2006

Assessing Epidermal Growth Factor Expression in the Rodent Hippocampus Following Traumatic Brain Injury

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ASSESSING EPIDERMAL GROWTH FACTOR EXPRESSION IN THE RODENT HIPPOCAMPUS FOLLOWING TRAUMATIC BRAIN INJURY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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May, 2006

Acknowledgements

I would first like to thank my advisor, Dr. Raymond Colello, for the support, encouragement, and insight that he has given me throughout my time in his laboratory. As both my professor and mentor, I have experienced his ability to captivate his students and audience with his enthusiasm for science, teaching skill, vitality, and sense of humor. Because of these qualities combined with his intelligence, patience, and ambition that I have witnessed in his laboratory, I feel honored and privileged to have had the opportunity to work with him.

I would also like to extend my thanks to the other members of my committee. Dr. Dong Sun has provided me with a great amount of time, assistance, advice, and continuous support throughout this year, and for that I am extremely thankful. I experienced her persistence combined with scientific knowledge, and it was a pleasure to work with her. Dr. John Bigbee has also served as a valuable resource, with the offering of his scientific insight being greatly appreciated. They both demonstrate exceptional examples of the genuine dedication of the faculty of the medical school community to helping students learn and appreciate scientific research.

The members of my laboratory, Woon Chow, Melissa McGinn, and Sarah Hagood have also been a source of support, assistance, insight, and encouragement. Their willingness to lend a hand and answer questions as well as share laughs and conversation made being in the lab that much more of an enjoyable experience.

Lastly, I would like to thank my family and friends for their continuous support and encouragement while I have studied at VCU. I especially thank my parents, Virgil and Marides Daus, for their unconditional love and support they have always given me.

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Hippocampal neurons are vulnerable to injury, as indicated by the prevalence of learning and memory deficits following traumatic brain injury. Research indicates that proliferation of neural precursor cells increases following brain injury, which implies that there is an endogenous response in the hippocampus to replenish neurons and restore cognitive function. Studies show that mitogenic growth factors may drive this proliferative response; one of which is epidermal growth factor. Because adults and the elderly manifest the most enduring deficits following TBI, it is critical to investigate how EGF expression following injury may relate to injury-induced cell proliferation and the degree of cognitive recovery observed with aging. In the current study, we assessed the temporal and spatial expression of EGF in the injured hippocampus with

age. Our results suggest that EGF expression increases following TBI, and this increase is more significant in the younger brain. Additionally, we investigated the phenotype and localization of cells that express EGF following injury.

Chapter 1

Introduction to Epidermal Growth Factor

Traumatic brain injury (TBI) is a leading cause of death and long-term disability in the United States, with millions of Americans sustaining a brain injury each year. The central nervous system (CNS) is highly vulnerable to injury due to its limited capacity to replenish neurons once they have been damaged or destroyed. Specifically, research indicates that loss of hippocampal neurons contributes to the cognitive deficits manifested in patients following TBI. Research is advancing to elucidate the biological basis and innate repair mechanisms underlying these cognitive impairments in hopes of developing an effective cure. The existence of neural stem and progenitor cells in the mammalian CNS suggests that the CNS retains some regenerative potential throughout life. These persistent cells are found in the two active germinal zones of the CNS: the subventricular zone (SVZ) lining the lateral ventricle and the dentate gyrus (DG) of the hippocampus. Research has revealed that these precursor cells proliferate and differentiate into neurons and glia both during animal development and into adulthood. Additionally, it has been found that the level of proliferation of these cells is significantly increased following injury. Observed levels of this heightened proliferation correlate with clinical findings. That is, research has shown that level of increased cell proliferation following TBI decreases with age, and juvenile patients tend to recover cognitive functioning with greater success than their older counterparts. Studies suggest that certain growth factors may play a role in regulating this injury-induced cell

proliferation. Epidermal growth factor (EGF) in the CNS has received recent attention because of the mitogenic response that neural precursor cells generate after exposure to this protein *in vitro* as well as *in vivo* following injury. Therefore, there is therapeutic potential in understanding the involvement of EGF in this innate proliferative response to injury. Developed therapies could mimic the growth factor microenvironment associated with the robust level of cell proliferation observed in juveniles following injury. By exploiting this endogenous proliferative response to injury, potential treatments could allow adult and elderly patients to achieve significant improvements in cognitive recovery following TBI.

For this thesis, it was hypothesized that EGF expression in the injured brain correlates to the level of enhanced cell proliferation observed following TBI. Specifically, we sought to determine if EGF expression increases following injury and if this expression decreases with aging. To test this, we compared the protein expression levels of EGF in juvenile, adult and aged rodents following TBI to age-matched sham controls using Western blot analysis. Additionally, we studied the localization and type of cells that make EGF following injury using immuno-histochemistry on whole brain sections of injured and sham adult rodents. Because the hippocampus is the most prevalent area injured following TBI, attention was directed to this structure of the CNS, where damage contributes to the characteristic deficits in hippocampal-dependent learning and memory functions following TBI. This thesis will first review the societal and clinical significance of TBI, followed by research on the proliferation of neural progenitor cells in the hippocampus and the role that these newly

generated cells may play in cognition. Focus will then be directed towards the enhanced proliferation of these cells that has been observed following brain injury. The potential involvement of growth factors in modulating this injury-induced cell proliferation will then be discussed. Specifically, this thesis will go through the structure and function of EGF and its receptor in the CNS to introduce how EGF may drive the enhanced cell proliferation observed following TBI and contribute to improvements in cognitive recovery.

Traumatic brain injury

Traumatic brain injury (TBI) continues to be of great concern because of its prevalence and severity in the United States. Each year, approximately 1.8 million Americans sustain a brain injury (McArthur et al., 2004), with the most common causes involving motor vehicle accidents, falls, sport-related injuries and violence (Bazarian et al.,2005, Thurman et al., 1999). Adolescents, young adults, and the elderly represent the populations at highest risk to TBI (McArthur et al. 2004, Thurman et al., 1999). Of those affected, 5.3 million Americans currently live with TBI-related disabilities, and approximately 50,000 Americans die annually (Thurman et al., 1999).

The pathology associated with TBI initially involves mechanical tissue damage followed by a series of secondary events that include ischemia, swelling, and metabolic failure, all of which may persist over a period of hours or days (Davis 2000). Studies have shown that secondary insults result in cell loss and dysfunction in the CA1 and CA3 subfields of the hippocampus (McIntosh et al., 1989). This accumulated loss and

disruption of hippocampal neurons contributes to the range of cognitive deficits observed in patients with TBI (Witgen et al., 2005). Severe brain injuries may result in patients having to live in a persistent vegetative state (McArthur et al., 2004). People who encounter mild and moderate forms of brain injury tend to exhibit memory loss, difficulty in information processing, and/or cognitive inflexibility (Hilton, 1994).

Cognitive impairments related to TBI can translate into costs at both societal and economic levels. People suffering from brain injury may find that difficulties in completing learning and memory tasks prevent them from returning to work, perhaps making it impossible (van der Naalt, 1999). Financial problems can mount when patients and their families lose a source of income while also having to pay for expensive hospital stays and medical treatments. Cognitive deficits following brain injury can also limit patients' ability to engage in social activities, hindering them further from becoming active members of society (Hilton, 1994, Thurman et al., 1999).

Most patients display some degree of cognitive recovery within weeks to months following injury (McArthur et al. 2004). However, clinical evidence suggests that age-related differences exist in the degree of cognitive improvement observed in patients after TBI. For example, a clinical study revealed that children who had suffered brain insults recovered cognitive functioning to a greater extent and exhibited more independence compared to injured young adults (Eiben 1984). Rutherford and colleagues reported that the numbers of post-concussion symptoms at both 6 weeks and 1 year after injury were correlated with age (Rutherford 1977).

These age-related differences have also been observed in experimental rodent

models of TBI. Numerous studies have utilized the fluid percussion injury (FPI) model on rats to mimic the pathological sequelae and subsequent cognitive deficits observed following traumatic insults to the human brain (Gorman et al., 1993, Hamm et al., 1996, McIntosh et al., 1989). Hamm and colleagues found that rodents experience an increase in neurological deficits and mortality rate with age following injury (Hamm, 1991). They also found that injured juvenile rats performed significantly better on hippocampal-dependent learning and memory functions than their aged counterparts on days 11 to 15 after FPI, as assessed by the Morris Water Maze test (Hamm et al., 1996). The molecular mechanisms underlying these age-related differences in cognitive recovery after injury are not fully understood. Nevertheless, this limited recovery reveals that the CNS has an inherent potential to regenerate and repair itself following injury. One potential mechanism in the CNS may involve the proliferation and differentiation of neural stem and progenitor cells located in specific germinal zones of the brain. A review of these cells, as well as their role in cognition, is necessary to understand how the enhanced proliferative response of these cells to injury may contribute to cognitive recovery following TBI.

Neural precursor cell proliferation

Cell proliferation and neuronal differentiation have been observed to persist throughout the lifespan of rodents (Gage, 2002, Picard-Riera et al., 2004). Research has established that endogenous cell proliferation in the adult CNS is restricted to the SVZ of the lateral ventricle and DG of the hippocampus, where reserves of multi-potent

neural precursor cells are located (Temple and Alvarez-Buylla, 1999, Peterson, 2002). In the adult DG, these neural precursor cells are born and subsequently proliferate in the subgranular zone (SGZ) at the border between the hilus and the granule cell layer (Kuhn et al., 1996). These precursor cells and their progeny migrate laterally to the granule cell layer where they differentiate and integrate into the existing circuitry as mature granular neurons (Parent, 2003, Kuhn et al., 1996). Additionally, studies using retrograde tracers have found that the mossy fibers of these granule cells form synapses and project their axons into their appropriate target, the CA3 subfield of the hippocampus (Markakis and Gage, 1999, Hastings and Gould, 1999). Research has established that neurogenesis is correlated to hippocampal-dependent learning and memory (Shors et al., 2004, Hallbergson et al., 2003). Therefore, this correlation between neural precursor cell proliferation and cognition may have therapeutic implications for TBI, which is characterized by learning and memory deficits (Hilton, 1994, Thurman et al., 1999). Studies have shown that a number of factors may enhance the proliferation of neural progenitor cells and possibly contribute to cognitive recovery, some of which include corticosteroids, glucocorticoids, growth factors and trophic factors (Bambakidis et al., 2005, Wong et al., 2006). Nonetheless, because of the prevalence of TBI and the cognitive improvements manifested in TBI patients, this thesis will first focus on the endogenous increase in proliferation of neural precursor cells in the hippocampal DG that may occur in response to brain injury.

Numerous studies have found that injury can trigger a heightened rate of cell proliferation of neural precursor cells (Chiumamilla et al., 2002, Sun et al., 2005, Dash

et al., 2001), suggesting that an innate reparative mechanism may exist within the CNS that could account for the cognitive recovery seen following TBI. For example, lesions to the granule zone of the hippocampus stimulate cell proliferation in the rat (Gould and Tanapat, 1997). Our lab observed an approximate 3-fold increase in cell proliferation in the DG in injured juvenile and adult rats as compared to sham controls, with maximum rate of cell proliferation observed in the SGZ and hilus 2 days post-injury (Figure 1.1) (Sun et al., 2005). Furthermore, x-irradiation studies have revealed that loss of neural precursor cell proliferation following TBI decreases cognitive recovery, as assessed by the Morris Water Maze test, which strongly supports the involvement of this proliferative response in cognitive recovery (Tada et al., 2000). However, in correlation with clinical findings in cognitive recovery after TBI, our lab has found age-related differences in the level of enhanced cell proliferation observed following injury, with injured juveniles exhibiting a more robust level of cell proliferation than adult and aged injured rats (Figure 1.2) (Sun et al., 2005). While these age-related differences in cell proliferation and subsequent cognitive recovery are not fully understood, research continues to investigate the micro environmental factors that may be involved in this possible mechanism of repair. Specifically, studies have shown that growth factors may regulate a variety of cellular activities in neural precursor cells, particularly proliferation and differentiation (Kelly et al., 2005, Hagg, 2005). We speculate that the enhanced proliferative response observed following injury is mediated by growth factor expression in the CNS. Research indicates that certain growth factors act as potent mitogens for neural precursor cells, suggesting that the expression of growth factors

Figure 1.1: BrdU-positive cells in the ipsilateral dentate gyrus in juvenile and adult rats. Coronal sections taken from injured juvenile (a) and adult (b) rats 2 days post injury showing an increased number of BrdU-positive cells in the DG of injured juveniles as compared to adults. BrdU-positive cells are clustered and concentrated in the subgranlar zone (SGZ). (c) Quantification of BrdU-positive cells in the SGZ. Graph shows that cell proliferation was significantly increased in injured juvenile and adult rats at both 2 and 7 days post injury as compared to age-matched sham controls (* $p< 0.05$, ** $p<0.01$). The total number of proliferating cells in injured juveniles was significantly higher than injured adult rats at 2 days post injury (*$p<0.05$), n = 4/group. (d) Nissl section of the hippocampus showing the area depicted in figure 1.1 (a) and (b) (red box). (Sun et al., 2005)

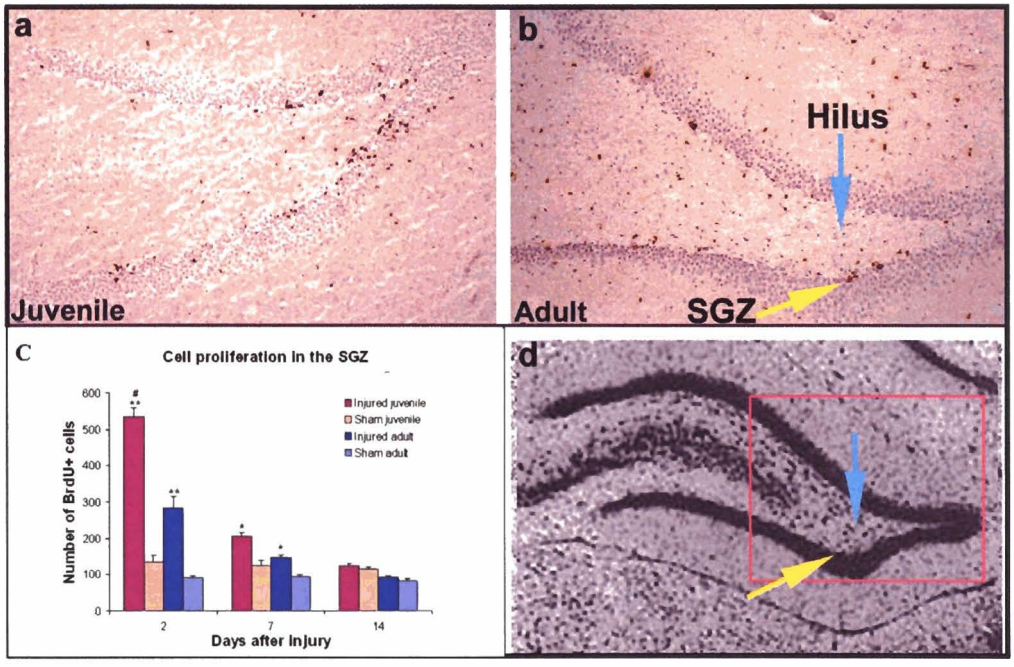
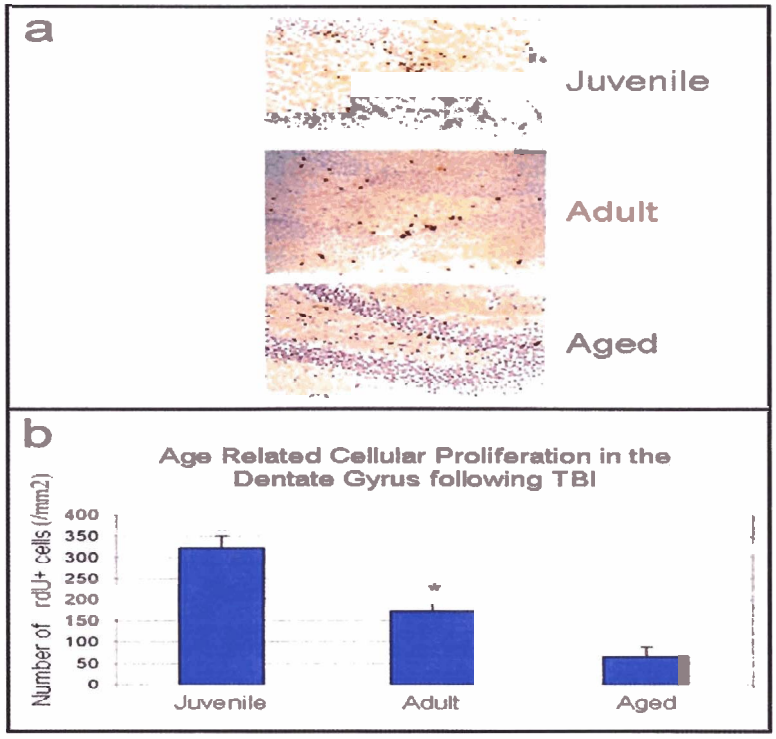

a
Juvenile
b
Hilus
Adult
SGZ
C
Cell proliferation in the SGZ
Number of BrdU+ cells
600
500
400
300
200
100
0
2
7
14
Days after injury
Injured juvenile
Sham juvenile
Injured adult
Sham adult
d

Figure 1.2: Cellular proliferation after TBI decreases with age. (a) Coronal section of the dentate gyrus of injured juvenile, adult and aged rats stained for BrdU showing the decreased level of cell proliferation with age. (b) Graph showing the reduction in total number of BrdU-positive cells with age found within the ipsilateral dentate gyrus, *$p<0.01$, $n = 4$/group. This graph represents a direct comparison of the injury-induced cell proliferative response between juvenile, adult and aged rats, after subtracting out the number of proliferating cells in sham rats of the corresponding age groups. (unpublished data)


a
Juvenile
Adult
Aged
b
Age Related Cellular Proliferation in the Dentate Gyrus following TBI
Number of rdU+ cells (/mm2)
400
350
300
250
200
150
100
50
0
*
Juvenile
Adult
Aged

following injury may underlie the injury-induced proliferative response of the CNS and subsequently contribute to cognitive recovery observed after TBI.

Growth Factors

Growth factors are polypeptides that coordinate cellular activities by inducing DNA synthesis and cell division via receptor-mediated cascades (Plata-Salaman et al., 1991). While the role of growth factors in non-neural tissues has received much attention (Varkey et al., 2006, Dziadzio et al., 2005), research in growth factor manipulation of the CNS is quickly expanding as the prevalence of neurodegenerative disease and injury continues to rise. While multiple classes of growth factors have been shown to act in the CNS, some of the more prominent ones include: Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Platelet-derived Growth Factor (PDGF), and Insulin Growth Factor-1 (IGF-1) (Aberg et al., 2006, Jin et al., 2003). Various cell types, such as neurons and glia, can synthesize growth factors and secrete them into the extra cellular environment where they act on local cells (Carpenter and Cohen, 1990). Growth factors stimulate and regulate diverse events such as cell, proliferation, differentiation, neurotrophic effects and survival (Alzerimer and Werner, 2002, Craig et al., 1996, Gensburger et al., 1987).

Based on studies in both injured and normal brains, observations were made that correlate endogenous growth factor expression levels with cell proliferation and neuronal differentiation (Aberg et al., 2006, Kelly et al., 2005). For example, studies indicate that many growth factors are expressed at high levels during brain

development, yet their expression is reduced in the normal adult brain, correlating to decreased levels of neurogenesis observed with aging (Caday et al., 1990). Furthermore, research shows that the level of growth factor expression increases significantly following trauma or other injury (Parent 2003). Studies have established that epidermal growth factor (EGF) is one particular factor that is widely distributed throughout the CNS and is involved in an array of cellular functions (Wong et al., 2004, Yamada 1997). Since EGF has been shown to act as a potent mitogen for CNS cells (McGinn et al., 2004, Plata-Salaman, 1991), research has directed greater attention towards the role that EGF may play in stimulating the proliferation of endogenous neural cells and replacing cells lost following injury.

Epidermal Growth Factor

First isolated in 1962, EGF was found as a contaminant of Nerve Growth Factor (NGF) that could induce eye openings and incisor eruption following injection into male mice (Carpenter and Cohen, 1990, Wong et al., 2004). Cohen concluded that these observations were caused by the stimulated epidermal growth and keratinization induced by this protein (Carpenter and Cohen, 1990). Urogastrone and human EGF were deemed to be the same molecule as the isolated EGF (Yamada et al., 1997). EGF can act in both paracrine and autocrine modes while it induces growth factor related activities in both ectodermal and mesodermal cells (Wong et al., 2004). Produced by the salivary and Brunner's glands of various body systems, it is found in saliva, cerebrospinal fluid (CSF), urine and breast milk (Plata-Salaman, 1991). Numerous

studies reveal the established role that EGF plays in non-neural tissues as well as in the brain.

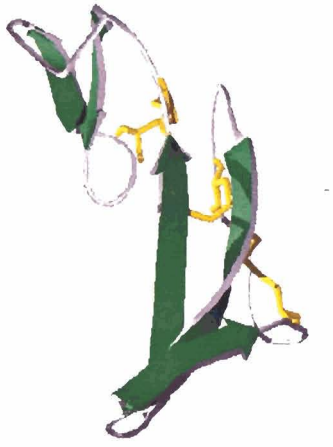
EGF-like proteins have been grouped into a single family. Members include: TGFα, Amphiregulin (AR), Heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BC), and Neu Differentiation Factor (heregulin) (NDF/HRG) (Yamada et al., 1997). These polypeptides all bind to the EGF receptor with high affinity and show mitogenic activity towards EGF-responsive cells (Yamada et al., 1997). Additionally, a primary structure of 50-60 residues with a characteristic 6 half cysteine motif is common to these proteins (Plata-Salaman, 1991, Wong et al., 2004). This cysteine motif is necessary to form the three disulfide bonds required for the EGF signaling through the EGF receptor (Plata-Salaman, 1991).

EGF Structure, Receptors, and Ligand Interactions

Found in vertebrates and invertebrates, EGF is a polypeptide made up of 53 amino acids (Carpenter and Cohen, 1990) (Figure 1.3). Six spatially conserved cysteine residues form intramolecular disulfide bonds necessary for the EGF biological activity through the EGF receptor (Wong et al., 2004, Yamada, 1997). Two sets of anti-parallel beta pleated sheets are also found in this protein (Carpenter and Cohen, 1990). It is first synthesized as a 140-150 kDa glycosylated transmembrane precursor that gets proteolytically cleaved in order to yield mature EGF (Plata-Salaman, 1991).

The EGF receptor, also known as ErbB1, is a 170 kDa membrane spanning protein consisting of a single polypeptide chain and N-linked oligosaccharides

Figure 1.3: Crystal structure of EGF and the EGF receptor. EGF (a) consists of 2 sets of anti-parallel beta-pleated sheets and six spatially conserved cysteine residues. These residues form essential intramolecular disulfide bonds needed for the activation of the tyrosine kinase domain and subsequent signaling cascades induced by EGF binding to its receptor (b) (Wong et al., 2004).



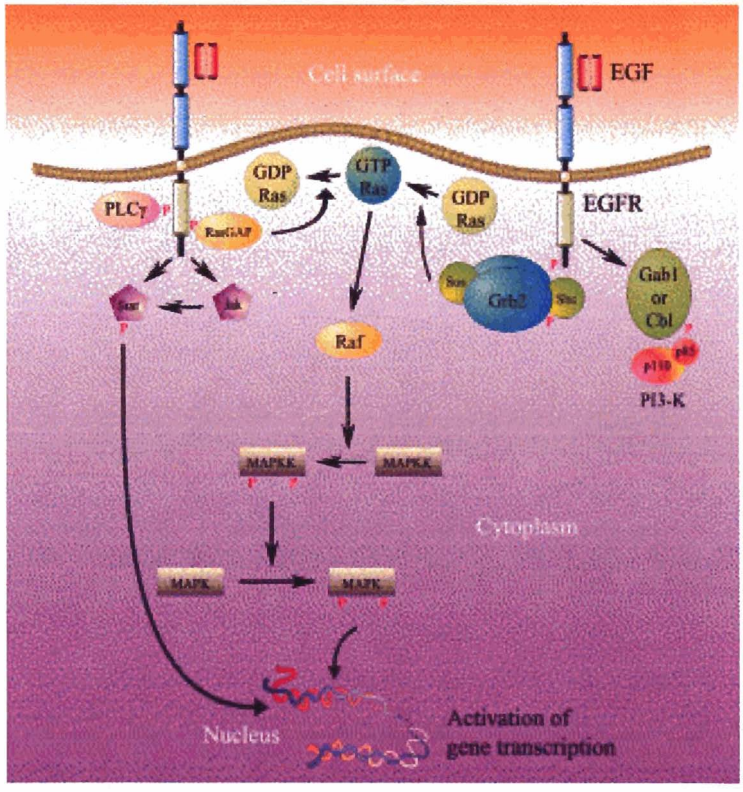
(b)

(Wong et al., 2004) (Figure 1.3). This receptor consists of four domains: an extracellular EGF binding domain, a cytoplasmic membrane spanning tyrosine kinase domain (Carpenter and Cohen, 1990), proximal cytoplasmic segment binding ATP, and a terminal cytoplasmic segment that undergoes phosphorylation (Plata-Salaman, 1991). The extra cellular domain has a high affinity to EGF and EGF-like ligands. The binding of these ligands causes dimerization of the EGF receptor and activation of the cytoplasmic tyrosine kinase domain (Carpenter and Cohen, 1990). The cysteine residues found on EGF form essential intramolecular disulfide bonds needed for the activation of the tyrosine kinase domain and subsequent signaling cascades induced by EGF binding (Wong et al., 2004). The cytoplasmic tyrosine kinase domain has high sequence homology to the erbB oncogene product of the avian erythroblastosis virus (v-ErbB) (Carpenter and Cohen, 1990, Wong et al., 2004). This similarity has implications for tyrosine kinase activity being involved in the regulation of cell proliferation (Carpenter and Cohen, 1990).

Following EGF receptor tyrosine kinase activation, a number of cellular processes can be stimulated, such as the regulation of intracellular calcium, sodium-hydrogen exchange, gene transcription, and down regulation of signaling (Plata-Salaman et al., 1991). For the purpose of this study, the focus will be upon the mitogen induced mechanisms yielding regulation of gene transcription and cell proliferation, specifically the MAP Kinase cascade (Figure 1.4).

Upon EGF binding, the EGF receptor is dimerized, activated, then autophosphorylated on its tyrosine residues (Wong et al., 2004). These phosphorylated

Figure 1.4. EGF activated MAP kinase cascade. The mitogen activated protein kinase cascade is the signal transduction pathway which is initiated by EGF binding and functions to up-regulate cell division. EGF binding causes the EGF receptor dimerization and auto-phosphorylation on its tyrosine residues which act as docking sites for SH2 signaling molecules. Grb2 interacts with the receptor tyrosine kinase through its two SH3 domains and binds to Sos. The Grb2/Sos complex recruits Sos to the plasma membrane where Ras is located and gets activated through the initiated exchange of its GDP for GTP. Activated Ras then initiates a kinase cascade involving (in order) Raf, MAP, MAPKK, and MAPK. Activated MAPK can translocate to the nucleus where it can phosphorylate and activate transcription factors involved in the regulation of cell proliferation. (Wong et al., 2004)


Cell surface
EGF
GDP
Ras
GTP
Ras
GDP
Ras
PLCγ
RasGAP
EGFR
Grb2
Gab1
or
Cbl
PI3-K
Raf
MAPKK
MAPKK
Cytoplasm
MAPK
MAPK
Nucleus
Activation of
gene transcription

tyrosine residues act as docking sites for signaling molecules with src homology 2 (SH2) domains (Wong et al., 2004). Specificity between signaling molecules and the EGF receptor is defined from a combination of the amino acid sequences immediately surrounding the phosphotyrosine residues on the receptor tyrosine kinase as well as the variant residues found within the SH2 motifs (Wong et al., 2004). Grb2 interacts with the receptor tyrosine kinase through its SH2 domain and is connected to the Ras/ MAPK cascade through its two src homology 3 domains (SH3) (Plata-Salaman et al., 1991). Grb2 binds to the Ras guanine nucleotide releasing factor Sos, and this Grb2/Sos complex recruits Sos to the plasma membrane where the Ras protein is located (Wong et al., 2004). This translocation of Ras causes the exchange of GDP to GTP on this protein which also activates it. The activated Ras then initiates a kinase cascade involving Raf (a serine/threonine kinase), MAP kinase kinase (MAP KK) (a dual specificity kinase), and MAP Kinase (MAP K) (a serine/threonine kinase) (Wong et al., 2004). Activated MAPK can translocate to the nucleus where it can phosphorylate and activate transcription factors (Wong et al., 2004). These transcription factors can then regulate cell proliferation and other cellular processes.

EGF Localization and Function

With the expression of EGF seen during both CNS development and throughout adulthood, implications can be made suggesting EGF's role in both normal and repair mechanisms for the brain. While EGF can be found in the CNS, blood and peripheral organs, a number of cells in the CNS are capable of synthesizing this protein (Wong et

al., 2004). These widespread cells include macrophages, glial cells and neurons (Plata-Salaman et al., 1991). Studies show that EGF may also enter the CNS from peripheral blood via the blood brain barrier (Plata-Salaman et al., 1991).

EGF has been detected throughout the CNS in the brainstem, hippocampus, cerebellum, diencephalon and telencephalon (Wong et al., 2004). EGF-immunoreactive neurons and neuronal fibers are also widely distributed throughout the CNS. With this wide ranging localization of EGF comes a variety of effects of EGF seen on neuronal and glial cells in the CNS including proliferation, differentiation, maintenance, prevention of hippocampal damage, regeneration, neuromodulation, and survival (Hermann et al., 2000, Plata-Salaman, 1991, Tarasenko et al., 2004).

Increased focus has been directed towards EGF because of its mitogenic properties that have been shown in both *in vitro* and *in vivo* studies (Kelly et al., 2005, McGinn et al., 2004, Gritti et al., 1999). Studies have revealed that EGF is a mitogen for neural precursor cells and can induce them to differentiate into both neurons and astrocytes *in vitro* (Mytilineou and Shen, 1992, Pan, 2003). Research also shows that through receptor tyrosine kinase cascades, EGF stimulates transcription of such genes as c-fos and c-myc (Mahimainathan et al., 2005). This mitogen-induced activation causes marked stimulation of DNA synthesis, measured by thymidine incorporation, and a subsequent rise in proliferation and differentiation in glial cells (McGinn et al., 2004). Specifically, by administering varying doses of EGF into the rodent striatum, our lab found that 0.05 ng of EGF delivered 48 hours earlier induced maximal proliferation, with the majority of newly generated cells differentiating into astrocytes

(McGinn et al., 2004). These collective findings support the notion that EGF may play a role in the endogenous proliferative response following injury. By studying the temporal and spatial expression of EGF following TBI, research can continue to investigate the relationship between injury-induced cell proliferation, growth factor expression, and cognitive recovery following brain injury. Efforts to further understand this relationship can create a more comprehensive picture of the factors involved in the enhanced level of cell proliferation observed following TBI. Since studies have shown that EGF acts as a potent mitogen to neural precursor cells and that growth factor expression in the brain declines with age, this thesis will focus on determining whether a correlation exists between the levels of EGF expression in the hippocampus and enhanced cell proliferation observed following injury. By gaining a greater understanding of the role that EGF plays in injury-induced cell proliferation, potential treatments could be developed which mimic the growth factor microenvironment that supports the robust level of cell proliferation of neural precursor cells observed in injured juveniles following TBI. These treatments could then harness growth factor driven cell proliferation in the hippocampus and subsequent neurogenesis in response to injury to help adult and elderly patients improve their degree of cognitive recovery following TBI.

Chapter Two

Epidermal Growth Factor Expression Following Injury

Hippocampal neurons are highly vulnerable to injury, as indicated by the prevalence of hippocampal-dependent learning and memory deficits following traumatic brain injury (TBI). Nevertheless, clinical and experimental evidence have revealed that, following injury, cognitive function can be restored to a limited extent. This observed cognitive recovery implies that the CNS has an innate capacity to repair itself once it has been damaged following TBI. One possible mechanism for this recovery involves the generation of new cells from multi-potent neural precursor cells located in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Research has shown that these precursor cells persist and proliferate throughout the mammalian lifespan. Additionally, there is a correlation between the proliferative response of these cells and subsequent neurogenesis with the restoration of cognitive function (Shors, 2004). Research has also shown that the level of proliferation of neural precursor cells is increased following a variety of brain insults, which implies that there is an endogenous proliferative response to injury in the hippocampus to replenish neurons and restore cognitive function following TBI (Sun et al., 2005). Furthermore, research has revealed that the level of enhanced proliferation after injury decreases with aging. This decreased proliferative capacity with aging correlates well with the diminished cognitive recovery observed in aged animals (Hamm et al., 1991). Therefore, it is vitally important to understand the underlying mechanism that drives

this reparative mechanism in order to develop therapeutic treatments that may improve cognitive recovery following TBI.

While the biological basis underlying these age-related differences is not fully understood, research has begun to investigate the microenvironment that stimulates the endogenous proliferative response generated by neural precursor cells following injury. Our lab and others speculate that this heightened cell proliferation following injury is mediated by growth factors. This hypothesis is supported by three primary observations: 1) growth factors are widely distributed and highly expressed in the developing CNS during the time of neurogenesis and their expression levels decrease with age; 2) neural precursor cells have been shown to proliferate in response to growth factors *in vitro*, and 3) a variety of brain insults induces a proliferative response of neural precursor cells and up-regulates growth factor expression.

One potential growth factor that may be driving this proliferative response is epidermal growth factor (EGF). This growth factor has been shown to be up-regulated during the time of CNS development, to induce the proliferation of neural stem cells *in vitro*, and is up-regulated following a variety of brain insults. With this in mind, we speculated that the temporal and spatial expression levels of EGF in the injured hippocampus can be correlated to the proliferative response seen following TBI. Moreover, the diminished response seen with aging following injury can also be correlated to decreased levels of EGF. To test this, western blotting analysis was conducted to assess the expression of EGF in the hippocampus of juvenile, adult and aged rodents following injury and their age-matched sham controls. Briefly, animals of

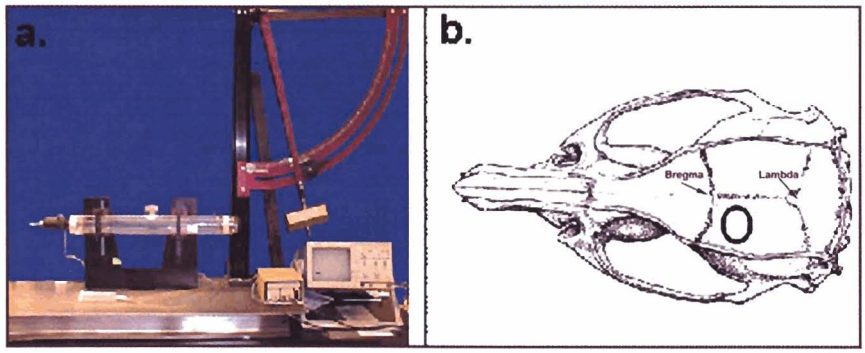
all age groups received a moderate lateral fluid percussion injury or sham surgery and perfused either 2, 7, or 14 days post-injury or sham surgery in order to compare levels of EGF expression to the levels of proliferation in the dentate gyrus observed following fluid percussion injury in previous studies from our lab. Additionally, immunohistochemistry analysis was conducted on whole brain sections to determine the type and localization of cells that express EGF.

Materials and Methods

Fluid Percussion Injury (FPI)

This model of injury was chosen because it reproduces the pathophysiological sequelae observed following TBI in humans (McIntosh et al., 1989). For this thesis, lateral FPI was used (Dixon et al., 1987) (Figure 2.1). We used Sprague-Dawley male rats aged at postnatal day 28 (juvenile), 3 months (adult) and 21-24 months (aged), which were supplied by Harlan, Inc., IN and Zivic Miller Lab, Inc, PA. Adult and aged rats were anesthetized in a Plexiglas chamber, using 3% isofluorane, intubated and ventilated with 2% isofluorane in a gas mixture (30% oxygen, 70% N2), and secured in a stereotaxic frame. Juvenile rats received continual anesthesia through a nose cone with the gas mixture described above. After a midline incision, the skull was exposed and a 4.9 mm craniotomy was made on the left parietal bone. A Luer lock fitting was then cemented to the skull. As described previously (Dixon et al., 1987), a moderate fluid pulse (2.1 ± .1 ATM) was administered, using a pre-calibrated fluid

Fig.2.1: Fluid percussion injury (**a**) Photograph of the fluid percussion injury device. (**b**) Illustration of the adult rat skull showing the site of the fluid percussion injury (black circle) in relation to the suture landmarks, bregma and lambda.


a.
b.
Bregma
Lambda
O

percussion injury device. After injury, the Luer lock filling was removed, the wound sutured, and the rats were returned to the vivarium after 3-hour observation. Sham animals were subjected to the same surgical procedure, but without the injury pulse.

Protein Isolation for Western Blotting Analysis

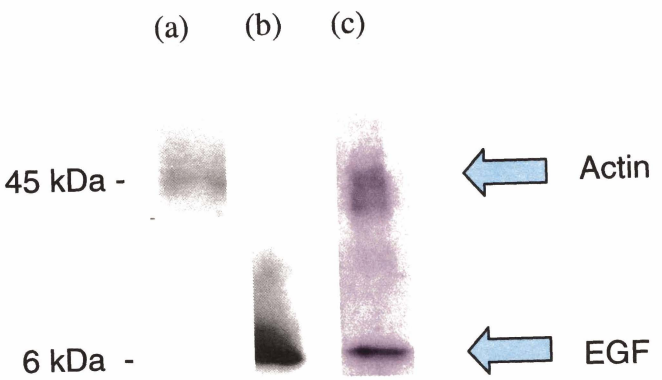
Following FPI or sham surgery, rats were sacrificed by an injection of sodium pentobarbital either at 2, 7, or 14 days post-injury/surgery and transcardially perfused with 200 ml ice-cold PBS (for all age groups, N = 4 for samples at 2 days post-injury/surgery, N = 2 at 7 days post-injury/surgery, and N = 2 at 14 days post-injury/surgery, except for the juvenile injured group at this time point, N = 1). Hippocampal tissue was dissected out on ice and immediately placed in ice-cold Ripa extraction buffer containing protease inhibitor cocktail and immediately homogenized with a Kontes pellet pestle homogenizer in a matching ependorf (Kontes, NJ). The homogenate was centrifuged at 12,000 RPM for 10 minutes, and the supernatant containing proteins was collected, centrifuged at the same speed, and then stored in -80° C for protein assay and Western blotting. The protein concentration was determined with the BCA protein assay method.

Western Blotting

Western blotting analysis was conducted to assess the expression of epidermal growth factor in the hippocampus of juvenile, adult and aged rodents following different time points post- injury or surgery. Frozen samples were first allowed to thaw

on ice. Appropriate amounts of protein (15 μg for 2 days post injury/surgery samples, 40 μg for 7 and 14 days post injury/surgery to obtain detectable signals) were mixed in 1:2 (volume) with Laemmli sample buffer (1:20 dilution of Laemmli and 2 Beta-mercaptoethanol) up to a 15 μl total volume and boiled for 5 minutes, allowed to sit at room temperature for 10 minutes and centrifuged. Samples were loaded onto a 7.5% Tris-HCl ready gel along with a Precision Plus protein dual color ladder. The gel was run in a non-denaturing buffer (pH 9.4) at 20mA for 120 minutes. After electrophoresis, gels were transferred to a PVDF (PolyVinylidine DiFluoride) membrane at 120V for 80 minutes in an ice bath, with constant stirring. Once transfer was complete, the membrane was washed twice for 10 minutes each in Tris-buffered saline with 0.1% Tween (TBST). TBST washes were followed by a 1 hour wash in blocking solution made of TBST and 5% reconstituted milk solids to prevent non-specific binding of antibody to the membrane. The membrane was incubated with primary antibodies (anti beta-actin: 1:2000 (loading control) (Sigma), anti-EGF: 1:5000 (Biomedical Technologies Incorporated)) in blocking buffer over night at 4° C with rotation. After washing 3 times for 10 minutes each with TBST, the membrane was incubated with a peroxidase-conjugated secondary antibody (anti-rabbit IgG-HRP, 1:10,000 (Santa Cruz) in blocking buffer). After further washing with TBST, the membrane was incubated with a chemiluminescent developing solution (Amersham), wrapped in Saran wrap, and exposed to the X-ray film. The presence of beta-actin and EGF bands was determined according to the band size (Figure 2.2). Quantification of total proteins was made by densitometry analysis using both the Versa Doc/

Figure 2.2. Visualized actin and EGF bands bands. Selected samples were first incubated with either antibody to (a) actin or (b) EGF alone, along with the appropriate secondary antibody, to assess the effect of (c) double-antibody incubation on protein separation. In subsequent western blotting tests, level of EGF expression in EGF bands determined by densitometry analysis was corrected against the actin loading control. For subsequent densitometry quantification of western blot films, volume boxes were drawn to include whole bands.


(a)
(b)
(c)
45 kDa -
6 kDa -
Actin
EGF

Quantity One (BioRad) and Adobe Photoshop/ Image J (NIH) image analysis programs.

Statistical Analysis

Data analysis was performed using a two-way analysis of variance for statistical significance (GraphPad), with * $p < 0.05$ or ** $p < 0.01$ used to indicate significant differences, followed by a Bonferonni's post-hoc test to compare differences within groups. Data was expressed as the mean ± SEM.

Immunohistochemistry

To determine the phenotype and localization of cells that express EGF after injury, 10 micron thick frozen rat brain tissues taken from an adult injured rat were cut coronally using a cryostat. Frozen sections were first allowed to air dry for 10 minutes in a staining tray. Sections were then fixed with ice-cold acetone for 15 minutes and twice washed with PBS for 3 minutes each. Endogenous peroxidase was quenched using 3% hydrogen peroxide for 15 minutes followed by PBS washes. Sections were blocked using a serum blocker consisting of 5% horse serum and 0.4% triton in PBS for 30 minutes at room temperature. Sections were then incubated overnight at 4° C with designated primary antibodies diluted in serum blocker (anti-rabbit EGF: 1:400, anti-rabbit GFAP: 1:500). Following primary antibody incubation, sections were washed 3 times in PBST (PBS and 0.4% triton) for 3 minutes each and incubated with HRP-conjugated anti-rabbit IgG (1:200) in PBST for 1 hour at room termperature. After PBS washes, DAB (diaminobenzidine tetrahydrochloride) (made with DAB in 0.1M

phosphate buffer and 30% hydrogen peroxide) was applied to sections for 10-15 minutes using a microscope to control the color reaction. Upon sufficient DAB staining, sections were washed with PBS and then water. Lastly, sections were placed in ethanol for 30 seconds and xylene for 5 minutes and subsequently cover-slipped with permount. For double-labeling, sections were rinsed with PBST and incubated overnight at 4°C with anti-rabbit EGF (1:400) and one of the following cell type specific markers: mouse anti-GFAP (1:500) to label astrocytes; mouse anti-NeuN (1:500) to label mature neurons, and mouse anti- ED-1 (1:500) to label macrophages and activated microglia. Sections were washed with PBS and incubated with Alexa Fluor 568 anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG (1:200) for 2 hours at room temperature. After washing with PBS and water, sections were coverslipped with Vectorshield (Vector Labs, CA) mounting media.

Results

Published data: TBI induces cell proliferation in the dentate gyrus.

In a previous study conducted by our lab (see Chapter 1), the endogenous stem cell response in the mammalian brain following TBI was examined using the fluid percussion injury (FPI) model. This model was chosen because it has been well characterized to produce the similar diffuse pathological sequelae and subsequent neurological deficits manifested in humans following TBI (Dixon et al., 1988). By using lateral FPI, our lab assessed the time course and extent of the proliferative response of endogenous stem cells in the juvenile rat hippocampus, as compared to the

adult, following injury. Specifically, juvenile (28 days) and adult (3 month old) male Sprague-Dawley rats were subjected to a moderate lateral fluid percussion injury at similar levels (2.1 +/- .8 ATM) as previously described by our group (Chirumamilla et al., 2002). Animals were then injected intraperitoneally with BrdU at 2, 7 or 14 days post-TBI and sacrificed twenty-four hours later. In both juvenile and adult rats, the peak period of the injury-induced cell proliferation was observed 2 days post TBI in the dentate gyrus ipsilateral to the injury. BrdU-positive cells, representing newly generated cells, were concentrated in the subgranular zone for both injured juvenile and adult rats. This proliferative response lasted for 7 days for both age groups, but its duration varied in the hilus. By 14 days post injury, levels of cell proliferation in the hippocampus of both age groups declined to the levels of proliferation observed in sham animals. Preliminary studies from our lab also revealed that level of proliferation at 2 days post injury decreased with aging, with level being lowest in aged injured rodents, as determined by the total number BrdU-labeled cells.

Present Study:

TBI increases expression of EGF

Since our lab previously observed age-related differences in the cell proliferative response to injury, we sought in the current study to assess the protein expression of EGF, a growth factor which may drive this injury-induced proliferative response, in the hippocampus of animals of various age groups. Specifically, juvenile (28 days), adult (3 month) and aged (21-24 months) male Sprague-Dawley rats sustained a fluid

percussion injury or sham surgery and were sacrificed 2, 7, or 14 days later, as previously described. Following protein isolation, we assessed expression of EGF in our samples using western blotting analysis. Prior to protein resolution by electrophoresis, each gel was loaded with equal amount of proteins samples obtained from 3 age groups of animals sacrificed at same post-injury time points. Two different image analysis programs (Versa Doc/Quantity One (BioRad) and Adobe Photoshop/ Image J (NIH)) were utilized for the densitometry quantification of the developed X-ray films of the blots. Versa Doc/Quantity One was utilized for its features of versatility combined with resolution, whereas Image J for its established use in obtaining data from 1-dimensional electrophoretic gels. By converting raw densitometry data into relative levels of EGF expression, measurements from repeated trials of the same samples could be averaged into a single value, independent of differences in time exposure of films. Specifically, within each gel and corresponding x-ray film, the EGF band representing "Juvenile Sham" served as an internal standard so that intensities measured in the subsequent EGF bands could be presented as percentages of the level of EGF expression found in the standard (Table 1). A two-way analysis of variance test, followed by a Bonferonni's post-hoc test, was conducted individually on each of the groups of samples corresponding to animals sacrificed either at 2 days, 7 days or 14 days post-injury/ surgery.

Regardless of imaging program used, our results suggest that level of EGF expression increases following TBI as compared to the level observed in sham controls (Figure 2.3, Table 2). Measurements of EGF expression from both programs revealed

Table 1. Expression of EGF relative to juvenile sham samples following TBI or sham surgery. Within each gel and corresponding x-ray film, the EGF band representing "Juvenile Sham" served as an internal standard so that intensities measured in the subsequent EGF bands could be presented as percentages of the level of EGF expression found in the standard. Table shows densitometry data reflecting EGF expression (after correction against actin) of one group, out of four, of samples at 2 days post injury, with two trials for each sample. After raw densitometry data was converted into relative expression of EGF, these values could then be averaged to yield a single value for EGF expression for a given sample in a certain group of proteins run on a gel.

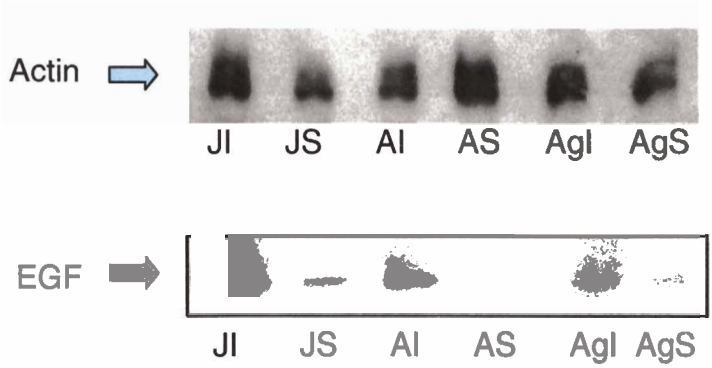
Image J Data	Expression of EGF, relative to Juvenile Sham	Averaged Relative Expression of EGF
Juvenile Injured		
0.5202817	584.69	
0.4912353	480.05	
		532.37
Juvenile Sham		
0.0889844	100	
0.1023291	100	
		100
Adult Injured		
0.62331	696.63	
0.5037477	492.28	
		594.455
Adult Sham		
0.0875573	98.88	
0.0597755	58.42	
		78.65
Aged Injured		
1.3180128	1483.15	
0.627198	612.92	
		1048.035
Aged Sham		
0.09809	111.26	
0.0898413	87.8	
		99.53

the most convincing findings at 2 days post-injury. At this time point, EGF expression was observed to be the highest in injured juvenile rats. Within juvenile animals, level of EGF expression was significantly higher in injured animals compared to their sham controls (Quantity One: *$p < .05$, Image J: ** $p < .01$). Differences between injured and sham animals in the adult and aged groups were not significant. Data from animals sacrificed at 7 (Figure 2.4) and 14 (Figure 2.5) days post-injury or surgery was less consistent between the two imaging programs. At 7 days post-injury/surgery, data from Image J revealed that EGF expression remains higher in injured compared to sham animals (not significant), whereas Quantity One did not reveal this trend. Additionally, with Image J, the mean level of EGF expression was found to be higher in adult injured animals than juvenile and aged injured animals, however, this finding was not significant. At 14 days, while it was found that EGF expression was higher in juvenile sham animals compared to injured juveniles, results from both programs revealed that level of EGF expression following injury tended to decrease towards levels observed in sham animals of all age groups.

Level of increased EGF expression following TBI decreases with age

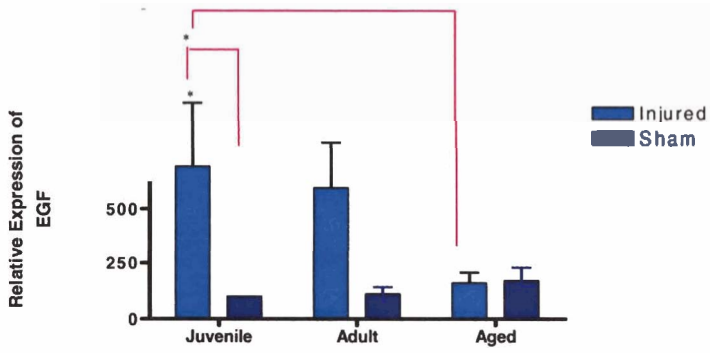
Together with the observation indicating that TBI increases level of EGF expression, results also point to decreased EGF expression following injury decreasing with age. Specifically, it was found that, at 2 days post injury, expression of EGF was significantly higher in juvenile injured rats than aged injured rats (Quantity One and Image J: * $p < 0.05$, Figure 2.3).

Figure 2.3: Relative expression of EGF 2 days after TBI. a) Western blot of gel loaded with proteins samples from animals perfused 2 days after fluid percussion injury or sham surgery. Level of EGF expression, with subtraction of actin, at this time point was found to be significantly higher in juvenile injured rats as compared to juvenile sham rats ((a) VersaDoc/Quantity One: *$p < .05$, (b) Image J: **$p < .01$). Legend: JI: Juvenile Injured, JS: Juvenile Sham, AI: Adult Injured, AS: Adult Sham, AgI: Aged Injured, AgS: Aged Sham.


Actin
JI
JS
AI
AS
AgI
AgS
EGF
JI
JS
AI
AS
AgI
AgS

(a)

(b)


Relative Expression of EGF
500
250
0
Juvenile
Adult
Aged
Injured
Sham

(c)

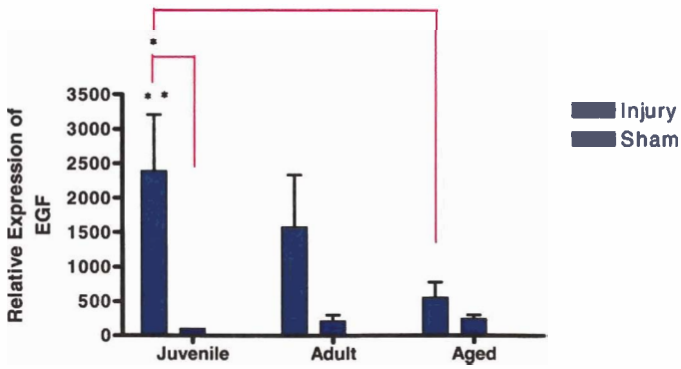

Relative Expression of EGF
3500
3000
2500
2000
1500
1000
500
0
Juvenile
Adult
Aged
Injury
Sham

Table 2. Relative expression of EGF at 2 days post-injury, corrected against actin-loading control. EGF expression was found to be significantly higher in Juvenile Injured as compared to Juvenile Sham rats at 2 days post-injury/surgery. Table provides the average values of relative expression of EGF obtained from averaged repeated trials of a given sample. These averages of averages from (a) VersaDoc/Quantity One and (b) Image J are displayed in chart form in Figure 2.3.

(a) VersaDoc/Quantity One

	Juvenile Injured	**Juvenile Sham**	**Adult Injured**	**Adult Sham**	**Aged Injured**	**Aged Sham**
1	158.46	100	206.03	104.88	220.17	92.55
2	590.86	100	522.73	123.02	89.25	241.3
3	507.25	100	470.49	183.99	259.1	297.04
4	1506.98	100	1177.92	37.55	79.28	57.17
Average	**690.89**	**100**	**594.29**	**112.36**	**161.95**	**172.02**
Standard Deviation	575.39		413.04	60.27	91.19	115.38
SEM	287.69	-	206.52	30.13	45.60	57.69

(b) Image J

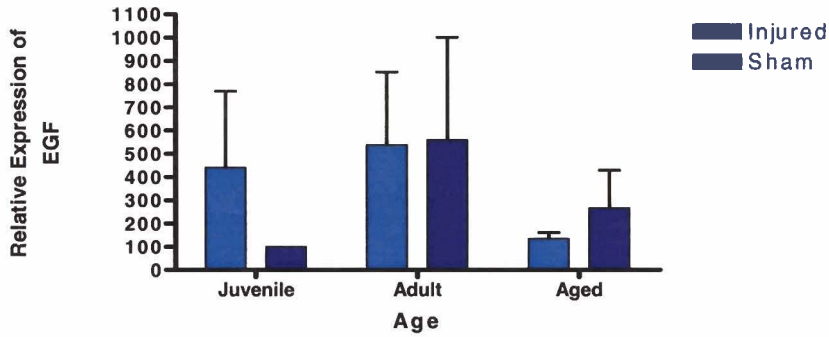
	Juvenile Injured	**Juvenile Sham**	**Adult Injured**	**Adult Sham**	**Aged Injured**	**Aged Sham**
1	532.37	100	594.46	78.65	1048.04	99.53
2	1652.98	100	1662.62	27.95	267.32	253.24
3	4205.85	100	3687.52	361.95	785.01	383.51
4	3185.51	100	352.62	374.77	112.55	241.97
Average	**2394.18**	**100**	**1574.31**	**210.83**	**553.23**	**244.56**
Standard Deviation	1625.22		1519.44	183.15	437.61	116.08
SEM	812.61	-	759.72	91.58	218.80	58.04

Figure 2.4. Relative expression of EGF at 7 days after TBI. (a) Western blot of gel loaded with protein samples from animals perfused 7 days after fluid percussion injury or sham surgery. Levels of EGF expression, corrected against actin-loading control, obtained through (b) VersaDoc/Quantity One and (c) Image J at this time point were not found to be significantly different. Legend: JI: Juvenile Injured, JS: Juvenile Sham, AI: Adult Injured, AS: Adult Sham, AgI: Aged Injured, AgS: Aged Sham.

(a)

JI JS AI AS AgI AgS

(b)



(c)

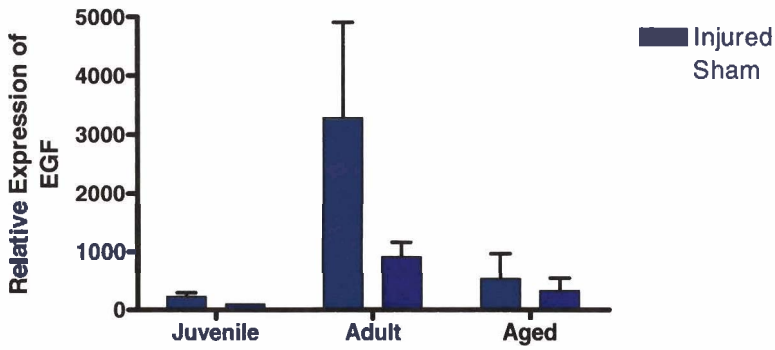
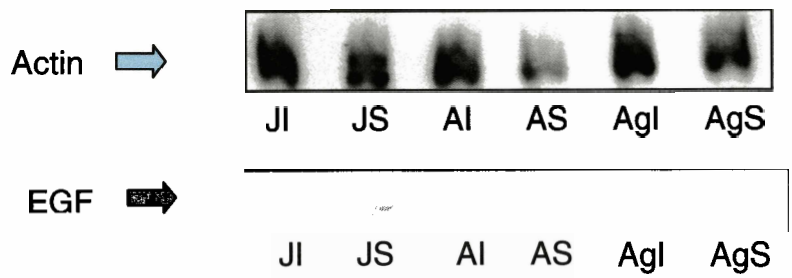
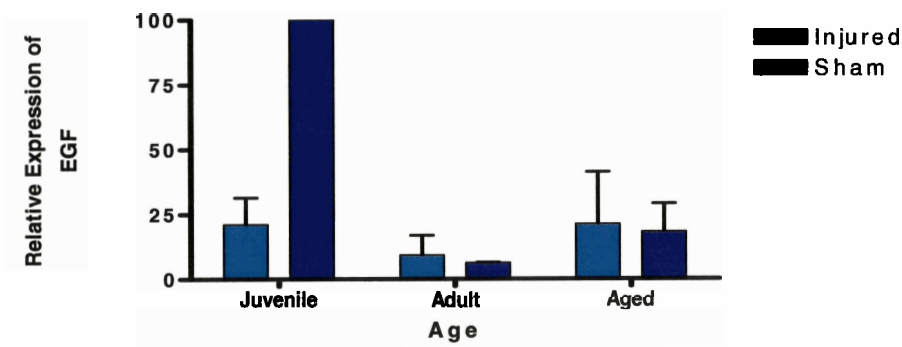


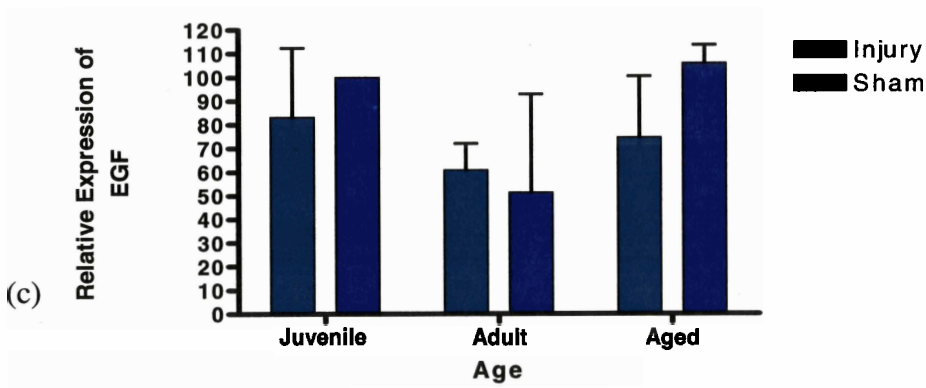
Figure 2.5. Relative expression of EGF at 14 days after TBI. (a) Western blot of gel loaded with protein samples from animals perfused 14 days after fluid percussion injury or sham surgery. Levels of EGF expression, corrected against actin-loading control, obtained through (b) VersaDoc/Quantity One and (c) Image J at this time point were not found to be significantly different. Legend: JI: Juvenile Injured, JS: Juvenile Sham, AI: Adult Injured, AS: Adult Sham, AgI: Aged Injured, AgS: Aged Sham.


Actin
JI JS AI AS AgI AgS
EGF
JI JS AI AS AgI AgS

(a)


Relative Expression of EGF
100
75
50
25
0
Juvenile
Adult
Aged
Age
Injured
Sham

(b)


Relative Expression of EGF
120
110
100
90
80
70
60
50
40
30
20
10
0
Juvenile
Adult
Aged
Age
Injury
Sham

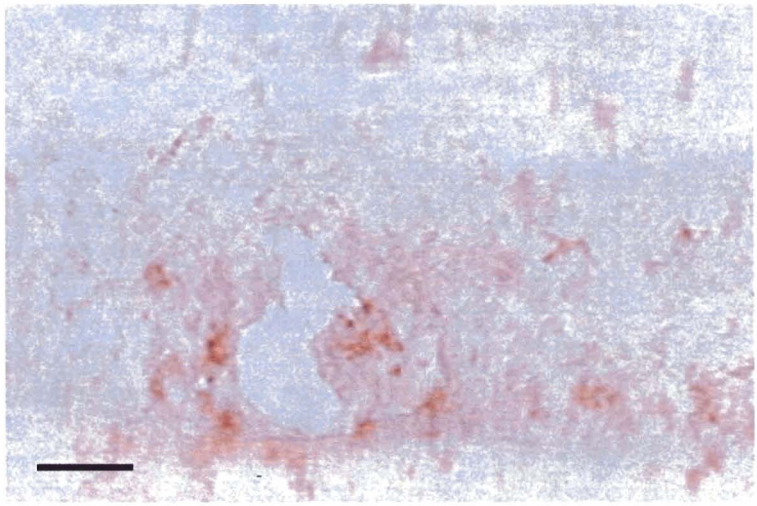
(c)

Results reflecting age-related differences in EGF expression at 7 and 14 days post injury in injured animals were less consistent across the two programs used. According to Quantity One data, aged injured animals expressed a lower level of EGF following injury compared to juvenile injured animals. However, with Image J, it was found that level of EGF expression was higher in adult injured animals compared to both juvenile and aged injured animals. By 14 days post-injury/surgery, age-related differences inincreased expression of EGF following injury were not evident; as most of the levels of expression in all of the age groups were similar to levels observed in sham animals.

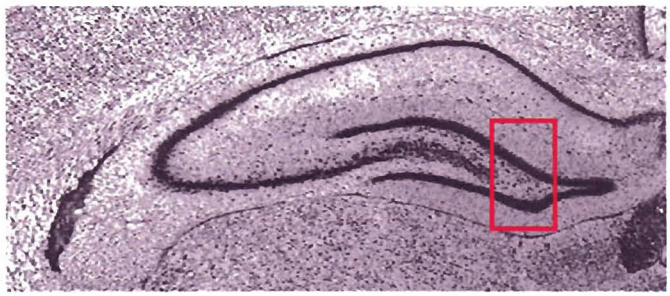
Immunohistochemistry and expression of EGF following injury

Since results indicated that expression of EGF increases following injury in the rodent hippocampus, we next sought to investigate the cellular and spatial expression of EGF following injury using immunohisotochemistry. We stained coronal whole brain sections obtained from an injured adult rat brain sacrificed at 2 days following FPI. After visualizing single-labeled EGF or GFAP sections, we found evidence of EGF positively stained cells exhibiting the characteristic morphology of astrocytes in the area of the cortical injury and hippocampus (Figure 2.6). Additionally, at the area of the injury site that had also contained areas that stained positive for EGF, we observed a large concentration of bi-concaved cells which we suspected to represent either red blood cells or macrophages. Studies have revealed that various cell types have the ability to synthesize EGF in the CNS such as intrinsic and blood-derived

Figure 2.6: Evidence of EGF staining with astroglial morphology. Micrographs of injured adult rat brain sections revealing immunostained cells exhibiting astrocyte morphology and staining positive for this mitogen, suggesting that astrocytes may express EGF following injury, particularly in the hippocampus. (a) Micrograph revealing the hippocampus ipsilateral to the injury showing a population of EGF+ cells. (b) Nissl section revealing area of the hippocampus represented in (a). Bars: 20 μm



(a)



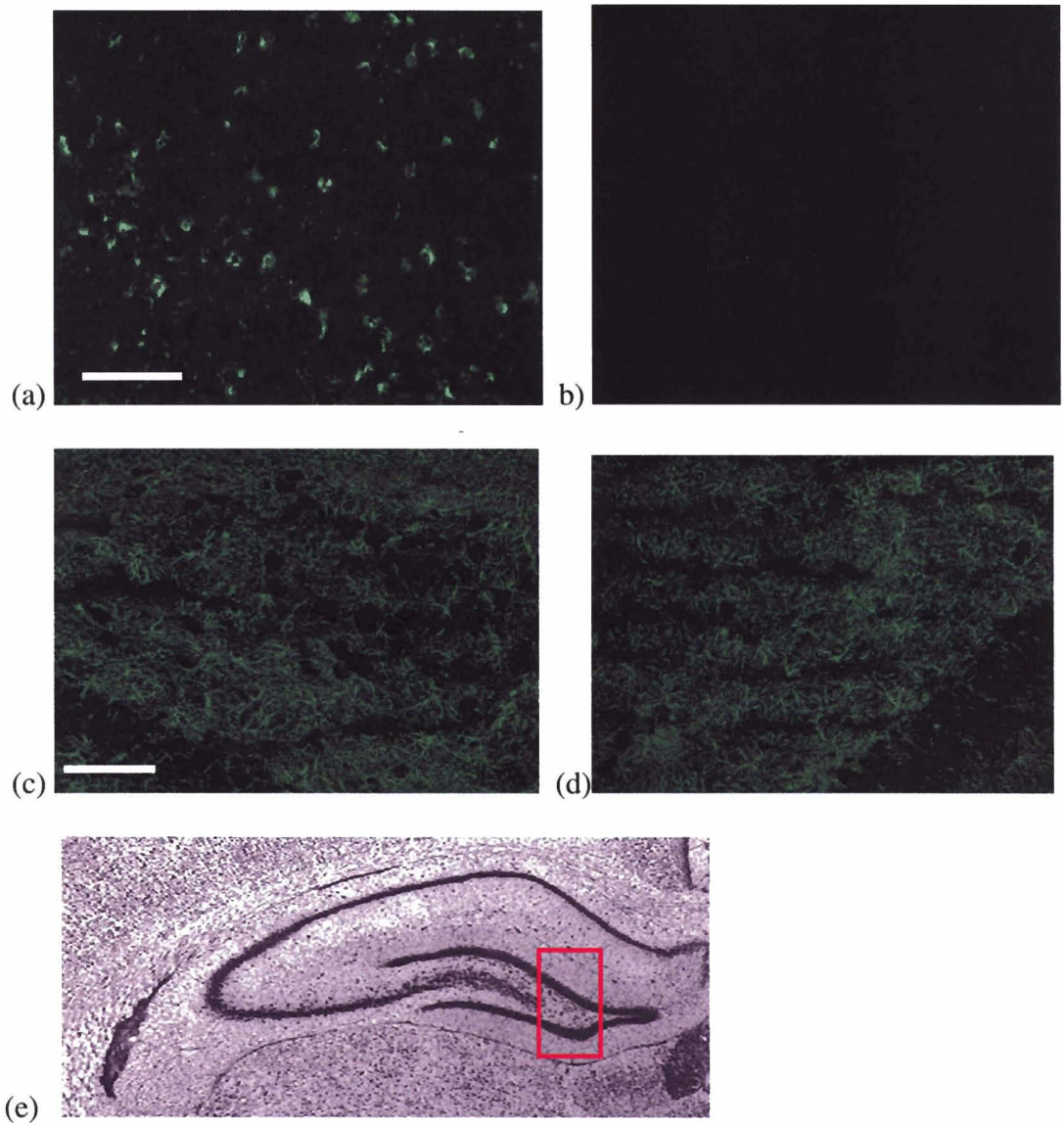
(b)

macrophages (Rappolee et al., 1988), glial cells (Schaudies et al., 1989) and neurons (Morrison et al., 1987). However, after exhaustive searching of double-labeled sections by confocal microscopy (Leica), we found no evidence of co-localization of EGF+ cells with either GFAP+, ED-1+, or NeuN+ cells, both at the site of injury or in the hippocampus. In all double-labeled sections, EGF staining (as visualized with Alexa Fluor 568) did not work, as evidenced by the observation of negative staining of ependymal cells which are characteristically known to express EGF. However, at the level of the hippocampus, distinct macrophage/microglia recruitment to the area ipsilateral to the injury was observed and a strong presence of astrocytes was found in both ipsilateral and contralateral sides (Figure 2.7). These findings provide preliminary evidence for the cellular phenotype and localization of EGF expression following injury.

Discussion

Traumatic brain injury is a debilitating condition which presents a challenge for the development of an effective cure due in part to the limited ability of the CNS to regenerate itself once it has been damaged. Research, however, suggests that the persistence and proliferation of neural stem and progenitor cells in the dentate gyrus of the hippocampus may contribute to functional recovery following TBI to a limited extent. Studies have also found that injury enhances the proliferation and subsequent neurogenesis of these cells, which has been correlated to improvements in hippocampal-dependent cognitive function (Shors 2004). Nevertheless, researchers are only

Figure 2.7: GFAP and ED-1 with EGF staining of cells in the hippocampus. Micrographs show 40x representations of an injured rat hippocampus with a concentration of cells stained positively for ED-1 at the side of the hippocampus (a) ipsilateral and (b) contralateral to the injury, and cells stained positively for GFAP were observed in both (c) ipsilateral and (d) contralateral sides. (e) Nissl section showing the area of the hippocampus represented in (a)-(d). Because EGF staining was insufficient, results are not conclusive for determining the phenotype of cells that express EGF following injury. Bars: 20 μm


(a)
b)
(c)
(d)
(e)

beginning to elucidate the complex microenvironment that drives this endogenous reparative mechanism in the CNS. In this study, we sought to investigate the temporal and spatial expression of the mitogenic factor, EGF, in the injured hippocampus, the most prevalent area of the CNS impacted by TBI. By assessing EGF expression in the hippocampus of various age groups, our results suggest that EGF expression increases following injury and that this heightened level of expression declines with age. In addition, we attempted to determine the cellular phenotype and localization of up-regulated EGF following injury in the CNS.

The significance of assessing the expression of EGF in the injured brain has therapeutic applications for harnessing an endogenous reparative mechanism in the addition, Previous studies from our lab have already begun to chronicle the innate proliferative response of neural stem and progenitor cells following injury and how EGF, a potent mitogen of the CNS, may stimulate this response. Specifically, we have observed in rats that injury-induced cell proliferation in rats peaks one week following injury, with the highest level observed in juvenile rats at 2 days post-injury (Sun et al., 2005). Additionally, we determined an EGF dose-response curve and found the amount of exogenous EGF that can peak cell proliferation in the adult rat striatum (McGinn et al., 2004). While numerous studies have revealed that expression of certain mitogenic growth factors tends to decrease in the aging brain under normal conditions (Aberg et al., 2006, Webster et al., 2002), mirroring observed declines in neuorogenesis and cognitive function with aging (Greenberg and Jin, 2006), relatively few studies have investigated the potential age-related differences in growth factor expression in the

brain following injury (Jin et al., 2003). Because the adult and elderly populations manifest the most enduring cognitive deficits following TBI, it is critical to investigate how differences in level of EGF expression following injury may relate to differences in injury-induced cell proliferation and subsequent degree of cognitive recovery observed with aging. In the current study, we provided preliminary evidence suggesting that alterations in EGF expression with age in the injured hippocampus correlate with age-related differences in the cell proliferative response to injury. Therefore, this study presents some of the first empirical observations regarding the time course and extent of EGF expression following injury, information that is essential to the future development of tailored growth factor therapies which recapitulate the exquisite microenvironment driving enhanced cell proliferation and subsequent neurogenesis following brain injury.

While our findings are important for the advancement of TBI research, there are a number of limitations to the interpretation of our observations that must be addressed. First, results from this study can only suggest that there is a correlation between injury-induced cell proliferation and EGF expression in the hippocampus following injury. In this study, we assessed level of EGF expression in the injured hippocampus in juvenile, adult and aged rodents using two different methods of densitometry analysis, BioRad's Versa Doc system with Quantity One software and Adobe Photoshop with Image J software provided by the NIH. Regardless of program used, we found that EGF expression significantly increased in juvenile rodents at 2 days post-injury and that increased EGF expression tends to decrease with age at this time point. Data at 7 and 14 days post-injury were less consistent across the different programs used.

We utilized the Versa Doc/Quantity One method because of the system's features of versatility combined with state-of-the-art resolution for obtaining data, either directly from blots or developed x-ray films. The latter option was chosen to ensure that tangible evidence of effective membrane blotting was obtained, due to low signal detection of samples from animals sacrificed 14 days after injury. Using this system, at 7 days post-injury, it was observed that levels of EGF expression following injury between juvenile and adult rats were equal, and that, interestingly, EGF expression was up-regulated in adult sham rats compared to adult injured rats as well as in aged sham rats compared to their injured counterparts at this time point. These findings were not consistent with previous research from our lab which indicated that, at 7 days post-injury, the proliferative response to injury remained higher both in injured animals and younger populations (Sun et al., 2005). The anticipated notion that injury induces higher levels of cell proliferation and subsequent level of EGF expression was also not supported in our observation that, at 14 days post-injury/surgery, while all measured EGF expression levels tended to be of similar value to the internal standard of juvenile sham animals, EGF expression was found to be greater in the aged sham rats compared to their injured counterparts. Nevertheless, it should be noted that while these levels of EGF expression observed at 14 days post injury were relatively low, EGF may still be serving some biological purpose.

On the other hand, Image J was utilized in this study because of its established reputation for providing immediate densitometry results following western blotting. Using this program, we found that level of EGF expression was higher in juvenile sham

rats at 7 and 14 days post surgery compared to juvenile injured rats at this time point. This program also indicated that EGF expression was markedly higher in the juvenile sham rats compared to juvenile injured rats, a finding that, as mentioned previously, is not consistent with the anticipated notion that cell proliferation is increased in response to injury and that levels of growth factor should correlate with this response.

While this study has revealed a number of counterintuitive findings, it should be acknowledged that these observations may hold some biological significance and that repeated trials would be necessary to assess the extent of that significance. Nevertheless, it is more likely that these findings were observed due to methodological error that may be remedied in the future with repeated trials and further investigation into the most optimal option to analyze data.

The differences in the results across these two imaging programs may stem from a combination of multiple factors from these experiments. In this study, these programs were detecting signals of varying intensities from previously developed films of the western blots. Therefore, it should be first recognized that these programs were detecting signals reflecting the conditions associated with a given western blotting run. Furthermore, each program may be more or less sensitive to the level of pixels that get counted in a given analyzed volume. Therefore, more trials will need to be conducted in order to determine which program most accurately addresses the aims of this research. Lastly, regardless of program used, in order to further support the hypothesis that EGF expression following injury correlates to age-related differences in the levels of injury-induced cell proliferation, future studies must increase n numbers for each of

the different types of animals investigated to reduce standard error of the mean and increase the power of statistical analyses.

Due to the experimental design of this study, we were not able to answer the question regarding how level of EGF expression differs across various time points post-injury. More specifically, in order to develop effective treatments for TBI which mimic the microenvironment that stimulates proliferation following injury, it is critical to fully understand the time course of EGF expression in the injured brain. For instance, information regarding the time window post-injury where EGF expression peaks would be essential for injecting an exogenous source of EGF following injury to enhance cell proliferation, subsequent neurogenesis, and, ideally, cognitive recovery. Because we ran gels that solely contained samples originating from animals sacrificed at similar time points post-injury, we could not assess how level of relative EGF expression following injury in different age groups varies at different time points post-injury. Future trials will need to include loading gels with samples from animals sacrificed at these various time points so that samples can be exposed to the same conditions and their band intensities legitimately compared relative to each other. By gaining this information, a more complete understanding of the time course and extent of EGF expression following injury can be obtained.

Lastly, there are limitations to the interpretation of our results regarding the cell phenotype and localization of EGF cells in the injured brain following immunohistochemistry analysis. First, because no evidence of double-labeled cells was found, whether in EGF/ED-1, EGF/GFAP or EGF/NeuN labeled sections, it can not be

concluded, based on these results, what the primary cellular sources of EGF are following injury. Future studies, however, should continue to investigate if these cells up-regulate EGF in the hippocampus for a number of reasons. We found that EGF staining did not work in our double-labeled sections, with tissue auto-fluorescing and not revealing any cellular morphology, implying that if trials were repeated with successful EGF staining, we may find evidence of double-labeling. Additionally, after viewing EGF/ NeuN sections under a microscope, we found gaps in our sections that could have stained with NeuN and represented neurons, indicating that NeuN staining also may have been unsuccessful. We found that astrocytes and macrophages/microglia were concentrated in the injured hippocampus, and past research has established that astrocytes and macrophages are a source of EGF.

While the results from this study only begin to suggest a correlation between EGF expression following injury and age-related differences in enhanced cell proliferation following injury, this research can contribute to the advancement of developing treatments that may significantly improve cognitive recovery in the adult and aged CNS following TBI. Studies have reported that newly generated granule cells in the adult hippocampus are involved in learning and memory (Shors et al., 2004). To further support the relationship between cell proliferation after injury and cognition, studies lab have revealed that cognitive recovery is significantly decreased by eliminating cell proliferation in the hippocampus using x-irradiation, as assessed by the Morris Water Maze (Tada et al., 2004). Studies have also shown that newly generated cells in the subgranular zone (SGZ) of the dentate gyrus following injury are able to

differentiate into neurons and send projections to their appropriate CA3 targets to be functionally integrated into the existing Hippocampal circuitry (Emery et al., 2005). Moreover, our lab has begun to directly test the relationship between growth factor expression, proliferation and cognitive improvement by assessing the extent to which an exogenous administration of EGF influences cell proliferation and cognitive recovery in adult rats following TBI (see Chapter 3).

Collectively, these results indicate a potential use for growth factors in treating traumatic brain injury. We provided evidence suggesting that epidermal growth factor expression increases following injury at 2 days post injury in the hippocampus of juvenile injured rats, similar to previous findings from our lab that level of enhanced cell proliferation following injury peaks at this time. Additionally, our findings suggest that level of increased EGF expression following injury decreases with age. Lastly, we attempted to determine the phenotype and localization of cells that express EGF following injury. In conjunction with other related studies, this research should lead to the development of growth factor therapies aimed at stimulating the enhanced proliferation of neural precursor cells following injury in order to regenerate cells lost to injury and improve functional recovery.

Chapter Three

Future Prospects: Exogenous Growth Factors on Hippocampal Cell Proliferation and Cognitive Recovery following TBI

Research suggesting a correlation between enhanced cell proliferation following injury and growth factor expression can be applied towards the development of treatments that may improve cognitive recovery following TBI. These treatments would be most beneficial for the adult and elderly populations, people who have been shown to manifest the most debilitating cognitive deficits following brain injury. However, while compelling evidence suggests that cell proliferation and the generation of new neurons underlie cognitive recovery after TBI and that growth factors mediate this proliferative response, a more complete understanding of the temporal profile of growth factor involvement must be obtained before applying this knowledge in a clinical setting. More importantly, research must determine whether a direct relationship exists between growth factor expression, cell proliferation and cognitive improvement following TBI.

Our lab has begun preliminary studies to directly test this relationship by determining the extent to which an exogenous application of EGF or bFGF influences cell proliferation and cognitive recovery in adult rats following TBI. In these studies, adult rats were subjected to a 2.1 atm fluid percussion injury and immediately received intraventricular infusion, via an Alzet Osmotic minipump, with either EGF or bFGF over a period of seven days. Furthermore, in order to label newly proliferating cells,

rats were given daily BrdU injections (50 mg/kg) for five consecutive days, starting at two days after injury. For controls, a group of sham and injured animals were infused with a saline vehicle. We observed that injured animals infused with growth factors exhibited a greater level of cell proliferation than those that were infused with saline vehicle alone, as determined by comparing total number of newly proliferating cells in the hippocampus of each group (Figure 3.1). These findings provided strong support that an exogenous application of growth factors can significantly enhance cell proliferation in the hippocampus following TBI. We next tested if similarly treated animals would display improvements in cognitive recovery using the Morris Water Maze test. By comparing differences in goal latency, we found that rats infused with either EGF or bFGF following TBI showed significant improvements in cognitive recovery compared to vehicle infused rats (Figure 3.2). Collectively, these preliminary studies have established a compelling link between growth factors, the neurogenic response, and cognitive improvement following TBI. Furthermore, our findings indicate that there is therapeutic promise in utilizing an exogenous application of growth factors to the injured brain to improve cognitive recovery following TBI. Nevertheless, further research is required to fully elucidate and recapitulate the microenvironment that can optimize the endogenous proliferative response in the CNS and improve cognitive recovery in adults and the elderly following TBI.

For instance, the role and differential expression of EGF receptors in the CNS with age has yet to be fully elucidated. The EGF receptor has been found to be widely distributed throughout the CNS and binds with high affinity to EGF ligands to initiate

Figure 3.1. Growth factor infusion enhances cell proliferation in the hippocampus following TBI. Quantitative analysis of the total number of BrdU-positive cells in the ipsilateral (ip) and contralateral (ct) DG at seven days following injury and EGF or bFGF infusion. In the ipsilateral SGZ, the total number of BrdU-positive cells was significantly increased in both EGF and bFGF-infused injured animals.

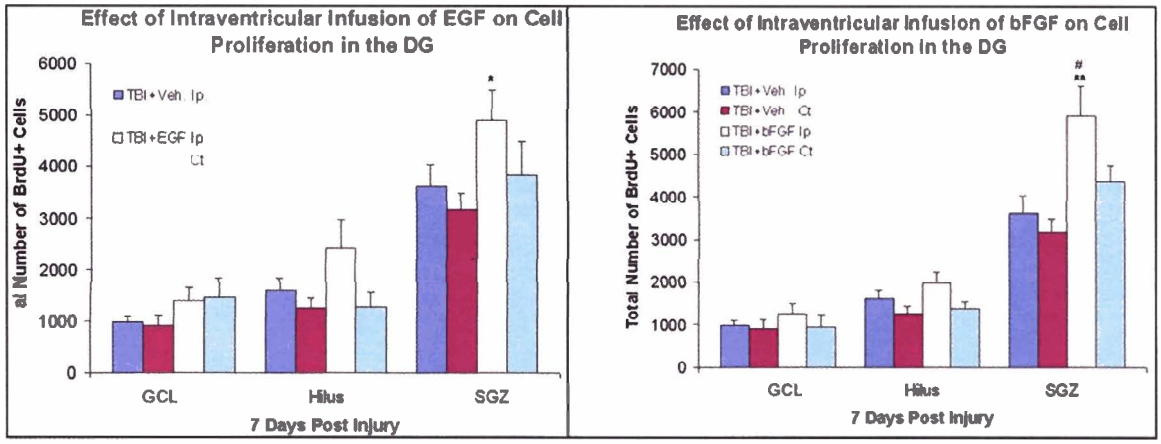
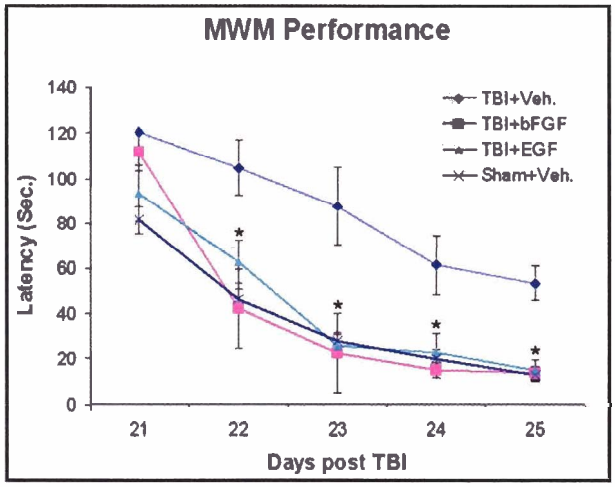

Effect of Intraventricular Infusion of EGF on Cell Proliferation in the DG
al Number of BrdU+ Cells
6000
5000
4000
3000
2000
1000
0
TBI + Veh Ip
TBI + EGF Ip
Ct
*
GCL
Hilus
SGZ
7 Days Post Injury
Effect of Intraventricular Infusion of bFGF on Cell Proliferation in the DG
Total Number of BrdU+ Cells
7000
6000
5000
4000
3000
2000
1000
0
TBI + Veh Ip
TBI + Veh Ct
TBI + bFGF Ip
TBI + bFGF Ct
#
**
GCL
Hilus
SGZ
7 Days Post Injury

Figure 3.2. Growth factor infusion improves cognitive recovery following TBI. Graph comparing MWM performance of injured rats infused with bFGF, EGF or saline vehicle, to sham animals infused with vehicle alone. Rats infused with EGF or bFGF following injury showed a significant improvement of cognitive recovery, as compared to injured, vehicle infused animals (*$p < 0.01$). This cognitive recovery, as measured by a shorter latency period in the water maze performance, reached similar levels to that observed for sham animals through days 22-25.


MWM Performance
Latency (Sec.)
140
120
100
80
60
40
20
0
21
22
23
24
25
Days post TBI
TBI+Veh.
TBI+bFGF
TBI+EGF
Sham+Veh.
*
*
*
*

a variety of signaling cascades and cellular activities, particularly proliferation and differentiation. However, research regarding whether the expression of EGF receptors decreases with age in the CNS has been inconsistent. One study conducted by Gomez-Pinilla and colleagues found that expression of EGF receptors first appeared in astroglia at postnatal day 16 and subsequently declined into adulthood (Gomez-Pinalla et al., 1988). However, in a more recent study, results from immunostaining indicated that EGFR expression was more prominent in the adult hippocampus compared to that seen at postnatal day 7 (Tucker et al., 1993). Yet, another study cited a lower level of EGF receptor expression in the aged subventricular zone (SVZ), the second active germinal zone of the CNS, and found that aged neural stem cells and their progeny displayed a decreased capacity to respond to EGF infusion and a complete but longer time course for repopulating the SVZ (Enwere et al., 2004). Taken together, these studies suggest that age-related differences in the level of growth factor receptor expression may also be related to the extent of the proliferative response to injury; however the biological explanation for these differences has yet to be fully elucidated.

Nevertheless, research has also established that not one, but rather a constellation of growth factors, is involved in stimulating cell proliferation in the injured brain. Numerous studies have been conducted to gain a more complete picture of how these different factors contribute to the neurogenic response in the hippocampus and cognitive improvement following TBI. Studies looking at the attenuation of cognitive deficits following exogenous administration of basic fibroblast growth factor and nerve growth factor represent only a fraction of the research that has been

conducted to further establish a link between growth factor expression, cell proliferation and cognitive improvement after injury (Monfils et al., 2005, Sinson et al., 1995). Additionally, our lab has started to utilize antibody microarrays to quantitatively analyze the expression of known growth factors found in the hippocampus following traumatic brain injury (Figure 3.3). Future studies will need to continue to address such critical issues as: optimal concentration and time window of growth factor administration to avoid toxicity, effect of administrating multiple growth factors, as well as ideal mode of administration.

In conclusion, this thesis has investigated the expression of epidermal growth factor in the injured hippocampus with age and the therapeutic implications of this information. By gaining a more complete understanding of growth factor expression following injury, treatments may be tailored to mimic the microenvironment that optimizes an endogenous proliferative response to injury and improve cognitive recovery in the adult and aged CNS following TBI.

Fig. 3.3: Protein microarray analysis. RayBio ™ antibody array highlighting a range of growth factors whose expression level can be analyzed in hippocampal tissue of rats following TBI.

A	B	C	D	E	F	G	H	I	J	K	L
POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF R α	PDGF R β	PDGF-AA	PDGF-AB	PDGF-BB	PlGF	SCF
IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF R α	PDGF R β	PDGF-AA	PDGF-AB	PDGF-BB	PlGF	SCF
SCF R	TGF-α	TGF-β	TGF-β 2	TGF-β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS
SC[redacted]	TGF-α	TGF-β	TGF-β 2	TGF-β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS

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