained in MEM containing 0.2 mM glutamine, 0.1% dextrose, 1% penicillin-streptomycin and 10% FCS. Immunocytochemical characterization of theses cultures showed the cultures to contain > 90% oligodendrocytes. The major contaminants were astrocytes (~5%) and unidentified cells.

Oligodendrocyte-Astrocyte Co-culture

For oligodendrocyte-astrocyte co-culture, confluent astrocyte monolayers were removed using 0.25% trypsin, 0.02% ethylene diamine tetra acetic acid (EDTA) and 2% DNase in 1X Hanks Balanced Salt Solution (HBSS). Dissociated cells were plated onto coated (10 μg/ml poly-ornithine) 16-well chamber slides (Nalge Nunc International, Naperville, USA) at a density of 5 x 10^4 cells/well. 72h following astrocyte plating, enriched oligodendrocytes were plated onto the astrocytes at a density of 5 x 10^4 cells/well. Oligodendrocyte-astrocyte co-cultures were maintained in oligodendrocytes medium, described above. Medium was changed at 48h and 120h.

Modified Cultures: Co-culture, Lone culture and Culture with ACM

For modified co-cultures, astrocytes were trypsinized, as described above, and plated onto 0.4 micrometer (μm) 6-well cell culture inserts (Becton Dickinson, Franklin Lakes, USA) at a density of 25 x 10^4 cells/insert. 48h following astrocyte plating, oligodendrocytes were plated in a droplet onto coated (10 μg/ml poly-ornithine) aclar coverslips at a density of 10 x 10^4 cells/coverslip. The coverslips were placed in 6-well plates and left undisturbed for 24h to allow for oligodendrocytes adhesion to the coverslips. 24h following plating, astrocyte-inserts were introduced into the 6-well plates (Figure 5); the plates were then flooded with oligodendrocyte medium, described above.

For modified lone culture and modified culture with ACM oligodendrocytes were plated in the same manner as those in modified co-culture. In modified lone culture, however, the plates were flooded with oligodendrocyte medium, described above. In modified culture with ACM the plates were flooded with ACM, described below.

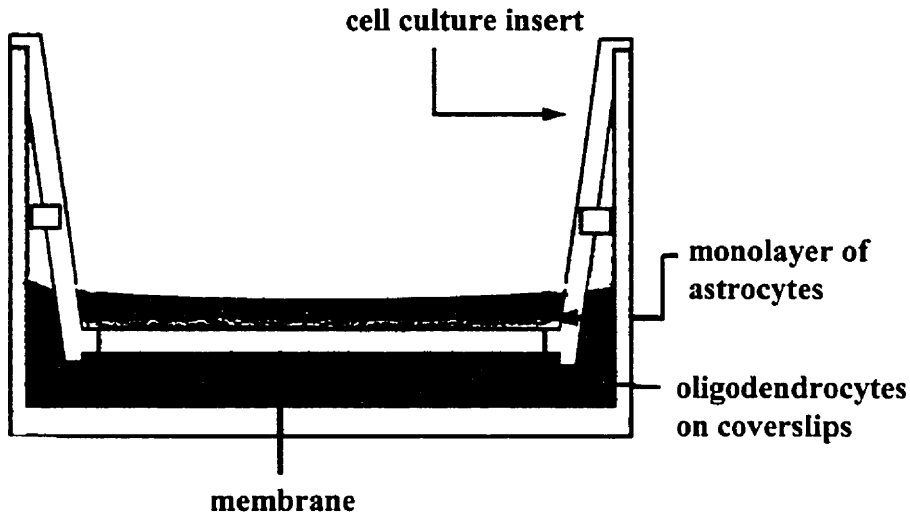

Figure 5. Modified oligodendrocyte-astrocyte co-culture

Generation of ACM and Astrocyte ECM

To generate ACM astrocytes were maintained in oligodendrocyte medium, described above. After 24h the medium was removed and centrifuged at 800 revolutions per minute (RPM) for 10 min to remove cellular debris. ACM was stored at 4° Celsius (C) and was used within one week of initial generation.

Astrocyte ECM was isolated from astrocytes plated in 16-well chamber slides. Astrocytes were lysed during a 1h incubation in distilled water leaving the ECM on the chamber slide (Oh and Yong, 1996).

Function Blocking Antibodies

Where indicated function blocking antibodies to growth factors were added to oligodendrocyte-astrocyte co-cultures at 1h, 24h and 48h following plating of oligodendrocytes. Mouse anti-rat CNTF (R&D Systems, Minneapolis, USA), mouse

anti-human basic FGF II (bFGF-II) (Upstate Biotechnology Institute, Lake Placid, USA), mouse anti-human insulin-like growth factor I (IGF-I) (Upstate Biotechnology Institute, Lake Placid, USA), mouse anti-human NT-3 (R&D Systems, Minneapolis, USA); these antibodies were used individually at 5 μg/ml or in combination. When used in combination the individual antibodies were used at 5 μg/ml each to give a total concentration of 20 μg/ml.

Kinase and Ras Inhibitors

Inhibitors of PI3-K, src kinases and PKC were added to oligodendrocyte/astrocyte co-cultures 48h following oligodendrocyte plating. All kinase inhibitors were used at concentrations in which the inhibitor activity is specific to the selected enzyme. LY294002 ([2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]) (Calbiochem) and Wortmannin (KY 12420) (Calbiochem) are selective inhibitors of PI3-K. Both LY294002 and Wortmannin act on the ATP-binding site of PI3-K. LY294002 has an IC_{50} of 1.4 μM (Vlahos et al., 1994). The IC_{50} of Wortmannin is 5nM (Okada et al., 1994). Bisindolylmaleimide I (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide) (GF109203X) (Calbiochem) is a selective inhibitor of PKC. Bisindolylmaleimide I act on the ATP-binding site of PKC and has an IC_{50} of 8 nM (Toulled et al., 1991). PP2 (4-amino-5-(4-cholorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) (AG1879) was used to inhibit the src family of tyrosine kinases. PP2 is a selective inhibitor of the src family. PP2 acts on the ATP-binding site of the src family of kinases and has an IC_{50} of 4 nM (Hanke et al., 1996). Farnesyltransferase inhibitor III (FT3) (Calbiochem) is an inhibitor of $p21^{ras}$ farnesyltransferase. FT3 inhibits

farnesyltransferase, an enzyme required for the processing of Ras. FT3 inhibits the recruitment of Ras; this inhibits the activation of Ras by membrane receptors. The IC_{50} of FT3 is 12 nM (Leftheris et al., 1994). Because FT3 inhibits Ras processing in cells and not Ras itself, FT3 was added earlier than the other kinase inhibitors. FT3 was added at 24h and 48h following oligodendrocyte plating.

Immunocytochemistry, Detection of DNA Fragmentation and Chromatin Condensation

Fixation of Cultures

Cultures were fixed in 4% paraformaldehyde at various time points. Depending on the experiment in question cultures were fixed at 24h, 48h, 72h, 120h or 7d following oligodendrocyte plating.

In Situ End Labeling of DNA Fragmentation

A terminal deoxynucleotidyl-transferase (TdT)-mediated nick end labeling (TUNEL) kit (Oncogene, Cambridge, USA) was used to detect DNA fragmentation. In a TUNEL assay, TdT catalyzes the addition of fluorescein-labeled nucleotides (deoxynucleotidyl triphosphate-fluorescein isothiocyanate) (dNTP-FITC) to the 3´OH ends of fragmented DNA.

The TUNEL assay was performed according to the manufacturer's instructions with minor modifications. Following fixation cells were incubated in buffer solution for 10 min at room temperature. After incubation in the buffer solution cells were incubated in TdT and dNTP-FITC for 1h at 37° C.

Immunocytochemistry

The glycolipid galactocerebroside (GalC) is a specific marker of oligodendrocytes (Bansal et al., 1989). Following fixation oligodendrocytes were incubated in mouse anti-GalC (Sommer & Schachner, 1981) for 30 min, followed by a 30 min incubation in goat anti-mouse Cy3 (Molecular Probes, Leiden, The Netherlands). GFAP was used as a marker of astrocytes; GFAP is an intermediate filament protein specific to astrocytes in the CNS (Norenberg, 1994). Following fixation astrocytes were incubated in 0.25% Triton-X 100 for 10 min, followed by a 30 min incubation in rabbit anti-GFAP (Dako, Glostrup, Denmark) and a 30 min incubation in goat anti-rabbit Alexa-488 (Molecular Probes, Leiden, The Netherlands).

Chromatin Condensation

Following immunocytochemistry cells were incubated in Hoescht 33852 (Sigma, Oakville, Canada) for 3 min at room temperature. Hoescht 33852 is a DNA fluorochrome that binds specifically to adenine-thymidine regions on DNA (McGarrity et al., 1983). Hoescht 33852 is routinely used as a marker of chromatin (Rivera et al., 1998; Levatte et al., 1998; Reed et al., 1995).

Analyses

Oligodendrocytes with DNA fragmentation (GalC+/TUNEL+ cells) were identified as dying oligodendrocytes. Oligodendrocyte death was expressed in percent as the number of dying oligodendrocytes/number of oligodendrocytes counted. A minimum

of 200 oligodendrocytes were counted per well or coverslip. All experiments were replicated at least three different times with n = 4 wells or coverslips each time. Statistical analyses were performed using SPSS, Version 8.0.

CHAPTER 3:

RESULTS

Mouse Oligodendrocyte Cultures

Mouse oligodendrocytes appeared to be quite healthy in culture. The majority of oligodendrocytes exhibit rounded cell bodies of approximately 8-10 μm in diameter; many oligodendrocytes displayed extensive processes and myelin sheaths (Figure 6).

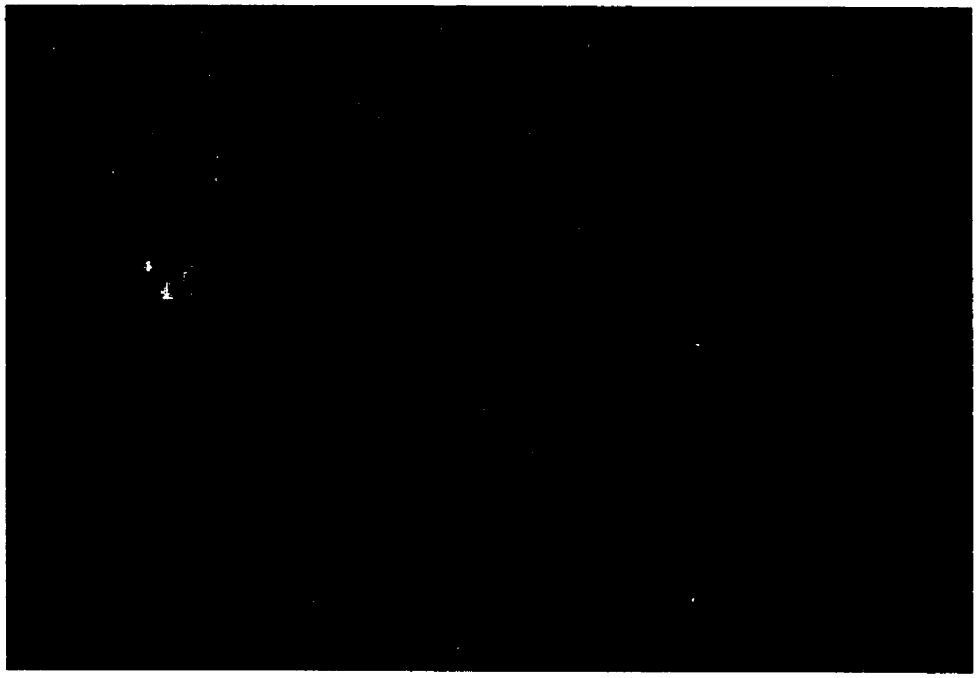

Figure 6. Mouse oligodendrocyte cultures
Oligodendrocytes are immuno-labeled for GalC (red), an oligodendrocyte-specific marker. Cell nuclei are labeled with Hoescht 33852 (blue). Oligodendrocytes exhibit uniform chromatin distribution characteristic of healthy cells.

Although the cultures appeared healthy the number of oligodendrocytes in culture decreased by nearly half over a 120h period (Figure 7). Closer examination revealed that many oligodendrocytes exhibited DNA fragmentation indicative of cell death. Using

TUNEL assay and immuno-labeling for GalC oligodendrocyte death was quantified over a period of 120h. After 24h in culture nearly 20% of mouse oligodendrocytes were positive for DNA fragmentation. From 48 to 120h oligodendrocyte death ranged from 24-28% (Figure 7).

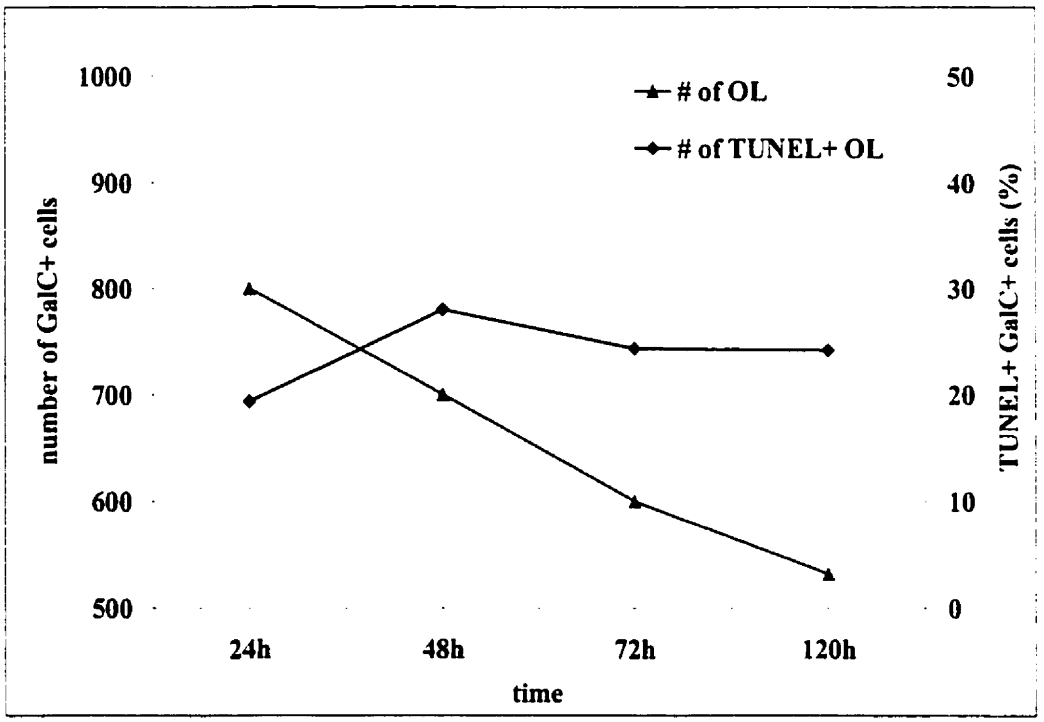

Figure 7. Mouse oligodendrocytes (OL) die in culture
Mouse oligodendrocyte cultures were harvested at several time points. GalC + TUNEL+ cells were identified as dying oligodendrocytes. Oligodendrocyte death is expressed in percent as the number of dying OL/number of OL counted. After 120h the number of oligodendrocytes in culture decreased by nearly half. This experiment was replicated three different times with n = 4 wells for each time point; a minimum of 200 oligodendrocytes were counted per well.

Dying oligodendrocytes exhibited morphological characteristics of apoptosis. Oligodendrocytes with DNA fragmentation appeared shrunken and compact. Hoescht 33852 stain revealed chromatin condensation in the nuclei of dying oligodendrocytes (Figure 8). Findings indicated that despite a healthy appearance mouse oligodendrocyte cultures contained a number of dying oligodendrocytes.

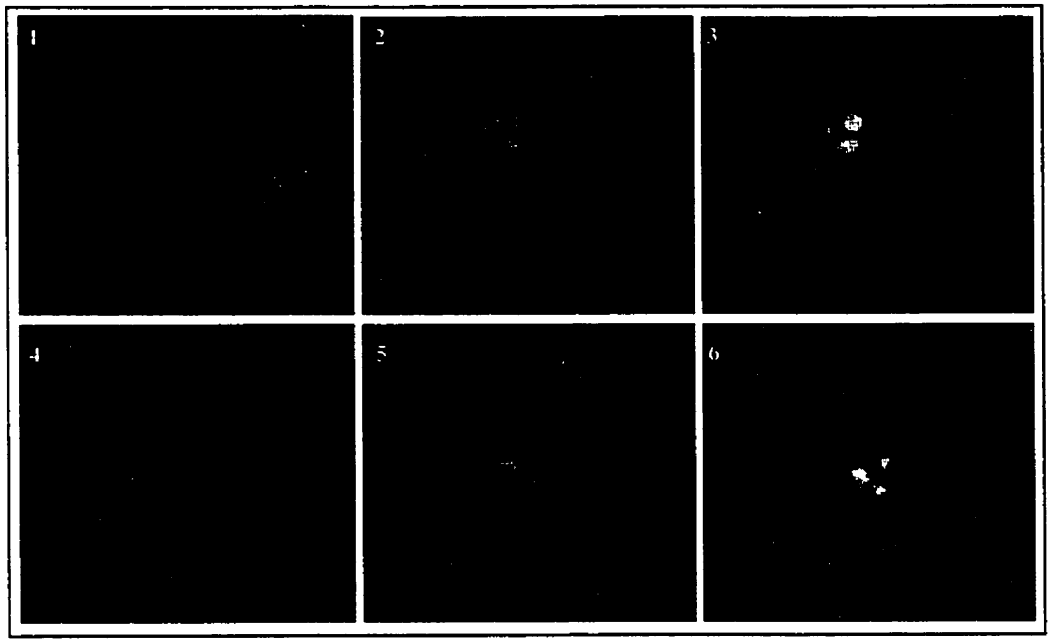

Figure 8. Dying oligodendrocytes exhibit chromatin condensation that is characteristic of apoptosis
Oligodendrocytes are labeled with GalC+ (red), TUNEL (green) and Hoescht 33852 (blue). Frames 1, 2 and 3: A dying oligodendrocyte found alongside a healthy oligodendrocyte. The dying oligodendrocyte exhibits chromatin condensation (Frame 1) and DNA fragmentation (Frame 2). Frame 3 demonstrates the co-localization of TUNEL and Hoescht staining. The healthy oligodendrocyte exhibits uniform chromatin distribution that is characteristic of a healthy, post-mitotic cell. Frames 4, 5 and 6: Another dying oligodendrocyte. The oligodendrocyte exhibits chromatin condensation (Frame 4) and DNA fragmentation (Frame 5). Frame 6 demonstrates the co-localization of TUNEL and Hoescht staining. The chromatin condensation is less extensive than that observed in Frames 1 and 3. It is likely that the cell is in an earlier stage of apoptosis.

Astrocyte Protection of Oligodendrocytes

Given the numerous beneficial properties of astrocytes we sought to determine whether co-culture with astrocytes could attenuate oligodendrocyte death. Co-cultures were generated by plating oligodendrocytes onto confluent monolayers of neo-natal mouse astrocytes (nmA) (Figure 9). Oligodendrocyte death was examined in co-culture

and lone-culture conditions. Death was quantified at 24h, 48h, 72h, 120h and 7d post-plating.

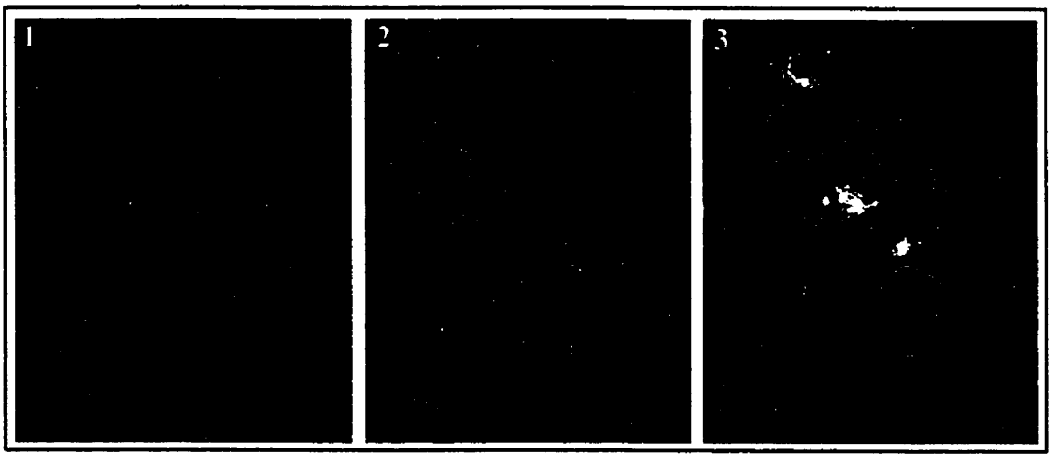

Figure 9. Oligodendrocyte-astrocyte co-culture
Oligodendrocytes are labeled for GalC+ (red), astrocytes are labeled for GFAP (green) cell nuclei are labeled with Hoescht 33852 (blue). Frame 1: Astrocytes Frame 2: Oligodendrocytes Frame 3: Frames 1 and 2 merged

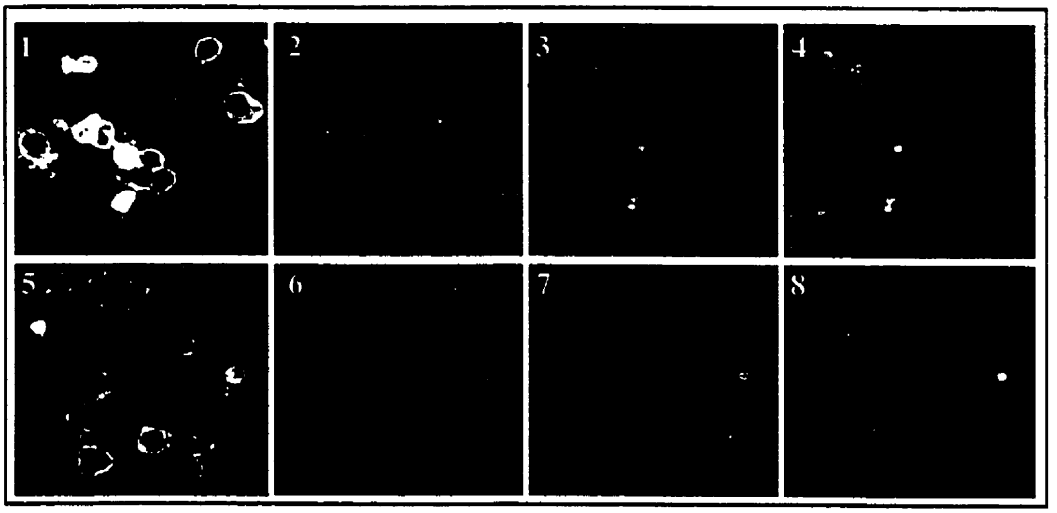

Figure 10. Co-culture with astrocytes attenuates oligodendrocyte death
Oligodendrocytes are labeled with GalC+ (white) (red), TUNEL (green) and Hoescht 33852 (blue). Frames 1-4: Oligodendrocyte death in lone culture. After 72h in lone culture more than 20% of oligodendrocytes exhibited DNA fragmentation. Frames 5-8: Oligodendrocyte death in co-culture. After 72h in co-culture with astrocytes less than 4% of oligodendrocytes exhibited DNA fragmentation. This experiment was replicated three different times with n = 4 wells in co-culture and lone culture conditions.

Co-culture with astrocytes attenuated oligodendrocyte death (Figure 10). At all time points examined co-culture with astrocytes significantly reduced oligodendrocyte death (Figure 11). Over a 7d period oligodendrocyte death in lone culture ranged from 20-30%; in co-culture oligodendrocyte death ranged from 2-13%.

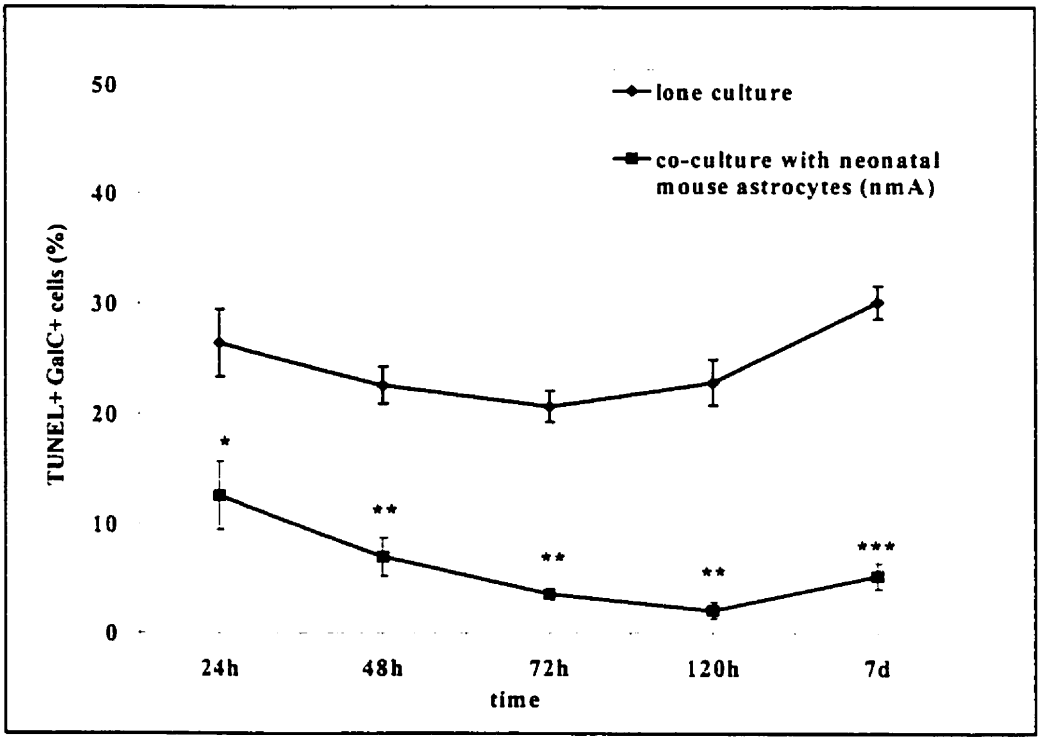

Figure 11. Time course of astrocyte protection
Oligodendrocyte death was examined in lone culture and in co-culture with astrocytes. At all time points examined oligodendrocyte death was significantly lower in oligodendrocyte-astrocyte co-culture than in lone oligodendrocyte culture. One-way ANOVA with Bonferroni post-hoc, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. This experiment was replicated three different times with $n = 4$ at each point on the graph.

After determining that neonatal mouse (nmA) astrocytes could attenuate oligodendrocyte death we investigated whether other cell types could also promote oligodendrocyte survival. We examined the roles of human fetal astrocytes (hfA) and mouse embryonic fibroblasts (meF). With respect to morphology, nmA, hfA and meF

are virtually indistinguishable (Figure 12B). 72h post-plating of oligodendrocytes we examined oligodendrocyte death in lone culture, co-culture with nmA, co-culture with hfA and co-culture with meF.

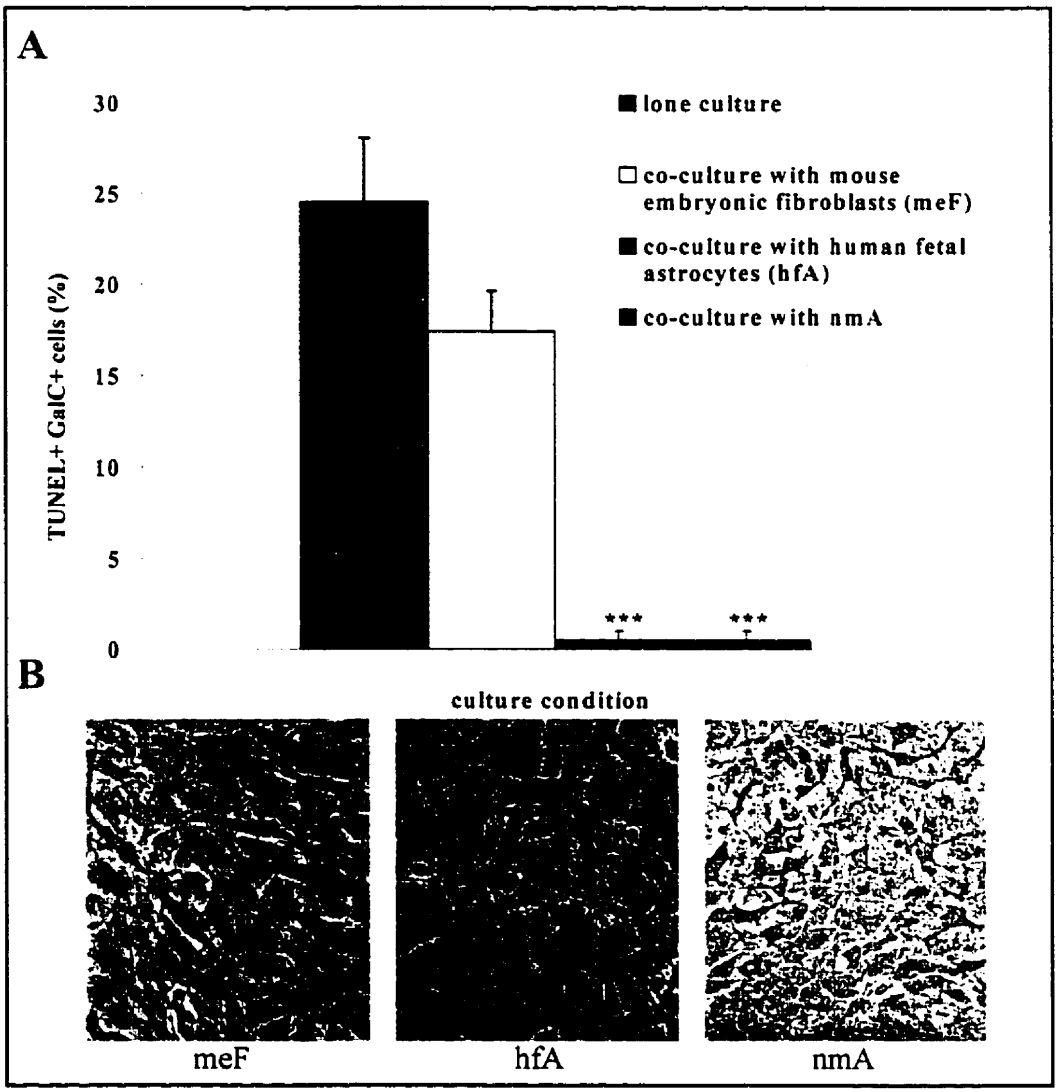

Figure 12. Astrocyte protection is cell type specific

A. Oligodendrocyte death was examined in lone culture and in co-culture with meF, hfA and neonatal mouse astrocytes (nmA). Co-culture with hfA and nmA attenuated oligodendrocyte death. Co-culture with meF did not prevent oligodendrocyte death. *** Value is significantly different than lone culture and co-culture with meF; one-way ANOVA with Bonferroni post-hoc, $p \leq 0.001$. This experiment was replicated three different times with $n = 4$ wells for each culture condition.

B. Phase contrast images of meF, hfA and nmA cultures. meF, hfA and nmA exhibit tremendous morphological similarities.

In comparison with lone culture, co-culture with hfA significantly attenuated oligodendrocyte death, $p \leq 0.001$. Co-culture with hfA provided the same amount of protection as co-culture with nmA. The finding that hfA protected oligodendrocytes suggested that astrocyte protection was not restricted to species; both mouse and human astrocytes attenuated the death of mouse oligodendrocytes. Co-culture with meF offered little protection and did not significantly reduce oligodendrocyte death (Figure 12A); this finding suggested that astrocyte protection was cell-type specific. Despite morphological and functional similarities to astrocytes, mouse fibroblasts did not attenuate oligodendrocyte death.

Mechanism of Astrocyte Protection: Soluble Factor or Contact Dependent Mechanism?

To elucidate the mechanism of astrocyte protection oligodendrocytes were cultured on astrocyte ECM or with ACM. After 72h in culture, the extent of oligodendrocyte death on ECM or with ACM was compared the extent of oligodendrocyte death in lone culture and in co-culture with astrocytes.

Neither ECM nor ACM protected oligodendrocytes. When plated on ECM oligodendrocyte cultures exhibited a similar amount of DNA fragmentation as lone oligodendrocyte cultures. When cultured with ACM oligodendrocyte cultures exhibited slightly less DNA fragmentation than lone oligodendrocyte cultures; this effect, however, was not significant. Co-culture with live astrocytes continued to attenuate oligodendrocyte death. In comparison with lone culture, ECM, and ACM, co-culture with astrocytes significantly reduced oligodendrocyte death, $p \leq 0.001$ (Figure 13).

Results indicate that astrocyte protection of oligodendrocytes is not mediated by astrocyte ECM or ACM. Astrocyte protection appears to require the presence of live astrocytes.

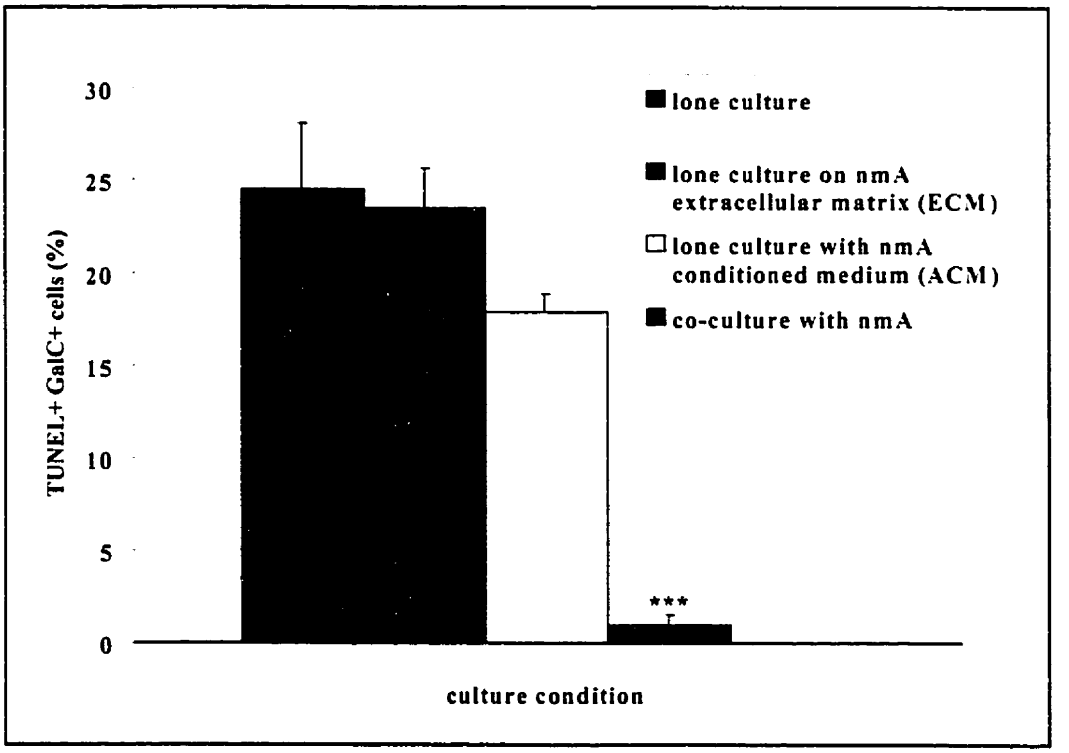

Figure 13. Astrocyte protection of oligodendrocytes requires the presence of live astrocytes
Oligodendrocytes were cultured alone, on astrocyte ECM, in ACM or in co-culture with astrocytes. Neither ECM nor ACM attenuated oligodendrocyte death. Co-culture with astrocytes reduced the amount of oligodendrocyte death. *** Value is significantly different than lone culture, culture on ECM and culture in ACM; one-way ANOVA with Bonferroni post-hoc, $p \leq 0.001$. This experiment was replicated three different times with $n = 4$ wells for each culture condition.

To resolve whether live astrocytes protected oligodendrocytes through a soluble factor or through cell-cell contact oligodendrocytes were cultured in a modified co-culture system. In modified co-culture oligodendrocytes were plated onto coverslips and astrocytes were plated onto cell culture inserts. The modified co-culture system allows oligodendrocyte-astrocyte interaction via soluble factors but prevented cell-cell contact. Oligodendrocyte death was quantified in modified co-cultures, lone cultures and lone

cultures with ACM. 72h following plating oligodendrocyte cultures exhibited extensive DNA fragmentation (Figure 14).

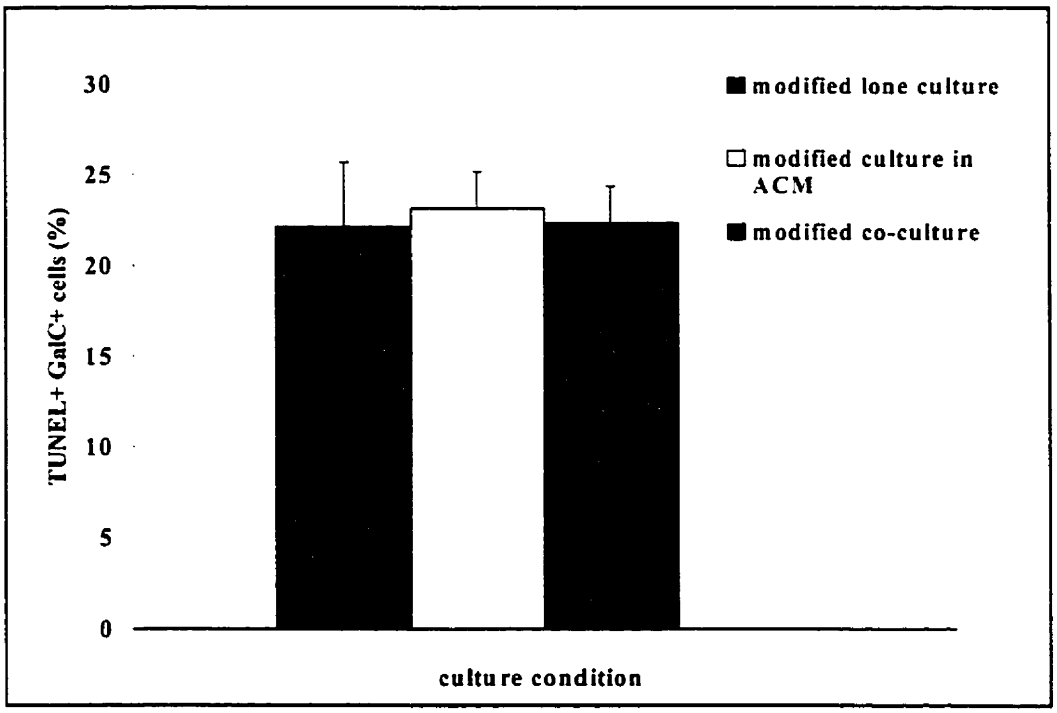

Figure 14. Astrocyte protection of oligodendrocytes requires cell-cell contact Oligodendrocytes were plated in modified co-culture, lone culture and culture in ACM. Cultures contained similar amounts of dying oligodendrocytes. In the absence of cell-cell contact astrocytes did not attenuate oligodendrocyte death. This experiment was replicated three different times with n = 4 coverslips for each culture condition.

There were no significant differences in oligodendrocyte death between any of the culture conditions. In the absence of cell-cell contact live astrocytes did not attenuate oligodendrocyte death. Thus it appears that astrocyte protection of oligodendrocytes requires cell-cell contact.

Involvement of Growth Factors

Many growth factors are anchored on the cell membrane; thus the requirement for cell-cell contact in astrocyte protection could be mediated by growth factors. To

determine if growth factors played a role in astrocyte protection, function blocking antibodies were added to oligodendrocyte-astrocyte co-cultures. Function blocking antibodies to CNTF, IGF, NT-3 and bFGF were used alone and in combination. The growth factors chosen represented four different families of growth factors. CNTF, IGF, NT-3 and bFGF are members of the IL-6 family of cytokines, the insulin-like growth factor family, the neurotrophin family, and the FGF family, respectively (Barres et al., 1992). Addition of function blocking antibodies alone or in combination had little effect on astrocyte protection of oligodendrocytes (Figure 15).

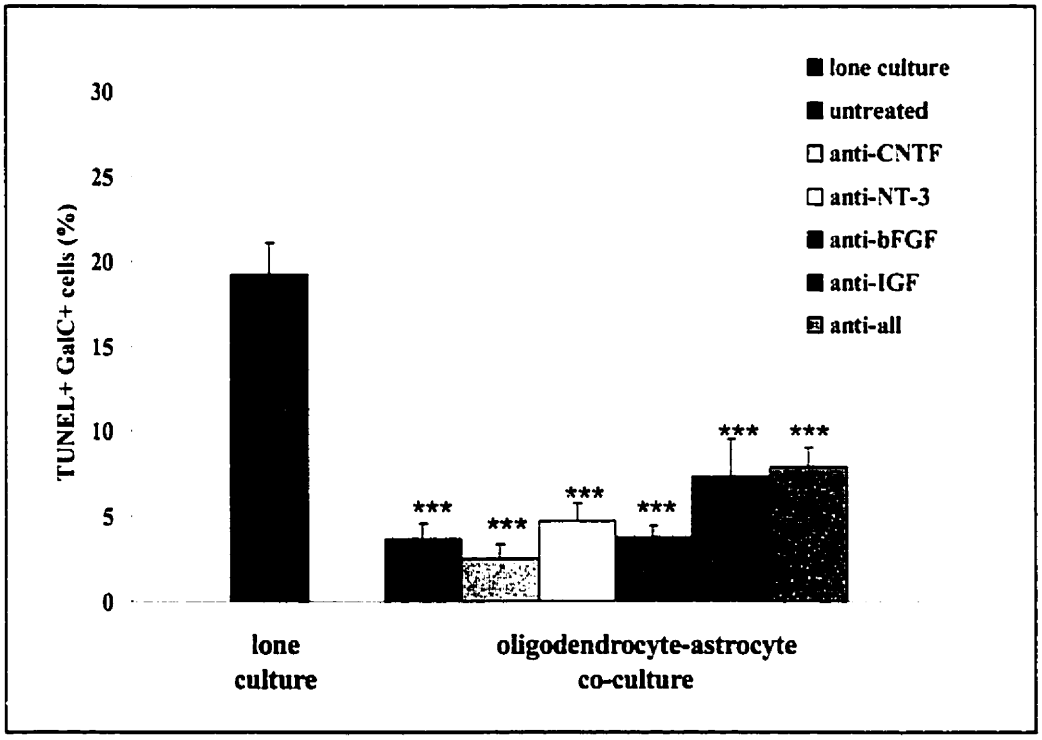

Figure 15. Growth factors do not appear to be involved in astrocyte protection of oligodendrocytes
Daily addition of function blocking antibodies did not affect astrocyte protection. *** Value is significantly different than lone culture; one-way ANOVA with Bonferroni post-hoc, $p \leq 0.001$. There were no significant differences in oligodendrocyte death between any of the co-culture conditions; one-way ANOVA with Bonferroni post-hoc.

Involvement of Intra-cellular Kinases and Ras

To elucidate possible intra-cellular mediators of astrocyte protection we investigated the effects of several kinase inhibitors. Inhibitors of PI3-K, src kinases, PKC and Ras were added to oligodendrocyte-astrocyte co-cultures.

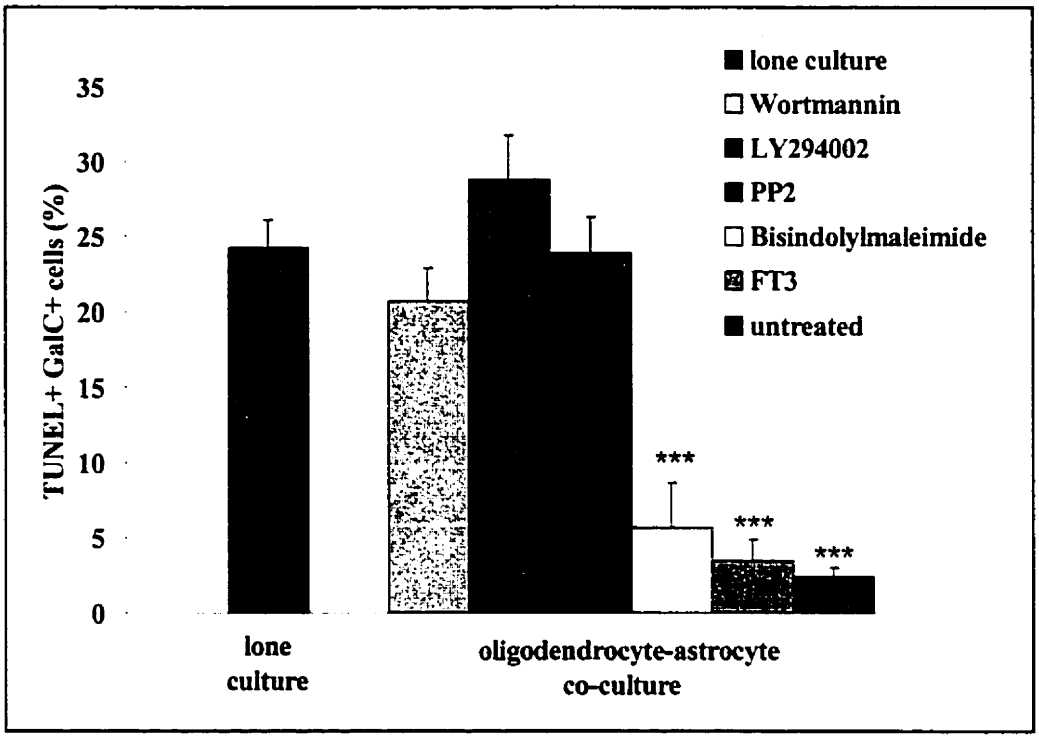

Figure 16. Effect of PI3-K, src kinases, PKC and Ras kinase inhibitors on astrocyte protection of oligodendrocytes
Inhibitors of PI3-K (Wortmannin and LY294002), src kinases (PP2), PKC (Bisindolylmaleimide), and Ras (FT3), were added to oligodendrocyte-astrocyte co-cultures. Treatment with 50nM Wortmannin, 50μM LY294002 or 5 μM PP2 negated astrocyte protection of oligodendrocytes. *** Value is significantly different than untreated lone cultures; one-way ANOVA with Bonferroni post-hoc, $p \leq 0.001$. Treatment with 3 μM Bisindolylmaleimide or 500 nM FT3 did not affect astrocyte protection. There were no significant differences in oligodendrocyte death between co-cultures treated with PP2, LY294002 or Wortmannin and untreated lone cultures; one-way ANOVA with Bonferroni post-hoc. This experiment was replicated three different times with n = 4 wells for each culture condition.

Treatment with the PKC inhibitor, Bisindolylmaleimide, or the Ras inhibitor, FT3, did not affect astrocyte protection of oligodendrocytes. Bisindolylmaleimide was used at 2

μM (Wu et al., 2000). FT3 was used at 500 nM (Hunt et al., 1996). PI3-K inhibitors, Wortmannin and LY294002, and an inhibitor of src kinases, PP2, significantly increased oligodendrocyte death in oligodendrocyte-astrocyte co-cultures (Figure 16). PP2 was added at 3 μM and 5 μM (Osterhout et al., 1999); the effects of PP2 were dose dependent (Figure 17). LY294002 and Wortmannin were used at concentrations of 50 and 10 μM and at 50 and 12.5 nM, respectively (Harvey et al., 2000, Wu et al., 2000). Wortmannin and LY294002 were only effective at the higher doses (Figure 18A and B). Kinase inhibitors did not appear to affect the integrity of astrocytes. Following treatment astrocytes maintained a healthy morphology and did not exhibit DNA fragmentation.

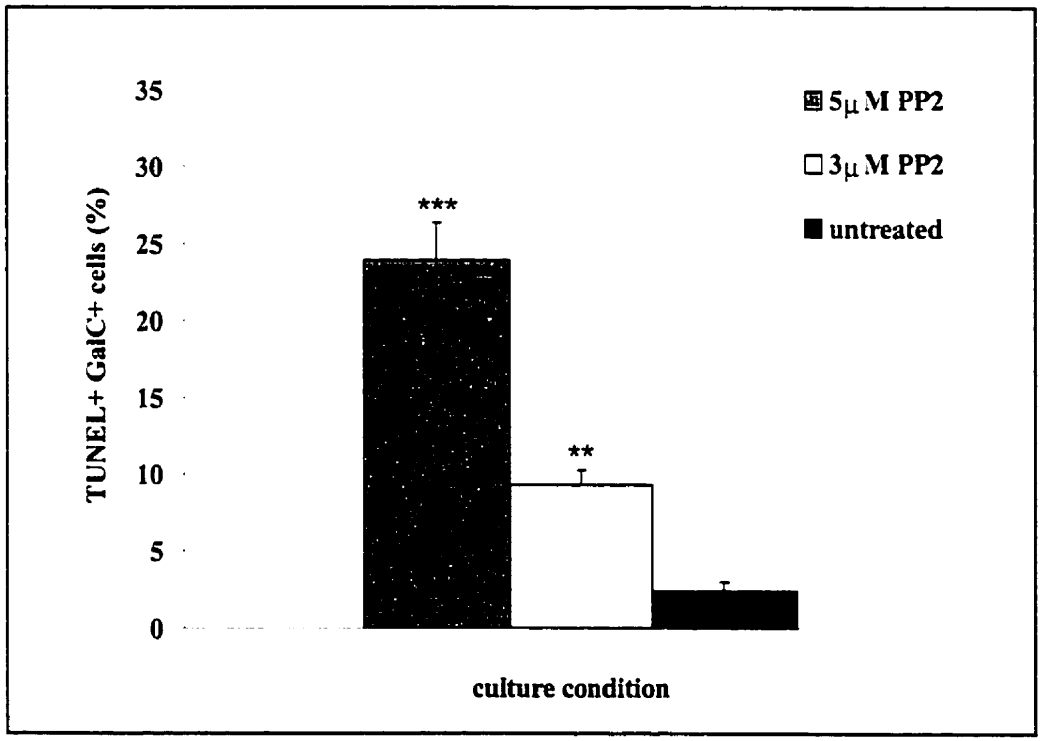

Figure 17. The src kinase inhibitor PP2 blocks astrocyte protection of oligodendrocytes.
5μM and 3μM PP2 increased oligodendrocyte death in co-culture with astrocytes. **, *** Value is significantly different than untreated co-cultures; one-way ANOVA with Bonferroni post-hoc, $p \leq 0.01$, $p \leq 0.001$. Experiments were replicated three different times with $n = 4$ well for each culture condition.

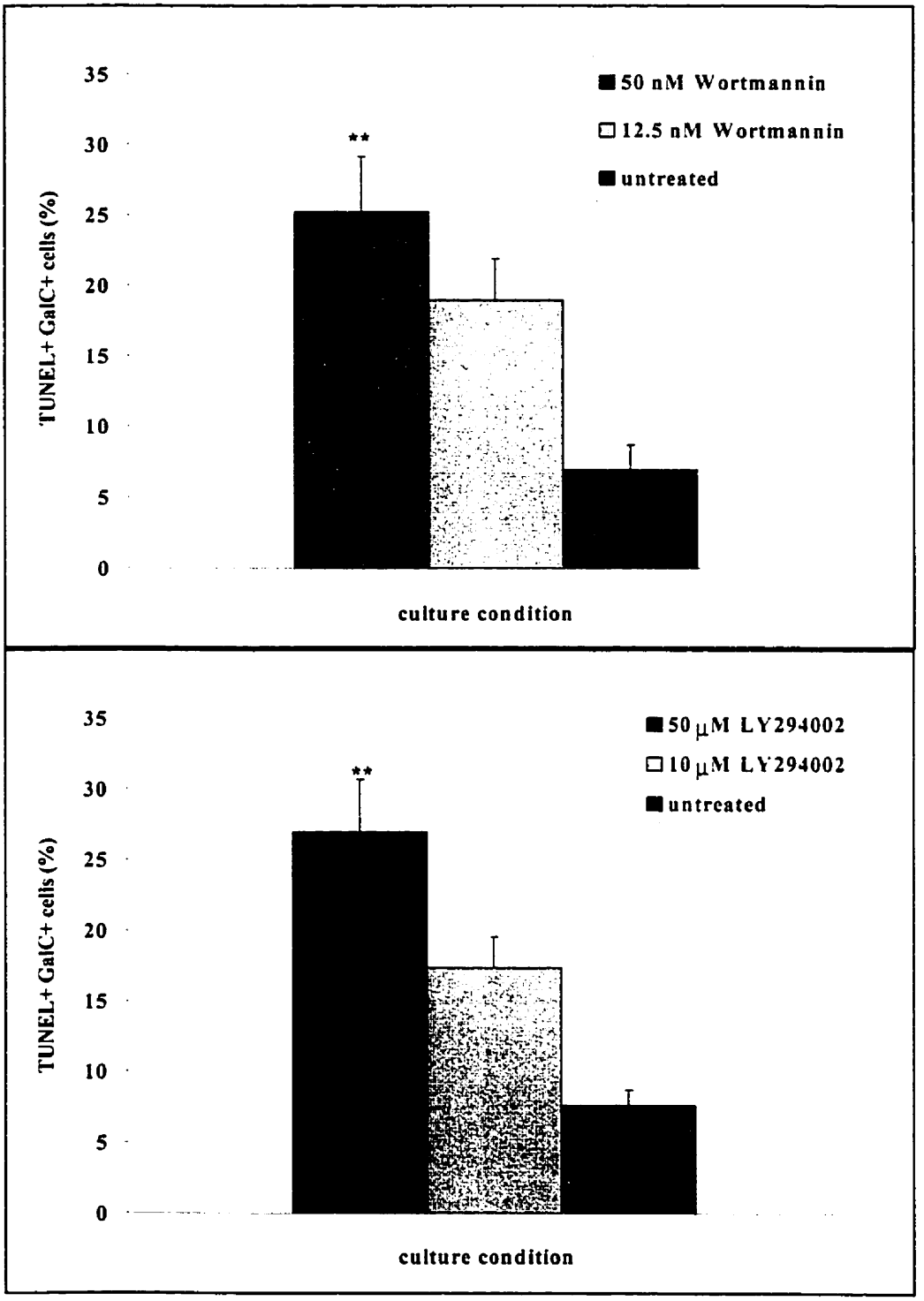

Figure 18. The PI3-K inhibitor Wortmannin blocks astrocyte protection of oligodendrocytes.
50nM and 12.5 nM Wortmannin and 50μM and 10μM LY294002 increased oligodendrocyte death in co-culture with astrocytes. *, ** Value is significantly different than untreated co-cultures; one-way ANOVA with Bonferroni post-hoc, $p \leq 0.05$, $p \leq 0.01$. Experiments were replicated three different times with $n = 4$ wells for each culture condition.

CHAPTER FOUR:

DISCUSSION

Oligodendrocyte death

In our mouse culture system oligodendrocytes undergo cell death (Figure7). Over a 7d period in culture oligodendrocyte death ranged from 20-30%. Oligodendrocyte death was accompanied by a substantial reduction in the number of oligodendrocytes in culture. Dying oligodendrocytes displayed morphological characteristics of apoptosis including DNA fragmentation and chromatin condensation (Figure 8).

The oligodendrocyte death we observed is consistent with reports of spontaneous death in culture. Essentially, spontaneous death is cell death that occurs in the absence of a known or intentionally applied insult. There are several reports of oligodendrocytes and neurons undergoing spontaneous death *in vitro* (Frost et al., 1999; Grinspan et al., 1996; Yasuda et al., 1995; Raabe et al., 1997; Ohgoh et al., 1998; O'Malley et al., 1994; Schamlenbach et al., 1993). There is evidence that spontaneous death results from either a lack of growth factors or a lack of cell-cell mediated signals. Addition of growth factors into the culture media can protect cells from spontaneous death. bFGF, IGF, PDGF, CNTF, and NDF can attenuate the spontaneous death of oligodendrocytes (Raabe et al., 1997; Grinspan et al., 1996; Yasuda et al., 1995). NT-3, NT-4, BDNF and GDNF can attenuate spontaneous death of neurons (Ohgoh et al., 1998). Cell-cell mediated survival signals also protect cells from spontaneous death. Neuron-oligodendrocyte contact can protect oligodendrocytes from spontaneous death (Frost et al., 1999) while astrocyte-neuron contact can protect neurons from spontaneous death (Schmalenbach et

al., 1993). Cells undergoing spontaneous death often exhibit signs of apoptosis. Raabe et al. (1997), Grinspan et al. (1996) and Yasuda et al. (1995) report morphological signs of apoptosis in oligodendrocytes undergoing spontaneous death.

It is likely that oligodendrocyte death in mouse cultures is the result of the absence of adequate survival factors including growth factors and/or cell-cell mediated signals.

Astrocyte protection of oligodendrocytes

Co-culture with astrocytes attenuates the spontaneous death of oligodendrocytes. Over a 7d period oligodendrocyte death in co-culture was significantly lower than oligodendrocyte death in lone culture (Figure 11). Astrocyte protection of oligodendrocytes appears to be cell type specific. meF did not protect oligodendrocytes from cell death. Astrocyte protection is not restricted to the mouse species. hfA offered the same amount of protection as neonatal mouse astrocytes (Figure 12).

The finding that astrocytes protect oligodendrocyte is not surprising. Astrocytes produce a number of factors that can promote oligodendrocyte survival including growth factors, antioxidants, and free-radicals scavengers. Astrocytes are also a major source of glutamate uptake in the CNS (Yong, 1998, Norenburg, 1994; Montgomery, 1994). In this regard it is likely that astrocytes can protect oligodendrocytes from growth factor deprivation, cytokine toxicity, oxidative stress, and glutamate toxicity. The protective capacities of astrocytes have been demonstrated in the neuronal literature. Live astrocytes and ACM protect neurons from a variety of insults including spontaneous death, serum deprivation, oxidative stress and glutamate toxicity (Ohgoh et al., 1998; O'Malley et al., 1994; Kilpatrick et al., 1993) (Dringen et al., 1999; Schamlenbach et al.,

1993; Drukarch et al., 1998; Blanc et al., 1998; Desagher et al., 1996; Tanaka et al., 1999; Ye et al., 1998). With respect to oligodendrocyte death astrocytes have been shown to protect oligodendrocytes from oxidative stress (Noble & Yong, 1995; Yonezawa et al., 1995). Here we demonstrate that astrocytes can also protect oligodendrocytes from spontaneous death.

Given the structural and functional similarities between fibroblasts and astrocytes it is surprising that meF are unable to attenuate oligodendrocyte death. Morphologically, fibroblasts and astrocytes are virtually indistinguishable (Figure 12B). Fibroblasts and astrocytes also share various functional similarities including growth factor production, glutamate uptake and expression of integrins and integrin binding molecules. Fibroblasts and astrocytes produce a number of the same growth factors including PDGF (Kawaguchi et al., 1999), NGF (Singh et al., 1997; Hattori et al., 1996; 1996), bFGF (Doan et al., 1999), and IGF (Yong, 1996). Fibroblasts can also produce hepatocyte growth factor (HGF) (Matsumoto et al., 1993; Miyazaki et al., 1998), LIF (Elias et al., 1994) and EGF while astrocytes also produce CNTF and NT-3. Fibroblasts and astrocytes also share a capacity for glutamate uptake (Cooper et al., 1998; Baclar et al., 1992). With respect to cell-cell and cell-ECM interactions fibroblasts and astrocytes express some similar integrins and integrin binding molecules. Fibroblasts and astrocytes both express $\alpha_i\beta_1$ (Herzhoff et al., 1999), $\alpha_2\beta_1$ (Herzhoff et al., 1999; Jenkins et al., 1998), $\alpha_3\beta_1$, $\alpha_5\beta_1$ (Dalton et al., 1999), $\alpha_6\beta_1$ (Jasiulionis et al., 1996), and $\alpha_6\beta_4$ integrins (Archelos et al., 1999). Fibroblasts and astrocytes express similar integrin binding molecules including ICAM (Pang et al., 1994; Yellin et al., 1995; Clayton et al., 1997; Sakurada et al., 1996; Iwasaki et al., 1999) and VCAM (Pang et al., 1994; Yellin et al.,

1995; Clayton et al., 1997; Lee & Benveniste, 1999) as well as several ECM proteins. Fibroblasts and astrocytes both produce fibronectin, laminin, tenascin, collagen and proteoglycans (Nunohiro T. et al., 1999; Kahari et al., 1996; Zhao et al., 1999; Nunohiro et al., 1999; Saika et al., 1996; Zhao et al., 1999; Goto et al., 1998; Dogic et al., 1999; Fischer et al., 1991; Kahari et al., 1996; Sakia et al., 1996; Fischer et al., 1991; Kao et al., 1990; Oh et al., 1995; McKeon et al., 1995; Laywell et al., 1992; Toru-delBauffe et al., 1992; Smith et al., 1995). While astrocytes and fibroblast do express some similar integrins and integrin binding molecules the configurations of these factors may differ. One function that astrocytes and fibroblasts do not appear to share is the production of antioxidants and free-radical scavengers. In 1999 Tanaka et al. demonstrated that fibroblasts could not protect neurons from oxidative stress. Astrocytes, however, were able to protect neurons; astrocyte protection was mediated by a soluble factor and astrocyte ECM. This paper demonstrates that while astrocytes and fibroblasts are similar cell types, there are differences in the functional properties of these cell types. It appears that astrocytes and fibroblasts secrete different soluble factors such as anti-oxidants or free radical scavengers. There is no evidence in the literature to suggest that fibroblasts produce either anti-oxidants or free radical scavengers. Furthermore while astrocytes and fibroblasts produce similar ECM proteins Tanaka et al. (1999) demonstrate that there are differences in fibroblast and astrocyte ECM. Astrocyte ECM protected neurons from oxidative stress; fibroblasts, on the other hand, did not exhibit this capacity. While fibroblasts and astrocytes share similar functional properties the results of this thesis and work by Tanaka et al. (1999) clearly indicate that fibroblasts lack the protective capacities of astrocytes. We have demonstrated that hfA share the protective capacities

of neonatal mouse astrocytes. To date there are no described differences in the structural or functional properties of astrocytes from different species.

Mechanism of astrocyte protection: soluble factor or contact dependent mechanism?

To elucidate the mechanism of astrocyte protection oligodendrocytes were cultured on astrocyte ECM or with ACM. Neither astrocyte ECM or ACM attenuated oligodendrocyte death (Figure 13).

To date in the literature, ECM proteins have not been shown to attenuate oligodendrocyte death. ECM proteins have been shown to promote neuronal survival. Laminin, fibronectin, and proteoglycans can attenuate neuronal death in response to various insults including spontaneous death, oxidative stress and glutamate toxicity (Drouet et al., 1999; Schmidt et al., 1995; Tanaka et al., 1999; Kappler et al., 1997; Nichol et al., 1995; Okamoto et al., 1994). ECM proteins have been shown to promote oligodendrocyte and neurite outgrowth. Astrocyte ECM as well as purified laminin and fibronectin can promote oligodendrocyte process extension *in vitro* (Oh et al., 1996, Buttery et al., 1999). Vitronectin, fibronectin, laminin, collagen, and proteoglycans can promote neurite outgrowth *in vitro* (Neugebauer et al., 1991; McKeon et al., 1995; Baldwin et al., 1996; Hirose et al., 1993). It should be noted that contrary reports have demonstrated that fibronectin and proteoglycans can also inhibit neurite outgrowth (Baldwin et al., 1996; McKeon et al., 1995; Smith et al., 1995). Furthermore other ECM proteins such as tenascin also inhibit neurite outgrowth (Powell et al., 1999).

Our finding that astrocyte ECM did not attenuate oligodendrocyte death is consistent with the literature on oligodendrocyte death; there are no reports of any ECM or ECM proteins attenuating oligodendrocyte death. Given the literature on astrocyte ECM and ECM proteins promoting oligodendrocyte process extension, neurite outgrowth and neuronal survival it seems plausible that astrocyte ECM could promote oligodendrocyte survival. It is possible that some of the astrocyte ECM proteins are conducive to oligodendrocyte survival while other proteins are not. The neuronal literature has demonstrated the duplicity of the ECM proteins. While the majority of ECM proteins are conducive to neurite outgrowth tenascin, fibronectin and proteoglycans can inhibit neurite outgrowth (Baldwin et al., 1996; McKeon et al., 1995; Smith et al., 1995; Powell et al., 1999). We have previously shown that astrocyte ECM generated in our laboratory contains fibronectin, vitronectin, and laminin; these ECM proteins were detected with Western Blot (Oh et al., 1996). It is possible that the some astrocyte ECM proteins are 'good' in that they promote oligodendrocyte survival while other ECM proteins are 'bad' in that they inhibit oligodendrocyte survival. In this regard it may be that the astrocyte ECM used in our experiments does not contain sufficient amounts of 'good' ECM proteins. Culture of oligodendrocytes on purified ECM proteins would elucidate the survival promoting properties of individual ECM proteins. Work in this thesis demonstrates that astrocyte ECM, which contains a number of ECM proteins, does not promote oligodendrocyte survival.

The finding that ACM did not protect oligodendrocytes suggests that soluble factors are not involved in astrocyte protection of oligodendrocytes in our culture system. Currently there is only one report of ACM attenuating oligodendrocyte death. In 1996,

Yonezawa et al. demonstrated that ACM could attenuate oligodendrocyte death induced by cystine depletion. The mechanism by which ACM attenuated oligodendrocyte death was not established. Presumably the ACM contained a soluble factor that could attenuate oxidative stress, this factor may have been an antioxidant or a free-radical scavenger. The protective capacities of ACM have been demonstrated in the neuronal literature; ACM has been shown to protect neurons from spontaneous death, serum deprivation and oxidative stress (Ohgoh et al., 1998; O'Malley et al., 1994; Kilpatrick et al., 1993; Dringen et al., 1999; Schamlenbach et al., 1993). In this regard it is likely that ACM can protect oligodendrocytes from similar insults. In our system ACM did not protect oligodendrocytes from spontaneous death. As mentioned earlier, spontaneous death of oligodendrocytes can be attenuated either through growth factors or through cell-cell mediated mechanisms. Given that ACM did not protect oligodendrocytes in our system astrocyte protection may involve cell-cell contact.

The finding that ACM does not attenuate oligodendrocyte death does not rule out the involvement of soluble factors. ACM does not allow for oligodendrocyte-astrocyte interaction. It is possible that oligodendrocytes need to signal to astrocytes to increase production of certain factors. Oligodendrocyte-astrocyte signaling could involve soluble factors or cell-cell mediated mechanisms. To resolve whether and oligodendrocyte-astrocyte interaction was needed for astrocyte protection we introduced a modified co-culture system. In the modified co-culture system oligodendrocytes were plated onto coverslips and astrocytes were plated onto cell culture inserts; this system allows oligodendrocyte-astrocyte interaction via soluble factors but prevents oligodendrocyte-astrocyte interaction via cell-cell contact.

In the absence of cell-cell contact, astrocytes did not attenuate oligodendrocyte death (Figure 14). This finding demonstrates that astrocytes promote oligodendrocyte survival through a cell-cell contact mediated mechanism. This finding does not eliminate the possibility that soluble factors are involved in astrocyte protection. Astrocyte-oligodendrocyte contact may promote oligodendrocyte survival by stimulating the production of soluble survival factors or by directly activating contact mediated survival signals. Direct protection through cell-cell contact could involve a survival signal through a membrane bound molecule such as a membrane bound growth factor or CAM. Regardless of downstream mechanisms of protection, astrocyte protection of oligodendrocyte is mediated by cell-cell contact. Oligodendrocyte-astrocyte contact can involve gap junctions, CAMs, growth factors and other membrane bound molecules (Zahs et al., 1998; Archelos et al., 1999; Hurwitz et al., 1992; Fok-Seang 1998; Lee & Benveniste, 1999). Because both growth factors and CAMs stimulate cell survival pathways we chose to focus on these factors.

Involvement of growth factors

To determine whether growth factors were involved in astrocyte protection of oligodendrocytes we treated astrocyte-oligodendrocyte co-cultures with function blocking antibodies to various growth factors. Function blocking antibodies to CNTF, NT-3, IGF and bFGF used alone or in combination did not negate astrocyte protection of oligodendrocytes (Figure 15). This finding does not eliminate the possibility that growth factors are involved in astrocyte protection. It is possible that the function blocking antibodies were not used in high enough concentrations; the amount of growth factors

secreted by astrocytes may be higher than that neutralized by the function blocking antibodies. Furthermore, astrocytes produce a large number of growth factors including PDGF, BDNF, NGF, FGF, NT-3, IGF, GDNF, CNTF, and LIF (Yong, 1996; Appel et al., 1997 Moretto et al., 1996; Aloisi et al., 1994). It is likely that these growth factors have redundant functions. In this regard negating only four of these growth factors does not eliminate the possibility that growth factors are involved. To elucidate the role of growth factors in astrocyte protection of oligodendrocyte it would be best to inhibit the function of many if not all astrocyte-derived growth factors.

The difficulties we encountered in investigating the role of growth factors also apply to the involvement of CAMs. Astrocytes express CAMs molecules from the integrin, cadherin, selectin and immunoglobulin superfamilies (Archelos et al., 1999; Hurwitz et al., 1992; Fok-Seang 1998; Lee & Benveniste, 1999). Each of these families contain a large number of CAMs. Given the number of growth factors and CAMs that astrocytes express, attempting to inhibit CAMs or growth factors individually or in combination did not seem like a very efficient approach to determining the factor(s) involve in astrocyte protection. With this in mind we decided to target downstream effectors of CAM and growth factors activation. In this approach it would be possible to inhibit large groups of CAMs and growth factors which could narrow our search of the factor(s) mediating astrocyte protection of oligodendrocytes.

Involvement of Intra-cellular Kinases and Ras

We investigated the role of several intra-cellular kinases and Ras in astrocyte protection of oligodendrocytes. Inhibitors of src kinases and PI3-K negated astrocyte

protection of oligodendrocytes. Inhibitors of PKC and Ras had no effect on astrocyte protection (Figure 16).

The kinases we investigated are involved in either growth factor or integrin signaling. Growth factors and integrins activate cell survival pathways; these survival pathways center around the involvement of PI3-K. Growth factors can activate PI3-K via Ras (Giancotti & Ruoslahti, 1999; Kumar, 1998). Integrins can activate PI3-K through src kinases (Giancotti & Ruoslahti, 1999) (Figure 3). PI3-K is involved in activating several factors within the cell such as Akt and PKC (Leevers et al., 1999; Prescott, 1999; Rameh & Cantley, 1999; Franke et al., 1997). Both Akt and PKC are involved in cell survival. Akt promotes cell survival through phosphorylation of bad, caspase-9 and Forkhead transcription factors or through activation of NFκ–B (Giancotti & Ruoslahti, 1999; Kumar, 1998, Datta et al., 1999) (Romashkova & Makarov, 1999) (Figure 3). PKC can promote cell survival through activation of NFκ–B (Wooten, 1999), activation of Akt and through upregulation of the anti-apoptotic gene bcl-2 (Genestier et al., 1995). It should be noted that PKC has a dual role in cell survival; PKC can be involved in both the induction of cell death and the promotion of cell survival (Lucas et al., 1995; Lavin et al., 1996).

Inhibiting components of growth factor signaling did not affect astrocyte protection of oligodendrocytes; the Ras inhibitor FT3 did not negate astrocyte protection of oligodendrocytes. Inhibitors of PI3-K did negate astrocyte protection of oligodendrocytes. To date there is no evidence that growth factors can activate PI3-K independent of Ras activation. It should be noted that only growth factors that activate tyrosine kinase growth factor receptors promote cell survival via Ras and PI3-K. Growth

factors that activate tyrosine kinase receptors include the IGF, neurotrophin, FGF, GDNF and PDGF families of growth factors (Butler et al., 1998; Segal & Greenberg, 1996; Bikfalvi et al., 1997; Malarkey et al., 1995). Astrocyte-derived growth factors that do not promote cell survival directly through Ras and PI3-K include CNTF and LIF. CNTF and LIF are both members of the IL-6 family of cytokine receptors; CNTF and LIF activate a janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway (Ip & Yancopoulos, 1996). It is possible that CNTF and LIF mediate astrocyte protection of oligodendrocytes. Results from the experiments on the effect of function blocking antibodies to growth factors, however, suggest that CNTF is not involved. Results of the kinase inhibitor experiments together with the results of the function blocking antibodies to growth factor experiments provide evidence that growth factors may not be involved in astrocyte protection.

Inhibiting components of integrin signaling did affect astrocyte protection of oligodendrocytes; the src kinase inhibitor PP2 as well as inhibitors of PI3-K, Wortmannin and LY294002, negated astrocyte protection of oligodendrocytes. These findings suggest that integrins may be involved in astrocyte protection of oligodendrocytes. It is possible that astrocyte protection involves astrocyte binding to oligodendrocytes via integrins. Activation of integrins on the oligodendrocyte may activate src kinase members. src kinase members, in turn, can activate PI3-K, which can promote oligodendrocyte survival (Figure 19).

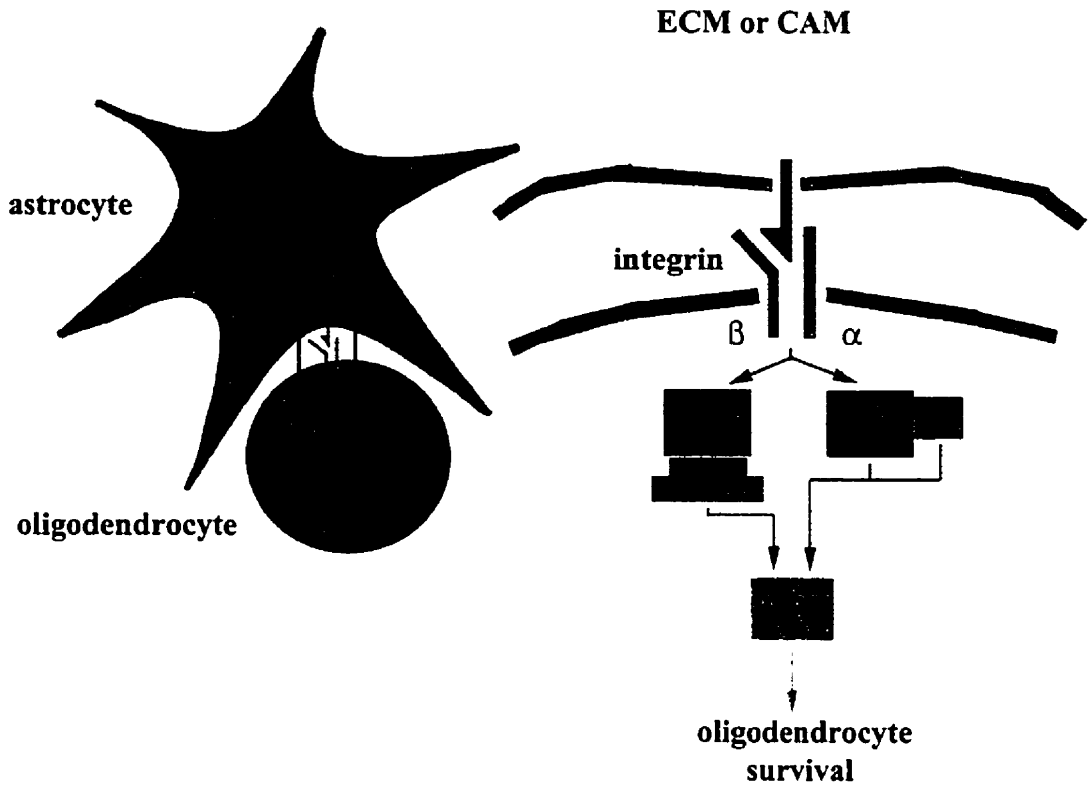

Figure 19. Astrocyte protection of oligodendrocyte may involve an integrin mediated mechanism
A. An astrocyte (green) and an oligodendrocyte (red) interacting through an integrin
B. Astrocyte-oligodendrocyte contact through integrins may promote oligodendrocyte survival

The finding that the PKC inhibitor Bisindolylmaleimide did not negate astrocyte protection provides some evidence of how PI3-K may be promoting oligodendrocyte survival. PI3-K can promote cell survival through activation of PKC or through activation of Akt (Leevers et al., 1999; Prescott, 1999; Rameh & Cantley, 1999; Franke et al., 1997). The finding that Bisindolylmaleimide did not effect astrocyte protection suggest that PI3-K is promoting cell survival either through Akt or through a novel mechanism. The kinase inhibitor experiments elucidated possible intra-cellular mediators

of astrocyte protection of oligodendrocytes. The finding that src kinase and PI3-K inhibitors negate protection allow us to theorize as to possible mechanisms of protection. Future directions of the described research should be aimed at determining the extra- and intra-cellular mechanisms involved in astrocyte protection of oligodendrocytes.

With respect to extra-cellular factors research demonstrated in this thesis suggests that integrins may mediate astrocyte protection. In this regard, future experiments should be aimed at determining which integrin(s), if any, mediate oligodendrocyte survival. To date, oligodendrocytes have been shown to express $\alpha_1\beta_1$, $\alpha_6\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins (Archelos et al., 1999). To demonstrate whether integrins are involved, function blocking antibodies to several α and β integrin sub-units, either alone or in combination, could be added to oligodendrocyte astrocyte co-cultures. Another possible experiment would be to determine whether activation of oligodendrocyte integrins can protect oligodendrocytes in lone culture.

We have proposed that astrocytes activate integrins on oligodendrocytes which activates src kinase members, PI3-K and Akt and promotes oligodendrocyte survival (Figure 19). There are several experiments that could confirm the intra-cellular mediators of astrocyte protection. The most obvious experiment would be to demonstrate that in co-culture with astrocytes oligodendrocytes exhibit increased activation of src kinases, PI3-K and Akt. This experiment would require assays for the activity of these kinases and comparisons of kinase activity in oligodendrocytes in lone culture and in co-culture with astrocytes. Kinase assays should also be done to confirm the inhibitory effects of the pharmacological kinase inhibitors that we have used. Pharmacological activators of src kinases, PI3-K and Akt would be useful; activators of these kinases

should promote oligodendrocyte survival in lone culture. Unfortunately pharmacological activators of src kinases, PI3-K and Akt are not available. In this regard adenoviruses expression dominant-negative and dominant-positive constructs of src kinase members, PI3-K and Akt would be very useful. With dominant-negative and dominant-active adenovirusal recombinants a possible experiment would be to introduce the adenoviruses into oligodendrocytes; if astrocytes protect oligodendrocytes via src kinases, PI3-K and Akt, astrocytes should not be able to protect oligodendrocytes that been infected with dominant-negative adenoviral recombinants to these kinases. A finding that astrocytes could not protect oligodendrocytes infected with dominant-negative adenoviral recombinants would support current data that pharmacological inhibitors of src kinase members and PI3-K negate astrocyte protection. In the same regard introducing adenviruses that express dominant-active constructs of either src kinase members, PI3-K or Akt should attenuate oligodendrocyte death in lone culture.

In summary this thesis demonstrates that astrocytes attenuate the spontaneous death of mouse oligodendrocytes. Astrocyte protection appears to be cell type but not species specific; while mouse fibroblasts cannot protect oligodendrocytes, human astrocytes share the protective capacities of mouse astrocytes. Astrocyte protection of oligodendrocytes is mediated by oligodendrocyte-astrocyte contact. It appears as though astrocyte protection of oligodendrocytes does not involve growth factors. Astrocyte protection of oligodendrocyte was not negated by function blocking antibodies to growth factors or by the Ras inhibitor FT3. Astrocyte protection was negated by an inhibitor of src kinases, PP2 and inhibitors of PI3-K, Wortmannin and LY 294002. We propose that astrocyte protection of oligodendrocytes is mediated by an integrin dependent mechanism

involving src kinases and PI3-K. Based on the finding that PKC inhibitor, Bisindolylmaleimide, did not negate astrocyte protection, it is likely that PI3-K promotes cell survival through activation of Akt. Future experiments will confirm both the extra- and intra-cellular mediators of astrocyte protection of oligodendrocytes

There is a long standing debate as to whether astrocytes play a beneficial or detrimental role in CNS function following injury. Following injury astrocytes form what is referred to as a glial scar. The formation of the glial scar has been proposed as a detrimental function of astrocytes following injury (Liuzii & Lasek, 1987). Glial scars are often associated with abnormalities in CNS function such as the generation of epileptic foci and impairments in neuronal regeneration (Davies et al., 1997; McKeon et al., 1995). In recent years it is becoming more and more evident that astrocytes play a beneficial role in CNS function following injury. As mentioned, following injury astrocytes exhibit increased production of growth factors, free-radical scavengers and anti-oxidants and increased uptake of glutamate. Astrocytes have also been shown to promote neuronal survival and the function of both neurons and oligodendrocytes. The finding that astrocytes attenuate oligodendrocyte death further supports the notion that astrocytes play a beneficial role in CNS function following injury.

With respect to oligodendrocyte death it is clear that while astrocytes possess the capacity to protect oligodendrocytes, oligodendrocytes still die in MS and CNS injury. Further knowledge of the mechanisms by which astrocytes protect oligodendrocytes will allow for manipulation of the protective capacities of astrocytes. Increasing the protective capacities of astrocytes in CNS injury and MS may enhance oligodendrocyte

survival; oligodendrocyte survival may prevent the demyelination and subsequent impairments in CNS function that occur in these conditions.

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