

SURVIVIN EXPRESSION AFTER TRAUMATIC BRAIN INJURY: POTENTIAL  
ROLES IN NEUROPROTECTION

By

ERIK ANDREW JOHNSON

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This document is dedicated to my wife, Karie, for her loving and unwavering support during this process.

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Erik Andrew Johnson

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In these studies, the expression profile and cellular localization of survivin, a novel anti-apoptotic and mitosis protein, following traumatic brain injury (TBI) in rats was examined. Specifically, survivin co-localization with the cell cycle protein PCNA, the apoptosis protease active caspase-3, and the DNA fragmentation label TUNEL was determined to reveal potential role of survivin in neuroprotection and to elucidate anti-apoptotic mechanisms. Levels of survivin mRNA and protein were increased in the ipsilateral, but not contralateral, cortex and hippocampus of rats after TBI, peaking at five days post injury. Similar temporal and spatial patterns of PCNA were also significantly enhanced in these brain regions. Immunohistochemistry revealed that survivin and PCNA were co-expressed in the same cells and had a focal distribution within the injured brain. Further analysis revealed a frequent co-localization of survivin and GFAP, an astrocytic marker, in both ipsilateral brain regions, while a much smaller subset of cells showed co-localization of survivin and NeuN, a mature neuronal marker. PCNA protein

expression was detected in both astrocytes and neurons of the ipsilateral cortex and hippocampus after TBI.

Western blot analysis revealed significant increases in the accumulation of active caspase-3 between five and fourteen days post injury. The percentage of survivin-positive and negative cells labeled with active caspase-3 at five or seven days post-injury was not significantly different. However, survivin-negative cells exhibited a significantly greater labeling with TUNEL compared to survivin-positive cells, thereby suggesting that expression of survivin may attenuate DNA cleavage and progression of apoptosis. Although a higher percentage of astrocytes accumulated active caspase-3 compared to neurons, these neurons showed significantly higher frequency of TUNEL labeling.

These novel data demonstrate that survivin is abundantly expressed in brain cortex and hippocampus of adult rats following TBI. Survivin accumulation occurs primarily in astrocytes and a sub-set of neurons. The occasional co-expression of survivin and PCNA coupled with the low frequency of TUNEL labeling in survivin expressing cells may suggest that survivin is primarily involved in attenuating apoptotic cell death and secondarily may play a role in regulation of neural cell proliferative responses after TBI.

## CHAPTER 1 INTRODUCTION

### **Traumatic Brain Injury Demographics**

Traumatic brain injury (TBI) is the leading cause of death and permanent disability for children and young adults in the United States. Currently, there are more than 5.3 million Americans, approximately 2% of the current U.S. population, living with TBI-related disabilities (Thurman et al., 1999a) with an estimated 1.5 million additional TBIs occurring each year (Sosin et al., 1996). Approximately 230,000 cases are severe enough that the victims require transport and hospitalization (Thurman et al., 1999b). Of these, approximately 50,000 victims die from their injuries, accounting for 33% of all injury-related deaths (Sosin et al., 1995). Of those severely injured survivors, 90,000 TBI victims must live with long-term disabilities (Thurman et al., 1999b). The U.S. economy loses an estimated \$56.3 billion a year through the direct and indirect costs associated with TBI (Thurman et al., 1999b).

Currently, most traumatic brain injuries result from motor vehicle accidents (48.9%) followed by falls (25.8%) and firearms/ assaults (19.2%) (Thurman et al., 1999b). Young males, ages 15 to 24, are the most “at-risk” demographic, a statistic that likely reflects lifestyle choices (Jennett, 1996; Thurman et al., 1999b). The magnitude of this problem led to the passing of Public Law 104-166, better known as the Traumatic Brain Injury Act of 1996, a bill designed to help prevent TBI and educate the public about the health consequences of this injury (Thurman et al., 1999b). While these educational efforts have decreased TBI-related deaths by an estimated 22% since 1980

(Sosin et al., 1995), the number of people *living* with TBI-related disabilities has risen (Thurman et al., 1999b).

To date, few pharmacological or treatment options are available to reduce these TBI-induced disabilities. Prevention remains the only effective “cure.” True advances in clinical treatment depend on understanding the underlying pathophysiology mechanisms that regulate both cell death and cell survival following TBI.

### **Traumatic Brain Injury Pathophysiology**

Traumatic brain injury is a complex injury that is comprised of an immediate primary injury and a progressive secondary injury cascade (Graham et al., 2000). The primary mechanical injury can be contusive or concussive and involves tearing and stretching of the neural tissues. Neurons and white matter tracts seem particularly vulnerable to the mechanical injury (Baldwin et al., 1997; Maxwell et al., 1997; McCullers et al., 2002; Grady et al., 2003). The secondary injury cascade is initiated by the primary mechanical injury and is defined by unrestrained biochemical and inflammatory reactions (Gennarelli, 1993). Though the brain is remarkably adaptive, damage sustained as the result of secondary injury prevents the brain from regaining pre-injury function.

While many of the biochemical processes seen after TBI occur under normal homeostasis, their collective dysregulation acts in a synergistic manner to contribute to the pathology associated with secondary injury. Some of the more prominent events include perturbations in blood flow (Graham et al., 1995; McIntosh et al., 1998; Raghupathi et al., 2000), ischemia (Lee et al., 1999; Passineau et al., 2000), excitotoxicity (Choi and Rothman, 1990; Gennarelli, 1993), calcium deregulation (Graham et al., 1995), free radical production (Kontos, 1989; Beckman et al., 1990; Maier and Chan, 2002),

inflammation (Povlishock and Kontos, 1985; Giulian, 1991; Morganti-Kossmann et al., 2001), edema (Choi, 1988; Bullock et al., 1991) and protease activation (Pike et al., 1998; Clark et al., 2000; Eldadah and Faden, 2000; Raghupathi et al., 2000; Knoblach et al., 2002; Larner et al., 2004). Ultimately, activation of these processes disrupts fragile homeostatic states and creates an inhospitable environment for neural cell survival.

### **Traumatic Brain Injury, Apoptosis and Caspase-3 Activation**

Cell death following TBI is distinguished by necrotic and apoptotic processes (Conti et al., 1998; Clark et al., 2000; Yakovlev and Faden, 2001). Necrosis and apoptosis lie on a continuum (Nicotera et al., 1999) wherein the mode of cell death is dictated by several factors including ATP availability (Green and Reed, 1998), calpain activity (Wang, 2000), intracellular calcium levels (Gwag et al., 1999; Zipfel et al., 2000), presence of anti-apoptotic factors (Raghupathi et al., 2000) and the presence of activated caspases (Denecker et al., 2001). Within hours of a TBI, neural cells around the contusion area exhibit classic signs of necrosis including cytotoxic edema, mitochondrial swelling, nuclear pyknosis, ruptured plasma membranes, organelle breakdown and vacuolated cytoplasm (Sutton et al., 1993; Dietrich, 1994; Denecker et al., 2001). However, as time progresses, many cells including neurons, astrocytes and oligodendrocytes begin to exhibit characteristics of apoptosis including chromatin condensation, cell shrinkage, apoptotic body formation and DNA laddering (Conti et al., 1998; Newcomb et al., 1999). It is well documented that apoptotic cell death continues for many months following injury, thereby making it a chronic contributor to post-TBI pathology (Cervos-Navarro and Lafuente, 1991).

Apoptosis has been well characterized following traumatic brain injury (Pike et al., 1998; Beer et al., 2000; Clark et al., 2000) and utilizes both the intrinsic and extrinsic

apoptotic pathways (Fig. 1-1). Each pathway involves a unique set of upstream and downstream cysteine specific proteases called caspases that cleave a variety of intracellular substrates and drive apoptosis. Synthesized as inactive zymogens, caspases require the cleavage of a pro-domain to become active. Caspase activation is achieved in multiple manners including proximity-induced autoproteolysis or cleavage by another caspase (Stennicke and Salvesen, 1999; Van de Craen et al., 1999). Both the intrinsic and extrinsic pathways lead to cleavage and activation of caspase-3, the most abundant executioner caspase in the brain (Chan and Mattson, 1999; Slee et al., 2001).

Among the numerous structural and regulatory protein targets of active caspase-3 are stress response proteins (*e.g.*, PARP, Rb and p21), signal transduction proteins (*e.g.*, phospholipase A2, NF $\kappa$ B and PKC), structural proteins (*e.g.*,  $\alpha$ -II-spectrin, actin and vimentin), nuclear matrix proteins (*e.g.*, lamins A, B1 and C) and mitochondrial proteins (*e.g.*, Bcl-2, Bcl-x1 and Bid) (Cohen, 1997; Chan and Mattson, 1999; Earnshaw et al., 1999; Wang, 2000). Cleavage of proteins such as iCAD/DFF45 (Enari et al., 1998; Liu et al., 1998b; Sakahira et al., 1998), poly (ADP-ribose) polymerase (PARP) (Ferrer and Planas, 2003), DNA-dependent protein kinase (DNA-PK) (Lazebnik et al., 1994) and acinus (Sahara et al., 1999) prevents DNA repair and promotes DNA condensation and fragmentation (Woo et al., 1998). When unregulated, even moderate activation of caspase-3 can rapidly lead to cell death. Therefore, nature has developed various mechanisms to temper the deleterious effects of caspase-3 over-activity and counter the progression of apoptosis.



### **Apoptosis Inhibition Following TBI**

While many pro-apoptotic proteins are expressed following TBI, there is a concomitant increase in pro-survival factors (Nowak and Jacewicz, 1994; Iwata et al., 1997; Buytaert et al., 2001; Hermann et al., 2001; Sanz et al., 2001; Alzheimer and Werner, 2002; Maroni et al., 2003). Of these pro-survival factors, a family of proteins known as inhibitor of apoptosis proteins (IAPs) can attenuate apoptotic cell death by directly binding to the active site of activated caspases such as caspase-3 (Tamm et al., 1998; Conway et al., 2000; Shin et al., 2001). The IAP family contains eight known members including survivin (Li, 2003). The proteins are highly conserved across species (LaCasse et al., 1998) and the expression of each IAP appears to be cell type specific. Each IAP has one to three baculovirus IAP repeat (BIR) domains that possess the ability to bind and directly inhibit active caspase-3 (Tamm et al., 1998; Conway et al., 2000; Shin et al., 2001), caspase-7 (Tamm et al., 1998; Shin et al., 2001) and caspase-9 (LaCasse et al., 1998; Deveraux and Reed, 1999). Mutation studies have demonstrated that the BIR domain is responsible for caspase interaction and is therefore necessary for anti-apoptotic action of the IAPs (Roy et al., 1997; Takahashi et al., 1998; Vucic et al., 1998; Muchmore et al., 2000). Although few IAPs have been extensively characterized in the context of TBI pathophysiology, increases in XIAP (Keane et al., 2001; Lotocki et al., 2003), NAIP (Xu et al., 1997; Hutchison et al., 2001; Thompson et al., 2004), cIAP-1 (Keane et al., 2001; Belluardo et al., 2002) and cIAP-2 (Keane et al., 2001) have been reported in neurons following brain injury. The potential role of survivin following TBI has not been investigated.

### **Cellular Proliferation Following TBI**

In addition to pro-survival factors, new cell production plays a pivotal role in the brain following injury. Large pools of neural progenitor cells have recently been identified in the germinal centers of the dentate gyrus subgranular zone (SGZ) and subventricular zones (SVZ) of the adult brain (Gage et al., 1998; Magavi et al., 2000; Gage, 2002; Sanai et al., 2004). Following both ischemia and TBI, these neural progenitor cells proliferate (Gould and Tanapat, 1997; Yagita et al., 2001) and differentiate into mature neurons (Gage et al., 1998; Doetsch et al., 1999; Magavi et al., 2000; Cameron and McKay, 2001; Dash et al., 2001; Kernie et al., 2001; Yagita et al., 2001; Peterson, 2002), astrocytes (Dash et al., 2001; Gould et al., 2001; Chirumamilla et al., 2002; Chen et al., 2003) and oligodendrocytes (Gould et al., 2001). Consistent with these findings, many cell cycle proteins (*e.g.*, cyclins A, B and D1, cdk4 and PCNA) are also up-regulated after brain injury (Miyake et al., 1992; Kaya et al., 1999a; Chen et al., 2003; McPherson et al., 2003).

Cellular proliferation following TBI can have both beneficial and detrimental consequences to the recovery of the damaged brain. These consequences can also vary by cell type. Neuronal progenitor cells have been shown to proliferate following brain injury (Parent, 1997; Hill-Felberg et al., 1999; Dash et al., 2001; Kernie et al., 2001; Yagita et al., 2001; Chirumamilla et al., 2002; Rice et al., 2003) but the functional viability and therefore significance of these newly formed neurons is not clear. New neurons appear to migrate away from the germinal centers of the subventricular zone (SVZ) and subgranular zones of the dentate gyrus (SGZ) but not towards areas of injury (Rice et al., 2003). Additionally, as many as 80% of all newly formed neurons undergo apoptosis within two weeks of their formation in normal conditions (Morshead and van

der Kooy, 1992; Morshead et al., 1994). The proliferation of neurons following TBI may be advantageous but their inability to survive and contribute to recovery requires additional clarification.

Glial cell proliferation, specifically astrocytes and oligodendrocytes, can serve to both support and inhibit natural recovery processes. Adult oligodendrocyte precursor cells (OPC) can develop into both astrocytes and oligodendrocytes following injury. Furthermore, their distribution in the adult brain is not as restricted as the neuronal precursor populations (Dawson et al., 2000). An increase in both the astrocyte and oligodendrocyte population may contribute positively to the post-injury milieu. Astrocytes metabolize extracellular glutamate, neutralize free radicals, modulate the immunological response by production of cytokines and modulating nitric oxide activity (Gabryel and Trzeciak, 2001; Bambrick et al., 2004; Heales et al., 2004). Similarly, oligodendrocytes can help re-myelinate damaged axons. Furthermore, glial cell proliferation may contribute to formation of the glial scar. This barrier can act to protect non-damaged brain regions from advancing secondary injury processes (Ridet et al., 1997; Bush et al., 1999; Smith et al., 2001). However, the glial scar also produces chondroitin sulfate proteoglycans which may then act to form an impermissible environment for axonal growth (Fawcett and Asher, 1999; Chen et al., 2002).

With developmental origins as hematopoietic cells, microglia are one of the few mature neural cell types that retain the ability to divide (Simard and Rivest, 2004). After various types of brain injury, microglia proliferate rapidly (Liu et al., 1998a; Csuka et al., 2000; Liu et al., 2000; Grady et al., 2003) to remove cellular debris, protect injured neurons and promote functional recovery (Giulian, 1991). However, microglia have been

documented as a major source of proteases and inflammatory cytokines following various CNS injuries (Nakajima and Kohsaka, 1993; Streit and Kincaid-Colton, 1995; Streit, 1996; Aldskogius et al., 1999; Fawcett and Asher, 1999; Gong et al., 2000).

Functional replacement of injured and dying cells may contribute to more complete recovery following TBI. Because the neural environment becomes hostile for new cells to survive during the injury state, the identification of proteins that promote both cellular proliferation and survival in compromised cellular environments may prove useful in treating the injured brain. A very delicate balance between proliferation and cell death inhibition is desired. Survivin is a protein that has recently been identified as having roles in both mitosis regulation and apoptosis inhibition in other non-central nervous system (CNS) pathological conditions and may contribute to this balance following TBI.

### **Survivin: Mitosis and Anti-apoptosis Protein**

Survivin was discovered in 1997 as a protein expressed only by rapidly dividing cells during development (Ambrosini et al., 1997). As its expression is prominent in apoptosis-resistant tumor cells, survivin became an intensely studied protein in cancer research. These studies demonstrated that survivin functioned to inhibit apoptosis and was essential for the proper completion of mitosis. Because it has an integral role in cellular proliferation and apoptotic cell death, both of which contribute to the pathophysiology of TBI, survivin may have an important role in the secondary injury cascade.

### **Survivin Protein Structure**

The survivin protein is composed of 142 amino acids with a molecular weight of 17 kDa per monomer (Ambrosini et al., 1997). Cellular survivin exists as a homodimer bound together by an intermolecular Zn<sup>+</sup> atom giving the complex a “bow-tie “

appearance and is the only IAP known to homodimerize in solution (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000) (Fig.1-2). Survivin is the smallest IAP to have anti-apoptotic properties, containing only a single BIR domain and microtubule-binding coiled coil domain (Ambrosini et al., 1997).

A distinct subcellular pool of survivin exists in the cytoplasm and nucleus of the cell (Conway et al., 2000; Li, 2003; Badran et al., 2004) with a ratio of 6:1, respectively (Fortugno et al., 2002). Recent evidence suggests that the subcellular localization of survivin may designate its role. The nuclear pool appears to be associated with cellular proliferation while cytoplasmic survivin appears to be more predictive of caspase inhibition (Moon and Tarnawski, 2003). Survivin is a relatively short-lived protein with a half-life of approximately 30 minutes (Zhao et al., 2000), though phosphorylation may enhance its stability (O'Connor et al., 2000a; O'Connor et al., 2002). Survivin is removed from the cell by polyubiquitination and proteasomal destruction (Zhao et al., 2000).

### **Survivin Expression and Mitosis**

As a protein found almost exclusively in apoptosis-regulated embryonic and fetal tissue (Adida et al., 1998; Kobayashi et al., 1999), survivin is not normally found in differentiated adult tissues. However, it is present at very low levels in adult cells with a high mitotic index (Ambrosini et al., 1997). The function of survivin during mitosis is intimately related to its ability to bind microtubules. Survivin is required for the assembly of a bipolar mitotic apparatus by controlling microtubule stability (Altieri, 2001, 2003b). Homozygous deletion of the survivin gene causes defects in microtubule assembly, mitotic spindle formation and cell division resulting in multi-nucleation and total lethality of the organism by E3.5-4.5 in knockout mice (Uren et al., 2000).

Beyond development, survivin is prominently expressed in many cancers and is linked to poor survival prognosis, higher rates of cancer reoccurrence and elevated mortality rates (Altieri, 2003a). Many neural derived cancer cell lines have been shown to over-express survivin including astrocytes (glioma), neurons (neuroblastoma) and oligodendrocytes (oligodendroglioma) (Shankar et al., 2001; Borriello et al., 2002; Sasaki et al., 2002; Kajiwara et al., 2003; Kleinschmidt-DeMasters et al., 2003; Jiao et al., 2004), indicating that mature, albeit abnormal, neural cells retain the ability to express survivin beyond differentiation. Additionally, proliferating neural stem cells express mitosis proteins after brain injury (Cameron and McKay, 1998; Doetsch et al., 1999; Cameron and McKay, 2001; Song et al., 2002) indicating that non-transformed neural cells may also express survivin following TBI.

### **Survivin and Apoptosis Inhibition**

The ability of survivin to inhibit apoptosis is known to occur in conjunction with the cell cycle but also has been shown to be independent of mitosis. For example, many tumor cells express survivin when not actively dividing and can inhibit apoptosis caused by chemotherapeutic agents (Li et al., 1998). Beyond cancer, survivin expression has been reported in non-proliferating, non-tumor cells after ischemic brain injury without activating mitosis and with the ability to inhibit cell death (Blanc-Brude et al., 2002; Tran et al., 2002; Conway et al., 2003). Therefore, survivin expression may occur without activation of the cell cycle.

It has been demonstrated repeatedly that survivin over-expression can inhibit apoptosis in cancer cells (Ambrosini et al., 1998; Grossman et al., 1999; Muchmore et al., 2000; Shin et al., 2001; Kim et al., 2004). Survivin expressing gastric and esophageal squamous cell cancers exhibit significantly lower rates of apoptosis compared to

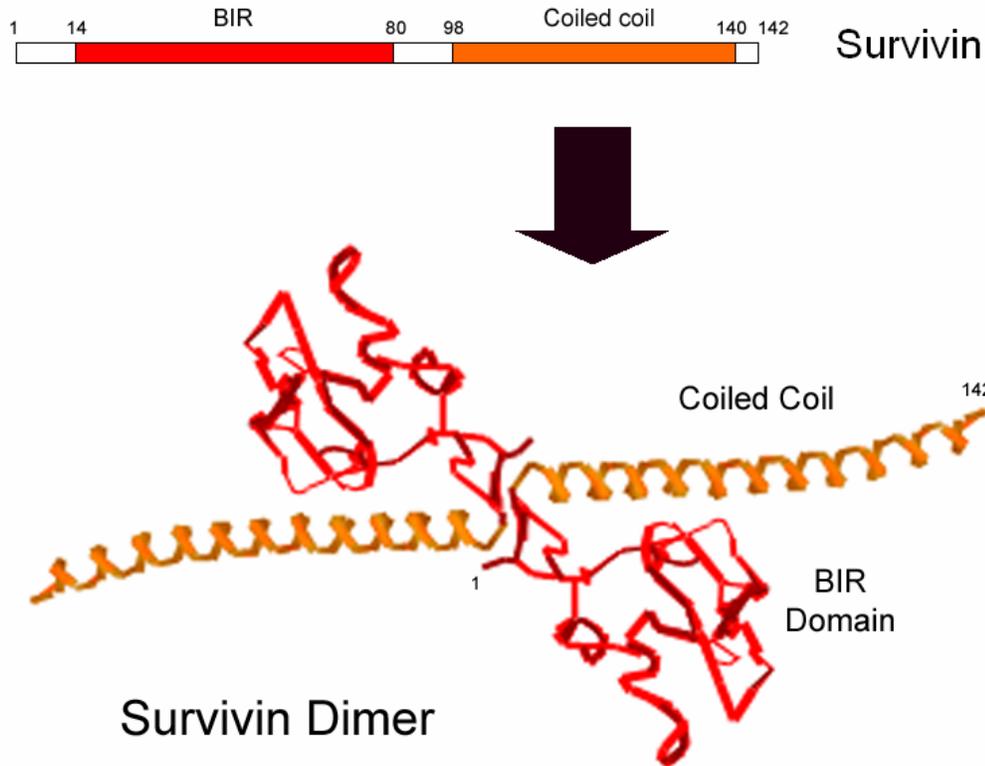


Figure 1-2: Survivin protein structure. Survivin is a 142 amino acid (17 kDa) protein that contains a single baculovirus IAP repeat (BIR) domain (red) and a C-terminus  $\alpha$ -helical coiled coil domain (orange). In solution, survivin homodimerizes and is held together by a zinc ion interaction. The survivin BIR domain has been shown to bind activated caspase-3 and inhibit apoptosis induced by many factors. The coiled coil domain can bind and stabilize microtubules during assembly of the bipolar mitotic apparatus and keep it in close proximity to caspase activity as mitosis progresses. 3-D survivin structure adapted from Verdecia et al 2000.

survivin-negative cancer cells (Lu et al., 1998). Molecular antagonists of survivin (*e.g.*, siRNA, antisense, dominant negative mutants) cause caspase-dependent cell death and magnify the effects of other pro-apoptotic signals *in vitro* and *in vivo* (Li et al., 1999; Kanwar et al., 2001; Kasof and Gomes, 2001; Shankar et al., 2001; Xia et al., 2002a; Zhou et al., 2002; Choi et al., 2003). In addition, survivin has been shown to protect cells from a variety of apoptotic stimuli including IL-3 withdrawal (Ambrosini et al., 1997), Fas stimulation (Tamm et al., 1998; Jiang et al., 2001), anoikis (Papapetropoulos et al., 2000), cytochrome c administration (Takahashi et al., 1998; Tamm et al., 1998), Bax

over-expression (Deveraux et al., 1997; Tamm et al., 1998), active caspase-3 (Tamm et al., 1998), active caspase-7 (Tamm et al., 1998; Jiang et al., 2001), Taxol (Li et al., 1998), and etoposide (Tamm et al., 1998; Jiang et al., 2001).

In these models, survivin appears to exert its anti-apoptotic effects by directly binding to active caspase-3 (Tamm et al., 1998; Kobayashi et al., 1999). It is possible, however, that survivin may also act at other, less clearly defined points in the apoptotic cascade (Suzuki et al., 2000; Grossman and Altieri, 2001; Grossman et al., 2001a; Fortugno et al., 2003) or outside of apoptotic caspase activation to prevent cell death (Shankar et al., 2001; Chakravarti et al., 2004).

### **Potential Role for Survivin in TBI Pathology**

There are currently no comprehensive studies of survivin in neural cells following CNS injury. Moreover, the potential involvement of survivin in TBI pathophysiology is unknown. The role of survivin in apoptosis inhibition and cellular proliferation in various *in vitro* and *in vivo* models supports the hypothesis that survivin may also contribute to the pathophysiology of TBI. Both apoptosis and cellular proliferation occur following traumatic brain injury and create an environment where survivin expression may be important in balancing two contrasting yet related processes. From the literature, it is clear that survivin is ubiquitously expressed by all cells early in development and that this expression may be restored in certain mature cells following CNS injury.

Therefore, based on the existing data described above from the areas of cancer, mitosis and apoptosis, a thorough investigation of survivin following TBI was warranted. Thus, the main goal of this work is to reveal and characterize potential roles for survivin in neural cell responses following traumatic brain injury. The general hypothesis of the study is that survivin is up-regulated following TBI and plays a role in anti-apoptotic and

cell cycle activation mechanisms to oppose TBI pathogenesis. Specifically, I propose that *(i)* survivin up-regulation inhibits caspase-3 mediated DNA fragmentation in a cell-specific manner following TBI, and *(ii)* survivin plays a role in cell cycle progression following TBI.

## CHAPTER 2 METHODOLOGY

### **Induction of Controlled Cortical Impact Brain Injury**

The surgical and cortical impact injury procedures were conducted as previously described (Dixon et al., 1991; Pike et al., 1998). Briefly, adult male Sprague-Dawley rats (250-300 g) were anesthetized with 4% isoflurane (Halocarbon Laboratories; River Edge, NJ) in 1:1 O<sub>2</sub>/ N<sub>2</sub>O for 4 minutes and maintained during surgery with 2.5% isoflurane. Core body temperature was continuously monitored using a rectal thermistor probe and maintained at 36.5-37.5° C using an adjustable heating pad. A unilateral craniotomy (ipsilateral to injury) was performed over the right cortex between the sagittal suture, bregma and lambda while leaving the dura intact. Traumatic insult was generated by impacting the exposed cortex with a 5 mm diameter aluminum tip at a velocity of 4 m/sec, a 150ms dwell time and 1.6 mm compression. Craniotomy control animals received the craniotomy but not the impact injury. All procedures were performed according to guidelines established by the University of Florida Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health (NIH). In the following studies, “ipsilateral” refers to the same side as the impact injury whereas “contralateral” refers to the opposite side of the injury. “Craniotomy control” refers to animals that received the craniotomy but did not receive the impact injury.

### **Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-PCR)**

Survivin primers were generated using GeneBank locus AF 276775: forward primer 5' TAAGC CACTT GTCCC AGCTT 3', and reverse primer 5' AGGAT GGTAC

CCCAT TACCT 3'. GAPDH: forward primer 5' GGCTG CCTTC TCTTG TGAC 3' and the reverse primer 5' CACCA CTTCG TCCGC CGG 3'. Cortical and hippocampal tissues from the ipsilateral and contralateral hemispheres were rapidly excised at either 1 day, 2 days, 3 days, 5 days, 7 days or 14 days and 'snap-frozen' with liquid nitrogen. Total RNA was isolated from the samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Final RNA concentrations were determined via spectrophotometry and were stored at -20° C in diethyl pyrocarbonate (DEPC) water for future cDNA preparation.

cDNA synthesis was performed using 1 µg of total RNA with the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen/Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Any DNA contamination was detected in the RNA samples by "no reverse transcriptase" reactions that were performed in conjunction with the cDNA synthesis reaction.

Q-PCR was performed as previously described (Tolentino et al., 2002) using the LightCycler-FastStart DNA Master SYBR Green I reaction mix (Roche Diagnostics, Indianapolis, IN) in combination with 0.5 µM primers, 2.5 mM MgCl<sub>2</sub> in the LightCycler rapid thermal cycler system (Roche Diagnostics, Indianapolis, IN). Briefly, the products were amplified then continuously quantified by online monitoring. Each PCR reaction has its kinetics represented by an amplification curve. Each amplification curve (fluorescence vs. cycle number) is assigned a crossing point value (CPV), which is the exact time point at which the logarithmic linear phase could be distinguished from the background. A lower CPV indicates a more rapid increase in the level of fluorescence indicative of a higher concentration of specific message present in the sample. Therefore,

those samples with a lower CPV have more amplified message than those with a higher CPV.

The survivin primer sets were subjected to serial dilution and linear regression analysis of the logarithm of the dilution factor vs. the CPV generated a standard curve for each transcript-specific template. The specificity of the amplified products were confirmed using melting curve analysis and gel electrophoresis. The relative amounts of RNA from the unknown samples were extrapolated from its calculated CVP in relation to the generated standard curve. Results are presented as percentage of craniotomy control. Data were analyzed by ANOVA with a post-hoc Bonferroni-test and are given as mean  $\pm$  SEM. Differences were considered significant at the level of  $p \leq 0.05$ .

### **Rat-Specific Survivin Polyclonal Antibody Production**

Commercially available survivin antibodies were not adequate to label survivin in tissue sections. Therefore, a new rat-specific antibody was developed for use in fluorescent immunohistochemistry. Two rat-specific survivin sequence peptides were synthesized using the protein sequence from GeneBank, accession number AF276775 (Swissprot Q9JHY7), for antibody production. The two peptides corresponded to regions in the conserved BIR domain (CPTENEPDLAQC) and from the C-terminus coiled coil domain (CFKELEGWEPDDNPIEE). The peptides used to develop the survivin antibody (R51) are specific to survivin and do not recognize other IAP family proteins according to SDSC Biology Workbench BLASTP (2.2.2) (Altschul et al., 1997) and CLUSTAL W (1.81) analysis (Higgins et al., 1992; Thompson et al., 1994) resulting in the survivin antibody's specificity. Alignment scores for CLUSTALW (1.81) were computed with the following multiple alignment parameters: Matrix: Gonnet, Gap Open Penalty: 10.00,

% Identity for Delay: 30, Penalize End Gaps: on, Gap Separation Distance: 0, Negative Matrix?: no, Gap Extension Penalty: 0.20, Residue-Specific Gap Penalties: on, Hydrophilic Gap Penalties: on, Hydrophilic Residues: GPSNDQEKR.

Rabbits were immunized with these peptides, allowed to produce antibodies to the peptides and finally serum was extracted from the immunized rabbits. The rat specific survivin antibodies were removed and affinity purified using a SulfoLink<sup>®</sup> kit (Pierce Inc; Rockford, IL) as per the manufacturers instructions.

### **Survivin Polyclonal Antibody Characterization**

The specificity of the survivin antibody (R51; Dr. G. Shaw) was compared to other commercially available survivin antibodies (Chemicon; Temecula, CA and Novus Biologicals; Littleton, CO) on western blots and in cell culture. On western blots using dividing cell culture lysates (HeLa and SY5Y) and injured tissue lysates, R51 and the Novus survivin antibody show a similar labeling pattern and recognized the 17 kDa monomer of survivin. For IHC, R51 showed characteristic staining of the cleavage furrow between dividing HeLa and SY5Y cells consistent with other reports (Li et al., 1998; Li et al., 1999; Uren et al., 2000) (Figure 2-1). In addition, dual-labeling in dividing cell cultures of both HeLa and SY5Y cells with R51 and the Chemicon survivin antibody showed co-localization at the cleavage furrow.

### **Western Blot Analyses**

The cortex and hippocampus from each set of brain tissues was excised, rinsed with cold PBS, snap frozen in liquid nitrogen and homogenized in ice-cold triple detergent lysis buffer containing a Complete<sup>™</sup> protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). Protein concentration was determined by bicinchoninic acid (BCA)

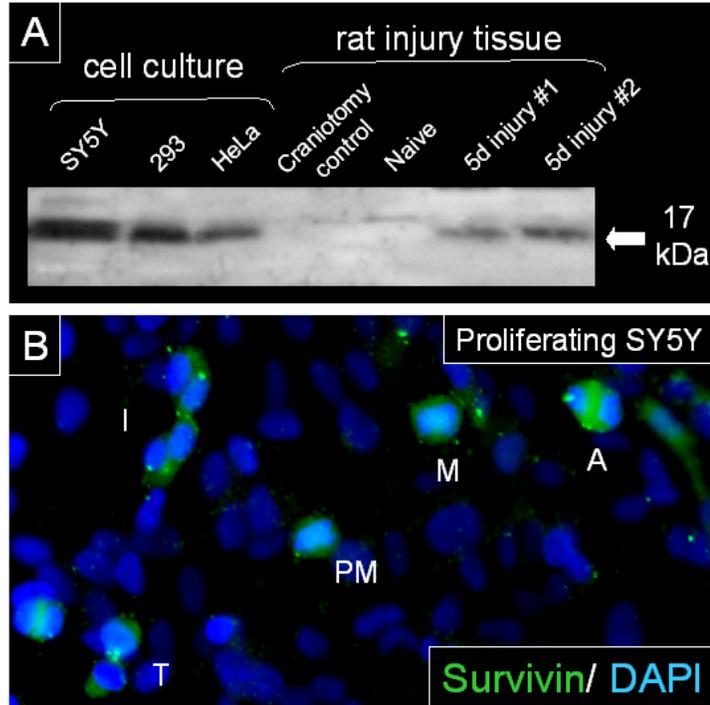


Figure 2-1: IHC characterization of the rat-specific survivin antibody. The survivin antibody (R51) reveals a characteristic and previously described labeling pattern on western blot (A) and in IHC (B). Western blot analysis revealed a classic 17 kDa band in cell culture lysates (lanes 1-3) and in injured rat tissue lysates (lanes 6-7) but not in un-injured rat tissue lysates (lanes 4-5). IHC using the survivin antibody revealed a well-characterized survivin (green) staining pattern around the nuclei (DAPI, blue) of proliferating SY5Y cells in various stages of mitosis including G2/M, interphase (I), pro-metaphase (PM), anaphase (A) and telophase (T).

micro protein assays (Pierce, Inc., Rockford, IL). Forty micrograms of protein per well was loaded and separated by SDS-PAGE, transferred to PVDF membranes and probed with either goat-anti-rabbit survivin antibody (Novus Biologicals; Littleton, CO; 1:1000) or goat-anti-rabbit active caspase-3 (Cell Signaling; 9661L; 1:100). After incubation with goat anti-rabbit HRP-labeled secondary antibody (Biorad, Hercules, CA), the membranes were developed using Enhanced Chemiluminescence Plus reagents (ECL Plus; Amersham, Arlington Heights, IL). For further PCNA analysis, developed PVDF membranes were incubated in stripping buffer, rinsed twice in TBST and incubated with PCNA (Santa Cruz Biotech; Santa Cruz, CA; 1:1000) antibody with goat-anti-mouse

HRP conjugated secondary antibody. Semi-quantitative, densitometric analysis was performed using the AlphaImager™ 2000 Digital Imaging System (San Leandro, CA). The blots were not labeled with an antibody, such as actin or GAPDH, to act as an internal standard because our previous studies found that many “stable” proteins are the targets of proteolytic cleavage and thus could not act as a proper internal control. Transformed data (experimental densitometry value/ craniotomy control densitometry value x 100) was evaluated by ANOVA and a post-hoc Dunnett-test. Values were expressed as percentage of craniotomy controls and are reported as mean ± SEM. Differences were considered significant at the level of  $p \leq 0.05$ .

#### **Preparation and Sectioning of Tissue for Immunohistochemistry (IHC)**

Tissue was prepared and sectioned for vibratome and cryostat sectioning. For vibratome sectioning, animals were transcardially perfused with 2% Heparin (Elkins-Sinn, Inc.; Cherry Hill, NJ) in 0.9% saline solution (pH 7.4) followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were post-fixed in 4% paraformaldehyde and stored in 0.1M PBS or cryobuffer. Sections were cut on a Series 1000 vibratome (Ted Pella; Redding, CA) at forty microns. For cryostat sectioning, animals were anesthetized with 4% isoflurane (Halocarbon Laboratories; River Edge, NJ) in 1:1 O<sub>2</sub>/ N<sub>2</sub>O for 4 minutes, then the head was removed. The brains were blocked in O.C.T. (Ted Pella; Redding, CA), snap frozen in liquid nitrogen and cut on a Leica CM3050 cryostat. Five micron sections were attached to Fropen (Ted Pella; Redding, CA)-treated coverslips, fixed in cold methanol for 20 minutes at -20° C.

### **Dual Label Fluorescent Immunohistochemistry (IHC)**

Sections were fluorescent immunolabeled with two primary antibodies in the following experiments: survivin (1:500)/GFAP for astrocytes (Sternberger; Lutherville, MD; 1:1000), survivin/NeuN for mature neurons (Chemicon; Temecula, CA; 1:1000), survivin/PCNA (Santa Cruz Biotech; Santa Cruz, CA; 1:200), PCNA/GFAP, PCNA/NeuN, active caspase-3 (1:100)/GFAP, active caspase-3 /NeuN and active caspase-3 /survivin (G. Shaw; 1:250). In addition, sections were labeled with the Apoptag<sup>®</sup> Cell Death Labeling kit (terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick-end labeling or TUNEL) to mark double stranded DNA breaks as per the manufacturer's instructions. This kit was used in conjunction with the following antibodies: TUNEL/GFAP, TUNEL/NeuN and TUNEL/survivin. The nuclear dye DAPI (in Vectashield; H-1200; Vector Laboratories; Burlingame, CA) was used to label the nuclei in all sections. The first primary antibody was incubated at 4° C for 24- 48 h in a 2% goat serum/ 2% horse serum/ 0.2% Triton-X 100 in 0.1 M PBS (block) solution followed by the second primary antibody at 4° C for 1 h in block solution. Fluorescent-tagged secondary antibody (Molecular Probes; Eugene, OR) was used for visualization. Sections were viewed and digitally captured with a Zeiss Axioplan 2 microscope equipped with a SPOT Real Time Slider high-resolution color CCD digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). A Bio-Rad 1024 ES confocal microscope was used to confirm single cell localization of the label pairings. The settings for these images were as follows: power = 100%; for the red field: iris = 2.7 – 5.2, gain = 1400, Blev = -3; for the green field: iris = 3.0 – 5.7, gain = 1400, Blev = -3. The number of animals used for each label pairing for dual-labeling IHC was four (n=4).

### **Dual Label Fluorescent IHC for Same-Species Antibodies**

Two systems were used for dual-labeling using same species antibodies; the tyramide signal amplification (TSA) kit (PerkinElmer Life Sciences, Boston, MA) and a biotin/streptavidin antibody protocol. Both techniques rely on steric hindrance to block same-species binding sites. Control sections showed the secondary/tertiary complex was sufficient for steric hindrance of same species sites for both protocols (Figure 2-2).

Tyramide signal amplification (TSA) was accomplished using the TSA kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions and as previously described (Stone et al., 2002). Biotin/streptavidin same species dual-labeling begins with an endogenous biotin blocking step (Vector Laboratories; Burlingame, CA) followed by incubation of the first primary antibody as described above followed sequentially by a biotin-conjugated secondary antibody and fluorescent-labeled streptavidin (Molecular Probes; Eugene, OR), both steps at room temperature for 1 h in block solution. The second antigen was then labeled as described above.

### **Experimental Group Sizes**

The number of animals used for western blot analysis is as follows (per time point): survivin = 6, PCNA = 6, active caspase-3 = 6. The number of animals used for dual-labeling IHC is as follows (presented as 5 days post injury or both 5 / 7 days post injury): survivin x PCNA = 4, survivin x GFAP = 6, survivin x NeuN = 4, PCNA x GFAP = 4, PCNA x NeuN = 4, active caspase-3 x survivin = 4/4, active caspase-3 x GFAP = 4/4, active caspase-3 x NeuN = 4/4, TUNEL x GFAP = 4/4, TUNEL x NeuN = 4/4 & TUNEL x survivin = 4/4.

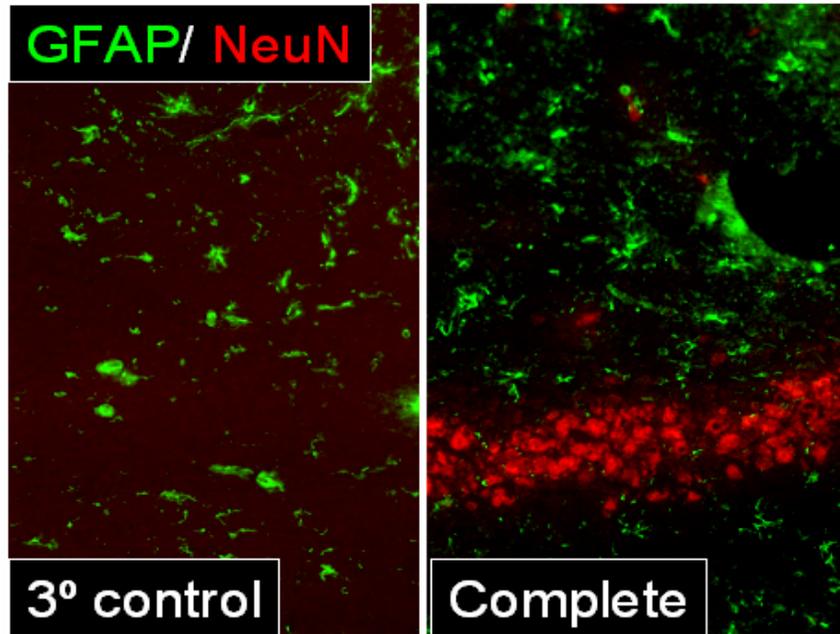


Figure 2-2: Control section for biotin/streptavidin same-species dual labeling IHC. Biotin/streptavidin same-species dual labeling IHC is a technique based on steric hindrance of same species binding sites to prevent the second fluorescent-labeled secondary from binding to the first primary antibody. To ensure that this process was sufficient, mouse antibodies for astrocytes (GFAP, green) and neurons (NeuN, red) were used to label a section of brain. These two protein targets were chosen because of their abundance and distinct labeling patterns in adult rat brain. On the left (3° Control), neurons were labeled with only the primary and biotin secondary with no fluorescent streptavidin tertiary antibody while astrocytes were labeled with a primary and fluorescent-tagged secondary antibody. On the right (Complete), neurons were labeled with a primary, the biotin conjugated secondary and a fluorescent-tagged streptavidin tertiary while astrocytes were labeled as described previously. The absence of red fluorescent labeling in the picture on the left indicates that the biotin secondary antibody was sufficiently large to prevent the green fluorescent-labeled secondary antibody to bind to it thus confirming steric hindrance of NeuN binding sites.

### Cell Quantification and Statistical Analysis

Cell counts were obtained by comparing the number of dual-labeled cells to total single-labeled cells in the following groups: survivin/NeuN positive cells to total NeuN positive cells, survivin/PCNA positive cells to total PCNA positive cells, PCNA/NeuN positive cells to total NeuN positive cells, survivin/GFAP positive cells to total GFAP positive cells, active caspase-3/NeuN positive cells to total NeuN positive cells, active caspase-

3/survivin positive cells to total survivin positive cells, active caspase-3/GFAP positive cells to total GFAP positive cells, TUNEL/NeuN positive cells to total NeuN positive cells, TUNEL/survivin positive cells to total survivin positive cells and TUNEL/GFAP positive cells to total GFAP positive cells. Percentages were calculated by dividing the number of dual-labeled cells with the total number of single-labeled cells. For each group, representative photomicrographs were selected and counted. Cells were counted in a total area of 188,000  $\mu\text{m}^2$  for each label pairing in both cortical and hippocampal regions. These numbers were then transformed into percentage of either total cells or total cell type. Individual comparisons between groups were made using an unpaired Student t-test. Results were considered significant at  $p < 0.05$ . Two additional blinded observers were used to count representative samples. Inter-rater reliability was calculated using the inter-rater reliability formula created by R.L. Ebel (Ebel, 1951). The intra-class correlation (ICC) value achieved was 0.97 indicating little variation between raters.

CHAPTER 3  
SURVIVIN EXPRESSION FOLLOWING TRAUMATIC BRAIN INJURY

**Induction of Survivin Expression After TBI**

Q-PCR analysis revealed an initial increase in survivin mRNA at 2 days post injury in the ipsilateral cortex and hippocampus. These transcripts remained elevated in both regions, reached maximum levels at day 5 post-injury and declined at 7 days in the cortex and at 14 days in the hippocampus. All experimental animals remained alive and exhibited slightly impaired motor and cognitive impairments (data not shown). Cortical mRNA levels reached a maximum of  $448 \pm 10.0\%$ , whereas hippocampal mRNAs attained  $606 \pm 10.0\%$  compared to craniotomy control values (Figure 3-1). To determine if the induction of survivin mRNA resulted in corresponding increases in survivin protein, western blot analysis was performed. Survivin (17 kDa protein) was readily detectable in the ipsilateral cortex and hippocampus of TBI rats, while it was negligible in contralateral cortex and hippocampus (Figure 3-2A). Survivin was expressed in a time-dependant manner with a maximum increase at 5 days after injury followed by a gradual decline by 14 days. Specifically, the levels of survivin in cortical tissue were at  $616 \pm 257\%$  at 3 days and at  $839 \pm 339\%$  at 5 days compared to craniotomy controls (Figure 3-2B). Similar increases of survivin protein in the ipsilateral hippocampus were detected at 3 days and 5 days post injury:  $464 \pm 196\%$  and  $545 \pm 102\%$  compared to craniotomy control, respectively (Figure 3-2C).

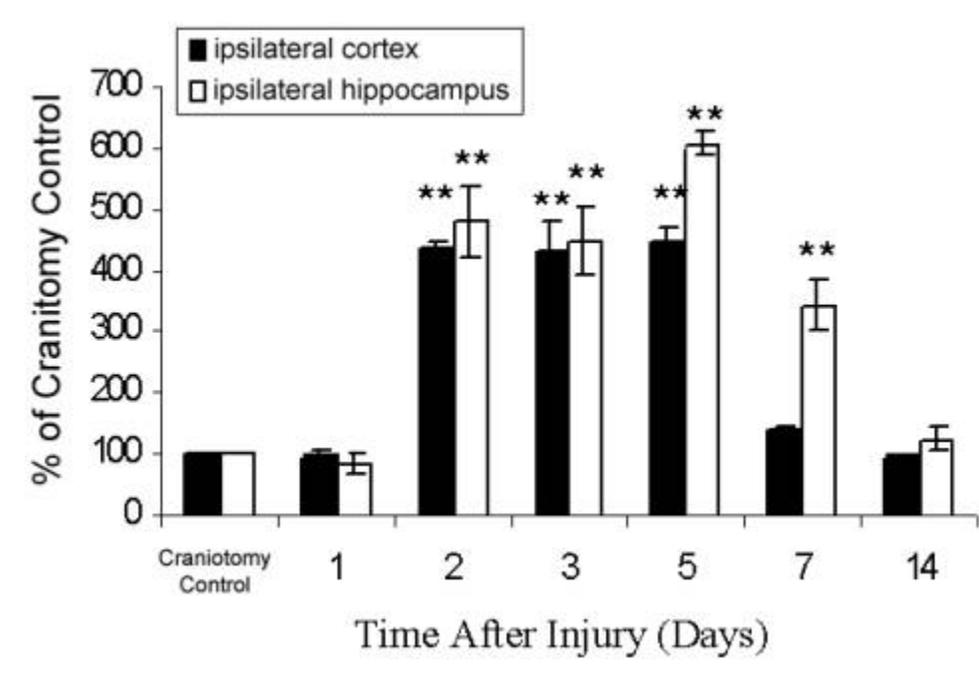


Figure 3-1: Survivin mRNA induction in rat brain after TBI. Rats were subjected to craniotomy followed by controlled cortical impact brain injury. Total RNA was isolated from injured (ipsilateral) cortex (ic) and hippocampus (ih) at indicated post-injury times. cDNA was synthesized, and quantitative PCR using survivin primers was performed as described in detail under Materials and Methods. Data are given as percent of survivin expression over craniotomy controls; each time point represents mean  $\pm$  SEM of 4 independent measurements in craniotomy control or TBI group. \*\*  $p < 0.01$  versus craniotomy control (one-way ANOVA test with *post hoc* Bonferroni analysis).

### PCNA Expression After TBI.

For detection of proliferating cell nuclear antigen (PCNA), PVDF membranes immunostained for survivin were stripped and re-probed using a PCNA-specific antibody. PCNA (36 kDa protein) was significantly detectable in the ipsilateral cortex and hippocampus of TBI rats, but only negligible amounts were observed in the contralateral cortex and hippocampus (Figure 3-3A). The temporal patterns exhibited by PCNA protein were similar to that of survivin protein. Namely, PCNA expressed in a time-dependant fashion with a maximum increase at 5 days after injury followed by a

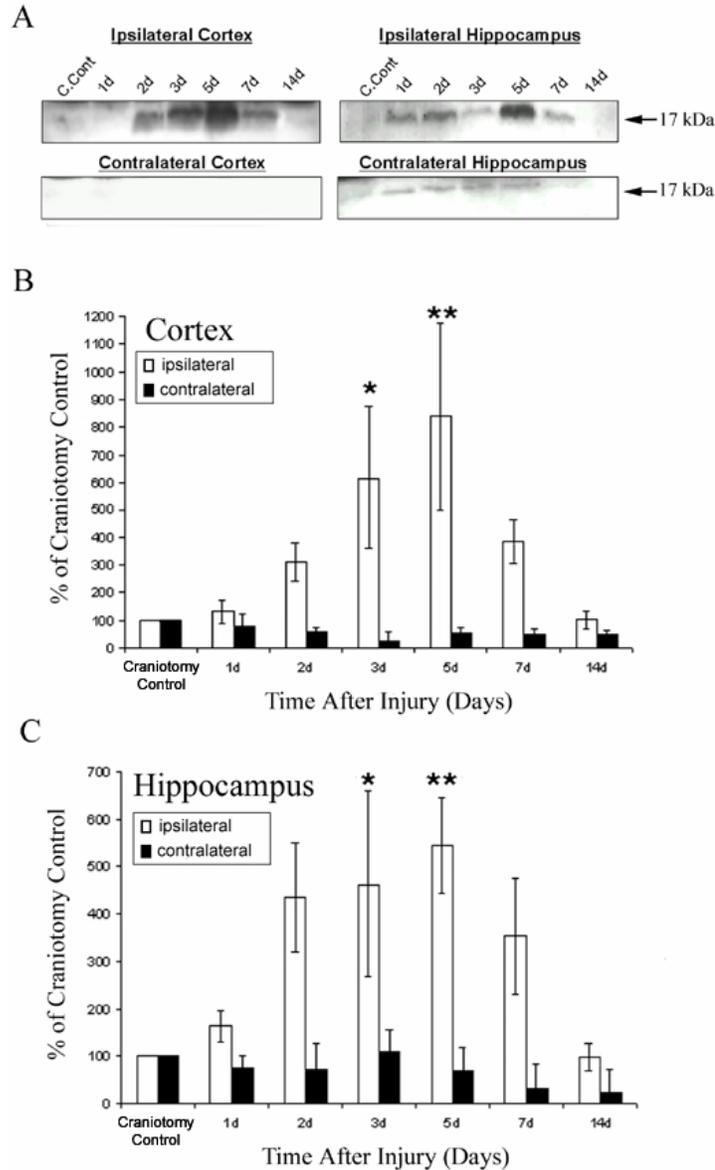


Figure 3-2: Expression of survivin protein after TBI in rats. Brain tissue homogenate proteins (40  $\mu$ g) were separated using SDS-PAGE, immunoblotted with survivin antibody and visualized as described in detail under Materials and Methods. A-Representative western blot of survivin (17 kDa protein) in ipsilateral cortex (ic) and hippocampus (ih), contralateral cortex (cc) and hippocampus (ch) obtained from injured rats, and from craniotomy control rats without cortical impact (craniotomy control.). Densitometry analysis representation of survivin-positive bands in ipsilateral (ic) and contralateral (cc) cortex (B) and ipsilateral (ih) and contralateral (ch) hippocampus (C) after TBI is shown as percent of craniotomy control values. Each data point represents the mean  $\pm$  SEM of 4 to 6 independent experiments. \* $p < 0.05$ , \*\* $p < 0.001$  versus craniotomy control (one-way ANOVA test with *post hoc* Bonferroni analysis).

gradual decline by 14 days. The levels of PCNA in ipsilateral cortical tissue were raised over craniotomy control by  $919 \pm 459\%$  at 3 days,  $2263 \pm 333\%$  at 5 days, and  $1035 \pm 356\%$  at 7 days post injury (Figure 3-3B). Similar increases of PCNA protein in ipsilateral hippocampus were detected at 5 days post injury with a maximum of  $1006 \pm 229\%$  compared to craniotomy controls (Figure 3-3C). No significant increase was found in the contralateral regions when compared to craniotomy controls (Figure 3-3A).

### **Co-Expression of Survivin and PCNA Following TBI**

To examine spatial co-localization of survivin and PCNA, dual-label immunohistochemistry was performed on five-day post injury brain tissue sections, when peak expression of these proteins was observed.

Survivin and PCNA immunoreactivity was found in the ipsilateral cortex (Figure 3-4A) and ipsilateral hippocampus (Figure 3-4B) consistent with data obtained using Western blot analyses. Within both regions, focal co-expression patterns of survivin and PCNA in single cells were detected, which was demonstrated by both separate fluorescent visualization of individual proteins and by merging the images of dual-stained slides (Figure 3-4C-E). However, the dual expression of survivin and PCNA occurred infrequently as survivin and PCNA immunoreactivity could readily be found separately (Figure 3-4C-E). Approximately 12% of the total number of PCNA-positive cells also labeled with survivin. The nuclear morphology of dual survivin and PCNA-positive cells was ambiguous as indicated by DAPI staining (Figure 3-4F). Therefore, DAPI staining was simply used for cell identification in all subsequent experiments.

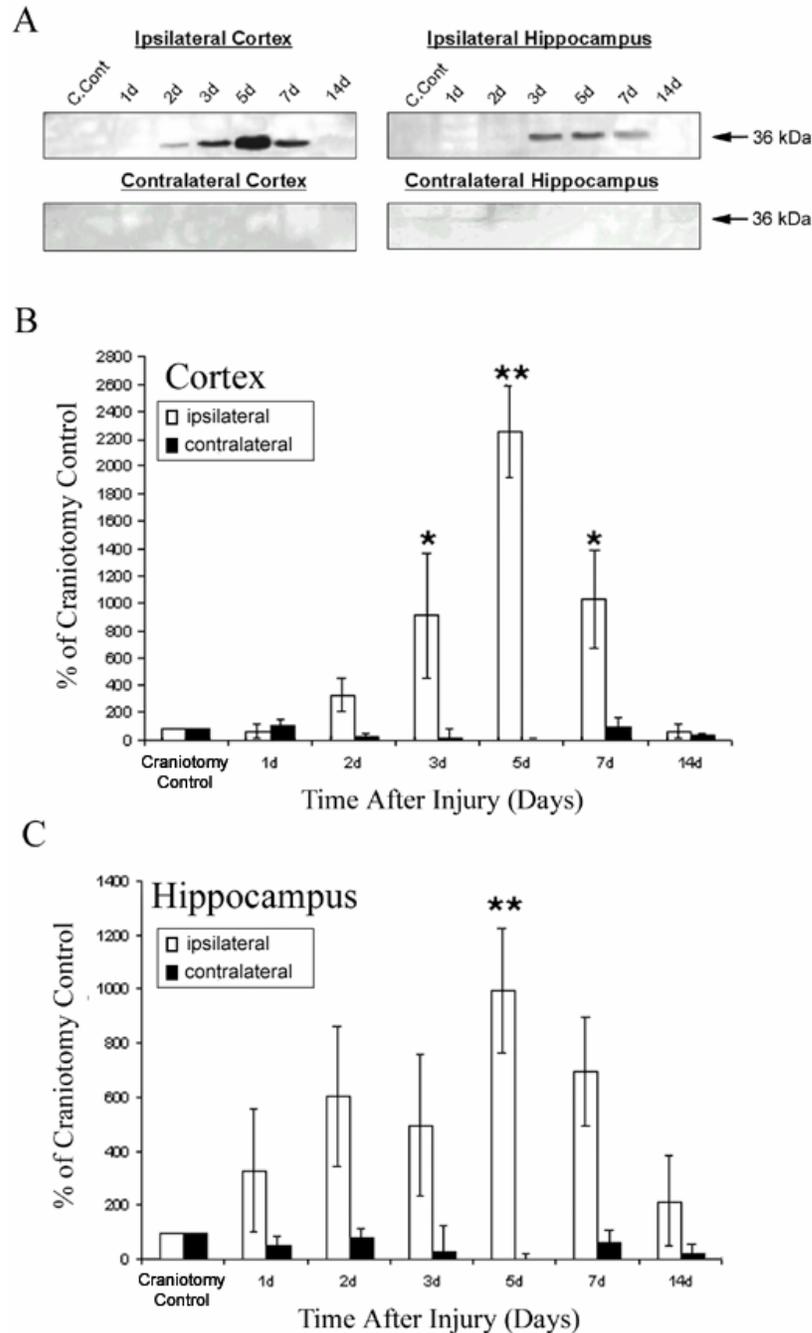


Figure 3-3: Expression of PCNA after TBI in rats. PVDF membranes visualized for survivin were stripped and re-probed with PCNA antibody as described in Materials and Methods. Representative western blots showing PCNA (36 kDa) (A) and densitometry analysis of PCNA-positive bands (B, C) are presented. Experimental conditions, sample size and abbreviations are identical to those in Fig. 3-2. \* $p < 0.05$ , \*\*  $p < 0.01$  versus craniotomy control (one-way ANOVA test with *post hoc* Bonferroni analysis). Values are mean  $\pm$  SEM with  $n=6$ .

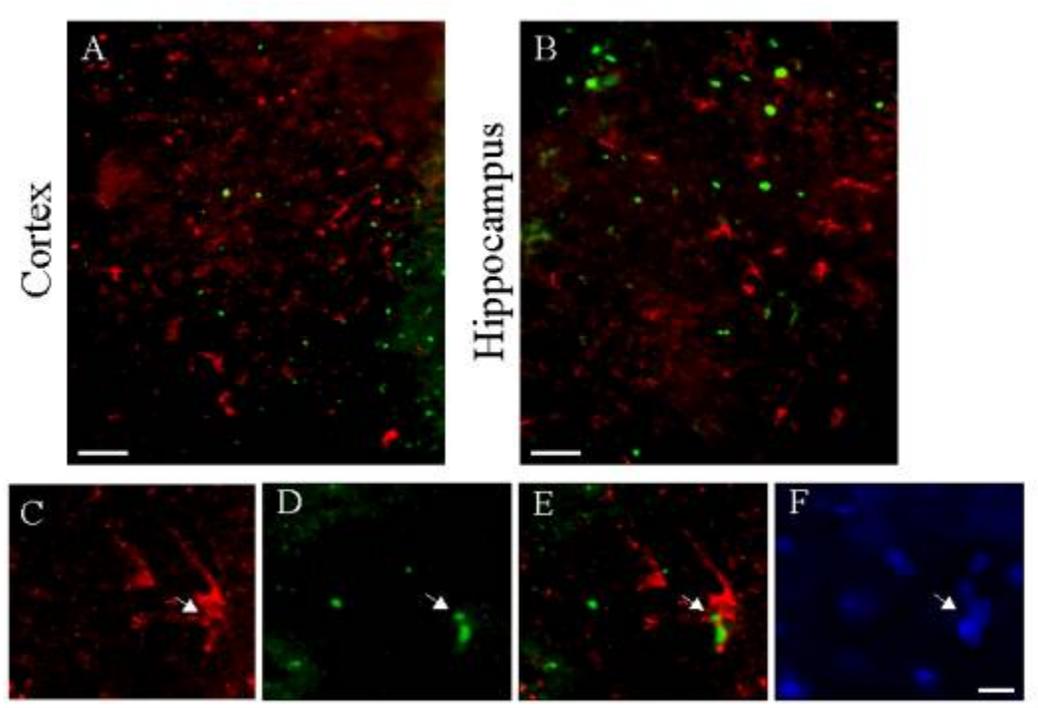


Figure 3-4: Immunohistochemistry of survivin and PCNA. Dual-label fluorescent immunostaining for survivin (red) and PCNA (green) was performed in the ipsilateral cortex (A) and hippocampus (B) at 5 day post-injury as described in detail under Materials and Methods. Survivin is expressed in the cytoplasm (C, red) while PCNA is expressed in the nucleus (D, green). The white arrow indicates the typical focal co-expression of survivin and PCNA as shown in merged survivin and PCNA images (E). PCNA expression was co-incident with DAPI staining (F, blue, white arrow). Magnification: 200x, scale bar 50  $\mu\text{m}$  (A and B); 400x, scale bar 20  $\mu\text{m}$  (C-F).

### **Survivin and PCNA are Expressed in Astrocytes After TBI**

To determine the cell types expressing survivin and PCNA, dual-label immunohistochemistry for these proteins and GFAP, a marker of astrocytes, was performed in five-day post injury tissue. In accordance with western blot data, survivin-positive immunoreactivity was observed in the ipsilateral cortex and hippocampus proximal to the injury cavity (Figure 3-5A & G, green) but not in the contralateral areas (Figure 3-5B & H). Survivin was co-localized with GFAP in the cells of injured cortex and hippocampus, which strongly suggested primary accumulation of survivin in cells of

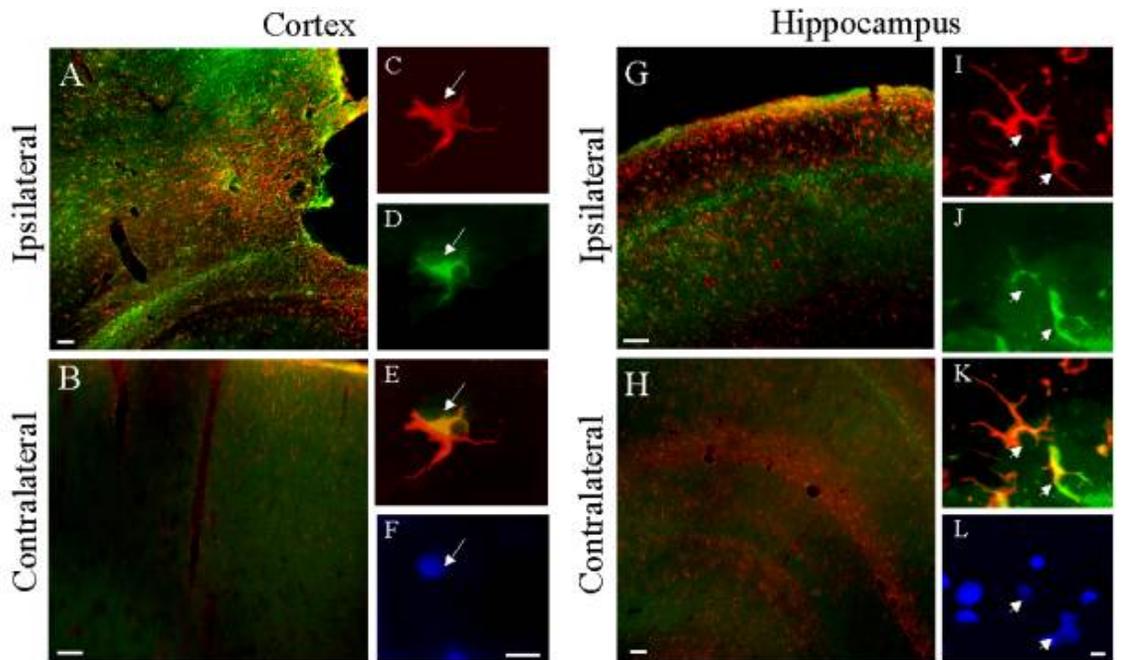


Figure 3-5: Co-localization of survivin and GFAP in brain tissue after TBI. Fluorescent immunohistochemistry for survivin (green) and GFAP (red) was performed in the ipsilateral and contralateral cortex (A, B) and in the CA1 and dentate gyrus regions of the hippocampus (G, H) at 5 day post-injury as described in Materials and Methods. The injury has completely destroyed the cortex in G leaving only the hippocampus in this picture. Survivin was expressed in the cytoplasm (D, J, green) of GFAP-positive astrocytes (C, I, red) of the ipsilateral cortex and hippocampus and was found to co-localize to these cells as shown in merged C/D and I/J images (E, K, respectively, yellow). White arrows indicate typical survivin-positive astrocytes. Nuclei are shown using DAPI (F, L, blue). Magnification: 100x, scale bar 50  $\mu\text{m}$  (A, B, G, H); 400x, scale bar 20  $\mu\text{m}$  (C – F, and I – L).

astrocytic lineage (Figure 3-5C-E, I-L). It was further observed that survivin was uniformly distributed in the cytoplasm and processes of astrocytes in both cortex and hippocampus (Figure 3-5D & J). DAPI staining is shown in Figures 3-5F & L. Approximately 88% of the total number of GFAP-positive cells also labeled with survivin.

PCNA-positive immunoreactivity staining was observed in the ipsilateral cortex (Figure 3-6A, green) and hippocampus (Figure 3-6G, green) of injured brain, while contralateral cortex and hippocampus exhibited negligible PCNA immunoreactivity

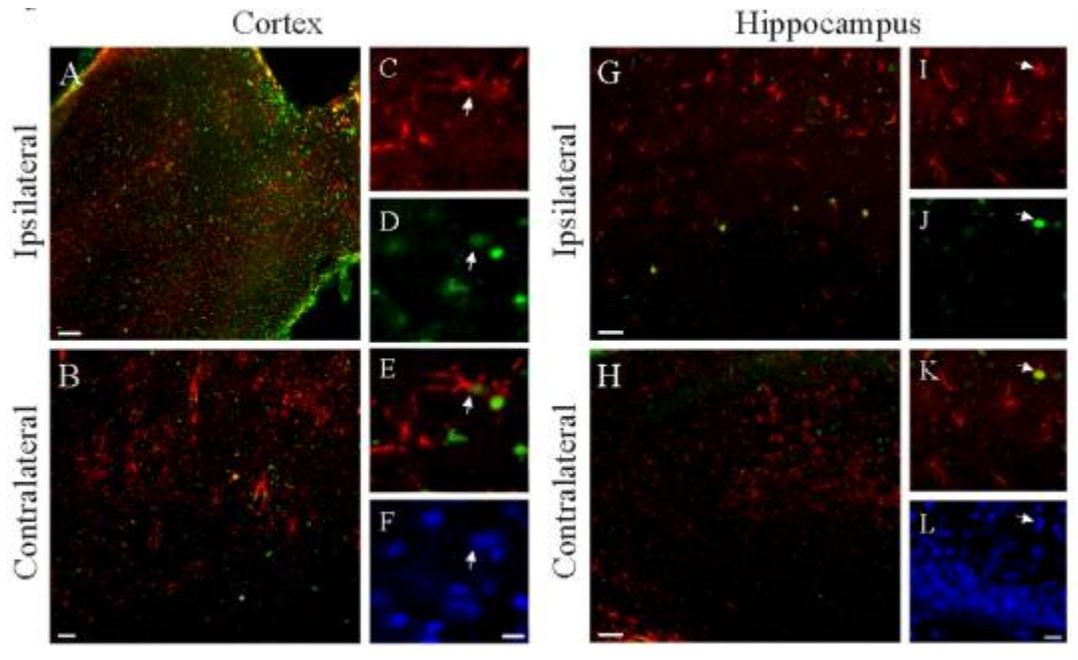


Figure 3-6: Co-localization of PCNA and GFAP in brain tissue after TBI. Dual-label immunostaining for PCNA (green) and GFAP (red) was performed in the ipsilateral and contralateral cortex (A, B) and the CA1 and dentate gyrus regions of the hippocampus (G, H) at 5 day post-injury. PCNA is present in GFAP positive cells of ipsilateral cortex (C, D) and, to a lesser extent hippocampus (I, J). E and K depict merged C/D and I/J, respectively. White arrows indicate typical PCNA-positive astrocytes. PCNA expression was co-incident with DAPI staining (F, L, blue). Magnification: 100x, scale bar 50  $\mu\text{m}$  (A, B, G, H); 400x, scale bar 20  $\mu\text{m}$  (C – F, and I – L).

(Figure 3-6B & H). PCNA (Figures 3-6C & I) was partially co-localized with GFAP (Figures 3-6D & J, red) in both regions, and was characteristically distributed in the nucleus of the cells in both cortex and hippocampus (Figures 3-6E & K). DAPI staining is shown in Figures 3-6F & L.

Taken together, dual-label immunohistochemistry data provides evidence that both survivin and PCNA can be detected in GFAP-positive astrocytes following traumatic insult. Since survivin and PCNA immunoreactivity was not exclusively localized in GFAP-positive cells, other cell types must also express survivin.

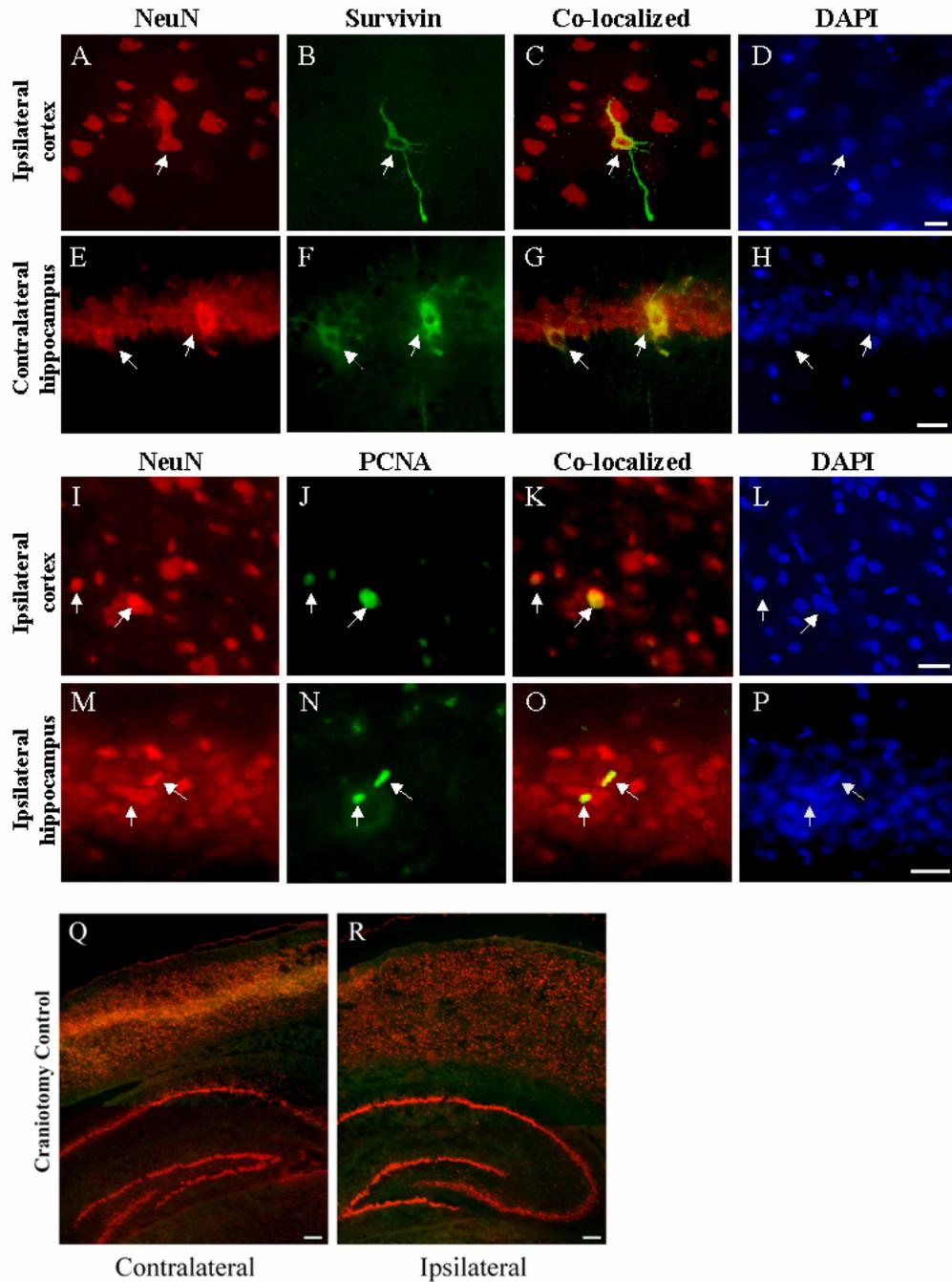


Figure 3-7: A sub-set of NeuN-positive neurons express survivin and PCNA after TBI. Dual-label fluorescent immunohistochemistry for survivin (green) and NeuN (red) in the ipsilateral cortex (A & B) and the CA1 pyramidal layer of the contralateral hippocampus (E & F) was performed as described in Materials and Methods. Survivin is expressed in the cytoplasm and, to a limited extent, in the processes of NeuN-positive neurons (merged images C & G). Dual staining for PCNA (green) and NeuN (red) is shown in the ipsilateral cortex (I & J) and the CA1 pyramidal layer of the ipsilateral hippocampus (M & N).

The nuclei are shown using DAPI staining (D & H, blue). PCNA is expressed in the nucleus of NeuN-positive neurons (merged images K & O). PCNA expression was co-incident with DAPI staining in these examples (L & P, blue). White arrows indicate focal co-localization of survivin/NeuN and PCNA/NeuN. Survivin/NeuN co-localization of survivin (green) and NeuN (red) was seen only in TBI rats as opposed to either hemisphere of craniotomy control (Q & R). (Magnification of A-P = 400x, scale bar = 20  $\mu\text{m}$ ; magnification of Q & R 50x, scale bar = 100,000  $\mu\text{m}$ ).

### **Survivin and PCNA are Expressed in a Sub-Set of Neurons After TBI**

As can be seen in Figure 3-7, survivin and PCNA were each co-expressed with NeuN, a marker of mature neurons. NeuN-positive cells were found to express survivin in the ipsilateral cortex distal to the injury cavity (Figure 3-7A-D) and in the contralateral hippocampus (Figure 3-7E-H). It should be noted, however, that NeuN-positive cells that also expressed survivin occurred infrequently. For example, the number of dual survivin/NeuN positive cells was estimated at 0.1% to 1.5% of the total number of NeuN-positive cells in these regions. Survivin immunoreactivity was negligible in either hemisphere of craniotomy control brains (Figures 3-7Q & R). No co-localization of survivin and NeuN was observed in ipsilateral hippocampus (data not shown). As can be seen in Figures 3-7B & F, survivin was predominantly localized to the cytoplasm and axons of NeuN-positive neurons. DAPI staining is shown in Figures 3-7D & H.

PCNA-positive neurons were found in the ipsilateral cortex (Figures 3-7I-L) and hippocampus after TBI (Figure 3-7M-P), whereas craniotomy control tissue exhibited only trace amounts of PCNA (data not shown). Similar to the survivin/NeuN co-localization data, dual PCNA/ NeuN immunostaining was a rare event accounting for approximately 4% of the total number of NeuN positive cells. PCNA was distributed in the nuclei of these neurons (Figures 3-7K & O) although the nuclear morphology of these cells was not clearly resolved by DAPI staining (Figures 3-7L & P).

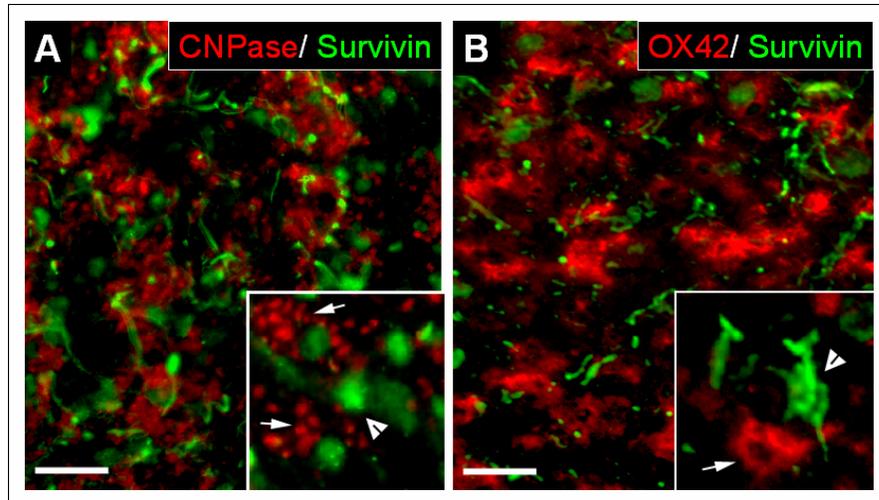


Figure 3-8: Survivin expression is absent in oligodendrocytes and microglia following TBI in rats. Dual-label fluorescent immunohistochemistry for survivin (green), CNPase (red, A) and OX42 (red, B) in the ipsilateral cortex and hippocampus was performed as described in Materials and Methods. Negligible co-localization was seen with survivin, CNPase and OX42 in the ipsilateral cortex (A, B respectively) and hippocampus (data not shown). Higher magnification photomicrographs (inset, A and B) show survivin-positive cells (white arrowheads) surrounded by oligodendrocytes (white arrows, A) and microglia (white arrows, B) that do not show co-localization. (Magnification of A and B = 400x, scale bar = 20  $\mu\text{m}$ ).

### **Survivin is Not Expressed in Microglia and Oligodendrocytes**

To further determine the neural cell types expressing survivin, dual-label immunohistochemistry for survivin, OX42, a marker of microglia, and CNPase, a marker for oligodendrocytes, was performed in five-day post injury tissue sections. No co-localization with survivin and either CNPase (Figure 3-8A) or OX-42 (Figure 3-8B) is observed following traumatic brain injury. In addition, attempts were made to localize survivin with the neuronal progenitor cell markers nestin, doublecortin,  $\alpha$ -internexin and  $\beta$ -III-tubulin. However, these antibodies did not prove to be of acceptable quality to use in western blot and IHC analyses in this model leaving proliferating progenitors undetected.

### Discussion of Chapter 3

Traumatic brain injury (TBI) initiates various biochemical cascades that induce neural tissue injury and cell death. To counteract these cascades, several proteins expressed in neural cells after TBI are directed to resist cell death and promote recovery in the injured CNS (Ridet et al., 1997; Chen and Swanson, 2003). Survivin is a multi-functional protein that inhibits apoptosis and is also required for the proper completion of mitosis. Anti-apoptotic and pro-mitogenic roles for survivin have been documented in proliferating cells of neural origin *in vitro*, such as in neuroblastoma and glioma cells (LaCasse et al., 1998; Tamm et al., 1998; Deveraux and Reed, 1999; Conway et al., 2000; Shin et al., 2001; Sasaki et al., 2002). However, no studies have investigated the potential role of survivin in the adult brain after TBI, when a sub-population of CNS cells may initiate a cell cycle-related process in response to injury.

These data demonstrate the induction of survivin expression in rat brain subjected to TBI. The expression of survivin was time-dependent, cell-specific and was present in astrocytes and, to a much lesser extent, in neurons in ipsilateral cortex and hippocampus. Induction of survivin in these cells was accompanied by occasional expression of PCNA, a cell cycle protein involved in mitotic G1/S progression. These data are the first to show that survivin mRNA and protein are significantly up-regulated after TBI in rats. PCNA expression after TBI has been described previously (Miyake et al., 1992; Chen et al., 2003), suggesting its role in mechanisms of brain recovery after injury. The concurrent up-regulation of survivin with a similar temporal profile as PCNA shown herein further suggests that survivin may play a role in cellular proliferation after TBI.

Brain injury evoked the expression of survivin and PCNA in a time-dependent manner (Figures 3-2 & 3-3). Western blot analysis revealed maximal co-expression of

both survivin and PCNA at five days post injury. Immunohistochemistry demonstrated co-localization of these proteins (Figure 3-4), although most cells were labeled separately with PCNA and survivin. In fact, only 12 % of the total number of PCNA-positive cells were also survivin positive. It has been reported that PCNA is expressed predominantly in G1/S (Bravo et al., 1987), while survivin is found at the G2/M phase of the cell cycle (Bravo et al., 1987; Otaki et al., 2000). Hence, a lack of strict co-localization of survivin and PCNA in this study may be explained by their expression at different points in the cell cycle. To determine if the differing expression patterns of survivin and PCNA contributed to lower incidence of co-localization, other cell cycle proteins were investigated including cdk4 (G1), cyclin B (G2), cyclin D (G1) and AIM-1 (M). Only PCNA provided clear results in both western blot and IHC analyses.

Survivin-positive and PCNA-positive astrocytes were observed in the proximal area of the injury and in the ipsilateral hippocampus. Proliferation of astrocytes is well documented after TBI as shown by cell labeling with BrdU as well as expression of PCNA (Latov et al., 1979; Dunn-Meynell and Levin, 1997; Carbonell and Grady, 1999; Norton, 1999; Csuka et al., 2000; Kernie et al., 2001; Chen et al., 2003). Because survivin and PCNA were expressed in astrocytes following TBI (Figures 3-5 & 3-6), it is possible that survivin plays an important role linking astrocyte survival and proliferation after traumatic insult. Astrocyte proliferation has been implicated in the formation of the glial scar observed after injury (Latov et al., 1979) and creates a non-permissive environment for repair (Sykova et al., 1999). However, glial proliferation may also enhance neuronal survival (Smith et al., 2001; Wei et al., 2001).

Of particular interest is a sub-set of NeuN-positive neurons found to express survivin only after TBI (Figure 3-7). These cells were much less abundant than survivin-positive astrocytes and their functional significance is currently unknown. However, both neurons and astrocytes have been documented previously to express cell cycle proteins after various insults such as exposure to  $\beta$ -amyloid activated microglia (Wu et al., 2000), TBI (Kaya et al., 1999a; Kaya et al., 1999b), chlorin  $e_6$  toxicity, (Magavi et al., 2000) or as a consequence of Alzheimer's Disease (Yang et al., 2001). The ramifications of cell cycle protein expression in mature neurons is still controversial and may be a marker of cell death rather than cell proliferation (Herrup and Busser, 1995; Li et al., 1997; Kaya et al., 1999a; Kaya et al., 1999b; Wu et al., 2000; Yang et al., 2001). These papers underscore the significant controversy that exists regarding the function of cell cycle proteins such as PCNA in neurons after different types of injury.

It should be noted that dual staining of survivin and PCNA could not be directly attributed to a specific cell type due to the technical difficulties of triple labeling antibody-based IHC. Therefore, other cell types, such as endothelial (Conway et al 2003), inflammatory cells (Hill-Felberg et al., 1999) or neural progenitor cells (Ignatova et al., 2002), may also contribute to survivin and PCNA expression after TBI. The appearance of survivin and PCNA separately in neurons (NeuN-positive) and astrocytes (GFAP-positive) along with co-localization of survivin with PCNA in the same cells provide correlative data to suggest an activation of cell cycle-like program in astrocytes and possibly in a small subtype of neurons after TBI. In these experiments, survivin co-localization with PCNA does suggest that survivin may be associated with a pro-mitotic process. In an attempt to clarify these protein's roles after TBI, the nuclear morphology

of survivin-positive cells was analyzed to define the apoptotic or mitotic architecture of nuclei. DAPI staining proved too ambiguous in identifying apoptotic versus mitotic phenotypes likely due to the thickness of the brain sections (40  $\mu\text{m}$ ). Further studies using direct markers of mitosis such as BrdU incorporation as well as simultaneous labeling with cell death related proteins is required to delineate anti-apoptotic and pro-mitotic activities of survivin and PCNA in these cells.

To summarize, an induction of survivin was found in rat brain cortex and hippocampus after TBI in a time-dependent fashion. Expression of survivin occurred predominantly in astrocytes and a sub-set of neurons but not in oligodendrocytes or microglia, and was occasionally accompanied by expression of PCNA. However, survivin expression is only found in 12% of PCNA positive cells, which suggests that the primary role of survivin after traumatic brain injury is not related to the cell cycle but rather to apoptosis inhibition. Thus, the next specific aim of this study was to examine the link between survivin, active caspase-3 and downstream DNA fragmentation following traumatic brain injury in rats.

CHAPTER 4  
SURVIVIN AND APOPTOSIS INHIBITION FOLLOWING TRAUMATIC BRAIN  
INJURY

**Caspase-3 is Activated in the Same Brain Regions as Survivin Following TBI**

To determine the temporal and regional profile of caspase-3 activation, western blot analysis was performed on cortical and hippocampal TBI samples. Active caspase-3 (19 kDa protein) was readily detectable in the ipsilateral cortex and hippocampus of rats subjected to TBI (Figure 4-1A). Caspase-3 activation occurred in a time-dependant manner in the ipsilateral cortex and hippocampus with prominent activation occurring between five and fourteen days post-injury, with peak accumulation occurring at seven days post-injury. In the ipsilateral cortex, significant increases in active caspase-3 levels reached  $3468 \pm 1088\%$  at five days,  $4019 \pm 1291\%$  at seven days and  $2984 \pm 1058\%$  fourteen days post-injury, compared with craniotomy controls. Similar increases in caspase-3 activation were detected in the ipsilateral hippocampus with increases of  $671 \pm 257\%$  at five days,  $2662 \pm 738\%$  at seven days and  $1487 \pm 405\%$  at fourteen days post-injury, compared with craniotomy controls (Figure 4-1B).

**Survivin Expression Correlates with Decreased TUNEL Labeling but not Active Caspase-3 Expression.**

Immunohistochemistry (IHC) was performed on brain sections at five days post injury to investigate the expression of active caspase-3 and TUNEL labeling at peak survivin expression (Johnson et al., 2004). IHC revealed moderate co-localization of survivin with active caspase-3 and TUNEL in the ipsilateral cortex (Figure 4-2A,F) and

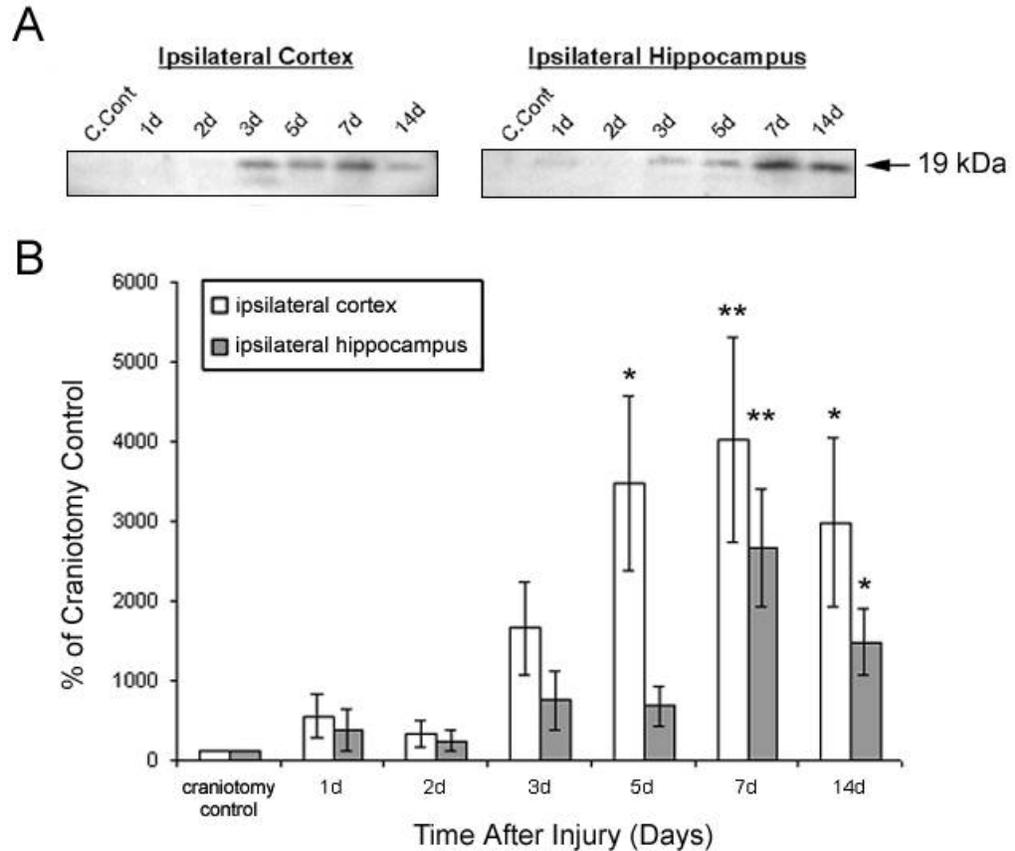


Figure 4-1: Caspase-3 Activation in rat brain after traumatic brain injury. Rats were subjected to craniotomy followed by controlled cortical impact brain injury. Brain tissue homogenate proteins (40  $\mu$ g) were separated using SDS-PAGE, immunoblotted with antibody specific for active caspase-3 and visualized as described in detail under Materials and Methods. Representative western blots of active caspase-3 (19 kDa) in ipsilateral cortex and hippocampus obtained from injured rats and from craniotomy control rats without cortical impact revealed the accumulation of active caspase-3 after TBI (A). Densitometry analysis of the active caspase-3 bands in ipsilateral cortex and hippocampus after TBI show significant increases in a time dependent manner (B). Data are given as percent of the active fragment of caspase-3 related to craniotomy controls; each time point represents mean  $\pm$  SEM of 6 independent measurements in craniotomy control or TBI group. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus craniotomy control (one-way ANOVA test with *post hoc* Dunnet analysis).

ipsilateral hippocampus (Figure 4-2B,G) of injured animals. These data are summarized in Table 1 (pg 45). Survivin was found primarily in the cytoplasm and to a lesser extent in the nucleus of the ipsilateral cortex (Figure 4-2C,H). A similar pattern was seen in the

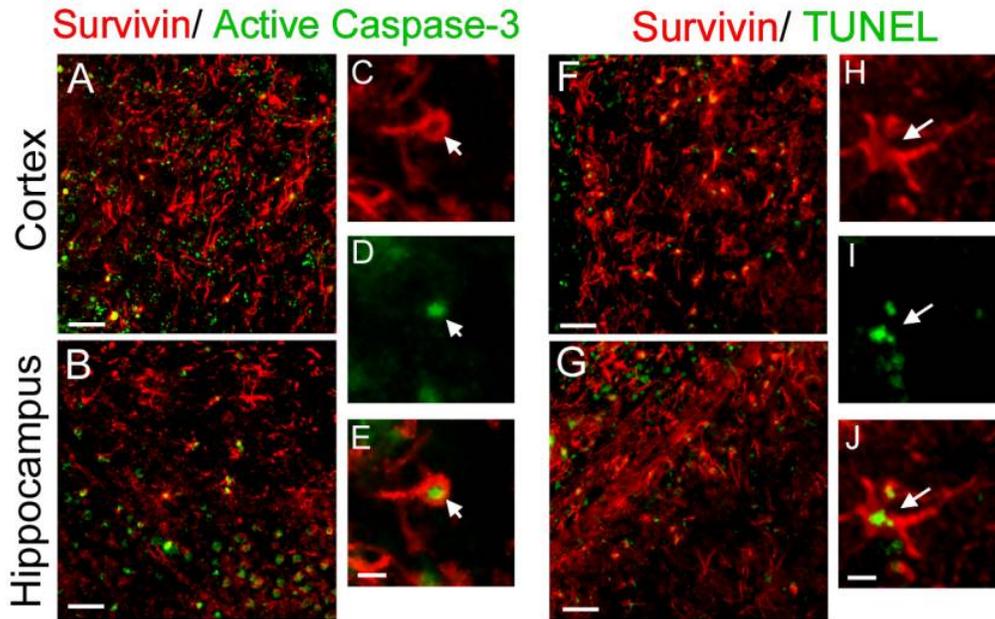


Figure 4-2: Co-expression of survivin and apoptosis markers following TBI in rats. Dual-label fluorescent immunostaining for survivin (red) and active caspase-3 (green, A-E) or TUNEL (green, F-J) was performed in the ipsilateral cortex (A, F) and hippocampus (B, G) at 5 day post-injury as described in detail under Materials and Methods. Survivin is expressed in both the cytoplasm and in the nucleus (C & H, red) while active caspase-3 (D, green) and TUNEL (I, green) label predominantly the nucleus. The white arrow indicates the typical focal co-localization of survivin and active caspase-3 (E) and TUNEL (J) as shown in the merged images. Magnification: 200x, scale bar 50  $\mu\text{m}$  (A, B, F, G); 200x, scale bar 10  $\mu\text{m}$  (C-E, H-J).

ipsilateral hippocampus (data not shown). Active caspase-3 (Figure 4-2D) and TUNEL (Figure 4-2I) labeling were both found in the nucleus in both regions. Survivin co-localization with active caspase-3 and TUNEL is shown at high magnification in Figure 4-2E and Figure 4-2J, respectively. Confocal microscopy was used to assure single cell co-localization of survivin, active caspase-3 and TUNEL (data not shown).

Quantitative analysis revealed no significant difference in the accumulation of active caspase-3 in survivin-positive cells compared to survivin-negative cells at five days post injury in either the cortex or hippocampus (Figure 4-3A,B). However, significantly higher percentage of TUNEL labeling was observed in survivin-negative

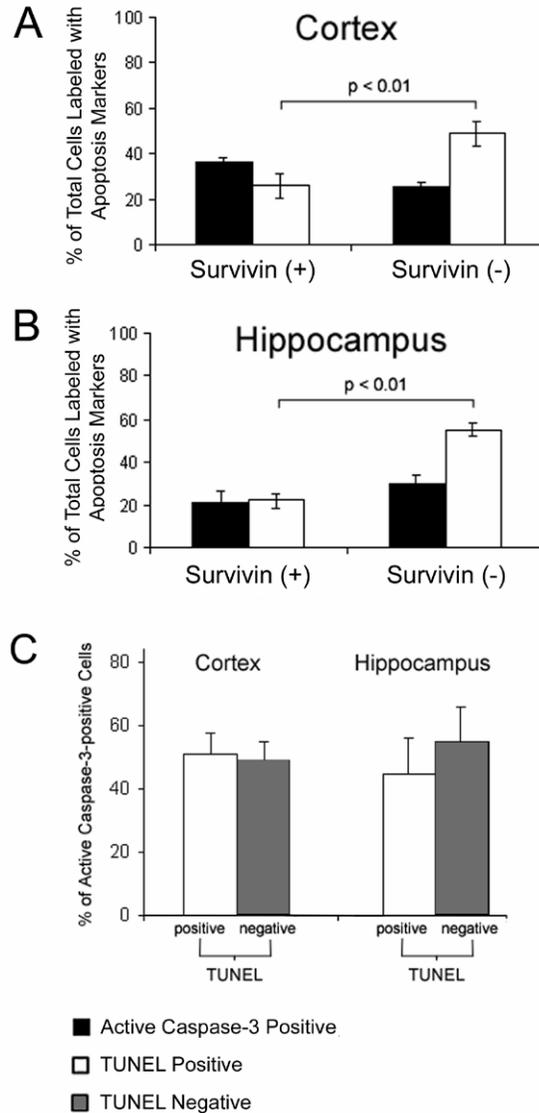


Figure 4-3: Survivin expression decreases the accumulation of TUNEL but not active caspase-3. Cells were quantified and visualized as described in detail under Materials and Methods. The percentage of cells expressing active caspase-3 and labeling with TUNEL was compared in two cell populations, survivin-positive and survivin-negative, in the ipsilateral cortex (A) and hippocampus (B). No differences were found in the percentage of survivin-positive and survivin-negative cells expressing active caspase-3 in either the cortex (A, black bars) or hippocampus (B, black bars). Significantly more survivin-negative cells were TUNEL-positive compared to survivin-positive cells in both the cortex ( $p < 0.01$ , A, white bars) and hippocampus ( $p < 0.001$ , B, white bars). No differences were found in the percentage of TUNEL-positive (C, white bars) or TUNEL-negative cells (C, gray bars) that also labeled with active caspase-3. Each bar represents mean  $\pm$  SEM of 4 (A, cortex) or 3 (B, hippocampus) independent measurements using an unpaired Student t-test for statistical analysis.

cells as compared to survivin-positive cells in both regions ( $p < 0.01$ ) (Figure 4-3A,B, Table 1A). At seven days post injury, there was no significant difference in the accumulation of both active caspase-3 and TUNEL labeling in survivin-positive cells compared to survivin-negative cells in either the cortex or hippocampus (Table 1B).

To verify that caspase-3 activation does not necessarily result in irreversible cell death, possibly due to survivin inhibition, quantification data was gathered on cells that accumulate active caspase-3 and are TUNEL label-positive and compared to active caspase-3-positive cells that do not label with TUNEL. Indeed, we found that the number of dual-labeled active caspase-3 and TUNEL cells was not statistically different from those cells labeled with active caspase-3 only (Figure 4-3C). This finding further supports the notion that several counteractive factors, including survivin, may inhibit active caspase-3 and diminish cell death following TBI.

#### **Astrocytes and Neurons Demonstrate Cell Specific Differences in Active Caspase-3 and TUNEL Labeling**

To determine cell types that express active caspase-3 or labeled with TUNEL, dual immunohistochemistry of these apoptosis markers was performed with GFAP, a marker of astrocytes, and NeuN, a marker of mature neurons. Co-localization of GFAP was observed with both active caspase-3 and TUNEL labeling in the ipsilateral cortex (Figure 4-4A,F respectively) and ipsilateral hippocampus (Figure 4-4B,G respectively). Higher magnification photomicrographs show a typical astrocyte expressing active caspase-3 (Figure 4-4C-E) or labeling with TUNEL (Figure 4-4H-J).

NeuN-positive cells exhibited modest accumulation of active caspase-3 and considerable labeling with TUNEL in the ipsilateral cortex (Figure 4-5A,F) and ipsilateral hippocampus (Figure 4-5B,G). These data are summarized in Table 1.

Table 1 Cell Quantification Data for Immunohistochemistry Labeling Pairs This table summarizes the results from all cell count experiments investigating survivin, cell type and the apoptosis markers. The numbers given are percentages calculated as described in the Materials and Methods. Each percentage represents the mean  $\pm$  SEM of 4 (cortex) or 3 (hippocampus) independent measurements at either five days post-injury (A) or seven days post-injury (B). \*\* p<0.01 versus survivin-negative cells, ## p<0.01, ### p<0.001 versus NeuN-positive cells, \$\$ p<0.01 versus TUNEL-negative cells (unpaired Student t-test).

A		Survivin (+)	Survivin (-)	GFAP (+)	NeuN (+)	TUNEL (+)	TUNEL (-)
Active Caspase-3 (+)	Cortex	37 $\pm$ 2	26 $\pm$ 6	50 $\pm$ 7###	7 $\pm$ 1	51 $\pm$ 6	49 $\pm$ 6
	Hippocampus	21 $\pm$ 5	22 $\pm$ 3	29 $\pm$ 4	15 $\pm$ 2	45 $\pm$ 11	55 $\pm$ 11
TUNEL (+)	Cortex	25 $\pm$ 2**	49 $\pm$ 5	11 $\pm$ 2###	46 $\pm$ 2		
	Hippocampus	30 $\pm$ 4**	55 $\pm$ 3	19 $\pm$ 0.2###	60 $\pm$ 3		
B		Survivin (+)	Survivin (-)	GFAP (+)	NeuN (+)	TUNEL (+)	TUNEL (-)
Active Caspase-3 (+)	Cortex	36 $\pm$ 2	39 $\pm$ 11	36 $\pm$ 3###	11 $\pm$ 1	48 $\pm$ 4	52 $\pm$ 4
	Hippocampus	37 $\pm$ 2	22 $\pm$ 6	31 $\pm$ 3##	15 $\pm$ 3	62 $\pm$ 5\$\$	38 $\pm$ 5
TUNEL (+)	Cortex	20 $\pm$ 5	25 $\pm$ 10	19 $\pm$ 1###	74 $\pm$ 5		
	Hippocampus	20 $\pm$ 3	31 $\pm$ 10	28 $\pm$ 7###	80 $\pm$ 2		

Specifically, both active caspase-3 (Figure 4-5D) and TUNEL (Figure 4-5I) were present in neuronal nuclei (Figure 4-5C,H), which was indicated by co-localization with NeuN (Figure 4-5E, J). Confocal microscopy was used to assure single cell co-localization of NeuN and GFAP with active caspase-3 and TUNEL (data not shown).

Quantitative analysis revealed a substantially different expression profile of active caspase-3 and TUNEL in neurons and astrocytes. A significantly higher number of astrocytes accumulated active caspase-3 as compared to neurons in the cortex, but not the hippocampus (p<0.001) (Table 1). Conversely, a significantly greater number of neurons were labeled with TUNEL compared to astrocytes in both the cortex and hippocampus (p<0.001) (Figure 4-6, Table 1A). A similar expression profile of active caspase-3 and

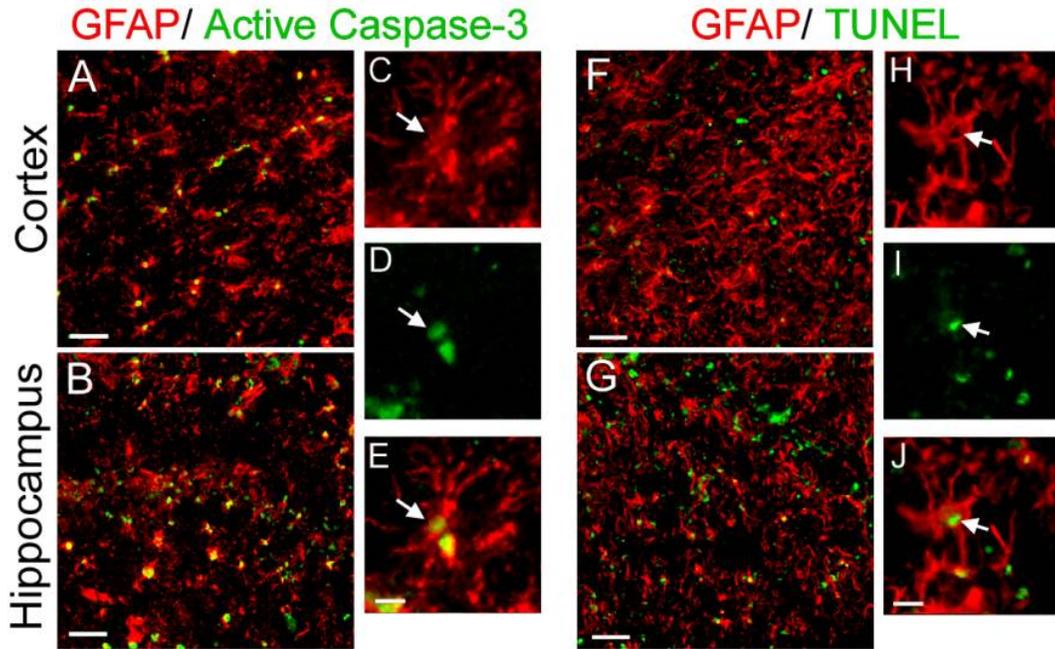


Figure 4-4: Astrocytes express active caspase-3 and label with TUNEL following TBI in rats. Dual-label fluorescent immunostaining for GFAP (red) and active caspase-3 (green, A-E) or TUNEL (green, F-J) was performed in the ipsilateral cortex (A, F) and hippocampus (B, G) at 5 day post-injury as described in detail under Materials and Methods. GFAP is expressed in the cytoplasm (C & H, red) while active caspase-3 (D, green) and TUNEL (I, green) are expressed in the nucleus. The white arrow indicates the typical focal co-expression of GFAP and active caspase-3 (E) and TUNEL (J) as shown in the merged images. Magnification: 200x, scale bar 50  $\mu\text{m}$  (A, B, F, G); 200x, scale bar 10  $\mu\text{m}$  (C-E, H-J).

TUNEL was observed in astrocytes and neurons seven days post injury (Table 1B).

#### Discussion of Chapter 4

Caspase-3 activation is a prominent feature of apoptosis and its role in DNA fragmentation after TBI has been well documented (Nicholson et al., 1995; Tewari et al., 1995; Pike et al., 1998; Tang and Kidd, 1998; Wolf et al., 1999; Beer et al., 2000; Buki et al., 2000; Clark et al., 2000). Survivin is an inhibitor of apoptosis protein (IAP), which can inhibit active caspase-3 and thereby moderate cell death in various tissues, including CNS (Shankar et al., 2001; Sasaki et al., 2002; Van Haren et al., 2004). However, no

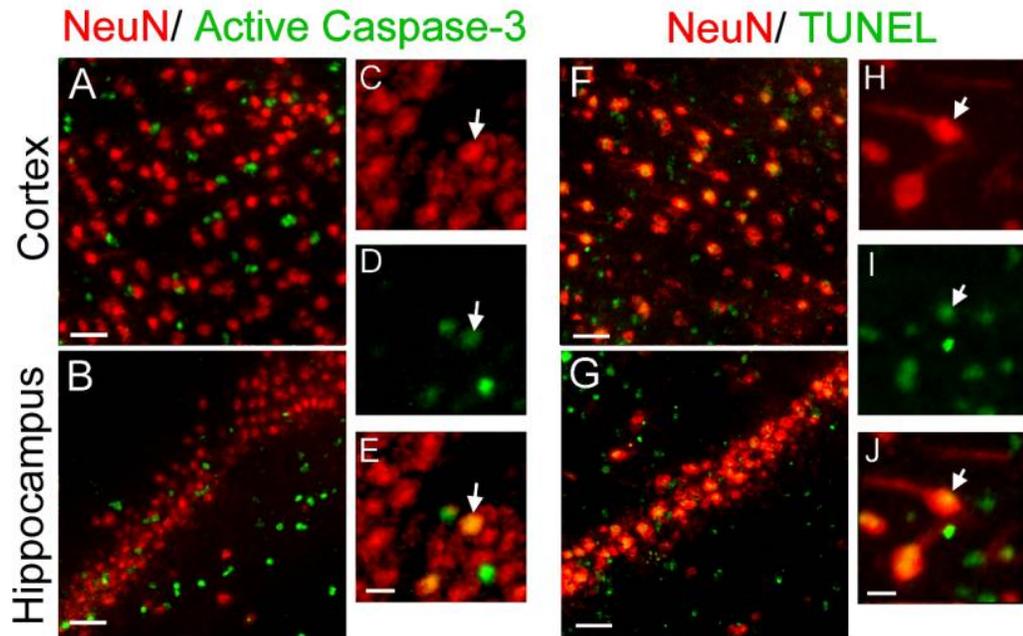


Figure 4-5: Neurons express active caspase-3 and label with TUNEL following TBI in rats. Dual fluorescent immunostaining for NeuN (red) and active caspase-3 (green, A-E) or TUNEL (green, F-J) was performed in the ipsilateral cortex (A, F) and hippocampus (B, G) at 5 day post-injury as described in detail under Materials and Methods. NeuN is expressed primarily in the nucleus but also in the cytoplasm (C & H, red) while active caspase-3 (D, green) and TUNEL (I, green) are found in the nucleus. The white arrow indicates the typical focal co-localization of NeuN and active caspase-3 (E) and TUNEL (J) as shown in the merged images. Magnification: 200x, scale bar 50  $\mu\text{m}$  (A, B, F, G); 200x, scale bar 10  $\mu\text{m}$  (C-E, H-J).

studies have investigated the potential anti-apoptotic role of survivin in the adult brain after TBI.

These data characterize the relationship between survivin expression and two apoptosis events: the accumulation of active caspase-3 and downstream DNA fragmentation (as shown by TUNEL labeling) in rat brain subjected to TBI. The use of TUNEL labeling in conjunction with active caspase-3 is considered a reliable tool to assess apoptosis progression (Lei et al., 2004; Marciano et al., 2004; Nakase et al., 2004). The appearance of active caspase-3 was time-dependent and region specific (Figure 4-1)

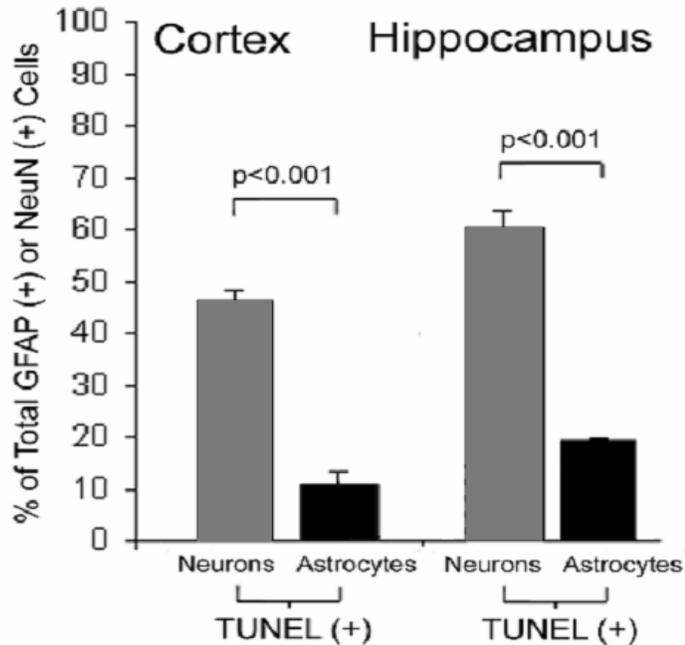


Figure 4-6: TUNEL labeling is cell specific following TBI in rats. Cells were quantified and visualized as described in detail under Materials and Methods. The percentage of cells labeling with TUNEL was compared in astrocytes (black bars) and neurons (gray bars) in the ipsilateral cortex and hippocampus. Significantly more TUNEL labeling was seen in neurons than in astrocytes in both the cortex and hippocampus ( $p < 0.001$ ). Each bar represents mean  $\pm$  SEM of 4 (cortex) or 3 (hippocampus) independent measurements using an unpaired Student t-test for statistical analysis.

with a pattern similar to survivin expression after TBI which suggests that survivin may inhibit caspase-3 activity to diminish the deleterious consequences of proteolysis after TBI (Tamm et al., 1998; Kobayashi et al., 1999; O'Connor et al., 2000b; Shin et al., 2001). Other IAPs are up-regulated in concert with activation of caspases after brain injury (Keane et al., 2001). In addition, survivin expression is up-regulated by the pro-survival PI3-kinase/Akt pathway which is activated after TBI (Kitagawa et al., 1999; Xia et al., 2002b; Kim et al., 2004).

Survivin-positive cells expressed active caspase-3 and were labeled with TUNEL (Figure 4-2) though survivin-positive cells showed no significant difference in accumulation of active caspase-3 compared to survivin-negative cells (Figure 4-3).

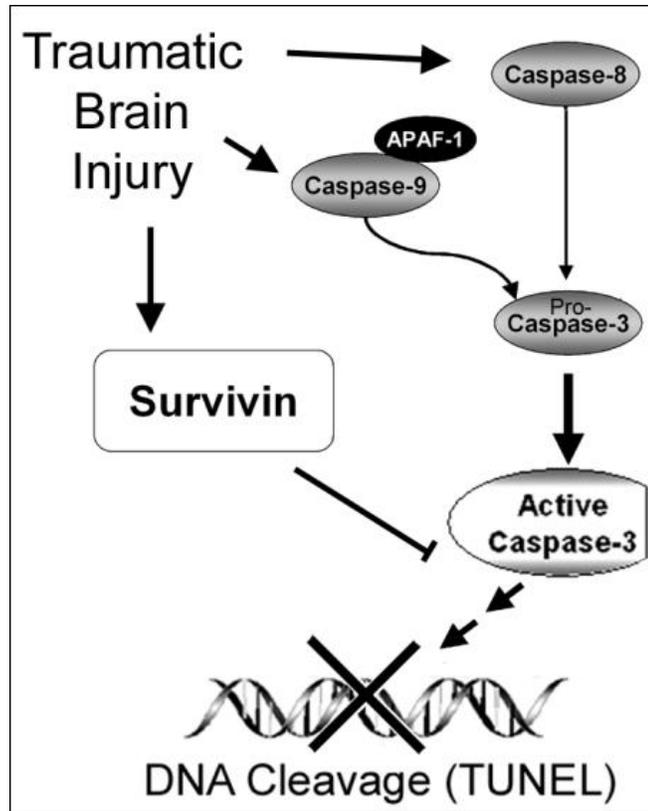


Figure 4-7: Putative mechanism of apoptosis inhibition by survivin following TBI. Traumatic brain injury induces activation of upstream caspases-8 and 9 that can process pro-caspase-3 to its active form. Once activated, caspase-3 can cleave several intracellular substrates and activate restrictases that may lead to DNA fragmentation. Concomitant survivin expression is up-regulated in response to the same TBI signals. Survivin has the ability to inhibit the activity of active caspase-3, which results in the attenuation of DNA fragmentation.

These data are in accordance with the ability of survivin (Tamm et al., 1998) and other IAPs (Shankar et al., 2001; Maier et al., 2002) to inhibit the activity but not the activation of caspase-3. In contrast, fewer survivin-positive cells exhibited DNA fragmentation (TUNEL labeling) compared to survivin-negative cells at five days post injury (Figure 4-3). These data suggest that survivin expression may attenuate the apoptotic cascade by inhibiting the cleavage of caspase-3 specific substrates that result in DNA fragmentation. Furthermore, the finding that active caspase-3 accumulation led to positive TUNEL

labeling in 51% of all active caspase-3-positive cells (Figure 4-3) is consistent with the observation that endogenous factors, including survivin, may inhibit active caspase-3 and attenuate DNA fragmentation following TBI. In an attempt to more directly investigate whether survivin could inhibit active caspase-3 activity, survivin was co-localized with markers of caspase-3 activity including the cleaved species of PARP, DFF45/iCAD and  $\alpha$ -II-spectrin (120 kDa). Unfortunately, these markers proved to be of unacceptable quality to use in western blot and IHC analyses in this model. Therefore, it remains unknown whether survivin correlates with a decrease in active caspase-3 activity as measured by caspase-3-specific breakdown products. The ability of survivin to inhibit DNA fragmentation has been shown in gastric cancer cells (Lu et al., 1998). In addition, survivin antisense treatment increases DNA fragmentation in neuroblastoma and oligodendroglioma (Shankar et al., 2001). Taken together, these findings suggest that survivin likely inhibits the proteolytic activity of caspase-3 after TBI to attenuate DNA cleavage.

The labeling patterns of active caspase-3 and TUNEL in astrocytes and neurons were investigated. Data described in Chapter 3 showed that a large majority of astrocytes but few neurons express survivin after TBI. These data show that both astrocytes and neurons expressed active caspase-3 and label with TUNEL post-injury but the prevalence of this labeling was drastically different (Figures 4-4, 4-5). A higher percentage of astrocytes accumulate active caspase-3 but fewer astrocytes label with TUNEL compared to neurons (Figure 4-6). It is unclear at present why these experiments revealed few neurons labeling with active caspase-3 (Table 1). Some active caspase-3-positive neurons may not have been detected due to caspase-3 mediated loss of NeuN antigenicity

(Unal-Cevik et al., 2004). Other groups have found prominent caspase-3 activation in apoptotic neurons after TBI (Beer et al., 2000). In addition, caspase-independent necrosis may also be a major contributor of neuronal cell death after TBI (Newcomb et al., 1999; Wennersten et al., 2003).

Cell specific survivin expression may contribute to the lower incidence of TUNEL labeling in astrocytes as compared to neurons (Table 1). Cell type specific expression of IAPs after TBI has been previously shown. For example, XIAP is expressed primarily by neurons (Lotocki et al., 2003) and a subset of oligodendrocytes (Keane et al., 2001) following brain injury. NAIP is expressed in neurons after ischemia (Xu et al., 1997) and TBI (Hutchison et al., 2001). Lastly, RIAP-2 is abundantly expressed in neurons as opposed to astrocytes after kainic acid treatment in rats (Belluardo et al., 2002). Figure 4-7 shows a putative mechanism for apoptosis inhibition by survivin. Following upstream caspase activation and cleavage of procaspase-3 to active caspase-3, survivin acts to attenuate the apoptotic cascade and finally DNA cleavage and cell death by inhibiting caspase-3 activity.

Taken together, data from Chapter 4 demonstrate that the activation of caspase-3 in the rat brain after TBI follows the same timeframe, regions and cell type expression patterns as survivin. Quantitative correlative analysis reveals that significantly fewer cells expressing survivin undergo final stage of apoptosis and cell death. These data suggest that DNA cleavage may be attenuated via inhibition of active caspase-3 by survivin after traumatic brain injury in a cell-specific fashion. Namely, astrocytes have significantly lower TUNEL labeling than neurons suggesting a more robust anti-apoptotic role for survivin in astrocytes. Collectively, these results suggest that survivin plays a

role in diminishing apoptosis and DNA fragmentation following traumatic brain injury in rats.

## CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

### **Conclusions**

Traumatic brain injury (TBI) remains a major health care and economic issue in the United States to this date. Despite continued education and improved first responder care, the Centers for Disease Control estimate that there are more than 5.3 million Americans living with disabilities from TBI with another 1.5 million new TBIs sustained in the U.S each year. Cognitive and memory deficits resulting from TBI are especially difficult to cure and no effective treatment options are available.

TBI is a complex injury that induces both apoptotic cell death and neural cell proliferation. Apoptosis is opposed by up-regulation of inhibitor of apoptosis proteins (IAPs) that attenuate apoptosis through direct inhibition of active caspases. Neural cell proliferation may contribute to neural tissue healing by repopulating damaged regions with new, functional cells but may also prevent normal recovery of the injured brain by forming impermissible barriers for axonal growth.

The recently discovered protein, survivin, may play a significant role in these processes following TBI. Survivin is a protein that is both integral for mitosis and is an IAP with anti-apoptotic properties. Though not normally found in quiescent adult tissues, survivin can be induced in certain mature, non-neural cells after CNS insult or cancer transformation to resist apoptosis. Furthermore, brain injury can induce stem cell proliferation, a process that likely requires the survivin protein for proper completion.

However, no study to date had investigated the temporal, regional and neural cell expression patterns of this unique protein following TBI.

Chapter 3 of this dissertation critically examined the transcriptional and translational expression of survivin following TBI in rats. This dissertation is the first to demonstrate that survivin mRNA and protein are expressed in neural cells following TBI and that this expression is cell type specific. QPCR analysis confirmed elevated levels of survivin following TBI that peaked at five days post-injury in the ipsilateral cortex and hippocampus. Immunoblot analysis confirmed survivin translation with peak expression at five days post-injury in both regions. Survivin localization at this time point was observed in approximately 88% of the astrocytes in the ipsilateral cortex and hippocampus. Survivin expression was also observed in a much smaller sub-set of neurons, where no more than 1.5% of neurons expressed survivin. Survivin expression was not observed in microglia and oligodendrocytes. Like other IAP proteins, expression of survivin appears to be cell type specific following TBI. In contrast to the IAPs XIAP, NAIP, cIAP-1 and cIAP-2 which are predominantly expressed in neurons following brain insult, survivin is expressed primarily in astrocytes. Attempts were made to localize survivin with the neuronal progenitor cell markers nestin, doublecortin,  $\alpha$ -internexin and  $\beta$ -III-tubulin. However, these antibodies did not prove to be of acceptable quality to use in western blot and IHC analyses in this model leaving proliferating progenitors undetected.

Survivin has two biochemically distinct functions, that of apoptosis inhibition and to properly separate DNA during mitosis. To reveal the relationship between survivin and cellular proliferation, several cell cycle proteins were investigated including cdk4,

cyclin B, cyclin D, AIM-1 and PCNA. Only PCNA provided clear results in both western blot and IHC analyses. Therefore, the regional, temporal and cell specific protein expression of the previously characterized cell cycle protein, PCNA and its co-localization with survivin was investigated. PCNA protein accumulated in the ipsilateral cortex and hippocampus peaking at five days post-injury. In addition, astrocytes and a small subset of neurons expressed PCNA in a pattern similar to survivin. However, only 12% of PCNA-positive cells also expressed survivin. The cellular identity of these dual-labeled cells remains unknown due to difficulties with triple-label IHC. Therefore, the ramifications of cell cycle protein expression in individual cell types remains unclear. However, these data seem to indicate that the primary function of survivin following TBI is not cellular proliferation. A more complete picture of survivin's role in cellular proliferation following brain insult would require co-localization with other cell cycle related proteins. Thus far, additional investigations with proteins such as AIM-1, cdk4, cyclin D and cyclin B1 have been inconclusive. Further studies will be required to determine the extent to which survivin acts as an indicator of cellular proliferation and the cell types that may be actively proliferating following TBI.

Chapter 4 revealed the relationship between survivin and apoptosis inhibition by investigating the accumulation of active caspase-3, the main executioner caspase in apoptosis, and the appearance of DNA fragmentation (TUNEL) following TBI. Immunoblot studies revealed that active caspase-3 did accumulate following TBI with significant accumulation at five, seven and fourteen days post-injury in the ipsilateral cortex and at seven and fourteen days post-injury in the ipsilateral hippocampus. Because survivin and active caspase-3 levels peaked on different post-injury days, IHC

was performed at the peak expression times of five days (survivin) and seven days (active caspase-3) post-injury. IHC analysis revealed that survivin and both active caspase-3 and TUNEL did co-localize to the same cells at these time points. Attempts were made to co-localize survivin with markers of caspase-3 activity to correlate the presence of survivin to an absence in caspase-3 specific breakdown products. Antibodies for the caspase-3-specific cleaved species of PARP, DFF45/iCAD and  $\alpha$ -II-spectrin (120 kDa) proved to be of unacceptable quality to use in western blot and IHC analyses in this model leaving markers of caspase-3 activity undetected.

Quantitative analysis revealed no significant difference in the accumulation of active caspase-3 in survivin-positive cells compared to survivin-negative cells at five days post injury in either the cortex ( $37 \pm 2\%$  v.  $26 \pm 6\%$ ) or hippocampus ( $21 \pm 5\%$  v.  $22 \pm 3\%$ ). Conversely, a significantly higher percentage of TUNEL labeling was observed in survivin-negative cells as compared to survivin-positive cells in ipsilateral cortex ( $49 \pm 5\%$  v.  $25 \pm 2\%$ ) and hippocampus ( $55 \pm 3\%$  v.  $30 \pm 4\%$ ). These data are consistent with my hypothesis that survivin expression may attenuate the apoptotic cascade by inhibiting the cleavage of caspase-3 substrates. However, by seven days post injury, there was no significant difference in the accumulation of either active caspase-3 or TUNEL labeling in survivin-positive cells compared to survivin-negative cells. It is unclear why this pattern was observed. In particular, the sharp decrease in survivin-negative, TUNEL-positive cells from five days to seven days post-injury was unexpected. This decrease in DNA fragmentation may be explained by death and removal of TUNEL-positive cells between five and seven days as evidenced by progressive increases in cavity size seen after brain injury (data not shown). The turnover from healthy to TUNEL-positive in the

survivin-negative cell population may not be steady, as indicated by the biphasic fluctuations of cell death indicators after brain injury (Holmin and Mathiesen, 1995; Domanska-Janik, 1996; Kampfl et al., 1996; Baskaya et al., 1997). Again because of the difficulty of triple-label IHC, the cell type of survivin dual-labeled cells could not be identified.

Because survivin is expressed in astrocytes and neurons, the accumulation of active caspase-3 and TUNEL-labeling in these cells was investigated. Both cell types accumulated active caspase-3 and labeled with TUNEL following TBI. Quantification studies revealed a significantly greater percentage of neurons labeled with TUNEL compared to astrocytes in both the cortex and hippocampus at both five and seven days post injury. Taken with the observation that a majority of astrocytes express survivin after TBI, it is possible that survivin expression contributes to the low DNA fragmentation observed in these cells despite prominent caspase-3 activation. The opposite appears to be the case with neurons. Few neurons express survivin following TBI and many of these cells show prominent DNA fragmentation. Neurons are particularly vulnerable to apoptosis signals following brain injury and absence of survivin expression may contribute to this vulnerability. Therefore the hypothesis does not seem to support an anti-apoptosis role for survivin in the majority of neurons following TBI.

Collectively, these data indicate that survivin, a developmental protein normally absent in adult tissues, is up-regulated at both the transcriptional and translational level following traumatic brain injury. Survivin is expressed predominantly by astrocytes and a small sub-set of neurons. In addition, this is the first study to provide indirect evidence that survivin functions as both an apoptosis inhibitor and as a cell cycle protein following

brain trauma. These data suggest that further studies are necessary to show the overall ramifications of survivin expression on the secondary injury cascade following TBI.

### **Future Directions**

Additional studies of survivin expression and function must be completed before survivin may be considered a potential therapeutic agent/target for brain injury treatment. First, survivin activities following TBI in the intact animal must be inhibited to observe the histological and behavioral changes associated with its expression. Of interest are heterozygous survivin knockout mice strains that appear to be particularly vulnerable to even mild apoptotic stimuli (Conway et al., 2002). TBI experiments comparing the histological and behavioral differences between heterozygous survivin knockouts and their wild type littermates would give more direct evidence of survivin function following brain insults. Additionally, a host of new techniques to inhibit survivin expression in the whole animal are currently being developed for cancer treatment and can be readily applied to a TBI model. Of practical interest to this model is “molecular antagonism” and pharmacological inhibition. “Molecular antagonism” using siRNA, antisense and dominant-negative survivin mutants are effective *in vivo* at interfering with survivin expression and function (Grossman et al., 2001b; Kanwar et al., 2001; Yamamoto et al., 2002; Williams et al., 2003). Pharmacological inhibition by flavopiridol (Zhai et al., 2002) or Purv.A (Gray et al., 1998) can increase survivin turnover and reduce its effectiveness as an apoptosis inhibitor by reducing the phosphorylation state of survivin (O'Connor et al., 2000a; Grossman et al., 2001b). Based on the findings in this dissertation, inhibition of survivin following TBI will likely increase apoptotic cell death, cavity size and have a negative impact on behavioral recovery.

Second, the activities of survivin must be enhanced to see if increased survivin has a beneficial role to the overall recovery of the organism. Both pharmacological and gene transfection can be used to accomplish this task. Gene transfection has been successfully used to temporarily up-regulate proteins of interest in the adult brain (Yenari and Sapolsky, 2004) and may be used to increase survivin expression following TBI. A more flexible approach may include pharmacological agents because they can be used in established wild-type animal models. Compounds that enhance Cdk phosphorylation activity such as PD0166285 (Li et al., 2002) may inhibit survivin turnover and promote cell survival by increasing the phosphorylation state of survivin. Based on the findings from this dissertation and work by other groups, enhancing survivin activity in the brain following TBI will likely decrease cell death, reduce cavity size and enhance behavioral recovery compared to untreated animals.

This set of proposed experiments will reveal the functional consequences of endogenous survivin expression in injured brain tissues. With this knowledge, it can be determined whether survivin is indeed a viable therapeutic agent for brain injury treatment.

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## BIOGRAPHICAL SKETCH

Erik Andrew Johnson was born in Louisville, KY, and raised in Kansas City, MO. He graduated high school from Lincoln College Preparatory Academy (Kansas City, MO) in 1994. He attended Macalester College (St. Paul, MN) where he received a Bachelor of Arts degree in 1998 with majors in biology, psychology and neuroscience. After a year of graduate study at the University of Texas-Houston, he transferred to the Interdisciplinary Program in Biological Sciences at the University of Florida (Gainesville, FL) to complete his doctorate in the laboratory of Dr. Ronald Hayes. Erik has been awarded top honors at the National and International Neurotrauma Society Student Poster Competition in 2001. In addition, Erik has twice been awarded the B.W. Robinson Research Endowment Grant-in-Aid Achievement Award in 2003 and 2004. Erik finished his doctoral work with two peer reviewed first author papers and five total papers to his credit.