

DOPAMINE AND CAMP REGULATED PHOSPHOPROTEIN, 32 KDA: A NOVEL
THERAPEUTIC TARGET IN TRAUMATIC BRAIN INJURY.

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Traumatic brain injury (TBI) represents a significant cause of death and disability in industrialized countries. Of particular importance to patients is the chronic effect that TBI has on cognitive function. Therapeutic strategies have been difficult to evaluate because of the complexity of injuries and variety of patient presentations within a TBI population. Experimental therapies based upon cortical and hippocampal neuroprotection have not translated clinically. However, pharmacotherapies targeting dopamine (DA) have consistently shown benefits in attention, behavioral outcome, executive function, and memory. Striatal damage causes deficits in executive function, learning, and memory. Dopamine and cAMP regulated phosphoprotein 32 (DARPP-32), expressed within striatal medium spiny neurons, is known to regulate several substrates of cognition. We found that controlled cortical impact injury in rats produces a chronic decrease in DARPP-32 threonine-34 phosphorylation and increase in protein phosphatase-1 activity. There is no effect of injury on threonine-75 phosphorylation or DARPP-32 protein. Amantadine has known benefits on post-TBI cognitive deficits and when given daily for two weeks reversed the DARPP-32 and protein phosphatase-1 changes. Amantadine also decreased the phosphorylation of threonine-75 consistent with activity as a partial N-methyl-D-aspartic acid receptor antagonist and partial dopamine agonist. FK-506, also known as tacrolimus, is a calcineurin inhibitor that has been shown to decrease cell death in the hippocampus following a fluid percussion experimental TBI. Calcineurin is also an important regulator of DARPP-32 phosphorylation in the striatum. We evaluated the effect of FK-506 on the hippocampus and DARPP-32 in the striatum to better detail its effects after a TBI. An acute administration of FK-506 following controlled cortical impact reversed the effects of TBI on DARPP-32 phosphorylation seen chronically. We then evaluated the effect of a combined drug therapy on cognitive deficits post TBI. An acute treatment with FK-506 post TBI followed by chronic Amantadine therapy demonstrated an improvement in both motor behavior and Morris water maze deficits seen following TBI. Neither drug produced benefit when given alone. These data demonstrate that DARPP-32 represents a promising new therapeutic target for TBI induced cognitive deficits.

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PREFACE

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1. INTRODUCTION

Traumatic brain injury (TBI) represents a significant cause of death and disability in industrialized countries. Of particular importance to patients is the chronic effect that TBI has on cognitive function. Therapeutic strategies have been difficult to evaluate because of the complexity of injuries and variety of patient presentations within a TBI population. However, pharmacotherapies targeting dopamine (DA) have consistently shown benefits in attention, behavioral outcome, executive function, and memory. Still it remains unclear what aspect of TBI pathology is targeted by DA therapies and what time-course of treatment is most beneficial for patient outcomes. Fortunately, ongoing research in animal models has begun to elucidate the pathophysiology of DA alterations after TBI.

1.1 TRAUMATIC BRAIN INJURY

TBI is the leading cause of death and disability in individuals less than 45 years of age in industrialized countries (Bruns and Hauser, 2003). Each year an estimated 1.4 million Americans experience a TBI and 80,000 to 90,000 suffer long-term substantial loss of function (Rutland-Brown et al., 2006). Clinical studies have shown that 10-15% of individuals with mild TBI have persistent cognitive and behavioral complaints. Outcomes from moderate TBI are much less favorable with some estimates suggesting that 50% of these individuals endure long-term injury-related disabilities (Kraus et al., 2005). This places an enormous economic burden on the U.S. healthcare system with an estimated cost of \$9-10 billion in acute care and rehabilitation annually. This cost is in addition to lost earnings, social services, and the cost to family members who must care for TBI survivors. TBI also represents a global healthcare crisis

with an estimated 2% of the world's population suffering from chronic symptoms of brain trauma, equating to more than 120 million individuals (NIH, 1998; Ragnarsson, 2002). For these reasons it has been a long sought goal of TBI researchers to understand the mechanisms of chronic disability after TBI to help develop treatment strategies that may assist patients with cognitive recovery.

However, researching chronic disability following TBI has posed a unique challenge to both clinical and experimental researchers. TBI is a highly variable and extremely complex phenomenon. Following the acute primary injury, which often consists of a focal contusion and more diffuse structural damage, there are a series of subsequent secondary responses, which include, but are not limited to, excitotoxicity, ischemia, oxidative stress, and ongoing structural and chemical alterations (Kochanek, 1993; DeKosky et al., 1998; Park et al., 2008). Traditionally, research in recovery of function after TBI has focused on preventing or manipulating early events in order to prevent chronic dysfunction. Drugs inhibiting apoptosis, blocking glutamate-induced excitotoxicity, or attenuating oxidative stress were designed to reduce cell loss with the premise that neuronal sparing would enhance recovery (Faden et al, 1989; Jennings et al., 2008). Unfortunately, the neuroprotective effects observed in the TBI laboratories have not translated successfully to the clinic (Gualtieri, 1988; Tolias and Bullock, 2004). In contrast, therapeutics used during the rehabilitative phase have shown more promise in addressing long term disability, although they don't necessarily demonstrate the same level of neuroprotection as drugs designed to inhibit apoptosis or block excitotoxicity (Gualtieri, 1988; Rees et al., 2007).

The failure of translating experimental preventative strategies to clinical efficacy has raised the question about what events in TBI are crucial for long-term outcome. The development of clinically relevant small animal models has greatly assisted the understanding of both acute and

chronic TBI-induced alterations in brain chemistry. The two most widely used models of TBI are fluid percussion (FP; Dixon et al., 1987) and controlled cortical impact (CCI; Lighthall, 1988; Lighthall et al., 1989; Dixon et al., 1991; Kline et al., 2001; Kline and Dixon, 2001). Both models produce clinically relevant brain pathology as well as behavioral and cognitive dysfunction in rats and mice (Dixon et al., 1987, 1991; Hamm et al., 1992, 1996a,b; Fox et al., 1998, 1999; Kline et al., 2002; Wagner et al., 2002, 2004; Kline et al., 2007a,b; Cheng et al., 2007, 2008; Hoffman et al., 2008a,b). Animal studies (Bramlett and Dietrich, 2002; Lifshitz et al., 2007) and human positron emission tomography (PET) imaging (Langfitt et al., 1986; Fontaine et al., 1999; Donnemiller et al., 2000) have shown that in addition to overt damage (e.g., cortical lesions and hippocampal cell loss), there exist areas of chronic dysfunction previously unappreciated, particularly in the striatum and thalamus, which are regions known to have important roles in cognitive, motor, and emotional processing (Vertes, 2006).

1.1.1 Introduction to dopamine in TBI

DA represents a unique signaling system within the central nervous system (CNS) due to its role as both a neurotransmitter and neuromodulator. Furthermore, DA receptors are abundantly expressed in brain areas known to be damaged after TBI, such as the frontal cortex and striatum, which are important for cognitive function (Seeman et al., 1978; Baron et al., 1985; McDonald et al., 2002; Chudasama and Robbins, 2006). The hippocampus, which is also critical for cognitive function does not have a high level of DA receptor expression, but is dependent on DA activity to modulate function (Lemon and Manahan-Vaughan, 2006; O'Carroll et al., 2006; Granado et al., 2008).

1.2. DOPAMINERGIC FUNCTIONAL ANATOMY: A TBI PERSPECTIVE

1.2.1 Persistent cognitive disability

Cognitive disorders experienced by TBI patients can present immediately after the initial injury or evolve during the subsequent months to years. Regardless of presentation, many patients live with sustained alterations in cognition and behavior for the rest of their lives (Millis et al., 2001). Non-pharmacological options for TBI patients experiencing cognitive and behavioral dysfunction are limited, with cognitive training paradigms often being the only consistent treatment provided. However, cognitive training effectiveness has not been fully validated, and program implementation can be variable (Turner-Stokes et al., 2005).

Persistent cognitive deficits can be categorized into one of three general domains: attention and processing speed, memory, and executive function (Gronwall, 1976; Levin and Grossman, 1978; Gronwall and Wrightson, 1981; Stuss et al., 1985; Binder, 1986, 1987; McMillan and Glucksman, 1987; Levin et al., 1988a,b; Gentilini et al., 1989; Stuss et al., 1989; Leininger et al., 1990; Binder, 1997; Binder et al., 1997; McMillan, 1997). Of these, memory difficulties are the most commonly reported and most difficult for patients and caregivers (Binder, 1987). In addition to cognitive difficulties, TBI survivors often experience behavioral difficulties characterized by enhanced emotional lability and alterations in affect (Fugate et al., 1997). Importantly, a prior TBI has been shown to be a risk factor for developing psychiatric and psychotic disorders (Koponen et al., 2002). Due to its often diffuse nature and the variety of cognitive disturbances experienced post-TBI, it has been difficult to localize a single disruption in neural function that could explain such an array of events. Indeed, persistent deficits experienced by TBI patients are probably due to a wide spectrum of different neural system dysfunctions. However, that does not preclude the possibility of utilizing a targeted therapeutic

strategy to enhance cognitive recovery after TBI. DA is a particularly important system to study in this context because it is known to have an important role in physiological events relevant to cognition and in numerous systems also affected by TBI, including the hippocampus, striatum, and frontal cortex.

1.2.2 Dopamine and TBI: The effect on cognition

Figure 1 is a depiction of the rat CNS demonstrating an overlay of injury processes that occur with a TBI and their direct relationship to DAergic pathways important to cognition. As illustrated, TBI can have widespread effects on brain anatomy and function within DAergic regions. What is not depicted in **Figure 1** is the indirect effect of TBI on DA signaling, including disruptions in glutamatergic cortico-striatal projections and striatal gamma-Aminobutyric acid (GABA)ergic outputs.

Figure 1

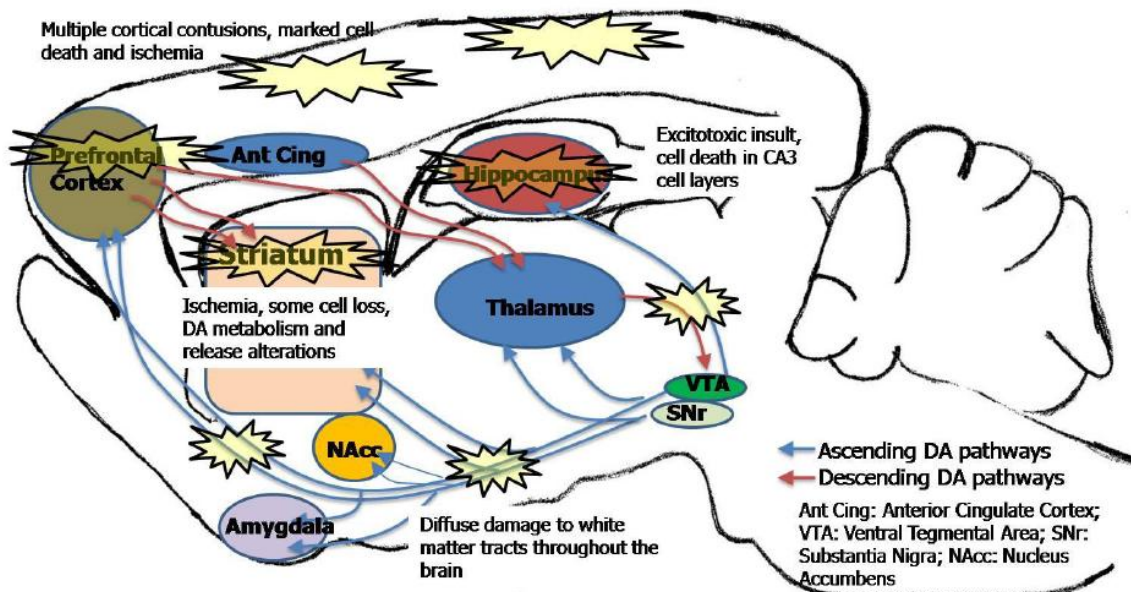


Figure 1: Depiction of TBI induced damage to DA systems in rat brain demonstrating widespread disruption in DA structures. Areas involved in both ascending and descending DAergic signaling pathways are damaged following a TBI. Not pictured is the extensive damage caused to other neurotransmitter systems and brain structures not directly involved in DAergic signaling pathways.

Ascending DAergic pathways in the CNS can be divided into two predominant systems: 1) the nigrostriatal pathway [substantia nigra (SN) innervating striatum], and 2) the mesocorticolimbic pathway [ventral tegmental area (VTA) projecting to the prefrontal cortex (PFC), hippocampus, amygdala, and nucleus accumbens (NAcc)] (Alexander and Crutcher, 1990; Graybiel, 1990). Projections from the mesocorticolimbic pathway are believed to be involved in modulating memory consolidation (Ploeger et al., 1991; Cools et al., 1993; Ploeger et al., 1994; Setlow and McGaugh, 1998; Coccorello et al., 2000), motivation (Mitchell and Gratton, 1994; Salamone, 1994; Baldo and Kelley, 2007), and drug reinforcement and addiction (Carelli, 2002; Schultz, 2004; Salamone et al., 2005; Berridge, 2006; Di Chiara and Bassareo, 2007; Ikemoto, 2007; Sutton and Beninger, 1999). Change in the release of DA in the mesocorticolimbic system is also associated with neuropsychiatric disorders, arousal, stress, and addiction (Tidey and Miczek, 1996; Viggiano et al., 2003; Sonuga-Barke, 2005). The nigrostriatal system is predominantly associated with voluntary movement (Hornykiewicz, 1966; Seeman and Niznik, 1990; Jackson and Westlind-Danielsson, 1994), but it has also been shown to be important for behavioral events including reward processing (Wickens et al., 2007) and acquisition of spatial learning and memory (Mura and Feldon, 2003). Research investigating Parkinson's disease (PD) has also shown that dysfunctional nigrostriatal signaling has implications for other cognitive functions including memory, executive function, and attention (Tamaru, 1997; Ridley et al., 2006).

The striatum, which includes the NAcc and caudate putamen, exists as part of an anatomic network that subserves functions associated with the dorsolateral PFC (DLPFC), but also receives inputs from numerous other brain areas including the hippocampus and limbic cortex. The DLPFC has dense projections to the head of the caudate and there exist reciprocal pathways back to the DLPFC through the thalamus (Middleton and Strick, 2000). Due to this complex relationship with surrounding cortical and subcortical structures through DAergic projections, the striatum is in a prime location for mediating human cognition. Studies have demonstrated that both the striatum and DLPFC are important for executive function and working memory (WM) (Crosson, 2003).

In Huntington's disease (HD), projections from the caudate to the frontal lobes are disrupted and result in significant motor, attentional, and executive dysfunction (Brandt et al., 1988; Zakzanis and Kaplan, 1999). Experiments producing lesions in the striatum suggest that the caudate, in particular, plays a specific role in cognition. Damage to the caudate produces deficits that resemble damage to corresponding projection targets of the PFC (Divac et al., 1967). In nonhuman primates, metabolic activity within the striatum has also been linked to specific changes in WM task performance (Levy et al., 1997). In a recent review by Grahn et al (2008) the authors examine the role of the basal ganglia relevant to learning and memory concluding that the goal-directed behaviors subserved by basal ganglia function are crucial to all forms of normal behavior.

Human neuroimaging studies also support the role of the striatum in cognition. PET studies using ¹⁸F-dopa in individuals with PD show a correlation between DA depletion and neuropsychological performance (Broussolle et al., 1990; Marie et al., 1999; Bruck et al., 2001; Duchesne et al., 2002). PET imaging studies in HD using C11-Raclopride indicate that striatal DA receptor subtype 2 (D2) binding is decreased and is sensitive to cognitive performance on a

variety of tasks including executive function, attention, and WM (Backman et al., 1997; Lawrence et al., 1998). While striatal dysfunction is associated with cognitive sequelae of HD and PD, DAergic system dysfunction within the PFC has been strongly tied to attentional and cognitive symptoms associated with schizophrenia and attention deficit hyperactivity disorder (ADHD; Heilman et al., 1991; Tassin, 1992; Knable et al., 1997; Tanaka, 2006).

These clinical studies strongly suggest that the striatum and PFC are functionally important for a variety of cognitive behaviors, and that the alterations in DA signaling appear to be the underlying cause of cognitive dysfunction in a variety of disease states. In TBI, it is known that both the striatum and PFC are vulnerable to damage. The known effects of TBI on the striatum include axonal degeneration (Ding et al., 2001), neuronal cell loss (Dunn-Meynell and Levin, 1997), and ischemia (Dietrich et al., 1994). Effects on the PFC include decreased glucose metabolism (Fontaine et al., 1999), changes in frontal lobe blood flow during memory tasks (Ricker et al., 2001), and hypoactivation with memory tasks (Sanchez-Carrion et al., 2008). Furthermore, experimental models of TBI have shown fairly robust effects on hippocampal neurons as demonstrated by significant loss in the CA2 and CA3 regions (Dixon et al., 1987; Hicks et al., 1993; Smith et al., 1994). While the hippocampus is not generally thought of in reference to DA signaling, it does have glutamatergic projections to the striatum that are important to the activity of GABAergic medium spiny neurons. Striatal medium spiny neurons also receive DAergic input from the VTA and SN (Meredith et al., 1990; Pennartz and Kitai, 1991). Furthermore, DA receptors in the hippocampus receive projections from the SN and have been shown to facilitate the maintenance of long term potentiation (LTP), which is hypothesized to be the physiologic basis for memory formation and consolidation (Li et al., 2003; Lemon and Manahan-Vaughan, 2006).

1.2.3 Attention, processing speed, working memory, and executive function after TBI

Following TBI, patients often demonstrate confusion as well as an inability to concentrate. They are also distracted, have difficulties performing more than one task at a time, and require increased time to perform tasks (Gentilini, 1989; Draper and Ponsford, 2008). While these tasks are often broad in nature, they share a commonality in the form of attention processing. TBI patients consistently show impairments on measures of processing speed, including the Symbol Digit Modalities Task and Digit Symbol Coding. Attentional processing is believed to be a widely distributed cognitive function that involves both cortical and subcortical pathways including striatal and thalamic inputs and reticular activation. However, it is generally accepted that DA function plays a significant role in the ability to focus attention (Cohen and Servan-Schreiber, 1992; Wise et al., 1996; Brennan and Arnsten, 2008). In ADHD, DAergic drug treatments have been shown to be effective in treating attentional disorders (Solanto, 1998). This does not rule out other possible neural dysfunctions as being the underlying cause of attentional difficulties after TBI, but it does provide compelling reasons to examine DAergic function in TBI.

Attention processing can be difficult to localize given that there are multiple modalities of attentional function, including auditory, tactile, and visual attention (Arciniegas et al., 2000; Spence and Gallace, 2007; Adair and Barrett, 2008). Furthermore attention can refer to a wide variety of different cognitive processes that are both voluntary and involuntary and involve several brain systems including the parietal cortices, basal ganglia, PFC, and anterior cingulate cortex (Raz, 2004). In contrast, the anatomy of memory function is comparatively better understood with specific memory processing events (e.g., retrieval, consolidation) ascribed to specific brain regions (Izquierdo and Medina, 1997; Izquierdo et al., 1997). In particular, damage to the hippocampus has historically been associated with reproducible deficits in spatial

and temporal memory processing (Buckley, 2005). In experimental TBI it is known that the hippocampus is exquisitely sensitive to both acute apoptotic events and excitotoxicity (Kotapka et al., 1991; Hicks et al., 1993; Dietrich, 1994; Smith et al., 1994). Regionalization of memory function and the reduction of hippocampal memory processing to spatial and temporal memory are simplistic views of memory processing in the CNS, but have helped guide research in the mechanisms of cognitive dysfunction following diffuse injuries such as TBI. However, at least one study (Lyeth et al., 1990) showed spatial memory impairments without clear neuronal loss in the hippocampus. Furthermore, strategies to reduce neuronal cell loss in the hippocampus using N-methyl-D-aspartic acid (NMDA) receptor antagonists and inhibitors of apoptosis have not always demonstrated behavioral improvements that correlate well with the level of neuronal sparing (Tolias and Bullock, 2004). These findings have challenged the practice of attributing cognitive dysfunction to discrete damage in specific brain regions after TBI and raised a new set of challenges for TBI research to look beyond anatomic damage and into functional studies.

Numerous studies have shown that after TBI there is a dysfunction in hippocampal LTP (Reeves et al., 1995; Sanders et al., 2000). The reasons for this impairment are not entirely clear. Faló et al (2006, 2008) have suggested that the impairments may be due to changes in synaptic composition and dysfunction in normal molecular processes that influence synaptic plasticity in the hippocampus, including dysfunction in scaffolding proteins. What has not been examined is the role that DA may have in this process. It is known that blockade of D1/D5 receptors in the hippocampus eliminates late LTP and can even affect early LTP, suggesting that synaptic plasticity in the hippocampus depends upon a synergistic interaction between glutamate and DA (Frey et al., 1991; Granado et al., 2008). This raises the question about what other structures and what other events, besides hippocampal neuronal loss, could also be relevant to memory function after TBI.

The PFC and corticostriatal DA signaling system have, in addition to the hippocampus, been shown to be important for memory formation. The aspect of memory generally assigned to the PFC has been that of WM. WM commonly refers to those cognitive processes that provide the capacity to maintain and manipulate a limited amount of information over a brief period of time (Baddeley et al., 1986; Baddeley, 1992). WM incorporates aspects of divided attention, which is vulnerable to disruption after TBI (Stuss et al., 1985; Levin, 1990; Ponsford and Kinsella, 1992; McDowell et al., 1997). For example, a study of patients one year following treatment for severe TBI consistently found that these individuals were significantly impaired on the Paced Auditory Serial Addition Test, a demanding task that recruits WM (Levin et al., 1990). WM deficits can be particularly disabling given the critical role of WM in overall intellectual functioning (Smith et al., 1996). WM processes are, arguably, an encoding process for long-term memory (Johnson, 1992). WM is also important for a wide variety of cognitive skills, such as problem solving, planning, and active listening (Jonides, 1995). Individuals with significant WM deficits have great difficulty recording features from a changing environment and keeping them in mind in order to guide behavior (Smith et al., 1996). As such, WM is a primary and critical component of all aspects of cognition, and impairment in this cognitive domain can be particularly disruptive for everyday functioning

TBI also causes an impairment in executive control or executive functioning, a critical aspect of cognition (Hanks et al., 1999; McDonald et al., 2002). Although individuals and clinicians often report or emphasize “memory” as being a primary functional concern, executive control dysfunction might actually be the most disabling aspect of cognitive compromise after brain injury (Mateer, 1999; Millis et al., 2001). It is known that impairments in executive control can compromise other aspects of cognition, such as memory for verbal information (Tremont et al., 2000) and visual information (Ricker et al., 1994; Lange et al., 2000; Ricker et al., 2001).

1.2.4 Emotional lability and behavioral alterations after TBI

Although not as well studied as cognitive deficits after TBI, it is widely recognized that TBI patients experience alterations in emotional control and general behavior (Oddy et al., 1985; Arciniegas et al., 2000). Dyer et al (2006) reported that of three groups, TBI, spinal cord injury, and non-injured, the TBI group was more likely to be rated worse in areas of impulsivity and verbal aggressiveness. Patients with TBI are also known to have increased rates of depression (Seel et al., 2003; Jorge et al., 2004; Moldover et al., 2004). The mesocorticolimbic DA signaling pathway has been implicated in emotional and behavioral disturbances (Mega and Cummings, 1994). In fact, in schizophrenia, many of the observed affective mood disorders are proposed to be due to changes in DA signaling within the mesocorticolimbic signaling pathway (Abi-Dargham and Moore, 2003).

1.2.5 The contribution of animal models in understanding persistent cognitive dysfunction after TBI

FP and CCI rodent models of TBI have been used extensively to produce deficits reminiscent of those seen clinically. Both models produce spatial learning and WM deficits in adult rats (Lyeth et al., 1990; Smith et al., 1991; Hamm et al., 1992, 1993; Hicks et al., 1993; Colicos et al., 1996; Dixon et al., 1996; Hamm, et al., 1996; Dixon et al., 1997; Scheff et al., 1997; Kline et al., 2000, 2002; Wagner et al., 2002; Kline et al., 2004, 2007a,b, 2008) and mice (Smith et al., 1994; Fox et al., 1998; Whalen et al., 1999) as tested in the Morris water maze (MWM). One concern when utilizing the MWM paradigm of memory testing is that this task represents a particularly stressful environment to animals. Furthermore, the MWM task is both a learning and memory task, and it is possible that dysfunction in MWM performance is, in part, due to defects in learning and coping strategies associated with damage in thalamic structures (Markowitsch, 1982; Aggleton

and Brown, 1999; Van der Werf et al., 2003). Importantly experimental TBI has also been shown to cause dysfunction in less stressful paradigms including open field exploration and radial arm maze tasks (Lyeth et al., 1990; Soblosky et al., 1996; Lindner et al., 1998; Enomoto et al., 2005; Wagner et al., 2007b). While open field exploration tasks typically assess mobility and anxiety, changes in the exploration of novel environments is considered an important indicator of learning and memory function in rats if other factors (anxiety, olfactory sensitivity, etc.) are properly controlled for (File, 2001; Christoffersen et al., 2008). Interestingly deficits post-TBI have also been observed in passive-avoidance tasks, which are typically considered to be a hippocampal independent measure of learning (Hogg et al., 1998; Milman et al., 2005). Due to its versatility and consistent results the MWM task remains a useful measure of memory dysfunction following experimental TBI. However, it is important to consider its limitations and the contribution of other cognitive processes to MWM deficits.

Analysis of the affected brain regions in animal models coupled with clinical studies have helped identify the brain areas thought to be responsible for cognitive processes commonly affected by TBI. The PFC, hippocampus, striatum, and limbic structures have all been shown to be sensitive to damage after TBI (Dixon et al., 1987; Lighthall, 1988; Lighthall et al., 1989). The degree of damage to these structures depends in large part on the localization and severity of TBI. Mild TBI models may only show diffuse white matter damage, cortical cell loss, and some hippocampal cell loss (Hicks et al., 1993; Sanders et al., 2001), while more moderate to severe injuries show greater degrees of both cortical and subcortical neuronal death and damage to structures beyond the contusion site including ischemic alterations in subcortical structures (Dietrich et al., 1994; Hellmich et al., 2005). There are also differences in structural damage depending upon the location of the insult (Lighthall et al., 1989, Thompson et al., 2005).

Animal studies of spatial learning and WM, coupled with clinical research examining executive function, attention, and behavior have consistently demonstrated prolonged cognitive dysfunction after TBI. Due to its importance to WM, executive function, behavior and emotion, and psychosis, DA represents a promising avenue of research in TBI therapy. TBI also shares some similarities to PD and other DA disorders. Similar to PD, TBI patients can experience memory impairment, bradykinetic motor dysfunctions, and decreases in cognitive processing speed. TBI patients also exhibit attention difficulties similar to ADHD, such as easy distractibility. Unlike pure disorders in DA function, TBI has its own unique set of concerns including inflammation, white matter damage, disruption of other neurotransmitter systems, and a unique series of temporal events in DA effects that are unlike other insults. For these reasons, understanding the DAergic signaling system and its relation to brain function is an important step in the proper utilization of DA targeted treatment strategies.

1.3. DOPAMINE AT THE CELLULAR LEVEL: GATEKEEPER OF COGNITION

In the brain, DAergic neurons arise from the VTA and SN and project to the striatum, cortex, limbic system, and hypothalamus (Graybiel, 1990). DA influences on a number of physiologic functions including hormone secretion, movement control, motivation, emotion, and cognitive processing (Jackson and Westlind-Daniesson, 1994; Floresco and Magyar, 2006).

The unique effects of DA at each of its terminal sites are mediated by membrane receptors belonging to the large seven transmembrane domain (7TM) G-protein coupled receptors. Activation of DA receptors leads to alterations in intracellular second messengers either through formation or inhibition. Cloning experiments have identified five different DA receptors that are divided into two groups based upon their structural and pharmacological properties; D1-like and D2-like receptors (Bunzow et al., 1988; Dal Toso et al., 1989; Dearry et al, 1990; Zhou et al.,

1990). Each of these receptor families have unique differences in regional CNS expression and unique intracellular signaling pathways that will be discussed briefly in the context of cognition. For a more complete review of the complex biochemical signaling pathways related to each receptor and the information gained from knockout mouse studies (D1-D5) see Gonon et al. (2000) and Tan et al. (2003).

In addition to different subsets of receptor populations throughout regions of the brain, DA effects are regulated through a complex control of DA release, re-uptake, and metabolism. Changes in a number of DA constituents, including the DA transporter (DAT) and tyrosine hydroxylase (TH), can effectively alter DA extracellular concentrations and its physiologic effect without directly affecting binding or receptor response.

1.3.1 DA release, metabolism, and the DA transporter

DA is synthesized first by the hydroxylation of the amino acid L-tyrosine to 3,4-dihydroxy-L-phenylalanine via the enzyme TH and is then decarboxylated by L-amino acid decarboxylase to DA. DA can be inactivated either by reuptake via the DAT and subsequent enzymatic breakdown by catechol-O-methyl transferase and monoamine oxidase or repackaged into vesicles for reuse.

The DAT is particularly important to presynaptic DA regulation, and differences in DAT expression due to genetic polymorphisms have been implicated in a number of diseases including bipolar disorder and ADHD (Greenwood et al., 2001; Thapar et al., 2005). Furthermore, DAT inhibition is the mechanism of action for a number of pharmaceuticals designed to enhance DA neurotransmission as well as drugs of abuse, such as cocaine (Hitri et al., 1994; Madras et al., 1994).

The DAT is a Na⁺/Cl⁻-dependent plasma membrane neurotransmitter transporter containing twelve transmembrane domains with both the amino and the carboxyl termini located on the intracellular side of the membrane (Hersch et al., 1997). DAT terminates the action of vesicular DA release at the synapse via re-uptake of extracellular DA (Torres et al., 2003) and acts as a reverse transporter of DA under basal conditions (Borland and Michael, 2004). Regional distribution of DAT has been found in areas of the brain with established DAergic circuitry including mesostriatal, mesolimbic, and mesocortical pathways (Ciliax et al., 1999). The rate at which DAT removes DA from the synapse has a profound effect on the amount of DAergic activity seen by the cell. This is best evidenced by the severe cognitive deficits, motor abnormalities, and hyperactivity seen in DAT knockout mice (Perona et al., 2008). DAT activity and expression has also been shown to change during normal aging (Bannon et al., 1992) and differs between males and females (Piccini, 2003).

There are many ways in which the DAT may be chronically regulated, including gene transcription, post-translational modifications, oligomerization, and trafficking (Doolen and Zahniser, 2001). Second messenger systems, in particular protein kinase C (PKC) activation, affect transporter activity (Huff et al., 1997). Activation of PKC via substrate and inhibitor binding appears to regulate DAT by altering its cellular distribution; specifically by altering levels of membrane bound DAT (Daniels and Amara, 1999). In addition, DA itself can regulate DAT via its interaction with the transporter or pre-synaptic autoreceptors (Williams and Galli, 2006). The DAT is also the target of several “DAT-blockers” including amphetamines and methylphenidate (MPD). These chemicals inhibit the action of DAT and, to a lesser extent, the other monoamine transporters. In contrast, amphetamines trigger a signal cascade thought to involve PKC or mitogen-activated protein kinase (MAPK) that leads to the internalization of DAT molecules, which are normally expressed on the neuron’s surface (Kahlig et al., 2004).

1.3.2 D1-like

The D1-like receptor family includes D1 and D5 receptors. For the purpose of this review, we will focus on the D1 receptor because the D5 receptor is less well studied in TBI. The distribution of the D1 receptor differs dependent upon the brain region that is being examined. Within the frontal cortex, D1 receptors are localized on post-synaptic dendrites of both pyramidal and non-pyramidal neurons. Similar structural localization of D1 receptors can be seen in the hippocampus and limbic cortex (Dearry et al., 1990; Monsma et al., 1990; Zhou et al., 1990). However, within the striatum, D1 receptors have been localized to non-synaptic dendritic spines suggesting that DA activity within the striatum is unique compared to cortical structures (Hersch et al., 1995; Caille et al., 1996).

D1 receptor activation is classically associated with the stimulation of adenylate cyclase (AC) and subsequent activation of cAMP. However, it has also been demonstrated that D1 receptors are able to activate phosphoinositide hydrolysis (Undie et al., 1994) and inhibit arachidonic acid release (Schinelli et al., 1994). These varied receptor signaling mechanisms demonstrate the wide range of effects that D1 agonism or antagonism can have in the CNS. Activation of phosphoinositide hydrolysis is known to affect intracellular trafficking, cell growth, differentiation, and survival (Undie et al., 1994, 2000; Ming et al., 2006; Liu et al., 2008). Arachidonic acid release is important for inflammatory signaling and neuronal functioning such as LTP and synaptic plasticity (Tassoni et al., 2008). While both of these D1 receptor actions are important to DAergic function and have the potential to mediate insults to DA containing neurons affected by TBI, it is D1 receptor mediated control over intracellular cyclic adenosine monophosphate (cAMP) signaling that represents perhaps the most pertinent aspect of D1 signaling to cognition. Manipulation of cAMP levels can affect cAMP response element binding (CREB) through both

MAPK dependent and MAPK independent pathways (Greengard, 1976; Schulman, 1995; Waltereit and Weller, 2003). In medium spiny neurons within the striatum, there is an intracellular signaling molecule, DA and cAMP-regulated phosphoprotein mKDa 32 (DARPP-32), which is known to mediate a number of important cellular signaling events and is intimately involved in cAMP signaling (Ouimet et al., 1984; Walaas and Greengard, 1984; Hemmings and Greengard, 1986; Greengard et al., 1999).

D1 knockout mice demonstrate spatial learning deficits (El-Ghundi et al., 1999), and D1a (a subtype of the D1 receptor) knockout mice show broad impairments in the initiation of learning cue related tasks (Smith et al., 1998). However, the idea of receptors being impaired versus not impaired may be a simplistic viewpoint of DA interaction at the D1 receptor. A review by Williams and Castner (2006) describes the mathematical relationship of tight DAergic control and its relation to dysfunction. They point out that D1 receptor mediation of PFC function is often dependent upon both concentration and the temporal sequence of DA release. A series of papers examining DA signaling and calcium regulation describe a paradigm in which there exists a difference in the kinetics of CREB phosphorylation that is dependent upon the length of incubation with a D1 agonist (Liu and Graybiel, 1996, 1998a,b). Ruskin and Marshall (1997) have shown that D1 receptor mediated induction of Fos in striatal neurons is dependent on D2 activation. Goldman-Rakic (1995) has described at least three possible cellular mechanisms for DAergic control of WM in the PFC; 1) direct synaptic control over pyramidal neuron activity, 2) nonsynaptic interaction with DA receptors located on distal spines of PFC pyramidal neurons, or 3) indirect modulation through GABAergic interneurons. Frey et al. (1991) have demonstrated that blockade of D1 receptors in the hippocampus impairs hippocampal late LTP maintenance, which may be another reason DA dysfunction impairs memory. This alteration in hippocampal LTP associated with D1 receptor changes is believed to contribute to spatial memory

deficiencies observed in D1 receptor knockout mice (Matthies et al., 1997; Granado et al., 2008).

Recent studies in PD (Cooper et al., 1991; Postle et al., 1997) have shown that WM dysfunction is often one of the first cognitive symptoms experienced by patients. This WM deficit is believed to be D1 receptor-mediated because D1 agonist treatment has been shown to alleviate impairments in young and aged non-human primates (Arnsten et al., 1994; Cai and Arnsten, 1997; Castner and Goldman-Rakic, 2004), as well as in other conditions characterized by prefrontal DA loss, such as chronic stress (Mizoguchi et al., 2000), chronic neuroleptic treatment (Castner et al., 2000), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment-induced delayed response performance (Schneider et al., 1994a,b). How hippocampal DA loss is associated with these processes is unclear, but in non-PD paradigms, the administration of D1 agonists has been shown to facilitate hippocampal LTP (Otmakhova and Lisman, 1996).

1.3.3 D2-like

The D2-like receptor family comprises D2, D3, and D4 receptors. For the purpose of this review, the focus will be on D2 receptors because D3 and D4 receptors are less well studied in TBI. D2 receptors are different from D1 in that they are associated both with terminal receptor activation and with presynaptic autoreceptor function and demonstrate a much greater expression in the striatum compared to other brain regions. However, like D1, D2 receptor expression in the striatum and frontal cortex are of particular interest in cognitive research. Interestingly, within the striatum, the pre and post-synaptic localization of the D2 receptors maintain a much closer proximity to actual synapses as opposed to the more diffuse location of D1 receptors on spines.

D2 receptor activation is more complex than D1 activation for a number of reasons. For example, D2 receptors can act through inhibitory G-proteins that lead to an inhibition of AC and cAMP (Cote et al., 1983; Onali et al., 1985; Weiss, 1985) or independently of cAMP pathways (Memo et al., 1986). D2 receptors have also been shown to inhibit phosphoinositide hydrolysis and subsequent Ca²⁺ mobilization (Vallar and Meldolesi, 1989; Picetti et al., 1997). An interesting caveat of D2 receptor activation is that in vitro studies utilizing co-immunoprecipitation methods and in-vivo studies utilizing fluorescent and bioluminescent energy transfer (FRET and BRET) analyses have shown that many D2 receptor systems exist in heterodimerized pairs (Canals et al., 2003; Fuxe et al., 2005). D2 receptors have been shown to form heterodimers with adenosine A2a receptors and metabotropic glutamate-5 (Mglut-5) receptors (Diaz-Cabirole et al., 2002; Fuxe et al., 2003; Hillion et al., 2002; Fuxe et al., 2005; Ferré et al., 2008). A2a and Mglut-5 heterodimers are of particular interest for TBI research due to their role in striatal LTP and long term depression (LTD) and subsequent plasticity of striatal connections (Ferré et al., 2002; Fuxe et al., 2003). The identification of heterodimer systems within the striatum indicate that D1 and D2 have control over CREB signaling within striatal neurons. Further, they modulate LTP and LTD dependent glutamatergic and adenosine transmitter systems (Calabresi et al., 2000, 2007; Centonze et al., 1999, 2003).

In PD, the contribution of neurotransmitters beyond DA is important to therapeutic manipulation as utilization of these other receptor systems provides an alternative way to manipulate DAergic signaling (Kase et al., 2000). D2-A2a interactions proposed by Fuxe et al. (2005) allow for precise alterations in intracellular signaling pathways that have been associated with cognitive function and striatal output. Mglut-5 receptors have also been implicated as important for control of DA signaling at the D2 receptor in a similar fashion to the A2a receptor. These heterodimer subtypes are highly concentrated on DAergic terminals within the striatum.

1.3.4 DARPP-32

DA signaling cascades in the striatum are involved in numerous physiologic functions including synaptic plasticity, movement regulation, and even the modulation of other neurotransmitter systems (e.g., acetylcholine, calcium, glutamate, and GABA). DARPP-32 is a cytoplasmic phosphoprotein found in 95% of the medium spiny neurons in the striatum (**Figure 2**), and it plays a central role in nearly all DA mediated events (Greengard et al., 1999; Calabresi et al., 2000; Svenningsson et al., 2005; Valjent et al., 2005). Two distinct phosphorylation sites, threonine-34 (T34) and threonine-75 (T75), make DARPP-32 a bifunctional signal transduction molecule that controls the activities of both protein phosphatase-1 (PP1) and protein kinase A (PKA) (Halpain et al., 1990; Nishi et al., 2002). The activity of both PP1 and PKA tightly regulates protein transcription related to numerous important cellular functions including neurotrophic factor production, regulation of synaptic plasticity, and cell homeostasis.

Figure 2

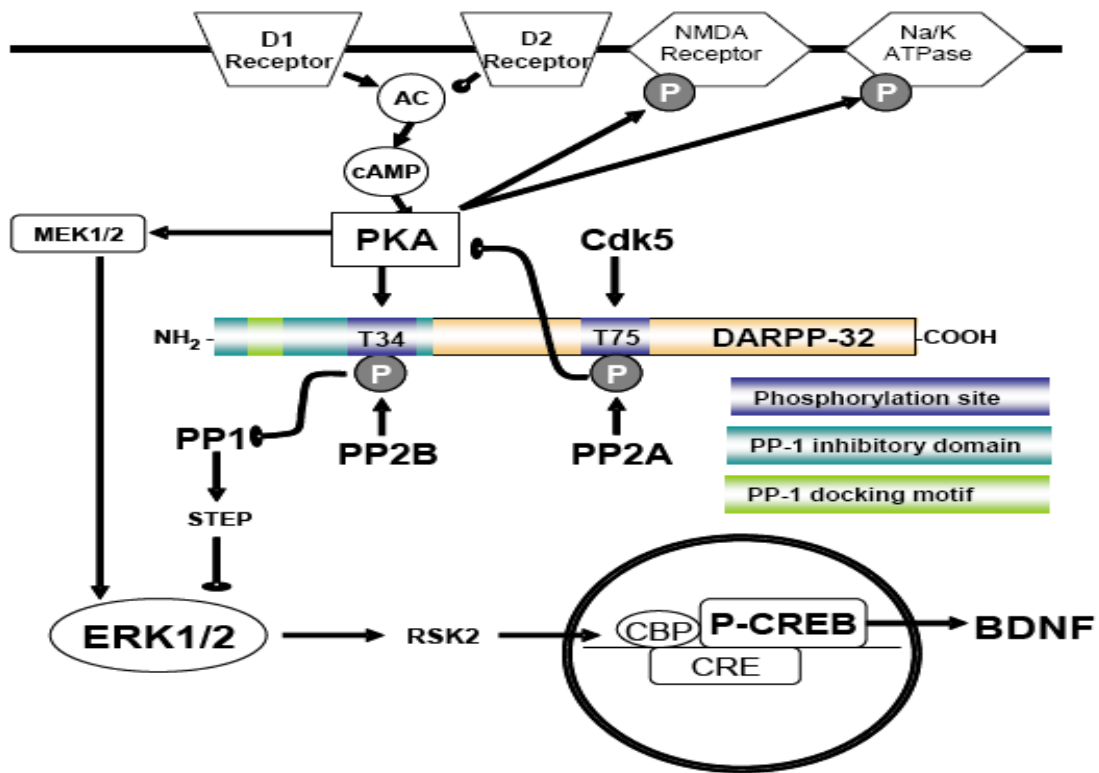


Figure 2: Schematic of DARPP-32 adapted from Nairn et al., 2004. DARPP-32 represents an important regulator of cell signaling within the medium spiny neurons of the striatum. Changes in phosphorylation state due to DA alterations effectively alter CREB activity and PKA activity, both of which are integral to cell homeostasis function

DA induced phosphorylation of ionotropic glutamate and GABA receptors are attenuated in DARPP-32 knockout mice, suggesting that DARPP-32 plays an important role in regulating excitatory neurotransmission (Yan et al., 1999; Flores-Hernandez et al., 2000, 2002). Ethanol reinforcement, which is known to act through regulation of NMDA receptors, is also reduced in DARPP-32 knockout models (Risinger et al. 2001; Maldve et al., 2002). Studies assessing the behavioral effects of other drugs of abuse, including cocaine and morphine, have shown that

DARPP-32 knockout mice demonstrate lower levels of psychomotor activation following drug administration compared to wild-type mice (Borgkvist et al., 2007). Further evidence using knockout mice indicates that DARPP-32 is necessary for the induction of striatal LTD and LTP, both important processes in models of memory acquisition and consolidation (Calabresi et al., 2007). Furthermore, after hypoxia-ischemia injury, DARPP-32 phosphorylation states were shown to be important to membrane potential, glutamate receptor activity, and oxidative stress (Yang et al., 2007).

DA acts through D1 receptor mediated increases in PKA to promote DARPP-32 phosphorylation at its Thr34 site, which leads to an inhibition of PP-1. Additionally, DA, glutamate, and adenosine act on protein phosphatase 2B (PP2B), also known as calcineurin, and protein phosphatase 2A (PP2A) to decrease phosphorylation at Thr34 and increase phosphorylation at Thr75. A reduction in phosphorylation at Thr34 subsequently removes the inhibitory effect of DARPP-32 on PP-1 (Greengard et al., 1999; Nairn et al., 2004; Svenningsson et al., 2004). The regulation of PP-1 and PKA by DARPP-32 allows convergent DA, glutamate, and adenosine signaling to alter the phosphorylation state of NMDA receptor subunits, the sodium and potassium adenosine triphosphatase (Na/K ATPase), and members of the extracellular regulated kinase (ERK) pathway (Bertorello, 1990; Blank, 1997; Fienberg et al., 1998; Snyder et al., 1998; Dudman et al., 2003; Hakansson et al., 2004).

Given the wide range of cellular processes mediated by DA, its interaction with DARPP-32 in the striatum, and the effect that DA action at its different receptor systems have on PFC and hippocampal function, it is clear that even minor disturbances in DA function could have significant implications for CNS function.

1.4. ALTERATIONS IN DAERGIC SYSTEMS AFTER TBI

The efficacy of DA receptor agonists suggests that TBI patients benefit from the promotion of central DAergic transmission. This could be a sign that DA release is suppressed after injury, that DA uptake is over active, or some combination of the two. Alternatively, it might be the case that DA activity remains normal after injury, but that basal DA activity is inadequate in the face of the injury-induced disruptions. Given that a few studies have also shown benefits with DA antagonists it must also be recognized that TBI possesses a complex series of temporally specific injuries to a wide range of different brain structures. It is important to acknowledge that any systemic treatment without a well defined window of therapy could be both beneficial and detrimental to the recovery process. For this reason, understanding the effect of TBI on DA transmission is crucial for proper therapeutic management and for promoting optimal recovery.

Evidence that DA systems are altered in humans after TBI is predominantly based on reports that neurostimulants are beneficial in attenuating cognitive deficits (Goldstein, 2003; McAllister et al., 2004) and data showing altered DA transporter binding after TBI (Donnemiller et al., 2000). Donnemiller et al., (2000) used single photon emission computed tomography (SPECT) to show that striatal DAT binding is decreased in patients 4-5 months after severe TBI, even in cases where no anatomical evidence of direct striatal injury exists.

After experimental TBI, alterations in catecholamine systems have been found in various brain regions and have been shown to be time-dependent (Huger and Patrick, 1979; Dunn-Meynell et al., 1994; McIntosh et al., 1994; Massucci et al., 2004). For example, regional increases in DA levels at acute time points after acceleration-deceleration brain injury were reported by Huger and Patrick (1979). A transient increase in DA in both the striatum, up to 6 hours, and the hypothalamus, up to 24 hours, have been identified utilizing microdialysis (McIntosh et al.,

1994). Interestingly, in the McIntosh et al., 1994 study cortical tissue DA levels are actually depressed for up to 2 weeks post FPI. In a CCI model of brain injury, there were significant increases in rat brain tissue DA levels and metabolism at 1 hour in the contralateral frontal cortex and 1 day in the ipsilateral frontal cortex (Massucci et al., 2004). Tissue DA levels were also elevated in both the ipsilateral and contralateral striatum at 1 hour compared to sham animals. Metabolism of DA was measured by the dihydroxyphenylacetic acid (DOPAC)/DA ratios demonstrating a bilateral increase in DA metabolism at 1 hr in the striatum of CCI injured rats (Massucci et al., 2004). TH activity and catecholamine increases (both DA and NE) have also been seen in the prelimbic and infralimbic cortices, areas critical to PFC function, up to 2 weeks following CCI (Kobori et al., 2006). Increases in human cerebrospinal fluid (CSF) DA and its metabolites post TBI have been shown to depend on both gender and genetic variations in the DAT. Furthermore, the systemic administration of DA as an inotropic agent in TBI patients was also associated with higher CSF DA (Wagner et al., 2007c). Increased DA metabolism may represent a compensatory response to increased tissue DA levels or may be due to further direct effects of TBI on DA regulation. The consequences of these changes can be either beneficial in that DA neurotransmission is restored after TBI or could potentially be deleterious due to DA-induced oxidative stress.

Figure 3

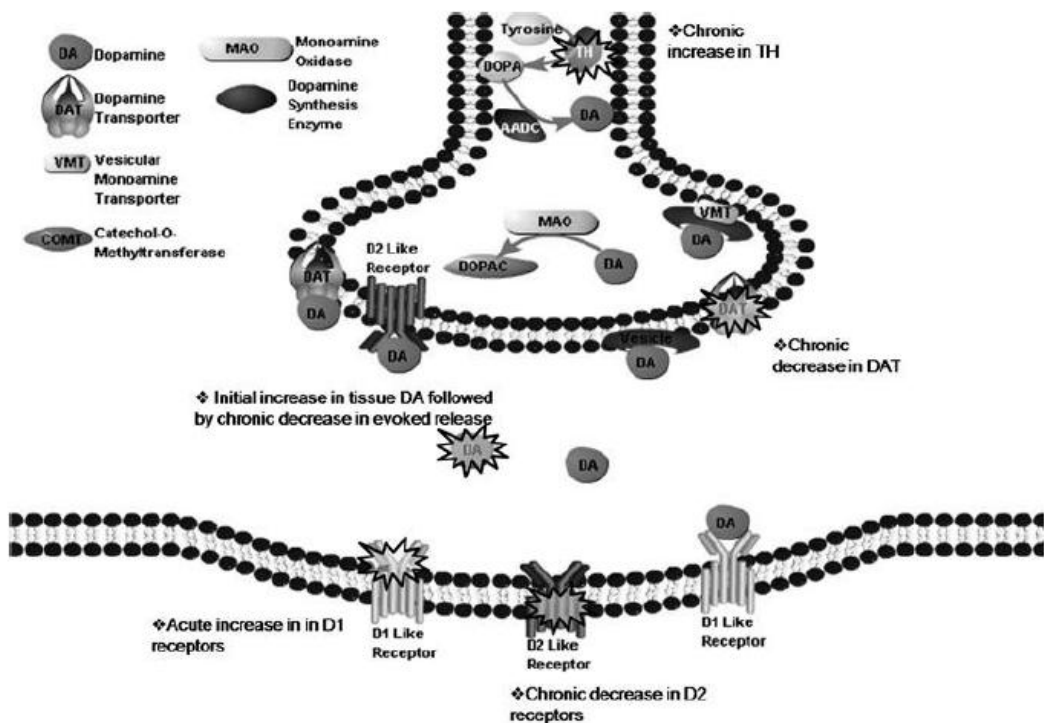


Figure 3 shows a summary of noted changes in DA at the cellular level after TBI in both the striatum and prefrontal cortex, which includes alterations in TH, DAT, and DA receptors. Schematic of alterations in DA cellular signaling following TBI. Changes in DA at the cellular level both pre and post-synaptically have been observed in the PFC and striatum of rats. These alterations include changes in tissue DA levels, DA receptors, TH, and the DAT. Current research suggests that acutely there is a transient increase in DA tissue levels followed by chronic DAergic hypofunction, as indicated by reduced DAT levels, and decreased evoked DA release. However, it remains unclear what the consequences of acute versus chronic changes are and how to best manage these changes in a clinical setting.

Given the importance of DA receptors to cognition, alterations in receptor expression has been another area of interest following TBI. Henry et al. (1997) reported a transient decrease in

striatal D1 receptors immediately after injury, followed by an increase at 1 day, and a subsequent return to pre-injury levels. No significant alterations in D2 receptor binding was reported in the study by Henry and colleagues. Direct analysis of DA D2 receptor protein via Western blots has shown no significant reduction in striatal D2 receptor or D1 receptor expression at 2 weeks in a rat model of TBI (Wagner et al., 2005, 2009). This finding suggests that DAergic dysfunction following TBI is not entirely mediated by changes in DA receptors.

In addition to temporal alterations in tissue DA levels there have also been changes observed in TH, the rate-limiting enzyme in catecholamine synthesis. TBI has been shown to increase TH protein in the rat frontal cortex at 28 days post-injury (Yan et al., 2001). Measured increases in TH protein are most likely due to enhanced synthesis as phospho-TH is also increased (Kobori et al., 2006). In contrast, DA beta hydroxylase protein levels were not altered after TBI suggesting that the increase in TH occurred predominantly in DAergic axons (Yan et al., 2001). The absence of a decrease in TH positive SN neurons further differentiates TBI from PD. Increases in TH protein have also been observed in the striatum with a similar temporal profile (Wagner et al., 2005; Yan et al., 2007; Wagner et al., 2009).

DAT is a crucial protein in the regulation of DA neurotransmission, playing a central role in determining the duration of action of DA by rapidly taking up extracellular DA into pre-synaptic terminals after release (Horn, 1990; Gainetdinov et al., 1998). Studies in animals lacking expression of the DAT gene (Gainetdinov, 2008; Wu et al., 2007) suggest that this protein is perhaps the single most important determinant of the extraneuronal concentration and duration of DA. Differences in the number of uptake sites (Nirenberg et al., 1997; Sesack et al., 1998) in different brain regions provide DA with different extracellular lifetimes (Garris et al., 1994). Regional decreases in total DAT expression have been reported after CCI (Wagner et al., 2005, 2009). Alterations in DAT expression suggest that improvements in cognition and

neurobehavioral recovery reported in experimental (Kline et al. 1994, 2000; Goldstein, 2003) and clinical (Whyte et al. 1997, 2004) TBI studies with the use of DAT inhibitors may, in part, confer their beneficial effects by increasing striatal extracellular DA in a post-injury environment where cortical influences on striatal DA neurotransmission may be impaired.

Recent work suggests that both frontal cortex and striatal decreases in total DAT expression post-TBI are gender specific and occur primarily in males (Wagner et al., 2005). Estrogen is known to have both a developmental and signaling role in DA systems so it is not surprising that TBI has different effects on DA signaling in males versus females. Specifically Wagner et al. (2005) showed that, when compared to gender-matched controls, male rats demonstrated a proportionally larger decrease in DAT expression compared to females. Furthermore, it was shown that environmental enrichment, which improves cognitive recovery (Hamm et al., 1996a; Passineau et al., 2001; Wagner et al., 2002; Kline et al., 2007b; Hoffman et al., 2008b) exerted bigger effects on post-injury DAT reductions in females compared to males. What remains unclear is whether the DAT changes that occur in males and females following TBI are beneficial or detrimental. Interestingly there is evidence that other members of the catecholamine metabolism system being altered post TBI. Specifically there have been noted increases in catechol-O-methyl transferase expression 24 hours post TBI that persists for up to 14 days in the microglia of the injured hippocampus, suggesting a possible compensation for observed changes in DAT activity and providing further evidence of DA dysfunction (Redell and Dash, 2007).

Studies examining DA neurotransmission have demonstrated reduced evoked DA overflow and altered kinetics of DA clearance in the striatum when assessed utilizing fast scan cyclic voltammetry and using a medial forebrain bundle stimulation paradigm (Wagner et al., 2005). Recent work suggests that daily treatment with MPD for two weeks after CCI reverses deficits in

DA neurotransmission. Interestingly, after two weeks of MPD treatment, there were no significant changes noted in DAT expression despite robust changes in DA neurotransmission and kinetics parameters (Wagner et al., 2009). Functional changes in DAT activity and trafficking, as well as other changes in DA receptor function may be responsible for the MPD mediated effects in DA neurotransmission observed.

What effect these alterations in DAT, evoked DA release, and TH increases have on DA signaling has just begun to be elucidated. Ongoing research into these molecular events will help researches develop targeted therapeutic strategies that specifically address TBI induced deficits in DA signaling.

1.5. DOPAMINE IN THE CLINIC: THE PAST AND FUTURE ROLE OF DA AGONISTS AND ANTAGONISTS

In 2006 the Neurotrauma Foundation (NTF) published an excellent review of current clinical recommendations for TBI management in both the acute and rehabilitative phases (Warden et al., 2006). As part of its review the NTF identified three drugs with DAergic effects as current viable options to assist with cognitive recovery. The identified pharmacotherapies were MPD, Amantadine hydrochloride (AMH), and bromocriptine. MPD was recommended to enhance attentional function and speed of processing. Both MPD and AMH were considered reasonable options to enhance general cognitive function after TBI. Bromocriptine was recommended to enhance executive function after TBI. The NTF also acknowledged that there remains limited clinical evidence for long term benefits with DAergic medications, but that initial clinical case reports and clinical studies have shown promise for stimulants (e.g., MPD), AMH, and bromocriptine. Both clinical data concerning the efficacy of DA agonists and a substantial

amount of literature in animal models demonstrate improved functional recovery with DA agonists post TBI.

1.5.1 CNS Stimulants (Amphetamine and Methylphenidate)

1.5.1.1: Mechanism of action. Stimulants are often employed to assist in the rehabilitation of individuals with TBI. The most commonly used stimulants are amphetamine (AMPH) and MPD. Both AMPH and MPD have been used as drugs of choice for the treatment of ADHD. AMPH is a CNS stimulant that possesses two main mechanisms of action. AMPH acts on presynaptic nerve terminals to inhibit the reuptake of serotonin, norepinephrine (NE), and DA by blocking monoamine transporters, including the DAT. AMPH also increases monoamine secretion via exchange diffusion and reverse transport (Haracz et al., 1998; Volkow et al., 2002a,b; Fleckenstein et al., 2007). In contrast MPD's predominant mechanism of action is via blockade of the DAT (Volkow et al., 2002a,b, 1998). Cognitive benefits on attention observed with AMPH and MPD treatment have been associated with the effective increase in DA caused by their administration (Volkow et al., 2001, 2004; Schiffer et al., 2006).

1.5.1.2: AMPH and MPD in the clinic. The administration of AMPH in the clinic to treat TBI is not as widely practiced as is the accepted pharmacotherapeutic strategy of providing MPD. Thus, this section will focus almost entirely on MPD. However, we would be remiss if we did not mention a report by Evans et al. (1987) who administered d-AMPH following closed head TBI and found that the treatment enhanced processing speed and improved memory. Regarding MPD, multiple studies have demonstrated its effectiveness in treating cognitive dysfunction after brain trauma (**Table 1**). In a double blind study, Gualtieri (1988) found that MPD treatment improved performance on measures of nonverbal fluency and selective attention as well as self-report measures in a subset of TBI "responders". Another clinical trial found that low dose MPD

treatment following moderate to severe TBI improved functional outcome at day 30 over controls as measured through the Disability Rating Scale (DRS) (Plenger et al., 1996). Kaelin et al. (1996) showed a trend toward improved DRS and a significant improvement in attention with low doses MPD treatment after TBI. Other recent studies have demonstrated beneficial effects of MPD on attention (Whyte et al., 1997) and information processing speed (Whyte et al., 2004) in individuals with TBI. However, a few studies have shown no effect on cognitive outcomes with MPD treatment (Mooney and Haas, 1993; Speech et al., 1993; Tiberti et al., 1998). It must be noted that for the Speech et al. (1993) study, the authors noted that statistical power was low, and in the Tiberti et al. (1998) study, the outcome measurement was memory function following organic amnesia, which may not be as profoundly influenced as attentional processing.

To better understand the mechanism of action responsible for the cognitive benefits gained with CNS stimulant treatment, specifically MPD, animal studies have examined both the behavioral and biochemical aspects of MPD treatment post TBI.

Table 1: Cognitive Outcomes with the use of Methylphenidate in Traumatic Brain Injury				
Citation	N	Phase During which Treatment Administered	Cognitive Outcomes Measured	Conclusion
<i>Clinical Studies</i>				
Gualtieri (1998)	15	Chronic	memory, attention	Benefit in memory
Mooney (1993)	38	Chronic	memory, attention, anger	Benefit in memory
Speech (1993)	12	Chronic	attention, learning, cognitive processing	No significant effect
Kaelin (1996)	10	Subacute	attention, Disability rating scale (DRS)	Improvement in attention recovery
Plenger (1996)	23	Recovery	attention, memory, vigilance, DRS	Enhanced rate of recovery
Whyte (1997)	19	Chronic	attention	Significant improvement in the speed of mental processing
Williams (1998)	10	Recovery-Chronic	attention, memory, behavior, processing speed	No significant differences between methylphenidate and placebo
Mahalick (1998)	14	Recovery-Chronic	attention	Significant improvement in attention and concentration
Whyte (2004)	34	Chronic	attention	Significant positive effects on speed of processing, caregiver ratings of attention
Kim (2006)	18	Chronic	working memory, visuospatial attention	Improves cognitive functioning following TBI. The effects were most prominent regarding working memory reaction time
Lee (2005)	10 (MPD group)	Subacute	memory, attention	Beneficial in improving cognitive function and maintaining daytime alertness
Pavlovskaya (2007)	6	Chronic	visuospatial attention	Difference in attentional shifts was significantly reduced during and following treatment

1.5.1.3: AMPH and MPD in animal models. Peak levels of MPD in the plasma and brain following intravenous injection occur within 20 minutes in awake animals and correspond with peak striatal DA levels as measured by microdialysis (Huff and Davies, 2002). Intraperitoneal and oral administration of MPD in rodents shows peak striatal DA levels after 40 minutes (Gerasimov et al., 2000). It has also been demonstrated that MPD has a shorter half-life in rats than in humans (Kuczenski and Segal, 2005). In order to overcome these limitations, doses are often larger to maintain drug levels at a therapeutic target over longer time intervals. Regardless of these limitations, experimental models utilizing a MPD treatment paradigm have been able to demonstrate cognitive benefit after both cortical ablation and TBI injuries (Kline et al., 1994, 2000). Specifically, a single administration of MPD followed by significant symptom relevant experience (i.e., beam walking experience) enhanced recovery of motor function following sensorimotor cortex lesions (Kline et al., 1994). Moreover, daily MPD treatments beginning as late as 24 hours after TBI in rats reveal significantly less spatial memory performance deficits versus saline treatment (Kline et al., 2000). Wagner et al. (2009) showed that daily treatment with MPD (5 mg/kg) post-CCI resulted in increased DA overflow and Vmax. There was no associated effect of MPD treatment on DAT localization, or DA receptor expression, however there was a significant increase in c-fos expression with MPD treatment.

Interestingly, while the exact mechanisms of MPD benefit on cognition after TBI are still not understood, gender differences have been observed. Wagner et al. (2007a) showed that injured males treated with MPD had no change in active behaviors and displayed significant improvements in a MWM task indicating that daily treatment with MPD results in enhanced cognition without noticeable motor enhancement. However, MPD treated female rats did not show significant improvement on the MWM task, but did exhibit increased swim speed, which was not observed in males. The researchers note that the dosing regimen was based on previous studies using male rats (5 mg/kg; Kline et al., 2000), and a lower dosing of MPD for

injured females may be required to augment learning and memory pathways without inducing stereotypical behaviors that may interfere with attention and learning processes. The finding of increased motor sensitivity to MPD in females may be due to hormonal influences on regional DAT densities post-TBI and/or differences in DAT modification/function (Bosse et al., 1997). In a previous paper Wagner et al. (2005) demonstrated gender specific alterations in DAT expression after TBI. Furthermore, estrogen is known to exert effects on DAergic neuron development (Kipp et al., 2006) and has neuroprotective properties independent of other drug treatments (Gibson et al., 2008). Estrogen has also been shown to act as a signaling molecule within the DA system (Kuppers and Beyer, 1999; Kuppers et al., 2000).

AMPH use in experimental models of TBI and selective cortical injury models has also been shown to accelerate recovery. The positive benefits of AMPH have been reported in FP (Dhillon et al, 1998) and selective lesion studies (Feeney et al 1981; Hovda et al., 1989; M'Harzi et al., 1988; Chudasama., 2005). AMPH treatment has been shown to reduce the accumulation of free fatty acids and lactate following FP in the cortex and hippocampus (Dhillon et al., 1998) and attenuate decreases in cerebral glucose utilization (Queen et al., 1997). AMPH treatment can also induce hippocampal brain derived neurotrophic factor (BDNF) following brain injury (Griesbach et al., 2008). This is not surprising given that AMPH treatment is known to induce use dependent plasticity and synaptogenesis (Butefisch et al., 2002) and has been strongly linked to plastic alterations following brain injury (Goldstein, 2003; Ramic et al., 2006). Interestingly, when combined with exercise, AMPH treatment no longer increased BDNF (Griesbach et al., 2008). This finding suggests that the positive benefits of AMPH treatment may be associated with its ability to enhance plastic responses in an injured brain and that combinational therapies are not necessarily more beneficial.

An important caveat to AMPH studies is that while AMPH treatment does increase levels of all monoamines (Fleckenstein et al., 2007), the beneficial effects of AMPH on motor recovery have only been reproduced by intraventricular administration of NE (Boyeson and Feeney, 1990). This does not rule out a positive role for DA facilitation with AMPH treatment on other cognitive processes, but simply suggests that DA mediated benefits on motor recovery may not be due simply to increases in DA release. This is supported by evidence that DA antagonists, such as haloperidol (which will be discussed later), can block the beneficial effects of AMPH treatment (Feeney et al., 1982; Hovda and Feeney, 1985).

1.5.2 Amantadine Hydrochloride

1.5.2.1: Mechanism of action. AMH is a water soluble salt that has the capability of crossing all cellular membranes including those of the CNS. AMH was originally used as an antiviral agent for influenza type A. Subsequent studies showed it to be effective in treating PD and multiple sclerosis (Godwin-Austen et al., 1970; Rinne et al., 1972; Cohen and Fisher, 1989). Though the mechanism of action for AMH treatment of PD and multiple sclerosis is not completely understood, biochemical studies have demonstrated that AMH increases extracellular DA concentrations by blocking reuptake and by facilitating the synthesis of DA (von Voigtlander and Moore, 1971; Bak et al., 1972; Gianutsos et al., 1985). In addition to acting at pre-synaptic targets, AMH has been demonstrated to act post-synaptically by increasing post-synaptic DA receptor density (Gianutsos et al., 1985) or altering their conformation (Allen, 1983). Evidence of a post-synaptic mechanism is clinically promising because the mechanisms of actions may not depend solely on the presence of surviving pre-synaptic terminals. Because the mechanism of action of AMH differs from other DA releasing drugs (see Gualtieri et al., 1989 for review), it is likely that the DAergic effects of AMH are a combination of pre-synaptic and post-synaptic effects.

1.5.2.2: AMH in the clinic. AMH has been found to be effective at treating cognitive dysfunction post TBI in both clinical trials and case reports (**Table 2**). While the level of evidence for AMH benefit is not as well developed as for MPD in adult TBI, clinical studies suggest general cognitive improvements with AMH administration after TBI. Zafonte et al (1998) and Wu & Garmel (2005) reported improved scores on the activities of daily living scales in case reports of patients treated with AMH. Case reports (Chandler et al., 1988; Kraus and Maki, 1997) also indicate general improvements in global functioning. In patients demonstrating indications of diffuse axonal injury after TBI, AMH appeared to be effective in improving cognition independent of the timing of administration (Meythaler, 2002). Kraus et al. (2005) showed that improvements in executive function measurements correlated with increases in left PFC glucose metabolism in TBI patients receiving AMH treatment. As reported with other pharmacotherapies (e.g., MPD) AMH did not confer benefits in all studies conducted (Schneider, 1999; Hughes et al., 2005). However, in a review of reports of AMH use after TBI, Sawyer et al., (2008) concluded that it appears to safely improve both arousal and cognition.

Table 2: Cognitive Outcomes with the use of Amantadine in Traumatic Brain Injury

Citation	N	Phase During which Treatment Administered	Cognitive Outcomes Measured	Conclusion
<i>Clinical Studies</i>				
Chandler (1988)	2	Acute	Agitation and aggression	Patient case report; reduced agitation and aggression in treated patients
Nickels (1994)	12	Recovery	arousal, orientation, sequencing skills, processing response time	All measures showed improvement
Kraus (1997)	7	Recovery	memory, attention	All patients showed some degree of positive response
Zafonte (1998)	1	Recovery	activities of daily living	Improvement in independent ADLs
Schneider (1999)	10	Acute	attention, cognition, agitation	Amantadine was no more effective than placebo at improving scores on cognitive tasks
Meythaler (2002)	35	Acute-Subacute	MMSE, FIM-cog, DRS	Amantadine appeared to be effective in improving cognition following TBI, independent of timing of administration
Saniova (2004)	74	Acute	GCS	Outcome GCS was higher in MPH treated patients
Wu (2005)	2	Acute	activities of daily living	Improvement in independent ADLs
Kraus (2005)	22	Chronic	executive function, prefrontal activity	Improvement in prefrontal function that correlated with increase in glucose utilization
Hughes (2005)	123	Acute	Recovery from coma	No significant decrease in coma duration
Saniova (2006)	32	Acute	GCS, orientation, glycaemia, lipid peroxidation, protein oxidation	Outcome GCS was higher in MPH treated patients, decreased lipid oxidation

1.5.2.3: AMH in animal models. The mechanisms of AMH effects are still poorly understood making animal studies of its effects an important step in improving clinical use. In humans, AMH has variable absorption rates and steady state plasma concentrations are typically reached within 4 to 7 days. Doses of AMH given to patients are also somewhat variable due to a lack of correlation between plasma concentrations and therapeutic effects (Aoki and Sitar, 1988). In-vitro studies have shown that doses required to affect DA uptake are higher than those used clinically with no significant difference in DA kinetics until concentrations of 40 mg/kg to 80 mg/kg were given (Baldessarini et al., 1972; Brown et al., 1976; Page et al., 2000). In brain injury patients the optimal dose of AMH has ranged from 50 to 400 mg/day given orally (Gualtieri et al., 1989). Unfortunately, AMH has not been as extensively researched in experimental models of TBI as CNS stimulants have. However, one study using daily treatment of AMH (10 mg/kg) did show significantly improved spatial memory performance compared to saline treated rats following TBI (Dixon et al., 1999).

1.5.3 Bromocriptine

1.5.3.1: Mechanism of action. Bromocriptine is a specific D2 receptor agonist that possesses a rather complex mechanism of action. At high doses (above 10 mg/kg) bromocriptine binds to both highly sensitive presynaptic D2 autoreceptors and less sensitive postsynaptic D2 receptors causing an expected inhibition in DA release and metabolism. Interestingly at lower doses (2.5 and 5 mg/kg) bromocriptine has been shown via microdialysis to increase extracellular DA levels in rats (Brannan et al., 1993). However, even at low doses of bromocriptine there is an associated delayed reduction in DA metabolites consistent with autoreceptor activation (Brannan et al., 1993; Pagliari et al., 1995). In vitro studies have suggested that at low concentrations bromocriptine can act as a partial D2 antagonist (Lieberman and Goldstein, 1985), which may explain an initial increase in DA release. In vivo studies have not been able

to provide conclusive evidence of bromocriptine antagonist activity. However, low doses of bromocriptine are characteristically associated with inhibition of DA neuronal firing (Jackson et al., 1990). Consequently, the reason behind this concentration dependent effect of bromocriptine remains unclear. It may be a consequence of D2 receptor location, affinity, or bromocriptine's activity as a partial D1 antagonist and mixed agonist-antagonist at D2 receptors (Lieberman and Goldstein, 1985; Tan and Jankovic, 2001). What has been shown consistently is that bromocriptine requires DA in order to produce any behavioral effects (Jackson et al., 1988), and alterations in DA concentration effectively alter bromocriptine's activity at pre versus post-synaptic D2 receptors (Maruya et al., 2003). Furthermore, a single administration of bromocriptine can cause an alteration in D2 receptor binding for further treatments (Jackson et al., 1988). These are important caveats to consider in TBI research evaluating bromocriptine as a potential treatment strategy.

1.5.3.2: Bromocriptine in the clinic. In humans bromocriptine has an oral availability of approximately 30-40% and reaches peak levels about 1-2 hours after administration. Following discontinuation, bromocriptine remains in the system for up to 12 hours. Low dose treatments for PD range from 5-30 mg/day while high dose treatments for more advanced PD are within 31-100 mg/day (Lieberman and Goldstein, 1985; Deleu et al., 2002). Bromocriptine is less well studied in clinical research compared to AMH and MPD (**Table 3**). Past case reports (Ben Smail et al., 2006; Karli et al., 1999) showed improvements in executive function after administering bromocriptine. McDowell et al (1998) also demonstrated improvements in executive function with a single 2.5 mg bromocriptine administration. Improvements in digit span, list learning, and motivation in bromocriptine treated patients (maximum of 10 mg per day) that persisted for at least two weeks after bromocriptine withdrawal were also reported (Powell et al., 1996). Bromocriptine did not appear to be effective in addressing moderate to severe TBI patients' attentional difficulties during a postacute phase of recovery at a dose of 5 mg twice

daily (Whyte et al., 2008). However, the study employed a relatively high dose of steady state bromocriptine at 10 mg per day for a more prolonged treatment timeline than previously studied in TBI (Whyte et al., 2008). It may be that the dosing of bromocriptine in head trauma patients needs to be specifically titrated given DAergic alterations caused by TBI. It is also possible that higher doses of bromocriptine for prolonged periods negatively impacts DA kinetics in this patient population.

Table 3: Cognitive Outcomes with the use of Bromocriptine in Traumatic Brain Injury

Citation	N	Phase During which Treatment Administered	Cognitive Outcomes Measured	Conclusion
<i>Clinical Studies</i>				
Powell (1998)	11	Recovery-Chronic	frontal cognition, reward, motivation	Bromocriptine treatment was followed by improved scores on all measures other than mood. Improvement was maintained after bromocriptine withdrawal in eight of the patients
McDowell (1998)	24	Recovery	prefrontal function	Demonstrated a selective beneficial effect of bromocriptine on cognitive processes which involve executive control
Passler (2001)	75	Recovery	DRS	Greater degree of recovery for TBI-vegetative state patients
Ben Smail (2006)	1	Recovery	ADL, executive function	Better executive function, improvement in ADL scores
Whyte (2008)	12	Recovery	Attention	Bromocriptine does not seem to enhance attentional skills

1.5.3.3: Bromocriptine in animal models. In rats, plasma concentrations of bromocriptine peak at 15-30 minutes with delayed maximal D2 binding occurring for a period of up to three hours (Maurer et al., 1983; Atsumi et al., 2003). Dosing for rats is generally lower than that for humans and is based on the findings from behavioral studies showing that higher doses (10-40 mg/kg) induce motor activation in normal rats (Jackson et al., 1988; Brennan et al., 1993). Rats receiving delayed (i.e., 24 hours post injury) and chronic (i.e., daily for 18 days) pharmacological treatment with bromocriptine (5 mg/kg) exhibited both enhanced WM and acquisition of spatial learning in a MWM task (Kline et al., 2002). In a follow up study, Kline et al. (2004) demonstrated that bromocriptine-treated rats exhibited enhanced spatial learning as they were

more adept at locating a hidden platform in a MWM task and also displayed increased hippocampal neuronal protection following TBI compared to vehicle-treated controls. Furthermore, the data showed that bromocriptine attenuated TBI-induced oxidative stress (Kline et al., 2004).

1.5.4 Other DAergic drugs

The administration of selegiline (L-deprenyl) once daily for seven days beginning 24 hr following FP injury has been reported to improve cognitive function in the MWM and enhance neuroplasticity (Zhu et al., 2000). L-deprenyl is used to enhance the action of DA by inhibiting its main catabolic enzyme in the brain, monoamine oxidase-B. Additionally, Newburn and Newburn (2005) showed that selegiline has potential clinical benefits in the treatment of post-TBI apathy.

Atomoxetine administered at a dose of 1mg/kg one day following lateral FP injury in rats showed improvement in Morris water maze performance compared to vehicle (Reid and Hamm, 2008). Atomoxetine is typically used as a non-stimulant drug for treatment of ADHD. While its mechanism of action is predominantly through inhibition of the NE transporter it has been shown to increase extracellular DA in the PFC (Bymaster et al., 2002).

1.5.5 The negative impact of DA antagonists following TBI

Antipsychotic drugs, in particular haloperidol and risperidone, have been administered to TBI patients to treat agitation and psychotic symptoms that may be related to the injury. Unfortunately there are very few clinical studies on cognitive effects in TBI patients following antipsychotic administration, which has limited conclusions on potential consequences of antipsychotic use in TBI populations (Elovic et al., 2008). However, animal studies have

demonstrated negative consequences of antipsychotic administration following TBI, in particular with administration of typical antipsychotics.

Haloperidol and risperidone have multiple CNS effects, but one of the predominant effects is a strong central DA receptor inhibition that can produce akinesia and pseudoparkinsonism. Given this profound DAergic component of these antipsychotics, the question about what effect they might have on the recovery process after TBI is important. Animal models have demonstrated that antipsychotics impair the recovery process and in some instances exacerbate the TBI-induced behavioral deficits. For instance, Feeney and colleagues demonstrated that even a single administration of haloperidol provided after TBI to adult rodents delayed motor recovery (Feeney et al., 1982). Moreover, administration of haloperidol after the rats were recovered, as indicated by normal beam-walking, led to a reinstatement of the deficits (Feeney et al., 1982). Similar findings were reported by Goldstein and Bullman (2002). Other studies have shown that antipsychotic drugs after brain trauma not only impair motor recovery, but also cognitive function. Wilson et al. (2003) showed that haloperidol led to slower acquisition of spatial learning in a water maze task. Interestingly there was no impairment noted with the administration of the atypical antipsychotic olanzapine following TBI by Wilson et al (2003). One possible explanation is the relatively low activity of olanzapine at the D2 receptor relative to haloperidol (Tauscher et al., 2004).

Recent studies from our laboratory have demonstrated that prolonged exposure to the typical and atypical antipsychotics, haloperidol and risperidone, respectively, after TBI impairs motor recovery and hinders the acquisition of spatial learning and memory retention (Kline et al., 2007; Hoffman et al., 2008; Kline et al., 2008). Risperidone and haloperidol also impaired performance in uninjured controls when given prior to behavioral testing, but not after, suggesting that sedation may play a role in performance deficits (Hoffman et al., 2008; Kline et al., 2008).

Haloperidol has also been shown to block the positive benefits of AMPH treatment (Hovda and Feeney., 1985). Interestingly, while single or multiple low doses of risperidone and haloperidol appear to be innocuous to recovery after TBI, chronic high-dose treatments are uniformly detrimental (Kline et al., 2007).

A number of studies have also shown positive improvements in WM and spatial memory with both early (Tang et al., 1997) and late (Kobori and Dash, 2006) administration of DA antagonists. For example, Kobori and Dash (2006) demonstrated that a single administration of the DA D1 antagonist (SCH23390) at 14 days post injury in rats improved WM for up to a week. Tang et al. (1997) showed an improvement in functional recovery with D2 receptor specific antagonists given immediately post injury and a synergistic effect when combined with D1 receptor antagonism in mice. Given that both haloperidol and risperidone have a higher affinity for the D2 receptors (Cohen, 1994; Reimold et al., 2007), it may be that specific blockade of D2 receptors is the event most associated with negative outcomes when antagonized at later time-points. This is not unreasonable as it has been shown that D1 receptor activation is also important for inflammatory and immunological responses including phosphoinositide hydrolysis and arachidonic acid release as discussed previously in this review. A potential explanation is that inhibition of D1 receptors after TBI beneficially affects the injury response while DA agonists at later time-points provide cognitive benefits.

1.6. FUTURE AVENUES OF DA RESEARCH IN TBI: DA REGULATION, SIGNALING, AND STRUCTURAL PLASTICITY

There remains a significant amount of work in TBI research to explore completely the realities and consequences of DAergic dysfunction after TBI. Are the observed alterations in DAT and TH protein levels a result of injury and ongoing biochemical damage or are they a response to

initial changes in DA levels in the cortex and subcortical layers? Furthermore, although no overt cellular damage has been identified within nigrostriatal and mesocortical DAergic pathways, there remains the possibility of axonal disruptions and biochemical alterations. Interestingly, in the PD literature it has been suggested that there exist subtle changes in oxidative stress related to DA signaling within the SN occurring prior to significant cell loss.

Animal models of TBI consistently produce widespread excitotoxic damage and increased amounts of oxidative stress in a number of different brain regions (Palmer et al., 1993; Rao et al., 1999). DA is known to possess excitotoxic properties (Olney et al., 1990), and DAergic fibers have been shown to modulate striatal glutamatergic excitotoxicity (Chapman et al., 1989; Filloux and Wamsley, 1991). The initial increases in DA observed post TBI may precipitate excitotoxic disruption and oxidative damage to DAergic cellular function that leads to the observed alterations in DA kinetics and decreased evoked DA release at later time-points.

Observed changes in DAT expression in TBI also raise an intriguing set of considerations. As discussed in the section concerning DAT activity, the DAT is regulated by a number of genetic factors and shows variation in activity and expression with both age and gender. Alterations in DAT expression can alter the kinetics of DA release as demonstrated in DAT knockdown models (Zhuang et al., 2001), as can changes in DAT cellular localization (Pristupa et al., 1998). Decreases in evoked DA overflow V_{max} following CCI may be explained by either changes in expression or changes in membrane bound DAT associated with DAT trafficking (Wagner et al., 2005, 2008). Given that a number of the current DA receptor agonist therapies act through a DAT mediated mechanism, it is necessary to fully understand the role of DAT changes in TBI in order to provide efficacious DA therapies.

In addition to documented biochemical alterations in DA signaling following TBI, there remains the possibility of structural changes. TBI is known to cause diffuse white matter injury and significant axonal disruptions throughout the CNS (Smith et al., 2003). However, it remains unclear if similar effects can be observed within DAergic systems. Increases in TH staining in both the PFC and striatum may represent regrowth of DAergic fibers that occur as a consequence of DA synapse or axonal pathology that occurred acutely following TBI.

Regrowth or collateral sprouting of catecholaminergic axons has already been demonstrated in experimentally induced lesions of adult CNS neurons (Katzman et al., 1971; Gilad and Reis, 1979; Fritschy and Grzanna, 1992). Moreover, in both PD patients and animals with experimental Parkinsonism, neural transplants or the supply of neurotrophic factors may promote regrowth of DAergic fibers in the striatum and reverse lesion-induced behavioral deficits (Kordower et al., 1991; Kopin, 1993; Tomac et al., 1995). The occurrence of spontaneous regrowth of DAergic fibers after partial nigrostriatal denervation has already been suggested (Onn et al., 1986) and a long-term increase in the amount of striatal TH has been observed after 6-hydroxydopamine injection in the SN pars compacta (Pasinetti et al., 1991; Blanchard et al., 1995). It is thus possible that the TBI-induced expression of TH in the nigrostriatal system might share similar mechanisms. However, further studies evaluating DA turnover, TH activity, DOPAC/DA and DA/TH in the nigrostriatal system after TBI are needed to confirm whether there is such a compensatory mechanism after TBI.

Furthermore the striatum is a heterogeneous region and a varied profile of DA kinetics has been reported within different areas of the dorsal (caudate and putamen) and ventral (NAcc) striatum (May and Wightman, 1989a,b; Bergstrom et al., 2001). It is possible that caudate subregions are differentially affected by CCI. Differences may also exist in damage to the NAcc core versus the shell. Subregion damage could also differentially affect PFC function given the regionally

specific cortical connections that exist within basal ganglia anatomy (Grahn et al., 2008). While many studies have also identified alterations in the PFC associated with TBI, future studies will likely need to evaluate DA neurotransmission in multiple DA regions outside the caudate putamen, as well as subregions within the caudate to characterize fully the effects of trauma on these heterogeneous structures.

DAergic dysfunction after TBI may not be limited to neurotransmitter release, concentration, and metabolism. Preliminary research in our group suggests that TBI can cause alterations in DARPP-32 phosphorylation altering a number of important intracellular signaling molecules. The importance of DARPP-32 to DAergic synaptic plasticity and modulation of other neurotransmitter systems has broad reaching implications for medium spiny neuron function in the striatum and represents another possible level of DA dysfunction following TBI.

Alterations in receptor expression also remain an area where further research is necessary. While studies to date have not demonstrated any overt alteration in DA receptors, there are a number of areas that require further consideration. D1 and D2 receptors depend upon a complex interplay with other receptor systems within the striatum and other DA systems. In particular, recent research in PD has identified a series of heterodimeric complexes of the D2 receptor with other neurotransmitter receptor systems including the adenosine A2a and Mglut5 receptors. It has been proposed in PD research that A2a antagonists can augment DAergic treatments. Interestingly, both A2a and Mglut5 receptors have also been shown to play heavily into DA's role on striatal plasticity and intracellular calcium signaling.

Given that TBI causes damage to areas known to be involved in DAergic processing and that research in other disease states have established that DA is important for cognitive outcomes, it is logical that TBI should produce clinical outcomes similar to what is observed in other

disorders of DAergic function. Indeed, the clinical picture of behavioral and cognitive dysfunctions after TBI shares a number of commonalities with other disorders of DA dysfunction, such as PD and HD.

To date, clinical studies have consistently demonstrated that pharmacotherapies that enhance DA post-TBI are beneficial to memory, attention, and executive function. However, clinical studies examining DAergic therapies have a number of limitations. Small patient populations, variations in treatment protocols, lack of proper controls including the absence of randomized clinical trials, and a poor definition of TBI make it difficult to identify who would benefit the most from DA enhancement therapy and the proper time-course of therapeutic intervention.

Ongoing animal and clinical studies have better characterized DAergic dysfunction following TBI, which is still difficult due to the diffuse nature and variable presentation of TBI. However, studies in both animals and humans have identified a series of temporally specific alterations in DA neurotransmission that occur after TBI (**Figure 3**). An acute hyperactive phase, characterized by increases in tissue DA levels and an initial increase in D1 receptors, is subsequently followed by hypofunction in DA signaling characterized by decreases in evoked DA overflow and alterations in both DAT and TH expression. Animal studies demonstrating the benefit of D1 antagonists and D2 agonists would suggest that the critical event in DAergic dysfunction following TBI is related primarily to signaling through the DA D2 receptor population.

Understanding the temporal alterations in DA following TBI and the mechanism of dysfunction at a cellular and systems level will allow DAergic therapies to be better tailored to specifically address the character of dysfunction in TBI populations during recovery. Furthermore, given the importance of genetic differences in DA kinetics and the role of gender in DA signaling, it is important to utilize animal models of injury to better understand how these factors affect

potential treatments. Doing so will help answer long standing questions in TBI rehabilitation in how to best optimize neuropharmacology strategies. Understanding the role of DA in cognitive recovery following TBI also adds another layer of consideration to the use of acute phase medicines that may affect CNS DA systems.

Effective treatment of cognitive dysfunction post-TBI remains a weakness in the care of TBI patients, in particular as critical care management improves and patient survival improves. Current rehabilitation strategies do not address the underlying deficits in CNS function that cause prolonged deficits in learning, memory, executive function, and behavior. Even given the limitations of current clinical research in DA therapies evidence indicates that the use of a DA enhancing drugs during rehabilitation benefits cognitive recovery. In particular the use of MPD, AMH, or bromocriptine to enhance learning, memory, attention, and executive function should be considered in TBI patients that present with deficits in these areas.

While some studies have suggested that administration of DAergic therapies may be beneficial even at timepoints beyond the recovery phase of TBI, typically within the first year post injury for humans and 2-4 weeks post injury in rats, in general early treatment within the recovery period has shown the most consistent benefits in both animals and humans. What remains unclear is at what dose and for what time period these drugs should be given. It is also unclear if there are patients who should not be given DAergic drugs due to potential negative side effects. Further, is it unclear how management should change if patients require other DA targeted therapies such as antipsychotics or pressors. Unfortunately, until these issues are resolved a large randomized therapeutic trial, even one that utilized different dosing protocols, would most likely provide more confusion than answers. Without understanding what is happening in our patients any results would be difficult to interpret particularly in such a heterogenous patient population.

To reach the level of an effective clinical trial three stages must occur in TBI DA research.

1) First there needs to be animal research to specifically identify temporal alterations in DA, the DAT and other metabolic proteins, DA anatomy, and DA responsive proteins within the hippocampus, striatum, and PFC. While striatal and PFC analysis of DA and DAT have been performed there remains a paucity of information regarding the hippocampus and other DA metabolic proteins. Furthermore, while other DA diseases such as PD have clearly described anatomical alterations what occurs post TBI in DAergic systems is unknown. In analyzing these four areas we will be able to fully understand the timecourse of DA changes post TBI, identify specific areas of potential manipulation that can be translated into drug studies, and most importantly begin to be able to appropriately analyze patient outcomes when DA therapies are employed.

2) Second, based upon our analysis of changes within animals we need to develop targeted treatment strategies that utilize both direct DA enhancement and indirect DA signaling manipulation. Ultimately, efforts need to be made to establish combination therapies that take advantage of the natural dichotomy in DA anatomy that is provided by D1 versus D2 receptor populations and functional location. Also by utilizing adjunct therapies that facilitate DAergic function without specifically altering DA tissue levels many of the negative consequences of DA therapies can be avoided.

3) Third, an understanding of DA effects within patient populations must be understood both following injury and in the presence of pharmacological agents. There must be efforts to promote small dosing studies in TBI patients to determine how providing DA therapy effects DA tissue concentrations and metabolites within human populations. In addition, efforts within TBI clinical studies need to be made to promote stratification of results based upon gender, genetic markers, in particular DAT expression profiles, and injury profiles.

1.7 SUMMARY AND AIMS

The first step in understanding the complexities of subcortical striatal signaling is to identify a common event that can be monitored to determine striatal dysfunction. Prior examination of the DA system following TBI has demonstrated acute and chronic alterations in DA release and metabolism. In addition pharmacotherapeutics whose action enhances DAergic neurotransmission within the CNS have been shown to enhance cognitive recovery in both animal models of TBI and clinical studies of TBI patients. While a number of neurotransmitter systems including glutamatergic, cholinergic, and GABAergic have been implicated in acute and chronic deficits; the DA system, through the interplay of DA receptors D1 and D2, has the ability to modulate each of the other systems.

This modulation is uniquely governed by the DAergic regulation of dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDA (DARPP-32) (**Figure 2**). The convergent properties of DARPP-32 are related to two distinct phosphorylation sites, Threonine 34 (Thr34) and Threonine 75 (Thr75), which act to regulate two key intracellular signaling molecules PKA and PP-1 (Greengard et al., 1999). While DARPP-32 has been predominantly characterized in the striatum, research has identified DARPP-32 expression in the frontal cortex. Alterations in DARPP-32 phosphorylation state influence NMDA receptor activity, structural plasticity, cell homeostasis, cell signaling, and general medium spiny neuron function (Greengard et al., 1999; Nairn et al., 2004).

The purpose of the studies presented in this thesis were to test the hypothesis that TBI produces a dysfunction in DA signaling pathways and subsequent alteration in DARPP-32

phosphorylation which is functionally responsible for chronic alterations in molecular signaling events in medium spiny neurons relevant to cognitive function, including alterations in ERK and CREB cell survival signaling. Furthermore we evaluated DARPP-32 as a potential therapeutic target for a combined drug treatment strategy following TBI.

We hypothesized that alterations in DARPP-32 following TBI are partially responsible for chronic cognitive deficiencies. We hypothesized that treatments aimed at reversing TBI induced dysfunctions in DARPP-32 signaling can provide alleviation of chronic cognitive dysfunction following TBI.

Aim 1. Determine the effect of TBI on DARPP-32.

The phosphorylation state of DARPP-32 at Thr34 is important to PKA and PP1 activity which are responsible for altering the phosphorylation state of numerous targets including ERK and CREB which are relevant to cell survival signaling, NMDA receptor subunits which are relevant to glutamate responses and synaptic plasticity, the Na/K ATPase which is relevant to maintenance of membrane potential and osmotic homeostasis, and structural proteins involved in synaptic density and dendritic complexity.

Aim 1a. Determine the effect and timecourse of TBI on the expression of DARPP-32 and the phosphorylation of DARPP-32 at Thr34 and Thr75.

Aim 1b. Determine the effect and timecourse of TBI on the expression and activity of the immediate DARPP-32 targets, PP1 and PKA.

Aim 2. Characterize the effect of TBI on injury relevant DARPP-32 responsive cell signaling systems.

Deficiencies common to tissue affected by TBI include alterations in cell osmotic homeostasis and membrane potential, cell survival, and changes in cell glutamate response. Measureable

representative molecular events include protein phosphatase-1 activity, ERK and CREB phosphorylation, and STEP expression and phosphorylation. Prior research has found that DARPP-32 phosphorylation state is important to each of the stated molecular events.

Aim 2a. Determine the effect and timecourse of alterations in ERK and CREB phosphorylation, protein phosphatase-1 activity, and striatally enriched protein phosphorylation.

Aim 3: Assess the contribution of the changes in phosphorylation of DARPP-32 following TBI to long term cognitive outcomes.

Effectively treating the persistent cognitive disorders seen in TBI sufferers will need to employ a more complete strategy that affects multiple potential therapeutic targets. DARPP-32 represents one target of therapeutic intervention with the potential to have multiple downstream benefits. However, if there is persistent structural damage to synaptic formations any treatment of molecular targets may not provide optimal recovery. Due to this we are proposing the utilization of two separate drug treatment paradigms to assess the role of clinically relevant pharmaceuticals on DAergic systems after TBI and cognitive recovery. Amantadine has been shown to be beneficial in TBI patients and effectively increases DAergic tone within the striatum and FC. FK506 has also been shown to provide benefit to outcomes in TBI patients, but is theorized to act predominantly through beneficial effects on structural damage.

Aim 3a. Determine the effect of Amantadine on DARPP-32 phosphorylation and cognitive outcome as assessed utilizing the Morris water maze (MWM).

Aim 3b. Determine the effect of FK506 on DARPP-32 phosphorylation and cognitive outcome as assessed utilizing the Morris water maze (MWM).

Aim 3c. Determine the effect of combined Amantadine and FK506 on DARPP-32 phosphorylation and cognitive outcome as assessed utilizing the Morris water maze (MWM).

These studies represent the first to examine DARPP-32 phosphorylation and its substrates in TBI. The concept that DARPP-32 phosphorylation and its substrates are altered following TBI is novel. A therapeutic approach to synergistically target DAergic dysfunction within the striatum represents a plausible combination therapy that may overcome current therapeutic limitations.

2. THE HIPPOCAMPAL CONUNDRUM: ALTERATIONS IN CALCINEURIN SUBUNITS POST TBI

2.1 INTRODUCTION

The hippocampus has been the predominant focus of TBI research into cognitive deficits. Learning and memory difficulties following TBI are thought to be caused by significant hippocampal cell loss, dysfunction in long term potentiation, and disruptions in normal hippocampal architecture. The hippocampus is a logical target for analysis of learning and memory disruptions given the history of hippocampal learning and memory deficits with ablation. Furthermore the hippocampus possesses a well characterized and understood form of synaptic plasticity that is disrupted following TBI and can be recovered with drug administration. The issue with a hippocampal focus in TBI is that histologic recovery of hippocampal neurons and a significant sparing of the hippocampus in injury models that would typically cause hippocampal loss are not consistently associated with cognitive benefit in experimental models of TBI. In fact numerous drugs have been utilized in TBI models that show significant neuroprotection, yet fail to translate to either improvements in animal behavior or benefits in clinical studies. The reasons for this particular failure is unknown, but is most likely due to a number of reasons. The hippocampus does not represent the sole brain region involved in cognitive processing. As mentioned in the introduction, a number of other brain areas are important to a variety of cognitive events that are significantly affected following TBI. Also, the sparing of neuronal bodies may not represent a significant recovery of normal hippocampal function.

Prior research into calcineurin inhibitors have demonstrated that both FK-506 and cyclosporine are able to provide cellular protection following TBI. In addition calcineurin inhibitors provide protection to structural damage and have positive benefits on post-TBI long term potentiation. These results have been shown predominantly in a FP model of TBI and have not been well examined in CCI.

TBI leads to significant behavioral, physical, and cognitive disability. Neuronal cell death occurs both at the site of contusion and in secondary areas due to excitotoxicity and oxidative stress (DeKosky et al., 1998; Kochanek, 1993). Animal models of brain injury have consistently demonstrated cellular death and dysfunction in the hippocampus (Colicos et al., 1996; Tehranian et al., 2008). In addition there is persistent dysfunction in hippocampal potentiation (Sanders et al., 2000). However, the cellular mechanisms which contribute to deficits are not well understood. Protein phosphorylation is a dynamic, rapidly reversible post translational modification, known to be involved in neuronal activation, plastic remodeling, and memory.

Calcineurin, also known as protein phosphatase-2B (PP2B), is a calcium/calmodulin dependent phosphatase that is highly sensitive to, and preferentially activated by, minor changes in intracellular calcium (Ca^{2+}) (Rusnak and Mertz, 2000). Calcineurin dephosphorylates several key cytoskeletal and synaptic vesicle proteins (Rusnak and Mertz, 2000), modulates the activity of the transcription factors, nuclear factor of activated T-cells (NFAT) and cAMP response element binding (CREB) (Yang and Klee, 2000), and regulates neuronal excitability through modulation of gamma-aminobutyric acid (GABA) (Huang and Dillon, 1998) and N-methyl-D-aspartic acid (NMDA) receptors (Tong et al., 1995).

The phosphorylation state of AMPA and NMDA receptors is regulated by the interaction of protein kinase A (PKA) and calcineurin (Banke et al., 2000; Beattie et al., 2000) which directly

affects long term potentiation (LTP) (Lee et al., 2003) and long term depression (LTD) (Lee et al., 2002); cellular functions linked to memory and learning (Li et al., 2003; Lemon and Manahan-Vaughan, 2006). Calcineurin, through its interaction with kinase-anchoring proteins (AKAPs) has also been shown to regulate structural proteins important for synaptic remodeling (Dell'Acqua et al., 2006).

Calcineurin is composed of a 57-61kDA catalytic subunit (CnA) and a 19kDA regulatory subunit (CnB) (Rusnak and Mertz 2000; Groth et al., 2003; Klee et al., 1979). Following neuronal activation, Ca²⁺ binds to high affinity EF hand Ca²⁺ binding motifs on the CnB subunit, allowing calmodulin (CaM) association with CaM binding domains on CnA (Griffith et al., 1995). This Ca²⁺ induced interaction of CnB with CnA results in conformational changes in the protein, removal of an autoinhibitory domain and exposure of the calcineurin active site needed for full activity of the phosphatase (Rusnak and Mertz 2000; Griffith et al., 1995; Guerini and Klee, 1989).

Pathologically, excessive calcineurin has been linked to mitochondrial dysfunction, and apoptosis (Asai et al., 1999). Increases in basal and maximal calcineurin activity (Kurz et al., 2005b) and alterations in calcineurin subunit A subcellular distribution (Kurz et al., 2005a) have been reported in the hippocampus following fluid-percussion (FP) TBI which can persist for 2-3 weeks following injury. Increases in calcineurin activity following TBI may be due to significant increases in intracellular Ca²⁺ that occurs early in brain injury (Fineman et al., 1993; Shapira et al., 1989). Calcineurin activity following TBI is generally considered to be pathologically altered and has been associated with increases in cellular death and dysfunction in both ischemia and TBI (Morioka et al., 1999). Inhibition of calcineurin activity is neuroprotective in ischemia and TBI models (Butcher et al., 1997; Friberg et al., 1998; Okonkwo et al., 1999; Okonkwo et al., 2003).

Both the CnA and CnB subunits consist of several different isoforms (CnA α , CnA β , CnA γ , and CnB1 and CnB2 respectively), which may offer unique substrate specificity, localization and/or recognition properties to the phosphatase.

The over-expression of CnA (Asai et al., 1999) has been shown to cause apoptosis through CN dependent dephosphorylation of BAD (Wang et al., 1999), and also cause mitochondrial dysfunction, elevated superoxide levels and increased production of reactive oxygen species (Manalan and Klee, 1983; Morioka et al., 1999; Wu et al., 2004). The levels of the CnA have also been found to change in synaptic membrane fractions for up 2 weeks following FP injury in the rat (Kurz et al., 2005a). Divergent changes were found in CnA α , CnA β after ischemia in the gerbil (Hashimoto et al., 1998) and in CnA α , β , & γ in schizophrenia (Liu et al., 2007). CnA subunits are particularly important for the initiation and maintenance of LTP (Wang and Kelly, 1996; Kayyali et al., 1997; Winder and Sweatt, 2001) and play an active role in structural plasticity of cortical circuits (Victor et al., 1995; Yakel, 1997). Mice, which have a selective, forebrain specific knockout of the CnB subunit show impaired LTD and working memory, with LTP, extinction, and memory for multi-trial tasks remaining intact (Zeng et al., 2001). Forebrain CnB knockout mice also show behavioral abnormalities similar to those found in schizophrenia (Miyakawa et al., 2003).

Although CnA isoforms have been located in brain tissue, little is known about the relative expression of the CnA isoforms in brain and how they are altered by trauma. The aim of this study was to determine how the CnA isoforms are distributed in the rat hippocampus and to elucidate how CnA isoforms are affected acutely and chronically following TBI. Furthermore, little is known about the expression of the CnB isoforms in the hippocampus and how they are altered by trauma. The aims of this study were also to determine how the CnB isoforms are

distributed in the rat hippocampus, to elucidate how CnB isoforms are affected acutely and chronically following TBI, and determine how a CCI injury affects calcineurin activity.

2.2 METHODS AND MATERIALS

2.2.1 Animals

Adult male Sprague-Dawley rats (n=36) were used in the study. Rats were purchased from Hilltop Laboratories (Scottsdale, PA, USA) and housed in pairs under a 12:12 light/dark cycle. Rats were given food and water with *ad libitum* throughout the study. All experiments were carried out in accordance with the University of Pittsburgh's guidelines for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.2.2 Surgery

On the day of surgery anesthesia was initiated with 4% isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL, USA) and 2:1 N₂O/O₂. Rats were intubated and maintained on 1.5-2% isoflurane during the surgical procedure. Following intubation, rats were placed on a thermal blanket to regulate body temperature (37°C) and the animals head placed in a stereotaxic frame. An incision was made down the midline of the skull and the soft tissues and periosteum deflected. A craniotomy was then performed over the right parietal bone to expose the dura. Controlled cortical injury (CCI, Pittsburgh Precision Instruments, Inc.) at a depth of 2.4mm at 4m/sec was carried out according to the Dixon method (1991). A total of 18 rats were injured, and the remaining 18 rats were shams. Righting reflex (Dixon et al., 1991) was monitored in the immediate post-surgical period to assess acute recovery.

2.2.3 Immunohistochemistry for the CnA subunit

Following a 2 hr (6 shams; 6 TBI) or 2 week (6 shams; 6 TBI) recovery period, animals were given an overdose of sodium pentobarbital (100 mg/kg; i.p), and perfused intra-aortically with 0.1M heparinized PBS in 4% PFA/0.1M PBS. Brains were dissected, submerged in increasing concentrations of sucrose, and stored at -80°C. Brains were then sectioned at 35µm in a cryostat, and free floating sections collected in tissue plate wells containing 0.1M TBS (pH 7.5).

All immunohistochemical procedures and incubations were carried out with agitation with the exception of the chromogen step. All treatment groups were stained together within each immunohistochemical session. Sections were matched by region, rinsed 3x 5 min in washing buffer (0.1%Triton-X in 0.1M TBS) and blocked in a mixture of 10% normal donkey serum in washing buffer for 2 hours at room temperature. Sections were then incubated overnight at 4°C in (one only) primary goat antibodies (Santa Cruz Biotechnology, CA) specific to CnA α (1:150), CnA β (1:150), CnA γ (1:100) subunit isoforms diluted in washing buffer/5% normal donkey serum. Following incubation, sections were then washed 3 x 8 minutes in washing buffer and endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol for 10 minutes. Following 5 x 5 minutes washing in TBS, sections were incubated for 1hr at RT in HRP conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch, PA) at 1:200 in 0.1M TBS. Immunoreactivity (IR) was then visualized using 0.01% 3' 3' Diaminobenzidine (DAB) after an extensive wash. The DAB reaction was terminated with dH₂O and sections were rinsed in 0.1M TBS, mounted onto slides, air dried and cover-slipped for light microscope analysis. All sections within the reaction were exposed to each of the reagents for the same time period. Control sections pre-adsorbed with their homologous peptides were negative for IR.

2.2.4 Immunohistochemistry for the CnB subunit

Following a 2 hr (6 shams; 6 TBI) or 2 week (6 shams; 6 TBI) recovery period, animals were given an overdose of sodium pentobarbital (100 mg/kg; i.p.), and perfused intra-aortically with 0.1M heparinized PBS in 4% PFA/0.1M PBS. Brains were dissected, submerged in increasing concentrations of sucrose, and stored at -80°C. Brains were then sectioned at 35µm in a cryostat, and free floating sections collected in tissue plate wells containing 0.1M TBS (pH 7.5).

All immunohistochemical procedures and incubations were carried out with agitation with the exception of the chromogen step. All treatment groups were stained together within each immunohistochemical session. Sections were matched by region, rinsed 3x 5 min in washing buffer (0.1%Triton-X in 0.1M TBS) and blocked in a mixture of 10% normal donkey serum in washing buffer for 2 hours at room temperature. Sections were then incubated overnight at 4°C in (one only) primary goat antibodies (Santa Cruz Biotechnology, CA) specific to CnB1 (1:100), CnB2 (1:100) subunit isoforms diluted in washing buffer/5% normal donkey serum. Following incubation, sections were then washed 3 x 8 minutes in washing buffer and endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol for 10 minutes. Following 5 x 5 minutes washing in TBS, sections were incubated for 1hr at RT in HRP conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch, PA) at 1:200 in 0.1M TBS. Immunoreactivity (IR) was then visualized using 0.01% 3' 3' Diaminobenzidine (DAB) after an extensive wash. The DAB reaction was terminated with dH₂O and sections were rinsed in 0.1M TBS, mounted onto slides, air dried and cover-slipped for light microscope analysis. All sections within the reaction were exposed to each of the reagents for the same time period. Control sections pre-adsorbed with their homologous peptides were negative for IR.

2.2.5 Western Blot Analysis

At 2 hours or 2 weeks (3 sham; 3 TBI per timepoint) animals were deeply anesthetized with pentobarbital (Nembutal, 80-100 mg/kg; Abbott Laboratories, North Chicago, IL). Animals were decapitated and the brains quickly removed and chilled on ice. Both the right and left hippocampi were exposed by careful dissection and the dentate gyrus from each side was excised. Following the removal of the dentate gyrus the remainder of the hippocampal formation was cut removing the CA1 region from the CA1-2/CA3 regions. The DG, CA1 region, and CA1-2/CA3 regions from three animals were combined and collected into separate tubes for each region. Tissue from both the hippocampus ipsilateral to injury and the hippocampus contralateral to injury was collected separately. Tissue was immediately placed into liquid nitrogen and then into a -80 oC freezer until processed. Tissue was homogenized in Lysis buffer (suspension buffer) which contains 0.1M NaCl, 0.01M Tris-Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), and protein concentrations were determined using a BCA protein Assay Kit (PIERCE, Rockford, IL). Samples containing 20 µg of protein were subjected to SDS-polyacrylamide gel electrophoresis through a 10% acrylamine gel, and then transferred to nitrocellulose membranes and immunolabeled with antiserum followed by donkey anti-goat immunoglobulin G conjugated to peroxidase (1:5,000; PIERCE, Rockford, IL). Proteins were visualized with a chemiluminescence detection system (SuperSignal, PIERCE, Rockford, IL). To assure equal loading, the membrane was restriped and reblotted with rabbit anti-actin antibody (1:15,000, Sigma, St. Louis, MO).

2.2.6 Calcineurin Activity Assay

Pooled protein samples prepared for western blot analysis were also utilized for the calcineurin assay. Calcineurin phosphatase activity was measured using the CALCIOCHEM Calcineurin

assay kit (EMD Biosciences, San Diego, CA). Each reaction (50 μ l total) contained 50 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mM CaCl₂, 1 μ M bovine calmodulin, and 0.3 mM RII phosphopeptide substrate. In wells where calcineurin activity was inhibited 2 mM EGTA was included in the reaction mixture. The amount of phosphate released was calculated using the standard curve with the equation; phosphate released = $(A_{620} - Y_{int})/\text{slope}$. Data is reported as the total amount of phosphate released in nMols.

2.2.7 Selection of Timepoints for Analysis

Two hours corresponds to acute alterations after TBI, including rapid increases in Ca²⁺ that occur immediately post injury and persist (Hovda et al., 1990; Shapira et al., 1989). Given the role of Ca²⁺ in the control of Calcineurin activity an acute examination of isoform alterations at 2 hours post injury was chosen to examine the affect of acute Ca²⁺ increases. Two weeks corresponds to the timepoint used to assess cognitive outcomes following CCI in rats (Yan et al., 2000; Kline et al., 2002).

2.2.8 Scoring for Wilcoxon-Mann-Whitney Rank-Sum Analysis

A six point scoring scale (0 = no IR, 6 = heavy IR) was utilized to assess the level of CnA and CnB isoform expression in DAB stained sections from separate subregions (CA1, CA1-2, CA3, dentate gyrus hilus, dentate gyrus hidden blade, dentate gyrus exposed blade) of the ipsilateral and contralateral hippocampi. To assess dendritic staining in the stratum radiatum of the ipsilateral and contralateral CA1 and CA1-2, a three point scoring scale was employed (1 = few, 3 = numerous). An independent observer blinded to timepoint and isoform stain scored each tissue section utilizing both scoring paradigms. Following scoring the data were organized by CnA or CnB isoform stain, timepoint (2 hour or 2 week), and ipsilateral or contralateral. Scores

were ranked (blinded to sham or TBI) from 1-12 (ipsilateral separate from contralateral) for each sub region and dendrite score. Following ranking, sham and TBI designations were revealed. Outcomes of sham versus injury were compared using the non-parametric Wilcoxon-Mann-Whitney (WMW) two-sample rank-sum test with a p value for significance set at $p \leq 0.05$. Comparisons were only made between comparable sham and injured regions in order to discuss regional alterations in injury.

2.3 RESULTS

2.3.1 Regional Calcineurin Activity in Rat Hippocampus Following a TBI.

Calcineurin enzyme activity was determined utilizing a commercially available Calcineurin activity assay kit that measures the amount of phosphate release when sample is incubated with the Calcineurin specific RII phosphopeptide. At 2 hours post injury there is about a two-fold increase in phosphate released (**Figure 4**) indicating increased Calcineurin activity in the CA3 and DG fractions of injured hippocampus compared to sham. The increase in activity is observed in both the contralateral and ipsilateral, with respect to injury, hippocampi. Within the contralateral DG, at 2 hours, there is nearly a four-fold increase in phosphate release relative to sham levels. Activity within the injured CA1 region is also increased relative to sham, but to a lesser degree than seen in the other two regions assayed.

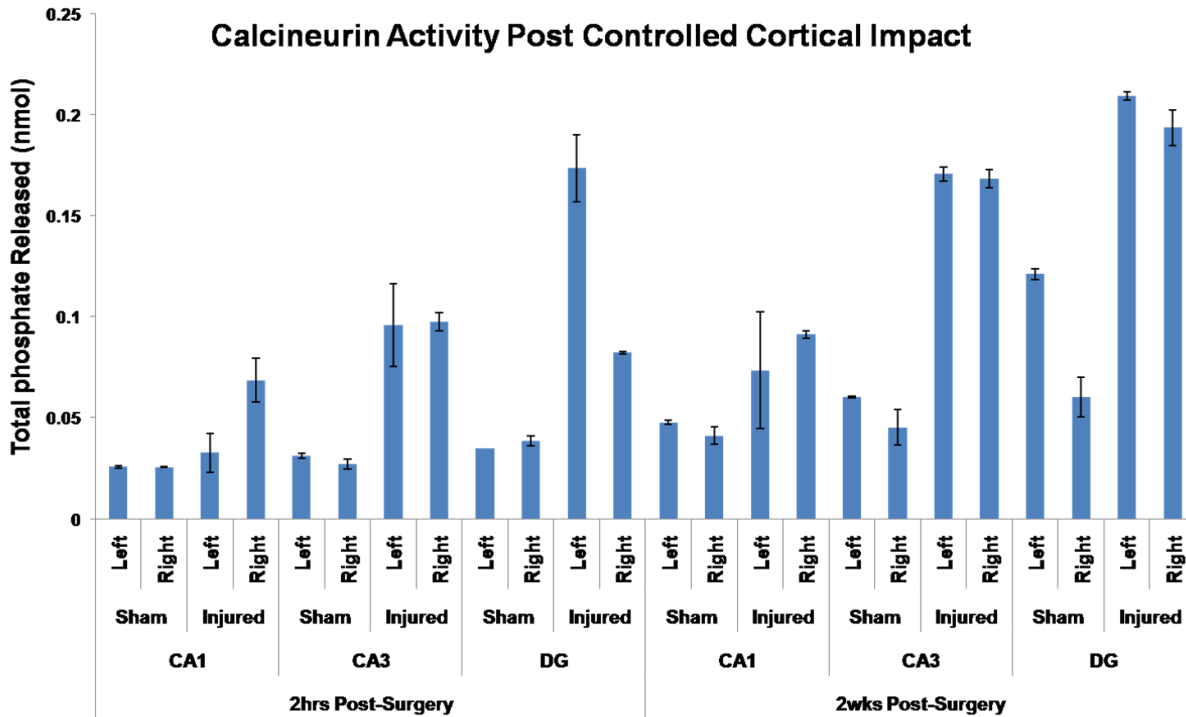


Figure 4: Qualitative analysis of calcineurin activity by measuring the amount of total phosphate released in the presence of the calcineurin specific RII phosphopeptide demonstrates an increase in calcineurin activity post injury compared to sham. Homogenates were made from pooled resection tissue from N=3 rats per group. Increases in phosphate release are detected at 2 hours post injury in all regions examined (CA1, CA3, and DG) in both the ipsilateral and contralateral, with respect to injury, hippocampi. At the acute timepoint (2 hours) the increase in activity is greatest in the contralateral DG. The injury induced increase in calcineurin activity persists chronically and can still be appreciated at 2 weeks post injury. At the chronic timepoint (2 weeks) the pattern of increase in phosphate release is maintained with increased calcineurin activity in all three regions (CA1, CA3, and DG) in both the contralateral and ipsilateral hippocampi. Error bars represent standard deviation of technical repeats. **Abbreviations:** DG = Dentate Gyrus.

The increase in phosphate release seen at the acute 2 hour timepoint is also present at 2 weeks post injury (**Figure 4**). Consistent with the results seen at 2 hours, the CA3 and DG regions show a two to three-fold increase in phosphate release relative to sham in both the ipsilateral and contralateral hippocampi. Also consistent with results seen at 2 hours, the CA1 region shows a lesser increase in phosphate release.

Samples incubated with RII phosphopeptide in the presence of EGTA showed no difference in nMol of phosphate released indicating that increases in Calcineurin activity were responsible for the increased amount of phosphate release in injured samples.

2.3.2 CnA Isoform Distribution in Rat Hippocampus.

CnA isoform distribution within the rat hippocampus was determined utilizing immunohistochemical staining in sham rats. Both isoforms CnA α and CnA β showed similar distribution patterns with higher expression in the CA1 neuropil and cell bodies than CA1-2 regions and noticeable columnar patterns of pyramidal neuron dendritic staining within the stratum radiatum of both the CA1 and CA1-2. Within the CA3 the CnA α isoform appears to be predominantly in the stratum lucidum with little expression within cell bodies of the stratum pyramidale layer. However the CnA β isoform is not as layer specific within the CA3 region and appears to have more widespread expression throughout the layers of CA3. In general the CnA β isoform shows relatively lower expression within the CA1, CA1-2, and CA3 regions of the hippocampus compared to the CnA α isoform. However, both isoforms show marked staining within the exposed blade of the dentate gyrus (DG) in both the cell bodies of the stratum granulosum and neuropil of the stratum moleculare layers. There was relatively little expression within the DG hidden blade or hilus of either the CnA α or CnA β isoforms. The CnA γ isoform showed no distribution within the rat hippocampus.

2.3.3 CnB Isoform Distribution in Rat Hippocampus.

CnB isoform distribution within the rat hippocampus was determined utilizing immunohistochemical staining in sham rats. Both isoforms CnB1 and CnB2 showed similar distribution patterns with higher expression in the CA1 and CA1-2 neuropil and cell bodies than CA3 regions. Within the CA3 the CnB1 and CnB2 isoforms appear to be predominantly in the stratum pyramidale with little expression within the dendritic and axonal layers of the stratum radiatum and stratum oriens. In general the two CnB isoforms show relatively similar expression within the CA1, CA1-2, and CA3 regions of the hippocampus. Both isoforms also show marked staining within the exposed blade of the dentate gyrus (DG) in both the cell bodies of the stratum granulosum and neuropil of the stratum moleculare layers. There was relatively little expression within the DG hidden blade or hilus of either the CnB1 or CnB2 isoforms.

2.3.4 TBI Induced Alterations in CnA Isoform Expression within the Rat Hippocampus

There were clear changes in CnA isoform distribution in the rat hippocampus at 2h post TBI with persistent alterations lasting until 2 weeks post TBI (**See summary: Table 4**).

CnAα	2 hours	2 weeks	CnAβ	2 hours	2 weeks
	<i>Injury Compared to Sham</i>	<i>Injury Compared to Sham</i>		<i>Injury Compared to Sham</i>	<i>Injury Compared to Sham</i>
C-CA1	NC	NC	C-CA1	NC	NC
I-CA1	NC	NC	I-CA1	NC	NC
C-CA1-SR	NC	NC	C-CA1-SR	NC	NC
I-CA1-SR	↓ U=3, n1=n2=6 p≤0.05	NC	I-CA1-SR	↓ U=0, n1=n2=6 p≤0.01	NC
C-CA2	NC	NC	C-CA2	NC	NC
I-CA2	NC	NC	I-CA2	NC	NC
C-CA2-SR	NC	NC	C-CA2-SR	NC	NC
I-CA2-SR	↓ U=0, n1=n2=6 p≤0.01	↓ U=5, n1=n2=6 p≤0.05	I-CA2-SR	↓ U=1, n1=n2=6 p≤0.01	NC
C-CA3	NC	NC	C-CA3	NC	NC
I-CA3	↑ U=5, n1=n2=6 p≤0.05	NC	I-CA3	NC	NC
C-DG-H	NC	NC	C-DG-H	NC	NC
I-DG-H	↑ U=0, n1=n2=6 p≤0.01	↑ U=3, n1=n2=6 p≤0.05	I-DG-H	NC	NC
C-DG-LB	NC	NC	C-DG-LB	NC	NC
I-DG-LB	↑ U=3, n1=n2=6 p≤0.05	NC	I-DG-LB	NC	NC
C-DG-MB	NC	NC	C-DG-MB	NC	NC
I-DG-MB	↓ U=1, n1=n2=6 p≤0.01	↓ U=0, n1=n2=6 p≤0.01	I-DG-MB	↓ U=1, n1=n2=6 p≤0.01	↓ U=0, n1=n2=6 p≤0.01

Table 4: Summary of statistical analysis utilizing Wilcoxon-Mann-Whitney rank-sum test to assess median rank differences of scored DAB stained histological sections. Analysis of regional alterations in CnA isoform immunoreactivity within the rat hippocampus demonstrates significant alterations in the regional immunoreactivity of both isoforms 2 hours following injury. Some of these alterations persist to 2 weeks following injury. **Abbreviations:** C = Contralateral, I = Ipsilateral, SR = Stratum Radiatum (Dendrite Counts), DG = Dentate Gyrus, H = Hilus, HB = Hidden Blade, EB = Exposed Blade, NC = No Change, ↓ = Denotes a Decrease, ↑ = Denotes an Increase.

2.3.5 TBI Induced Alterations in CnB Isoform Expression within the Rat Hippocampus

There were changes in CnB isoform staining within regions of the rat hippocampus at 2 hours post TBI with persistent alterations lasting until 2 weeks post TBI as determined by sham versus injury comparison using the Wilcoxon-Mann-Whitney two-sample rank-sum test with a p value for significance set at $p \leq 0.05$. (See summary: Table 5).

TABLE CnB1	2 hours	2 weeks	CnB2	2 hours	2 weeks
	Injury Compared to Sham	Injury Compared to Sham		Injury Compared to Sham	Injury Compared to Sham
C-CA1	NC	NC	C-CA1	NC	NC
I-CA1	↑ U=1, n1=n2=6 p≤0.01	↑ U=0, n1=n2=6 p≤0.01	I-CA1	↑ U=1, n1=n2=6 p≤0.01	↑ U=3, n1=n2=6 p≤0.05
C-CA1-SR	NC	NC	C-CA1-SR	NC	NC
I-CA1-SR	NC	NC	I-CA1-SR	NC	NC
C-CA1-2	NC	↑ U=1, n1=n2=6 p≤0.01	C-CA1-2	NC	↑ U=3, n1=n2=6 p≤0.05
I-CA1-2	↑ U=0, n1=n2=6 p≤0.01	↑ U=0, n1=n2=6 p≤0.01	I-CA1-2	↑ U=0, n1=n2=6 p≤0.01	↑ U=3, n1=n2=6 p≤0.05
C-CA1-2-SR	NC	↑ U=3, n1=n2=6 p≤0.05	C-CA1-2-SR	↑ U=1, n1=n2=6 p≤0.01	↑ U=3, n1=n2=6 p≤0.05
I-CA1-2-SR	NC	NC	I-CA1-2-SR	↑ U=3, n1=n2=6 p≤0.05	↑ U=3, n1=n2=6 p≤0.05
C-CA3	NC	NC	C-CA3	NC	NC
I-CA3	↑ U=0, n1=n2=6 p≤0.01	NC	I-CA3	↑ U=0, n1=n2=6 p≤0.01	NC
C-DG-H	NC	↑ U=5, n1=n2=6 p≤0.05	C-DG-H	NC	NC
I-DG-H	↑ U=5, n1=n2=6 p≤0.05	NC	I-DG-H	↑ U=5, n1=n2=6 p≤0.05	↑ U=0, n1=n2=6 p≤0.01
C-DG-HB	NC	↑ U=5, n1=n2=6 p≤0.05	C-DG-HB	NC	NC
I-DG-HB	NC	NC	I-DG-HB	NC	NC
C-DG-EB	NC	NC	C-DG-EB	NC	NC
I-DG-EB	↓ U=0, n1=n2=6 p≤0.01	↓ U=1, n1=n2=6 p≤0.01	I-DG-EB	↓ U=1, n1=n2=6 p≤0.01	↓ U=5, n1=n2=6 p≤0.05

Table 5: Summary of statistical analysis utilizing Wilcoxon-Mann-Whitney rank-sum test to assess median rank differences of scored DAB stained histological sections. Analysis of regional alterations in CnB isoform immunoreactivity within the rat hippocampus demonstrates

significant alterations in the regional immunoreactivity of both isoforms 2 hours following injury. Many of these alterations persist to 2 weeks following injury. **Abbreviations:** C = Contralateral, I = Ipsilateral, SR = Stratum Radiatum (Dendrite Counts), DG = Dentate Gyrus, H = Hilus, HB = Hidden Blade, EB = Exposed Blade, NC = No Change, ↓ = Denotes a Decrease, ↑ = Denotes an Increase.

2.3.6 TBI Resulted in Acute Regionally Specific Alterations in CnA α Distribution

CnA α staining in the injured ipsilateral CA1 region was in cell bodies within the stratum pyramidale (SP) layer and in the stratum oriens (SO) layer without localization to cell bodies and no clear dendritic distribution (**Figure 5**). There was a loss of the columnar distribution of CnA α staining within dendrites of the stratum radiatum (SR) layer compared to sham (**Figure 6**). Within the contralateral CA1 of injured animals there is a noticeable difference in cellular distribution of the CnA α isoform (not pictured) with significant staining of dendrites and cell bodies throughout the SR and SP layers that is similar to sham animals. There appears to be a general decrease in contralateral expression of CnA α within the CA1 and an increase in contralateral expression of CnA α within the exposed blade of the DG in injured versus sham animals that did not reach significance (**Figure 5**).

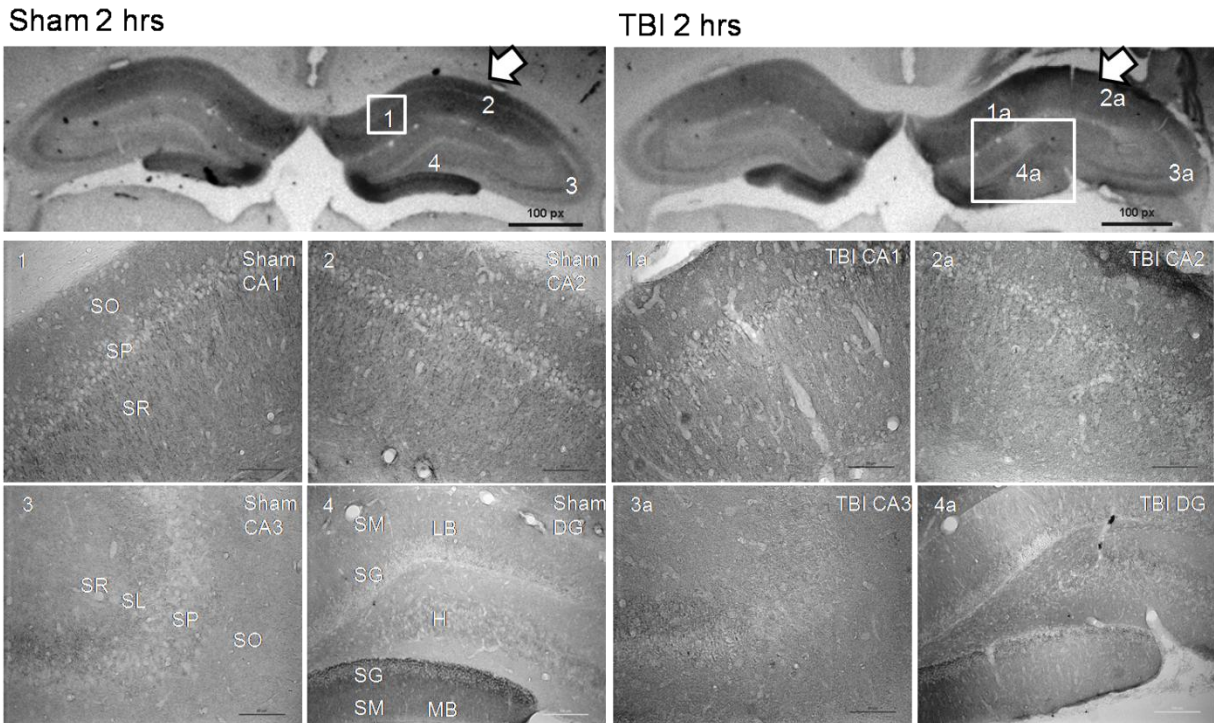


Figure 5: CnA α immunohistochemistry demonstrating alterations in CnA α isoform distribution 2 hours following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a noticeable loss of dendrite staining within the SR of CA1 and CA1-2 in TBI (1a and 2a) versus sham (1 and 2). There is a significant loss of CnA α expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

The majority of CnA α staining in ipsilateral CA1-2 and CA3 regions of injured animals are in the surrounding neuropil of the SO and SR, with a few cell bodies within the SP showing expression that is not seen in sham animals. There was also decreased dendritic staining in the SR of the CA1-2 region compared to sham. Increases in staining within the hidden blade and hilus of the DG are predominantly in the neuropil with some localization to cell bodies (the stratum

granulosum (SG) in the hidden blade). Decreases in CnA α staining within the exposed blade of the DG is localized to cell bodies within the SG layer and neuropil of the stratum molecular (SM) layer (**Figure 6**). The loss of CnA α isoform expression within the exposed blade of the DG is predominantly lateral to midline with a relative sparing of expression in the genu of the DG.

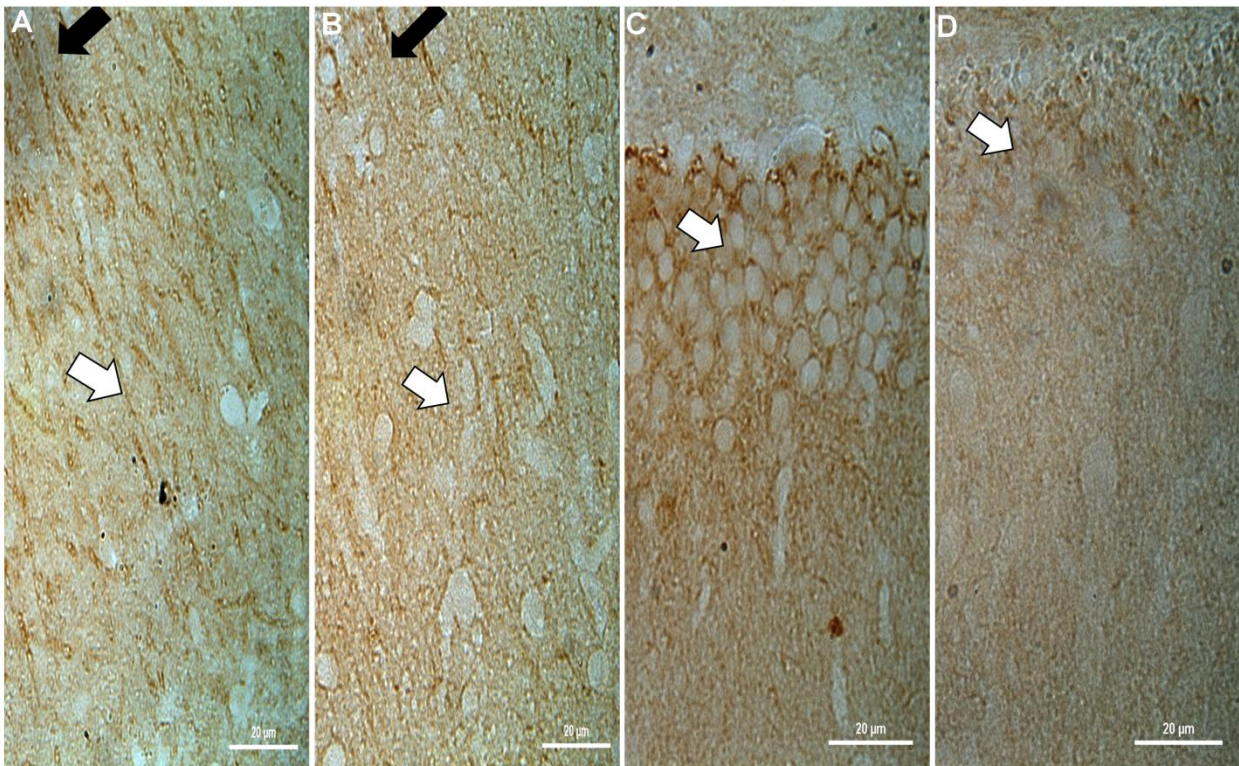


Figure 6: Representative higher power images demonstrating the loss of isoform dendrite staining in the deep SR of TBI animals (**B – white arrow**) compared to sham (**A – white arrow**) at 2 hours post injury, with a relative sparing of dendrites immediately adjacent to the neuronal somas (**A and B – black arrows**). There is also a noticeable decrease in the exposed blade of the dentate in the SG cell body layer as seen in TBI animals (**D – white arrow**) compared to sham (**C – white arrow**). There appears to be some cell loss in the dentate which may explain some of the decreases with both isoforms.

2.3.7 TBI Resulted in Acute Regionally Specific Alterations in CnB1 Distribution

CnB1 staining in the injured ipsilateral CA1 region was in cell bodies within the stratum pyramidale (SP) layer and in the stratum oriens (SO) layer without localization to cell bodies (Figure 7). The columnar distribution of CnB1 staining within dendrites of the stratum radiatum (SR) layer remained intact in injured animals compared to sham and there appeared to be increased expression (Figure 8). CnB1 staining within the contralateral CA1 of injured animals did not differ from sham animals (not pictured) with significant staining of dendrites and cell bodies throughout the SR and SP layers.

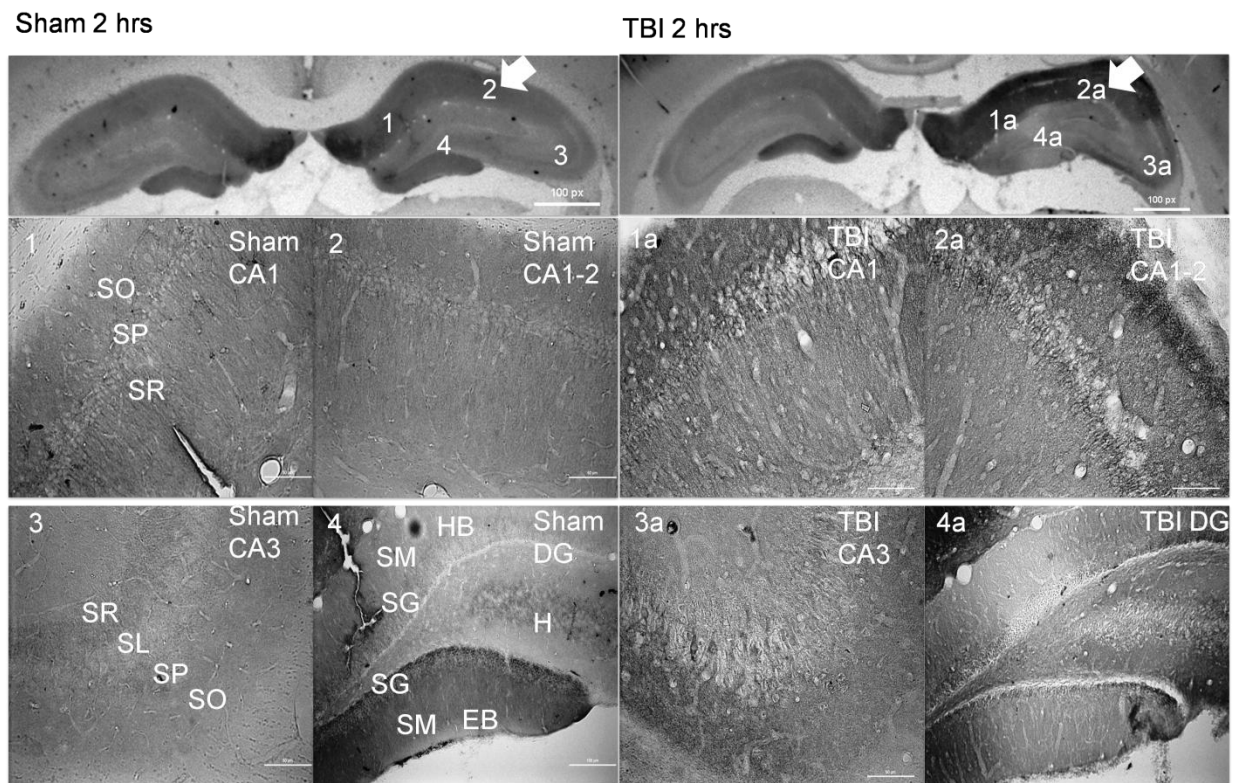


Figure 7: CnB1 immunohistochemistry demonstrating alterations in CnB1 isoform distribution 2 hours following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a noticeable increase of staining within CA1 and CA1-2 in TBI (1a and 2a)

versus sham (1 and 2). There is a significant loss of CnB1 expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

The majority of CnB1 staining in ipsilateral CA1-2 and CA3 regions of injured animals are in the surrounding neuropil of the SO and SR. There was no change in dendritic staining in the SR of the CA1-2 region compared to sham. Increases in staining within the hilus of the DG are predominantly in the neuropil with some localization to cell bodies. A significant decrease in CnB1 staining within the exposed blade of the DG was localized to cell bodies within the SG layer and neuropil of the stratum molecular (SM) layer (**Figure 8**). The loss of CnB1 isoform staining within the exposed blade of the DG is predominantly lateral to midline with a relative sparing of staining in the genu of the DG.

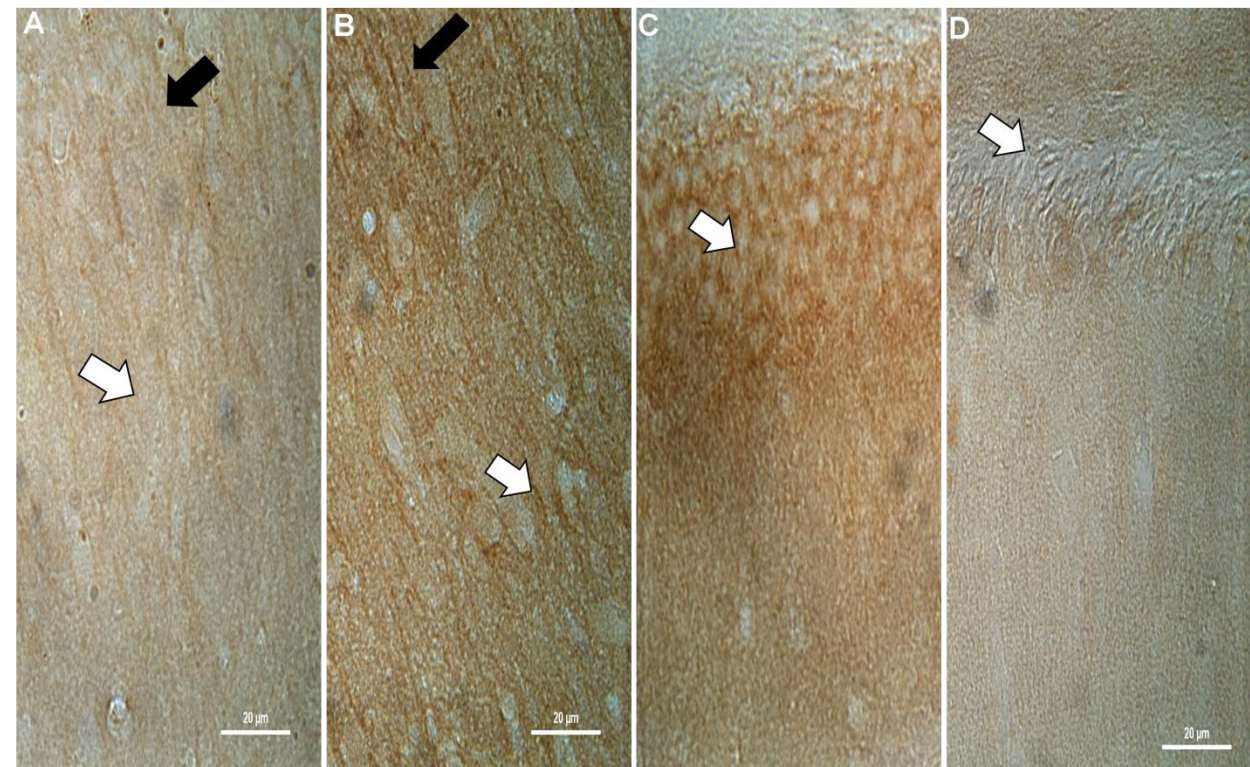


Figure 8: Representative higher power images demonstrating the increase of CnB1 isoform staining in CA1 of TBI animals (**B – white arrow**) compared to sham (**A – white arrow**) at 2 hours post injury, with a relative sparing of dendrites (**A and B – black arrows**). There is also a noticeable decrease in the exposed blade of the dentate in the SG cell body layer as seen in TBI animals (**D – white arrow**) compared to sham (**C – white arrow**). There appears to be some cell loss in the dentate which may explain some of the decreases with both isoforms.

2.3.8 TBI Resulted in Chronic Regionally Specific Alterations in CnA α Distribution

At 2 weeks post injury staining within the ipsilateral CA1 appears to be localized to the SP layer within the cell somas (**Figure 9**). In many sections there is a loss of CnA α dendritic expression within the ipsilateral CA1 consistent with what occurs at 2 hours post injury, however the variability in dendritic expression among animals indicates no statistical significance in SR dendrite staining of the CA1 in injured hippocampus compared to sham. CnA α expression in injured CA1-2 and CA3 regions of the ipsilateral hippocampus is in the neuropil of the SO and SR. Similar to the ipsilateral CA1-2 region at 2 hours at 2 weeks very little CnA α staining of dendrites can be appreciated within the SR. There is also a loss of staining within the stratum lucidum (SL) layer of the ipsilateral CA3 region at 2 weeks compared to sham that is not seen at 2 hours.

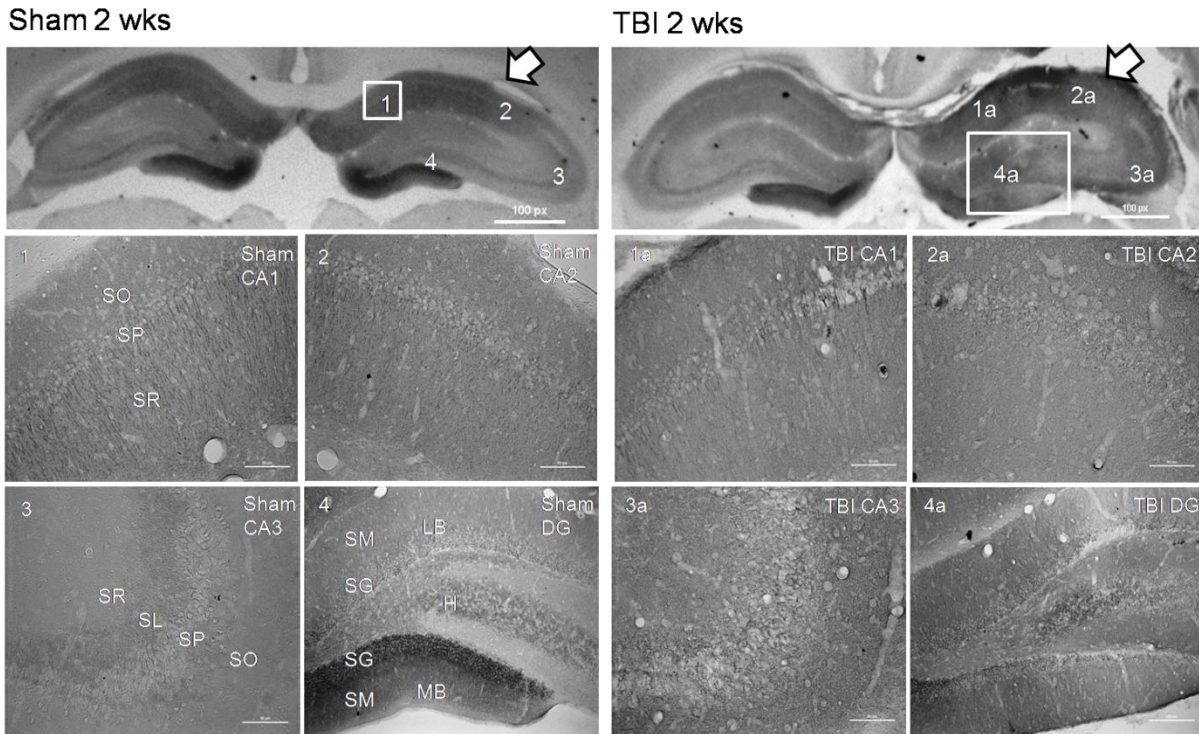


Figure 9: CnA α immunohistochemistry demonstrating alterations in CnA α isoform distribution 2 weeks following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a loss of dendrite staining within the SR of CA1 and CA1-2 in TBI (1a and 2a) versus sham (1 and 2) that did not reach significance. There is a significant loss of CnA α expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

There is higher expression in the hilus and hidden blade of the injured ipsilateral DG in both cell somas and surrounding neuropil compared to sham, however it does not reach statistical significance. In the exposed blade of the ipsilateral DG CnA α demonstrates reduced expression predominantly within cell somas of the SG with some decreases in the surrounding neuropil

compared to sham. Similar to 2 hours post injury there remains relatively high expression of CnA α within the genu of the ipsilateral DG.

2.3.9 TBI Resulted in Chronic Regionally Specific Alterations in CnB1 Distribution

At 2 weeks post injury staining within the ipsilateral CA1 appears to be localized to the SP layer within the cell somas and dendrites of the SR layer (**Figure 10**). CnB1 staining is increased in injured CA1-2 region of the ipsilateral hippocampus in the neuropil of the SO and SR. There is also an increased amount of CnB1 staining within the contralateral CA 1-2 and an increase in dendritic staining within the SR which was not present at 2 hours post injury compared to sham.

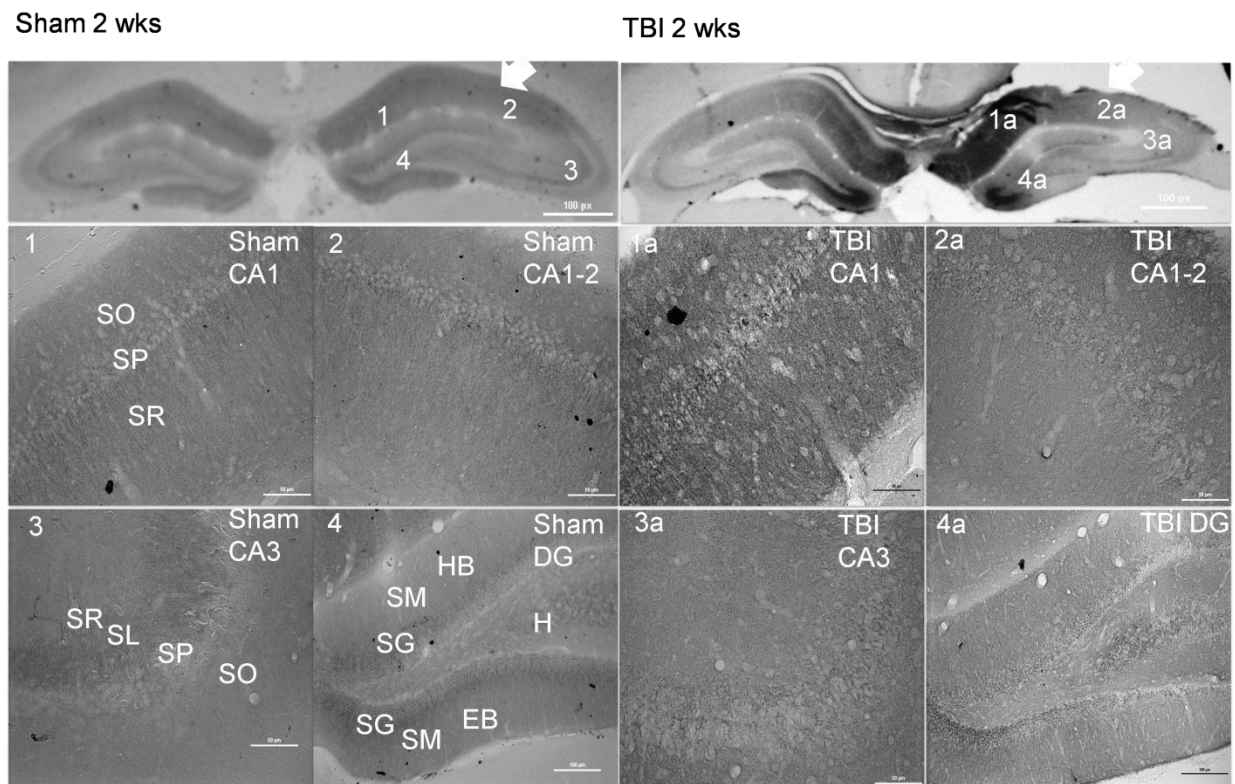


Figure 10: CnB1 immunohistochemistry demonstrating alterations in CnB1 isoform distribution 2 weeks following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham

surgery. There is an increase in staining within the CA1, CA1-2, and CA3 in TBI (1a, 2a, 3a) versus sham (1, 2, 3). There is a significant loss of CnB1 expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μm for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

In the exposed blade of the ipsilateral DG CnB1 demonstrates reduced staining predominantly within cell somas of the SG with some decreases in the surrounding neuropil compared to sham. Within the contralateral DG there is an increase in CnB1 staining within the hilus and HB not seen at 2 hours post injury compared to sham (**Figure 10**).

2.3.10 TBI Resulted in Acute Regionally Specific Alterations in CnA β Distribution

Acute changes in staining of the CnA β subunit are similar to alterations seen with CnA α subunit staining. CnA β staining is in the neuropil of the SO and SR of both the CA1 and CA1-2 regions with a noticeably less dendritic staining in the SR of both regions (**Figure 11**). Unlike CnA α there does not appear to be as much distribution of CnA β to the cell somas of the SP layer in either CA1 or CA1-2. CnA β distribution within the CA3 region is distinct from that seen with CnA α as there is more CnA β staining within the cell somas of the SP layer of CA3 that is not as apparent following injury, however it is not a significant difference. There are non-significant decreases in CnA β staining within the SL of CA3 and increases within the SO layer. There is less CnA β staining of both the cell somas of the SG and neuropil of the SM layer in the exposed blade of the injured ipsilateral DG compared to sham. Similar to CnA α there is also a non-significant decrease in CnA β expression within both the CA1 and CA1-2 regions that only appears to occur at acute timepoints.

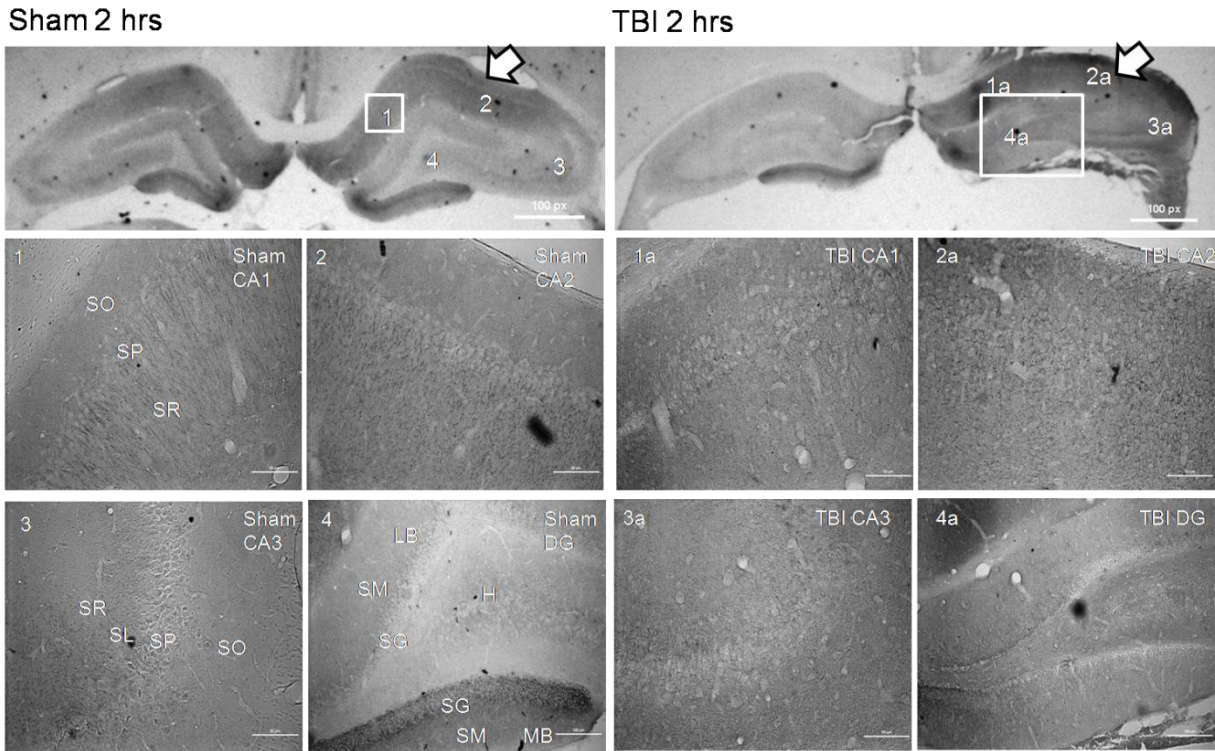


Figure 11: CnA β immunohistochemistry demonstrating alterations in CnA β isoform distribution 2 hours following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a significant loss of dendrite staining within the SR of CA1 and CA1-2 in TBI (1a and 2a) versus sham (1 and 2). There is a significant loss of CnA β expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

2.3.11 TBI Resulted in Acute Regionally Specific Alterations in CnB2 Distribution

Acute changes in staining of the CnB2 subunit are similar to alterations seen with CnB1 subunit staining. CnB2 staining is in the neuropil of the SO and SR of both the CA1 and CA1-2 regions with a noticeable increase in staining in the SR of the CA1-2 region (**Figure 12**). There is less

CnB2 staining of both the cell somas of the SG and neuropil of the SM layer in the exposed blade of the injured ipsilateral DG compared to sham (**Figure 12**).

Unlike CnB1 there are significant increases in CnB2 in contralateral CA1-2 SR dendritic staining acutely (not pictured).

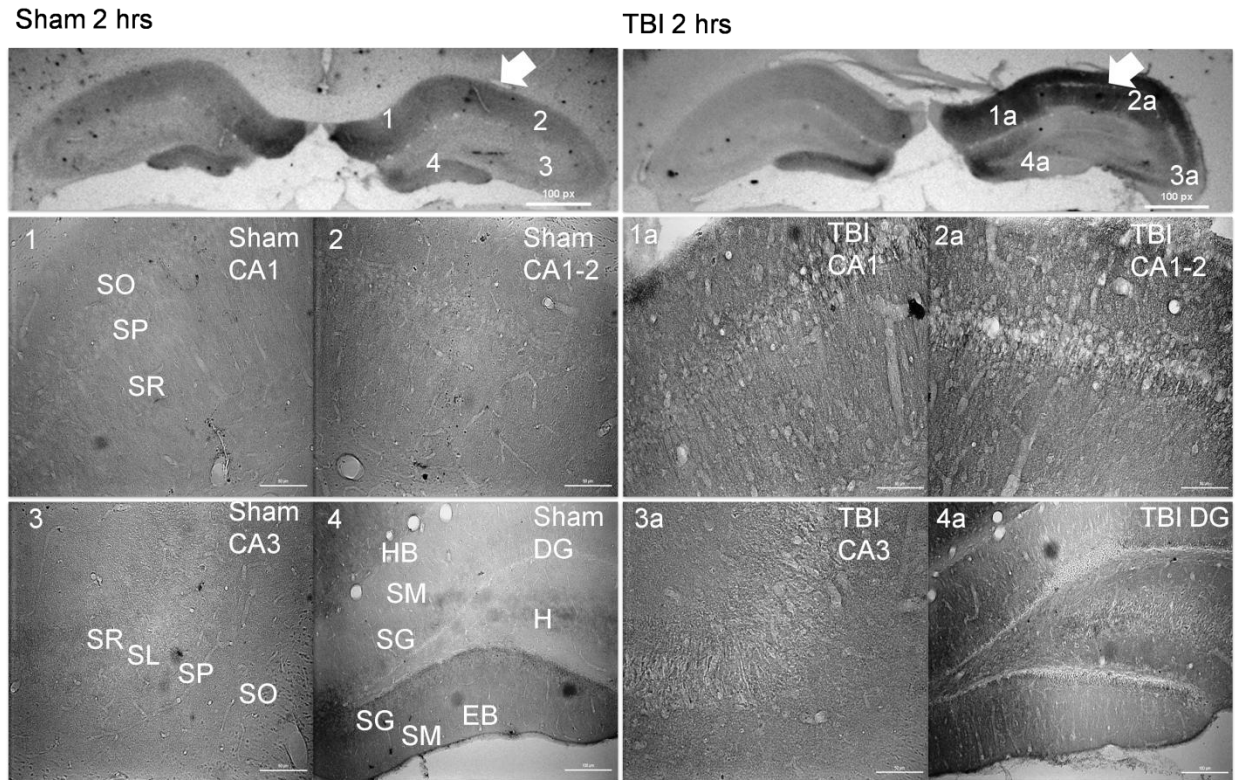


Figure 12: CnB2 immunohistochemistry demonstrating alterations in CnB2 isoform distribution 2 hours following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a significant increase in staining within the CA1 and CA1-2 in TBI (1a and 2a) versus sham (1 and 2). There is a significant loss of CnB2 expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

2.3.12 TBI Resulted in Chronic Regionally Specific Alterations in CnA β Distribution

In sham animals at 2 weeks post injury the staining of the CnA β subunit in the CA1 region of both hippocampi appears to be less than that appreciated in the 2 hour post injury sham animals. There is less CnA β staining within dendrites of the SR of both the CA1 and CA1-2 regions, although neither reaches significance owing to the variability of dendrite expression in injured CA1 and CA1-2 at 2 weeks post injury (**Figure 13**). At 2 weeks changes within the CA3 region of the CnA β subunit mirror those seen at 2 hours with apparently less staining within the cell somas of the SP layer and increases within the neuropil of the SO and SR layers which do not reach statistical significance. Similar to 2 hour post injury there is less CnA β expression within the exposed blade of the injured ipsilateral DG compared to sham, predominantly in the SG layer.

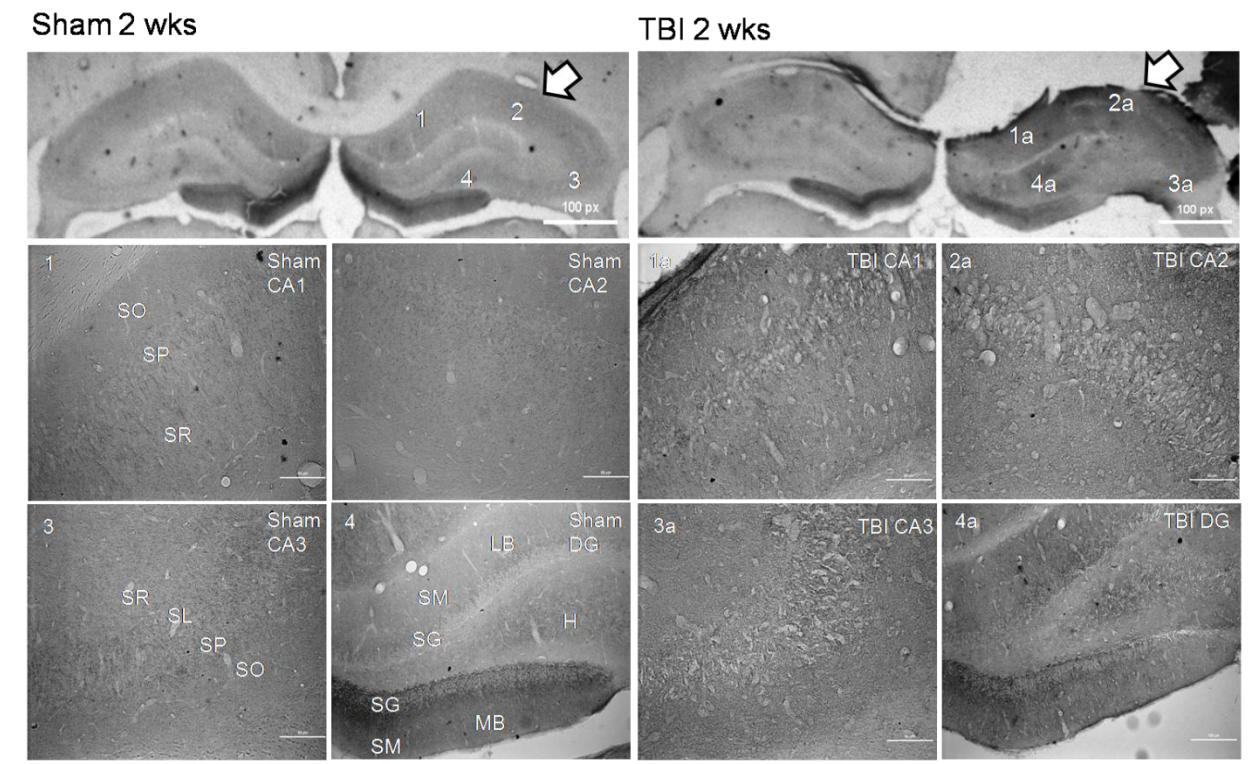


Figure 13: CnA β immunohistochemistry demonstrating alterations in CnA β isoform distribution 2 weeks following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a significant loss of CnA β expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

2.3.13 TBI Resulted in Chronic Regionally Specific Alterations in CnB2 Distribution

There is more CnB2 staining within dendrites of the SR of both the ipsilateral and contralateral CA1-2 as well as increases in CnB2 staining within the SO and SP regions of both the ipsilateral CA1 and CA1-2 regions (**Figure 14**). At 2 weeks changes within the CA3 region of the CnB2 subunit seen at 2 hours are no longer present. Similar to 2 hours post injury there is less CnB2 staining within the exposed blade of the injured ipsilateral DG compared to sham, predominantly in the SG layer, and an increase in CnB2 staining within the ipsilateral hilus of injured animals compared to sham.

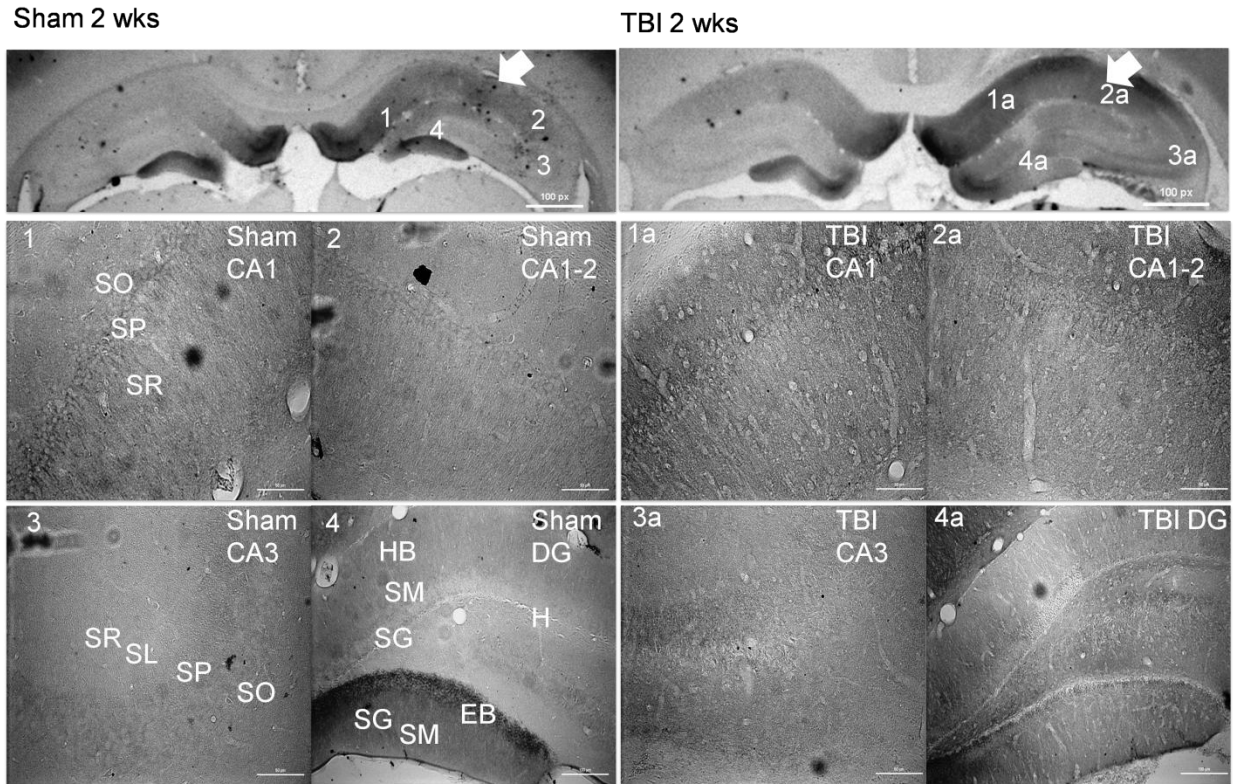


Figure 14: CnB2 immunohistochemistry demonstrating alterations in CnB2 isoform distribution 2 weeks following TBI (right panels). Arrows indicate side of cortex ipsilateral to injury or sham surgery. There is a significant increase in CnB2 isoform expression within both the CA1 and CA1-2 regions in injured (1a and 2a) compared to shame (1 and 2). There is a significant loss of CnB2 expression within the EB of the DG in TBI (4a) versus sham (4). Scale bars, 50 μ m for regional sections. **Abbreviations:** SO= Stratum Oriens; SP= Stratum Pyramidale; SR= Stratum Radiatum; SL= Stratum Lucidum; SM= Stratum Moleculare; SG= Stratum Granulosum; HB= Hidden Blade; H=Hilus; EB= Exposed Blade.

2.3.14 Changes in CnA Subunit Staining Appreciated with Immunohistochemistry are not Due to Changes in Protein Concentration within Hippocampal Regions

Western blot analysis of ipsilateral and contralateral DG, CA1, and CA1-2/CA3 regions indicates no difference between sham and injured animals in the protein concentrations of the CnA α isoform relevant to each region. This suggests that the alterations in CnA α isoform staining seen in immunohistochemistry is due predominantly to a redistribution in the regions of interest and not due to altered protein synthesis (**Figure 15**). However, there is an apparent decrease in protein expression of CnA β in the ipsilateral DG which is consistent with the immunohistochemistry. There are no other apparent differences in CnA β protein expression in other regions.

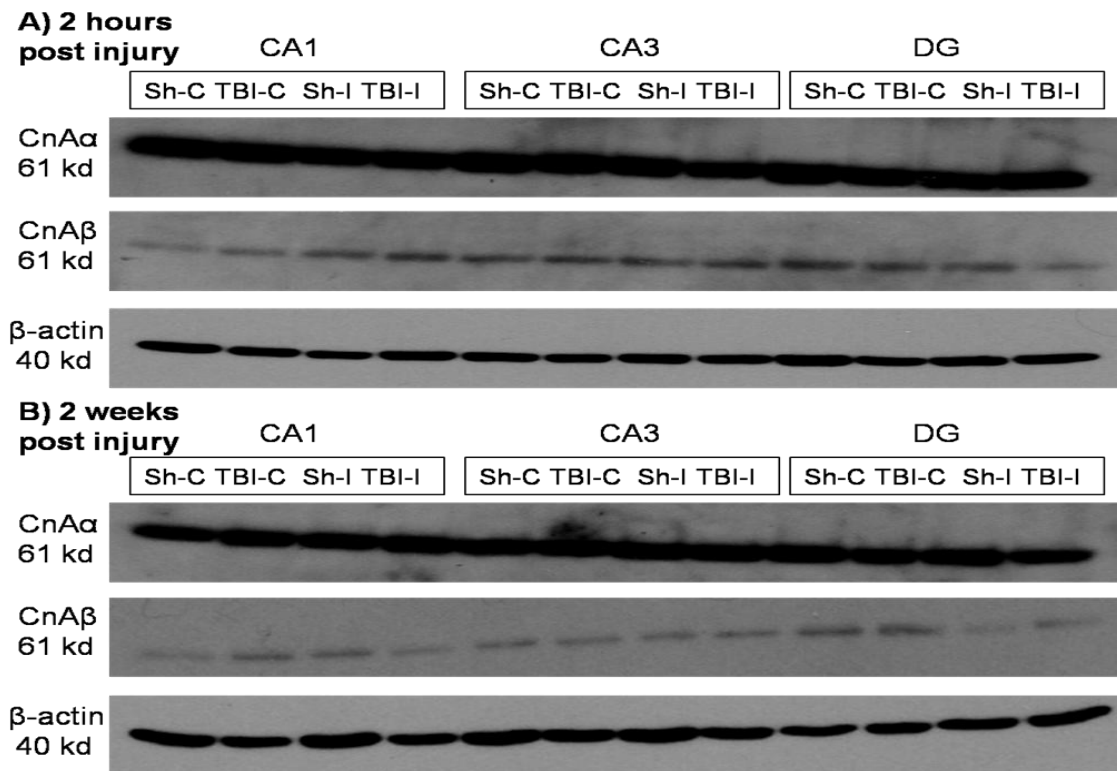


Figure 15: Qualitative western blot analysis of specific hippocampal regions. Homogenates were made from pooled resection tissue from N=3 rats per group. There is no change in CnA α protein expression in injured versus sham at either 2 hours (15a) or 2 weeks (15b) post injury.

However, there does appear to be a relative decrease in the expression of CnA β in the injured DG ipsilateral to injury compared to sham at both timepoints. **Abbreviations:** Sh-C= Sham Contralateral; TBI-C= Traumatic Brain Injury Contralateral; Sh-I= Sham Ipsilateral; TBI-I= Traumatic Brain Injury Ipsilateral.

2.3.15 Changes in CnB Subunit Staining Appreciated with Immunohistochemistry are in Part Due to Changes in Protein Concentration within Hippocampal Regions

Western blot analysis of ipsilateral and contralateral DG, CA1, and CA1-2/CA3 regions indicates a visible difference between sham and injured animals in the protein concentrations of the CnB1 and CnB2 isoforms relevant to each region. At both 2 hours and 2 weeks post injury there is an increase in the expression of both CnB isoforms within the injured contralateral and ipsilateral CA1 regions compared to sham. There is also a decrease in both isoforms expression within the injured contralateral and ipsilateral DG compared to sham at 2 hours and 2 weeks. This suggests that the alterations in CnB isoform staining seen in immunohistochemistry is due in part to changes in protein expression and possibly also to a redistribution in the regions of interest (**Figure 16**).

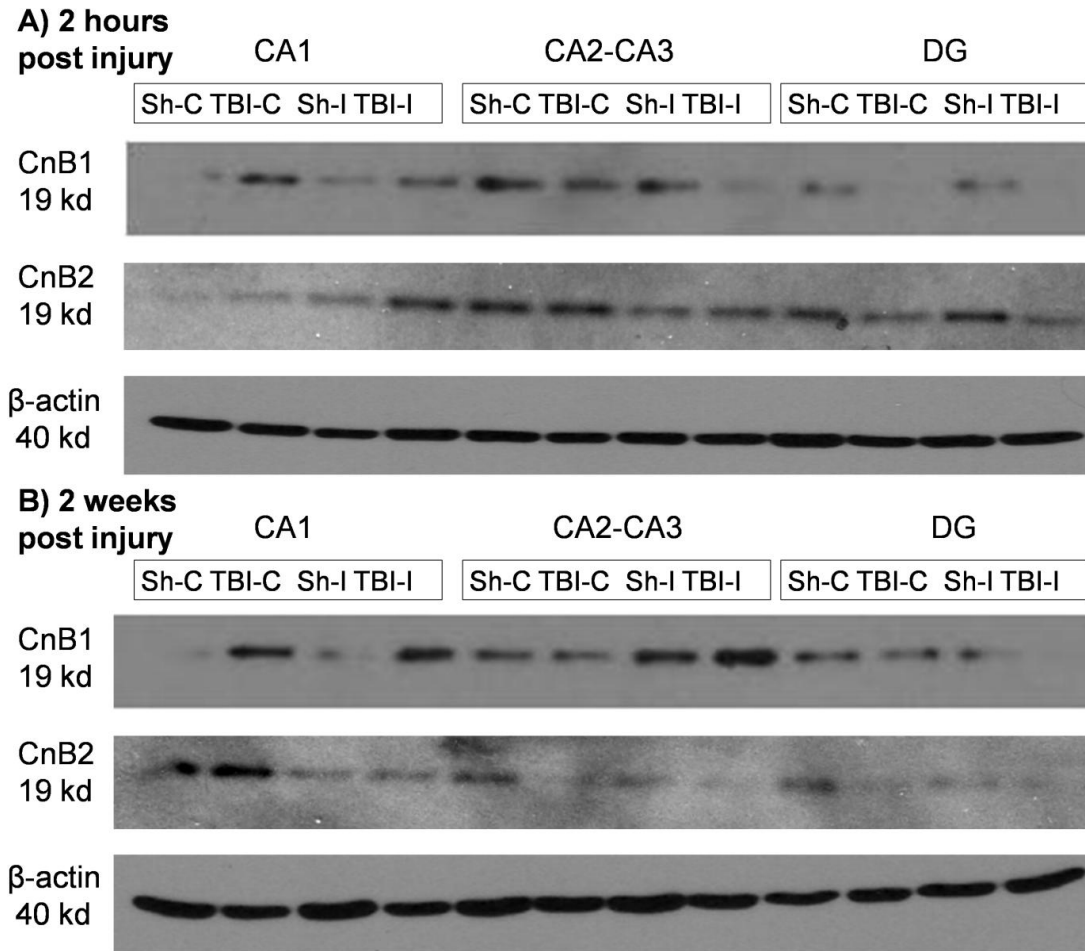


Figure 16: Qualitative western blot analysis of specific hippocampal regions. Homogenates were made from pooled resection tissue from N=3 rats per group. There is a noticeable increase in both CnB1 and CnB2 expression within the CA1 region at both timepoints examined. There is also a noticeable decrease in both CnB subunit isoforms expression within the DG of injured animals compared to sham, which is consistent with immunohistochemistry. Results of western blot analysis of the CA2-CA3 region do not completely agree with immunohistochemistry suggesting that there may be an alteration in distribution within these regions as well as changes in total expression. **Abbreviations:** Sh-C= Sham Contralateral; TBI-C= Traumatic Brain Injury Contralateral; Sh-I= Sham Ipsilateral; TBI-I= Traumatic Brain Injury Ipsilateral.

2.4 DISCUSSION

Following CCI there are regionally specific alterations in CnA subunit isoform distribution. Acutely there is a loss of both CnA α and CnA β expression within the exposed blade of the DG and a decrease in dendritic expression within both the CA1 and CA1-2 regions of the ipsilateral hippocampus in CCI versus sham. Alterations in expression within the DG persists chronically as do changes in dendritic expression, although not consistently.

Following CCI there are also regionally specific alterations in CnB subunit isoform distribution. Acutely there is decreased IR in both CnB1 and CnB2 within the exposed blade of the DG and an increase in IR within the CA1, CA1-2, and CA3 regions of the ipsilateral hippocampus in CCI versus sham. Alterations in IR within the DG persist chronically as do changes in the CA1-2 region. There are also significant increases in the IR of the CnB2 isoform in the contralateral CA1-2 region that persists chronically.

Furthermore, consistent with research in FP (Kurz et al., 2005b), CCI produces an increase in calcineurin activity in all regions examined in injured hippocampus compared to sham. The increase in calcineurin activity was seen acutely and persisted chronically (**Figure 4**). The greatest increase in calcineurin activity occurred in the DG, both ipsilateral and contralateral, compared to sham. In nearly all areas examined, even in the contralateral side of the hippocampus there were increases in calcineurin activity suggesting that alterations in calcineurin function are not limited to the injured side of the brain.

In the acute phase after TBI, elevations in Ca²⁺ (Shapira et al., 1989) can lead to abnormal neuronal activation leading to excitotoxicity, increased superoxide levels, oxidative stress and cell

death (Hovda et al., 1992). Although calcineurin appears to be a key mediator in many of these processes there needs to be a greater understanding of how calcineurin is specifically modulated after injury, particularly with regard to the individual calcineurin subunits and isoforms. The present study showed that CnA isoforms are differentially modulated in the hippocampus in a regionally specific manner after TBI. Indeed, both up- and down-regulation of the same CnA isoform can occur in regionally distinct areas of the hippocampus within the same post-injury time period. This suggests that individual calcineurin isoforms may have different roles within select hippocampal areas in the post-injury period. Whether these changes are a short term response to injury, the initiation of long term neuronal destabilization mechanisms or a counter-regulatory mechanism awaits further studies conducted in a temporal manner using specific calcineurin inhibitors.

Beyond alterations in the catalytic subunit, the changes in regulatory subunit expression are important given the increased use of drugs designed to block Calcineurin activity which typically inhibit activity by limiting the ability of the regulatory subunit (CnB) to bind to and activate the catalytic subunit (CnA). The present study showed that CnB isoforms are differentially modulated in the hippocampus in a regionally specific manner after TBI. This suggests that individual Calcineurin regulatory isoforms may have different roles within select hippocampal areas in the post-injury period and may have implications for regional effects of Calcineurin inhibitors. This study also shows that following a CCI there is nearly a two-fold increase in Calcineurin activity in all regions of the hippocampus, both ipsilateral and contralateral, in injured animals versus sham. This increase in Calcineurin activity persists to the two week timepoint indicating that there is ongoing Calcineurin dysfunction in both regulatory subunit regional expression and in activity.

The present study illustrates that changes in Calcineurin appear as early as 2 hours post TBI and persist until at least 2 weeks post injury. Changes in hippocampal expression as assessed utilizing immunohistochemical staining of CnA isoforms were noticeable in all but the CnA γ isoform, which had little to no expression within the hippocampus. Western blot data suggest that the visualized alterations in expression were not associated with changes in protein concentrations within hippocampal regions, but rather in cellular distribution or perhaps in divergent expression within subsets of different cell types throughout the hippocampus (**Figure 15**). Changes in hippocampal IR, as assessed utilizing immunohistochemical staining, were noticeable in both of the CnB isoforms. Western blot data suggest that the visualized alterations in IR were associated with changes in protein concentrations within gross hippocampal regions, however this does not rule out possible changes in cellular distribution or perhaps in divergent expression within subsets of different cell types throughout the hippocampus (**Figure 16**).

The expression of the CnA subunit has been linked to mitochondrial dysfunction and cell death signaling through BAD (Asai et al., 1999). CnA isoform expression, specifically CnA α , is known to be important in both oxidative stress and mitochondrial dysfunction which may play a role in both acute and chronic dysfunction within the hippocampus following TBI (Uchino et al., 2008). The level of cellular death was not assessed in this injury paradigm. However, given the regionally specific and variable alteration of CnA cellular distribution within the CA1, CA1-2, CA3 and the hilus and hidden blade of the DG acutely and chronically, there may be broader implications than simply cell death signaling (**Figures 5,9,11,13**).

While the expression of the CnA subunit has been linked to mitochondrial dysfunction and cell death signaling through BAD (Asai et al., 1999), alterations in the expression of the regulatory CnB subunit have not. Rather, changes in CnB expression have been shown to cause alterations in hippocampal cellular potentiation and depression (Zeng et al., 2001) and have

been implicated in schizophrenia (Miyakawa et al., 2003). Mbye et al., 2009 demonstrated that a non-Calcineurin inhibitory analog of cyclosporin A demonstrates cellular protection by acting upon mitochondrial integrity. This suggests that the cellular death and cytoskeletal degradation seen post TBI may be independent of Calcineurin activity. The increases in Calcineurin activity seen predominantly within the CA3 and DG fractions in both ipsilateral and contralateral hippocampi (**Figure 4**) also suggest that Calcineurin is playing a role beyond cell death signaling post-TBI given that, in the CCI model, cellular death is reported within the ipsilateral CA1 region with dystrophic neurons seen in the ipsilateral CA3 and DG (Colicos et al., 1996).

Another possible explanation for changes in Calcineurin isoform subunit distribution is related to the structural function of hippocampal cell signaling (Buzsaki, 1996; Muller et al., 1996). The entorhinal cortex sends projections through two separate pathways into the hippocampus. The perforant pathway synapses within the DG and CA3 regions which then send projections into the CA1 region while another separate pathway from the EC directly synapses within the CA1 region. The CA1 then sends its axonal outputs back into the deep layers of the EC.

Both the DG and CA1 dendritic field receive input from the EC through two separate pathways. Alterations in isoform expression in these regions may be due to excitatory glutamate release from cortical structures occurring post injury. Isoform alterations in contralateral isoform expression appreciated acutely in both the CA1 and DG (**Figures 5 and 11**) that are no longer present at 2 weeks post injury (**Figures 9 and 13**), suggest a potential cortical component. Given that there is extensive crossover signaling between the right and left EC and hippocampi, excitotoxic glutamate release could explain isoform alterations in the areas that receive cortical input either ipsilateral or contralateral to injury (the DG and CA1 regions) (Buzsaki, 1996). Sustained low levels of Ca^{2+} caused by increased cellular excitability due to increased glutamate release in the postinjury period may activate CnA and be responsible for the

persistent changes in isoform expression in the CA1, CA1-2, and DG regions. Alterations in Ca^{2+} concentration and flow, both acutely and chronically, have been appreciated within the hippocampus following TBI (Deshpande et al., 2008; Sun et al., 2008).

At two weeks alterations in CnA isoform distribution within the SR of CA1 and CA1-2 did not reach significance (**Figures 9 and 13**). This may indicate that after a period of recovery there is a reversal of initial alterations in isoform distribution and a possible recovery of Calcineurin subunit function chronically. This may be partially responsible for the recovery post TBI. This was not seen in the dentate, however, and acute alterations in isoform distribution persisted into the chronic phase. What consequences regionally specific changes have on global hippocampal function is unclear, however given the extensive cortical connections throughout the dentate it is possible that persistent ipsilateral dentate loss of CnA is related to changes in synaptic inputs from cortical neurons.

Several studies have suggested that subunits may possess action independent of Calcineurin activity (Kayyali et al., 1997; Asai et al., 1999; Zhou et al., 1999). The CnA subunit can become constitutively active if proteolytic cleavage removes the autoinhibitory site (Manalan et al., 1983) and the expression of the CnA α isoform has been shown to be important to LTP and synaptic plasticity of the CA1 and CA1-2 pyramidal neurons (Victor et al., 1995; Zhuo et al., 1999; Zeng et al., 2001; Groth et al., 2003). Following TBI there is a chronic failure in LTP induction (Sanders et al., 2000) that can be ameliorated with Calcineurin inhibition (Albensi et al., 2000). Alterations in Calcineurin subunit distribution may provide a potential mechanism for this loss of synaptic plasticity. Following TBI there is a persistent dysfunction in learning and memory (Dixon et al., 1996; Hamm, et al., 1996; Dixon et al., 1997; Scheff et al., 1997; Kline et al., 2000, 2002). Alterations in LTP (Reeves et al., 1995), LTD (Albensi et al., 2000) following TBI could

be, in part, due to rapid, persistent alterations in Calcineurin A subunit expression demonstrated in this study.

Changes in CnA expression profiles throughout the hippocampus have important implications for cellular function independent of cellular death pathways. There is a consistent loss in dendrite staining of both isoforms, within the ipsilateral CA1 and CA1-2 regions at 2 hours, which partially reverses by 2 weeks (**Figures 5,9,11,13**). This is in contrast to prior results demonstrating persistent increases in CnA α isoform staining within the apical dendrites of CA1 neurons (Kurz et al., 2005a). However, it must be noted that the previous study used a FP model and there may be a different alteration in Calcineurin distribution with CCI. It is also important to recognize that while there is reduced dendritic staining in the deep layers of the CA1 and CA1-2 SR there remains significant CnA α expression in areas closer to the cell soma which is consistent with the results presented by Kurz et al (2005a).

Differences in CnA α distribution alterations compared to CnA β have implications for isoform specific actions following TBI. CnA β staining appears to be primarily localized to dendrites within the SR of the CA1 and CA1-2 and within the exposed blade of the dentate suggesting that CnA β function may be more important to plastic events relevant to synaptic connections within these regions. Prior developmental studies have suggested that both CnA α and CnA β have important roles in synaptic organization and synaptogenesis (Eastwood et al., 2005).

There were decreases at both 2 hours and 2 weeks in both CnA isoforms expression within the ipsilateral exposed blade of the DG (**Figures 6,9,11,13**). Given the projections of the DG through CA3 and into the CA1 region, decreases in isoform expression may not be due to acute alterations in hippocampal regions, but rather a loss of expression throughout projecting neurons. This would be consistent with recent experimental results demonstrating significant

axonal loss throughout the gray matter of the hippocampus (Hall et al., 2008). Losses in projecting dendrites and subsequent alterations in synaptic targets would also provide a possible explanation for the reduction of CnA isoform expression within the SR of the CA1 and CA1-2 regions given that a loss of synaptic input would preclude the necessity for Calcineurin activity at synapses.

Changes in CnB distribution and expression profiles throughout the hippocampus have important implications for cellular potentiation and depression. Unlike the CnA subunit, CnB isoforms are generally upregulated throughout the CA1 and CA1-2 regions ipsilaterally and the acute changes in the regulatory CnB subunit isoforms nearly all persist into the chronic phase (**Table 5**). The CnB2 isoform also shows a significant increases in dendritic IR within the CA1-2 region both acutely and chronically (**Figures 12 and 14**). The TBI related up-regulation of CnB IR in the present study may be involved in modulating synaptic depression, given that a knockout of CnB impairs LTD. Zeng and colleagues (2001) suggest from their studies that the selective loss of the CnB subunit hypothetically impairs bidirectional signaling mechanisms between LTP and LTD during very specific memory tasks. The increase in the regulatory subunit expression coincides with increases in Calcineurin activity in the CA1 and CA3 regions suggesting a potential link between subunit alterations and activity. However, Calcineurin activity is highest within the DG following injury (**Figure 4**) which coincides with a significant decrease in the expression of the regulatory subunit (**Table 5; Figure 16**) and decrease in CnA distribution (**Table 4; Figure 6, 13**). The persistent change in regulatory subunit expression and catalytic subunit distribution cannot account for the persistent increases in Calcineurin activity within the DG, but may play a role in the ongoing deficiencies in hippocampal potentiation after TBI within the CA1.

The down-regulation of the CnB regulatory subunit isoforms (**Figure 16**) may be a counter regulatory response to eliminate non-specific neuronal activity after TBI occurring within the DG. This may be more pertinent in TBI given that following a brain injury the CnA subunit can become constitutively active if proteolytic cleavage removes the autoinhibitory site (Manalan et al., 1983). Perhaps downregulation of the regulatory subunit is a cellular response to the increases in catalytic Calcineurin activity seen both in FP (Kurz et al., 2005b) and shown for the first time in CCI in the present study (**Figure 4**). This would suggest that there may be a two-fold dysfunction in Calcineurin following a TBI, both an increase in potentially harmful activity, and an alteration in subunit distribution important for synaptic function, in particular altering dendritic stability (Halpain et al., 1998) and affecting the growth of neurites (Chang et al., 1995).

For normal Calcineurin activity it is necessary, in the presence of Ca²⁺, for the regulatory CnB subunit to bind to its binding site on the catalytic CnA subunit (Griffith et al., 1995). Both of the calcineurin inhibitors, FK-506 and cyclosporin A, rely upon the disruption of this interaction to limit Calcineurin activity (Kay et al., 1989; Wiederrecht et al., 1993). Regionally distinct changes in the two subunits create a situation whereby these two drugs may have different effects within different regions of the hippocampus, which have yet to be appreciated. Given the increase in activity within the DG (**Figure 4**), but loss of regulatory expression (**Figure 16**), Calcineurin inhibitors may not be effective in curtailing DG damage post TBI. Understanding how Calcineurin activity and structure is altered post injury may help to better develop and target treatment strategies for TBI.

In summary, Calcineurin subunit isoforms are differentially modulated by injury in a regionally specific manner. Regional alterations in CnA and CnB isoforms within the hippocampus ipsilateral to injury persist chronically. Further studies are needed to examine exactly how isoform changes relate to increases in Calcineurin activity in regions of the hippocampus. It is

also unclear what the functional implications are of specific subunit isoform alterations. While studies have shown cellular potentiation and depression dependent upon CnB expression, it remains unclear what the CnB subunit's function is independent of the activity of the Calcineurin heterodimer. These regionally specific alterations in isoform expression also have important implications for the clinical use of Calcineurin inhibitors in TBI. This study is the first step in identifying these alterations and characterizing specific Calcineurin subunit changes following TBI that will allow for better targeted therapeutic strategies to assist in patient recovery from TBI.

2.5 CONCLUSION

Cellular death within the hippocampus is a well described feature of experimental TBI (Lighthal et al., 1989; Sanders et al., 2001; Jennings et al., 2008). The causes of cell death are many including oxidative stress, glutamatergic excitotoxicity, mechanical damage, and persistent apoptotic cell signaling activation (Kochanek, 1993). We examined calcineurin subunits within the hippocampus to evaluate how CCI alters calcineurin post TBI. There was an acute alteration in the distribution of both the regulatory and catalytic subunits that persisted into the chronic phase post TBI throughout the hippocampal architecture. In addition there was an increase in activity in all areas examined both acutely and chronically. The level of injury chosen for this study was mild-moderate in severity in order to preserve the hippocampus. A more severe CCI has the effect of causing significant hippocampal loss, as seen in chapter 4 of this thesis. Even with a milder injury the hippocampus still shows significant ongoing cellular pathology. This suggests that neuroprotection strategies designed to spare hippocampal architecture may preserve cellular numbers, but not reduce cellular pathology. It is for this reason that it has become critically important to look beyond the hippocampus to other brain structures in order to effectively treat behavioral dysfunction after TBI.

3. DYSFUNCTION IN STRIATAL SIGNALING PATHWAYS FOLLOWING TBI

3.1 INTRODUCTION

TBI causes persistent cognitive deficiencies. A complex etiology makes the identification of therapeutic targets difficult in TBI (Kochanek, 1993; DeKosky et al., 1998). Subcortical regions represent novel areas for therapeutic targeting in TBI given their importance to functional deficits and integration with other brain regions (Donnemiller et al., 2000; Vertes, 2006). To date few studies have examined the effect of TBI upon subcortical neuronal signaling and potential treatment targets remain unknown.

The basal ganglia are involved in executive function, emotional control, and memory and learning (Ridley et al., 2006; Baldo and Kelley, 2007). The striatum represents the major entry into the basal ganglia. Striatal medium spiny neurons (MSN) receive glutamatergic inputs from the cerebral cortex and DAergic innervation from the midbrain (Pennartz and Kitai, 1991). The interaction of glutamate and DA within MSNs modulate the plasticity of corticostriatal synapses (Calabresi et al., 2007).

Acutely, following a TBI there is an increase in glutamatergic release leading to excitotoxicity and oxidative stress (Palmer et al., 1993). Alterations in glutamate receptor subunit composition and expression contribute to persistent dysfunction in plasticity (Osteen et al., 2004). What effect glutamatergic signaling alterations has in the striatum is unknown, however DA signaling in striatal neurons has been examined. Dysfunctions in DAergic signaling after TBI include increases in DA (Massucci et al., 2004; Kobori et al., 2006), alterations in dopamine transporter

(DAT), chronic reductions in evoked DA release (Wagner et al., 2009), and alterations in tyrosine hydroxylase (Yan et al., 2007). Pharmacotherapies that enhance DA have clinical (McAllister et al., 2004; Warden et al., 2006) and experimental (Kline et al., 2000; Dixon et al., 1999) benefit in TBI. The exact mechanism of many of these therapeutic strategies, however, remains unknown.

The DA and cAMP regulated phosphoprotein-32 (DARPP-32) is a cytoplasmic phosphoprotein found in 95% of MSNs. Two phosphorylation sites, threonine-34 (Thr34) and threonine-75 (Thr75), make DARPP-32 a bifunctional signal transduction molecule that controls the activities of protein phosphatase-1 (PP-1) and protein kinase A (PKA) (Greengard et al., 1999; Nishi et al., 2002; Valjent et al., 2005).

DA acting at D1 receptors promotes DARPP-32 phosphorylation at Thr34, leading to inhibition of PP1. DA and glutamate act on protein phosphatase 2B (PP2B) and protein phosphatase 2A (PP2A) to decrease phosphorylation at Thr34. A reduction in phosphorylation at Thr34 removes the inhibitory affect of DARPP-32 on PP1 (Nairn et al., 2004; Svenningsson et al., 2004). The regulation of PP1 and PKA by DARPP-32 allows DA and glutamate to regulate striatal neuronal signaling, protein transcription, and potentiation (Fienberg et al., 1998; Hakansson et al., 2004).

Given the importance of DARPP-32 to striatal function and the role it plays as an integrator of multiple signaling pathways, DARPP-32 represents a unique therapeutic target in TBI. Understanding the role of DARPP-32 in post-TBI striatal dysfunction is necessary to understanding subcortical effects of TBI and designing effective therapeutic interventions.

This study represents the first to examine DARPP-32 phosphorylation in TBI. We hypothesize that following TBI there will be a decrease in the phosphorylation of DARPP-32 at Thr34 and

subsequent increase in PP-1 activity within the striatum that persists. We also predict that the increase in PP-1 activity post TBI will be associated with alterations in downstream signaling including decreases in ERK 1/2 and CREB phosphorylation.

3.2 METHODS AND MATERIALS

3.2.1 Animals

Adult male Sprague-Dawley rats ($N = 188$) were used in the study. Rats (300-325 g) were purchased from Hilltop Laboratories (Scottsdale, PA, USA) and housed in pairs under a 12:12 light/dark cycle. Rats were given food and water with *ad libitum* throughout the study. All experiments were carried out in accordance with the University of Pittsburgh's guidelines for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

3.2.2 Surgical Procedures for TBI

On the day of surgery anesthesia was initiated with 4% isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL, USA) and 2:1 N₂O/O₂. Rats were intubated and maintained on 1.5-2% isoflurane during the surgical procedure. Following intubation, rats were placed on a thermal blanket to regulate body temperature (37°C) and the animals head placed in a stereotaxic frame. An incision was made down the midline of the skull and the soft tissues and periosteum deflected. A craniotomy was then performed over the right parietal bone to expose the dura. Controlled cortical injury (CCI, Pittsburgh Precision Instruments, Inc.) at a depth of 2.6mm-2.8mm at 4m/sec was carried out as previously reported (Dixon et al., 1991; Yan et al., 2000). A

total of 91 rats were injured, and the remaining 97 rats were shams. Righting reflex⁴⁴ was monitored in the immediate post-surgical period to assess acute recovery.

3.2.3 Tissue Preparation, Immunohistochemistry, and Immunofluorescence Staining

Animals ($N = 3$ in each group for each time point for a total $N = 30$) were given an overdose of sodium pentobarbital (100 mg/kg; i.p), and perfused intra-aortically with 0.1M heparinized PBS in 4% PFA/0.1M PBS. Brains were dissected, submerged in increasing concentrations of sucrose, and stored at -80°C . Brains were then sectioned at $35\mu\text{m}$ in a cryostat, and free floating sections collected in tissue plate wells containing 0.1M TBS (pH 7.5).

All immunohistochemical procedures and incubations were carried out with agitation with the exception of the chromogen step. All treatment groups were stained together within each immunohistochemical session. Sections were matched by region, rinsed 3 x 5 min in washing buffer (0.1%Triton-X in 0.1M TBS) and blocked in a mixture of 10% normal donkey serum in washing buffer for 2 hours at room temperature. Sections were then incubated overnight at 4°C in (one only) primary rabbit antibodies specific to DARPP-32 (1:1000; Cell Signaling), p-DARPP-32-Thr34 (1:600; Chemicon International), p-DARPP-32-Thr75 (1:600; Chemicon International) diluted in washing buffer/5% normal donkey serum. Following incubation, sections were then washed 3 x 8 minutes in washing buffer and endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 10 minutes. Following 3 x 5 minutes washing in washing buffer, sections were incubated for 2hrs at RT in biotin conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, PA) at 1:400 in 5% normal donkey serum in washing buffer. Sections were then washed 3 x 5 minutes in washing buffer and incubated in ABC reagent (Vector Industries) for 30 minutes. Immunoreactivity (IR) was then visualized using 0.01% 3' 3' Diaminobenzidine (DAB) following 5 x 5 minutes washing in washing buffer. The DAB reaction

was terminated with dH₂O and sections were rinsed in 0.1M TBS, mounted onto slides, air dried and cover-slipped for light microscope analysis. All sections within the reaction were exposed to each of the reagents for the same time period.

For double labeling, sections ($N = 4$ in each group for each timepoint for a total $N = 32$) were incubated with primary antibody (DARPP-32 1:100; Cell Signaling; p-DARPP-32-Thr34 1:50; Chemicon International; p-DARPP-32-Thr75 1:200; Chemicon International) in 10% normal donkey serum in washing buffer with mouse anti-NeuN (1:2000) at 4°C for 16-24 hours. Alexa fluorescent dyes (488, 594) conjugated to goat anti-rabbit or donkey anti-mouse secondaries, respectively, were used for double labeling of DARPP-32/p-DARPP-32-Thr34/p-DARPP-32-Thr75 and NeuN. At least 3 sections of brain tissue through the striatum were processed for each experiment. Control experiments omitting primary antibodies were run in parallel to confirm antibody specificity. Images were captured using a confocal microscope (LSM 510; Zeiss, Jena, Germany).

Semi-automated computer assisted threshold cell count analysis of DAB stained immunohistochemistry sections: Pictures of mounted sections were taken using a Nikon microscope. Two sections from each animal for a total of six pictures per group were used for analysis. Based upon techniques presented in (Brey et al., 2003; Leal et al., 2006) a semi-automated, computer-assisted, cell count based upon a set threshold of DAB IR was used to count positive cell bodies in the two regions of interest (Nucleus Accumbens; NAcc, and Dorsolateral Striatum; DLStr). For detailed description of methodology see **Appendix A: Supplemental figure 1**. Briefly, the DLStr and NAcc were identified with visual inspection and pictures of each area were taken utilizing an appropriate magnification (20x for DLStr and 10x for NAcc). For the NAcc section the shell of the NAcc was outlined and cell counts were limited to the core and shell. Images were captured as tiff files. ImageJ (NIH, USA) software for

windows was utilized to analyze images. The blue image was used for analysis as previous studies have shown that DAB IR provides the greatest contrast in the blue spectrum. Images were converted grayscale and then to binary data by setting a threshold for positive DAB staining selected by an examiner blinded to injury. Once thresholds (Typically the top 15-20% of most intense staining) were selected for a specific area (DLStr or NAcc) using a randomly selected section image these thresholds were maintained throughout each section for that particular area ensuring that the criteria for a DAB positive cell body remained consistent throughout all examined sections. Following conversion to binary images, ImageJ (NIH, US) analysis software was used to count positive pixels defined as having a size of 100-500 for DLStr and 50-300 for the NAcc; which limited artifacts while identifying positive neuronal cell bodies (**Supplemental figure 1**).

3.2.4 Western Blot Analysis

Animals ($N = 6$ in each timepoint for each group for a total $N = 60$ for DARPP-32 and p-DARPP-32 timecourse analysis; $N = 6$ for each group for a total of $N = 42$ for Amantadine hydrochloride studies). After deeply anesthetized with pentobarbital (Nembutal, 80-100 mg/kg; Abbott Laboratories, North Chicago, IL), animals were decapitated and the brains quickly removed and chilled on ice. Tissue from the striatum was excised and frozen in liquid nitrogen. Tissue was stored at -70°C until used for analysis. Striatal tissue was homogenized in 400 μl of Lysis buffer containing 0.1M NaCl, 0.01M Tris-Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 $\mu\text{g}/\text{ml}$ aprotinin, 100 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitor cocktail 1 (Sigma, St. Louis, MO) was added to samples used for western blot. Protein concentrations were determined using a BCA protein Assay Kit (PIERCE, Rockford, IL). Samples containing 20-100 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis through a 10% acrylamide gel, and after were transferred to nitrocellulose membranes. Membranes were

blocked with 5% bovine serum albumin (Sigma, St. Louis, MO) in tris-buffered saline (TBS-T). Following block membranes were washed 3 x 10 minutes in TBS-T and immunolabeled with antibodies (1:10,000 DARPP-32; 1:2000 p-DARPP-32-Thr34; 1:2000 p-DARPP-32-Thr75; Cell Signaling, Danvers, MA) in 5% BSA in TBST for 18-24 hours. Following primary incubation membranes were washed 3 x 10 minutes in TBS-T and incubated for 2 hours in by goat anti-rabbit immunoglobulin G conjugated to peroxidase (1:10,000; PIERCE, Rockford, IL). Proteins were visualized with a chemiluminescence detection system (SuperSignal, PIERCE, Rockford, IL). To assure equal loading, all membranes were striped and re-blotted with rabbit anti-actin antibody (1:20,000, Sigma, St. Louis, MO). Blots were exposed to autoradiographic X-ray film for 10 s to 2 min and bands were semi-quantified using ImageJ (NIH, USA) software. Values are given as a ratio (percentage change) of optical density of injured samples versus sham control within individual blots. All optical densities were normalized to β -actin.

3.2.4.1 Preparation of nuclear extracts used in western blot analysis. Nuclear fractions were prepared utilizing a nuclear extraction kit (Active motif, Carlsbad, CA). Briefly, frozen tissue was homogenized on ice with 250 μ l of 1x Hypotonic buffer with DTT and detergent. Homogenized tissue was centrifuged for 10 minutes at 850 x g at 4°C. Cells were resuspended in 250 μ l of 1x Hypotonic buffer by pipetting up and down and incubated on ice for 15 minutes. 15 μ l of detergent was added and the suspension was vortexed for 10 seconds. Suspension was then centrifuged for 30 seconds at 14,000 x g at 4°C. Supernatant was collected (cytoplasmic fraction). The remaining pellet (nuclear fraction) was resuspended in 100 μ l of complete lysis buffer and vortexed for 10 seconds. Suspension was incubated for 30 minutes on ice on a rocking platform. Suspension was then vortexed for 30 seconds and centrifuged for 10 minutes at 14,000 x g at 4°C. Supernatant was collected (nuclear fraction). Fractions were used for western blot analysis as described above.

3.2.5 Protein Phosphatase-1 Activity Assay

Animals ($N = 6$ in each timepoint for each group for a total $N = 42$). Striatal tissue was prepared following the same protocol as used for western blots, with the exception of the use of the phosphatase inhibitor cocktail in the lysis buffer. The following protocol utilized a modified PP-2A activity assay kit (Millipore, Temecula, CA) (**Appendix A: Supplemental figure 2**). 100 μg of protein, determined via BCA assay, was added to an eppendorf tube with 25 μl of agarose A/G beads and 6 μl of anti-PP-1 δ rabbit antibody (Millipore, Temecula, CA). Following overnight incubation in 4°C and 3 min spin down at 10,000 g, beads were washed 3 times with ice cold TBS and once with Ser/Thr phosphopeptide buffer. Beads were spun down in between each wash for 1 min in 4°C. Following the final wash, 30 μl of phosphopeptide and 20 μl of Ser/Thr phosphopeptide buffer was added to the eppendorf tube. The eppendorf tube was then incubated for 10 minutes in a water bath at 30°C. 25 μl of supernatant was placed in a 96 well plate (in duplicates) and malachite green detection assay was utilized to visualize free phosphates. Plate was read on a plate-reader at 650 nm. A standard curve was calculated and the level of phosphatase activity is reported in picomoles of phosphate released per 25 μl of supernatant. 10 μl of TBS and 10 μl of 2x loading buffer were then added to the eppendorf. The eppendorf tube was then heated to 95°C for 5 minutes and western blots were run to determine the relative amount of PP-1 protein in each tube. Final values are normalized to western data to ensure equal protein for each sample.

3.2.6 Protein Kinase A Activity Assay

To analyze PKA activity the PepTag PKA assay was employed (Promega, Madison, WI). For each sample 5 μl of reaction buffer, 5 μl of the A1 peptide, 5 μl of the PKA activator, and 10 μl of protein sample were added. For a positive control the catalytic subunit of cAMP protein kinase

was added, for a negative control sample and the catalytic subunit were excluded. Mixed samples were incubated at room temperature for 30 minutes. Reaction was stopped by placing the reaction tubes on a heating block at 95°C for 10 minutes. Samples were then run for 30 minutes at 100 volts on a 0.8% agarose gel (mixed in 50 mM Tris-HCL). Pictures were taken with a kodak blacklight image camera. Density of bands was analyzed utilizing ImageJ analysis software (NIH) and corrected for the concentration of protein loaded per sample. Data is represented as percentage of density versus sham \pm standard deviation and was analyzed using ANOVA with significance set at $p \leq 0.05$.

3.2.7 Statistics

Cell counts using the semi-automated cell count method were analyzed according to analysis of variance (ANOVA) with a significance level set at $p \leq 0.05$. Cell count data is expressed as the mean \pm standard error (SEM) of the mean. Optical density of western blot bands from each group were compared according to a two-factor ANOVA (time versus injury) followed by Bonferroni post-hoc comparison with a significance level of $p \leq 0.05$. Western blot data are expressed as the group means \pm standard error (SEM) of the mean. Phosphatase and kinase activity data are expressed as the group means \pm standard deviation of injury compared to the appropriate sham. Statistical evaluations were performed according to ANOVA with a significance level of $p \leq 0.05$ used for all tests. Two-way ANOVA was not run on phosphatase or kinase activity assays due to the limited timepoints analyzed. The effect of interest was any alterations in activity at each timepoint assessed compared to sham.

3.3 RESULTS

3.3.1 TBI Causes a Specific Decrease in the Phosphorylation of DARPP-32 at Threonine-34

Within the Striatum

Following a TBI there is an acute decrease in DA tissue levels and chronic increase (McIntosh et al., 1994; Massucci et al., 2004). Alterations in DA have been shown to affect the phosphorylation state of DARPP-32 in MSNs (Nishi et al., 2003). We therefore examined whether, following a TBI, there was a change in DARPP-32 phosphorylation state at the Thr34 and Thr75 phosphorylation sites (**Figure 17**). TBI caused an acute decrease in the phosphorylation of DARPP-32 at Thr34 (p-DARPP-32-Thr34) as assessed utilizing western blots (N=6 for each group) (**Figure 17**) (At 6hrs $p < 0.05$). This decrease in p-DARPP-32-Thr34 persisted through all timepoints examined in the ipsilateral striatum (1Day $p < 0.0001$; 1week $p < 0.05$; 2week $p < 0.01$; 4week $p < 0.05$) and at 2 and four weeks in the contralateral striatum (2week $p < 0.01$; 4week $p < 0.05$), relevant to injury (**Figure 17**) indicating that TBI causes a persistent dysfunction in striatal intracellular signaling that persists beyond the initial insult. The decrease in p-DARPP-32-Thr34 was not due to changes in DARPP-32 expression (**Figure 17**). Furthermore, this was a site specific alteration that was not accompanied by any change in the phosphorylation state of the Thr75 site (p-DARPP-32-Thr75) (**Figure 17**), indicating that the loss of p-DARPP-32-Thr34 was not due to non-specific increases in phosphatase activity upon DARPP-32. To assess the regionality of this alteration we also examined the frontal cortex. Examination of the frontal cortex demonstrated decreases in p-DARPP-32-Thr34 at 1 week and 2 weeks post injury (**Appendix A: Supplemental figure 3**), however the decrease in p-DARPP-32-Thr34 was only identified in the ipsilateral cortex, relative to injury, did not appear acutely, and did not persist past 2 weeks. No alterations in DARPP-32 protein expression or p-DARPP-32-Thr75 were appreciated in the frontal cortex.

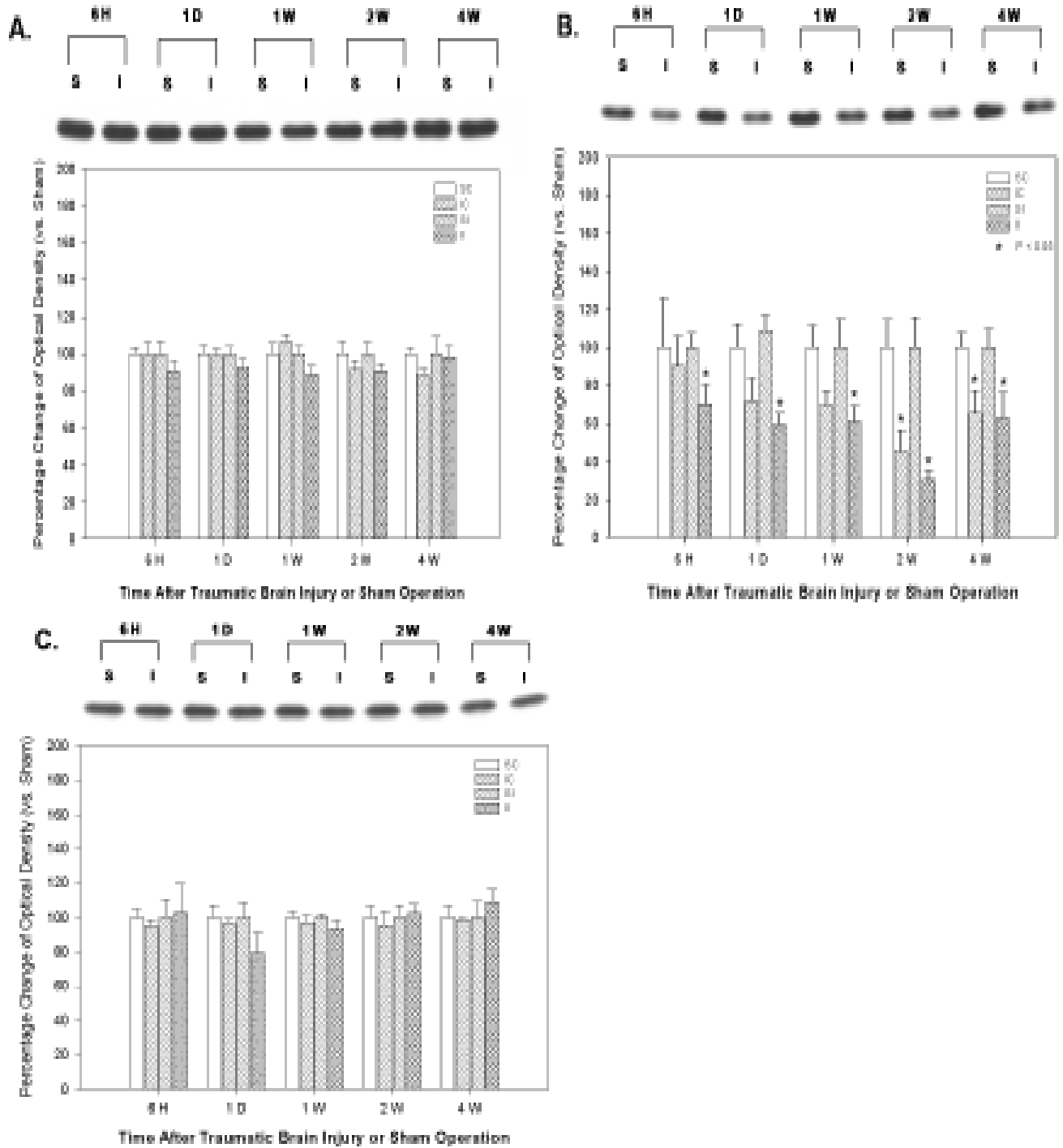


Figure 17. Timecourse of TBI effect on DARPP-32 protein expression and phosphorylation state in rat striatum (N=6 per group at each timepoint). **A**, Representative western blot of ipsilateral striatum (above) and optical density (below) of DARPP-32 protein expression showing no significant effect of TBI on DARPP-32 expression. **B**, Representative western blot of

ipsilateral striatum (above) and optical density (below) of p-DARPP-32-T34 showing a significant reduction in p-DARPP-32-T34 in the striatum ipsilateral to injury at all timepoints examined and in the striatum contralateral to injury at 1 day-4 weeks post injury. **C**, Representative western blot of ipsilateral striatum (above) and optical density (below) of p-DARPP-32-T75 showing no significant change in p-DARPP-32-T75 following TBI. * $p \leq 0.05$ normalized to β -actin and compared to sham; ANOVA with Bonferroni correction. Data represented as a percentage of sham following normalization to β -actin \pm SEM. *Abbreviations: SC = Sham Contralateral to Injury; IC = Injured Contralateral; SI = Sham Ipsilateral to Injury; II = Injured Ipsilateral; S = Sham; I = Injured.*

3.3.2 Striatal Sub-Region Analysis of DARPP-32 Phosphorylation Following TBI

The striatum in rats is comprised of regions that differ in functional relevance to cognitive and motor tasks. The NAcc is important for reward learning (Jones et al., 1990) and addiction (Piazza et al., 1991). The DLStr is important to motor learning and motor control (Nakamura et al., 2001). The phosphorylation state of DARPP-32 plays a critical role in these striatal specific tasks (Meyer-Lindenberg et al., 2007). Utilizing DAB immunohistochemistry of free-floating rat striatal slices we examined the effects of TBI on various sub-region p-DARPP-32-Thr34 (**Figure 18**) levels to assess region specific alterations and validate the decreases in p-DARPP-32-Thr34 identified by western blot analysis (**Figure 17**). Immunohistochemistry for non-phosphorylated DARPP-32 demonstrated no alteration in regional immunoreactivity of DARPP-32 confirming that following TBI there is no change in DARPP-32 expression at examined timepoints (**Figure 18**). Immunohistochemistry for p-DARPP-32-Thr34 showed noticeable reductions in immunoreactivity in the injured ipsilateral and contralateral DLStr compared to sham at 1 day and 2 weeks (**Figure 18**). There appears to be significant decrease in p-DARPP-32-Thr34 reactivity in the NAcc of TBI versus sham, however immunoreactivity in the VMStr

appears increased in injured versus sham (**Figure 18**). In order to better understand the cellular distribution of p-DARPP-32-Thr34 changes following injury an unbiased, computer assisted, threshold, cell counting method was used to characterize the level of cell soma staining in injured versus sham (**Appendix A: Supplemental Figure 1**). Analysis of positive p-DARPP-32-Thr34 in various subregions (N=3 brains, 2 sections from each for a total N=6 for each group) demonstrated no difference between cell staining in the DLStr of injured versus sham, however there was a decrease in the number of p-DARPP-32-Thr34 positive cells in the NAcc of injured versus sham (**Figure 18**) (1day $p < 0.01$; 2week $p < 0.01$.) No p-DARPP-32-Thr34 cell bodies were appreciated in either sham or injured VMStr. Immunohistochemistry for p-DARPP-32-Thr75 showed no changes between sham and injured animals in any striatal sub-region assessed. The computer-assisted, cell counting method did not show any difference in positive DARPP-32 (**Figure 18**). DAB immunohistochemistry confirms the specific alteration in p-DARPP-32-Thr34 seen by western blot (**Figure 18**) following TBI and demonstrates striatal sub-region specific alterations that may have significance for specific persistent striatal deficits after a TBI. No significant alteration in the number of DARPP-32 positive cells indicates that the difference between sham and injured p-DARPP-32-Thr34 cell counts is most likely due to the decrease in DARPP-32 phosphorylation and not due to cellular loss within the NAcc.

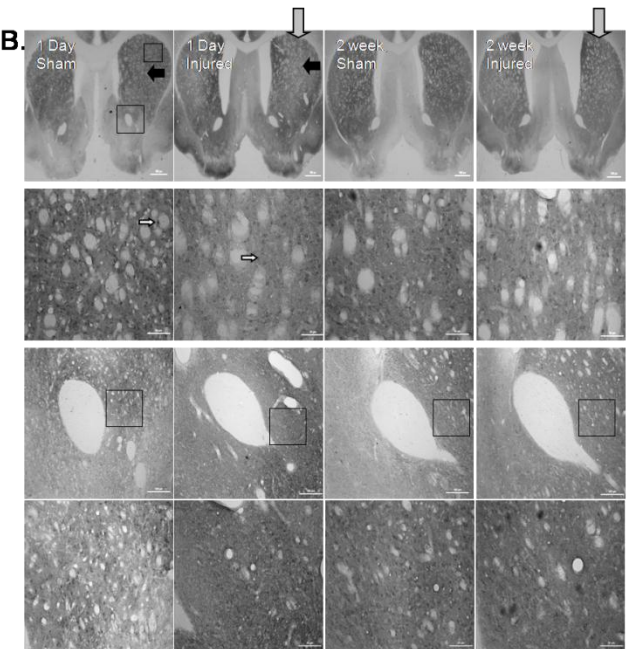
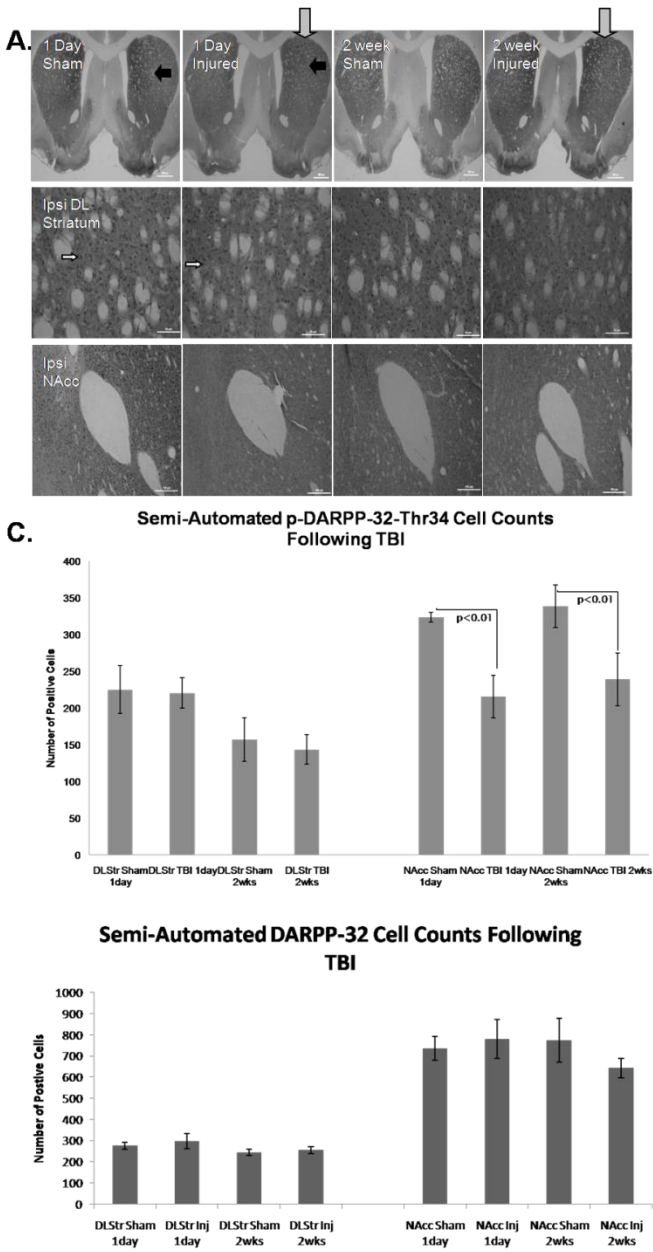


Figure 2. Timecourse of TBI effect on DARPP-32 and p-DARPP-32-T34 DAB immunoreactivity in rat striatum (N=3 per group at each timepoint; boxes indicate area of higher power view shown below). **A**, Representative sections of rat striatum at 1 day and 2 weeks post injury showing no effect of TBI on DARPP-32 expression in either the DLStr or NAcc. **B**, Representative sections of rat striatum at 1 day and 2 weeks showing a reduction in p-DARPP-32-T34 in the neuropil of the DLStr (indicated by black arrows) both contralateral and ipsilateral to injury (side indicated by grey arrow). There also appears to be reduced IR in DLStr MSN cell body staining (indicated by white arrows) in both the DLStr and NAcc at 1 day and 2 weeks post injury. **C**, Semi-automated cell count analysis of 1 day and 2 week timepoints in the ipsilateral DLStr and Nacc showing no significant difference in the number of DARPP-32 positive cells at either timepoint in the DLStr or NAcc. There is a significant difference in the number of p-DARPP-32-Thr34 positive cells in the NAcc in injured compared to sham at both 1 day and 2 weeks post injury. Data represents means \pm standard deviation. $p < 0.01$ compared to sham; single factor two-way ANOVA. Abbreviations: *ipsi* = ipsilateral to injury; *DL* = Dorsolateral; *NAcc* = Nucleus Accumbens.

Figure 18. Timecourse of TBI effect on DARPP-32 and p-DARPP-32-T34 DAB immunoreactivity in rat striatum (N=3 per group at each timepoint; boxes indicate area of higher power view shown below). **A**, Representative sections of rat striatum at 1 day and 2 weeks post injury showing no effect of TBI on DARPP-32 expression in either the DLStr or NAcc. **B**, Representative sections of rat striatum at 1 day and 2 weeks showing a reduction in p-DARPP-32-T34 in the neuropil of the DLStr (indicated by black arrows) both contralateral and ipsilateral

to injury (side indicated by grey arrow). There also appears to be reduced IR in DLStr MSN cell body staining (indicated by white arrows) in both the DLStr and NAcc at 1 day and 2 weeks post injury. C, Semi-automated cell count analysis of 1 day and 2 week timepoints in the ipsilateral DLStr and Nacc showing no significant difference in the number of DARPP-32 positive cells at either timepoint in the DLStr or NAcc. There is a significant difference in the number of p-DARPP-32-Thr34 positive cells in the NAcc in injured compared to sham at both 1 day and 2 weeks post injury. Data represents means \pm standard deviation. $p \leq 0.01$ compared to sham; ANOVA. Abbreviations: *Ipsi* = Ipsilateral to injury; *DL* = Dorsolateral; *NAcc* = Nucleus Accumbens.

3.3.3 Changes in DARPP-32 Phosphorylation in Sub-populations of Medium Spiny Neurons

Following a TBI

Within the striatum there are two independent outflow tracts comprised of two morphologically indistinct MSN populations (Bertran-Gonzalez et al., 2008). It has been shown that these two subpopulations of MSN respond differently to drugs that induce alterations in DARPP-32 phosphorylation (Bateup et al., 2009). We used confocal immunofluorescence to assess if there appeared to be a difference in MSN p-DARPP-32-Thr34 response to TBI (**Figure 19**). At all timepoints post injury there is a noticeable decrease in the immunofluorescence of p-DARPP-32-Thr34 within the injured ipsilateral DLStr compared to sham (**Figure 19**).

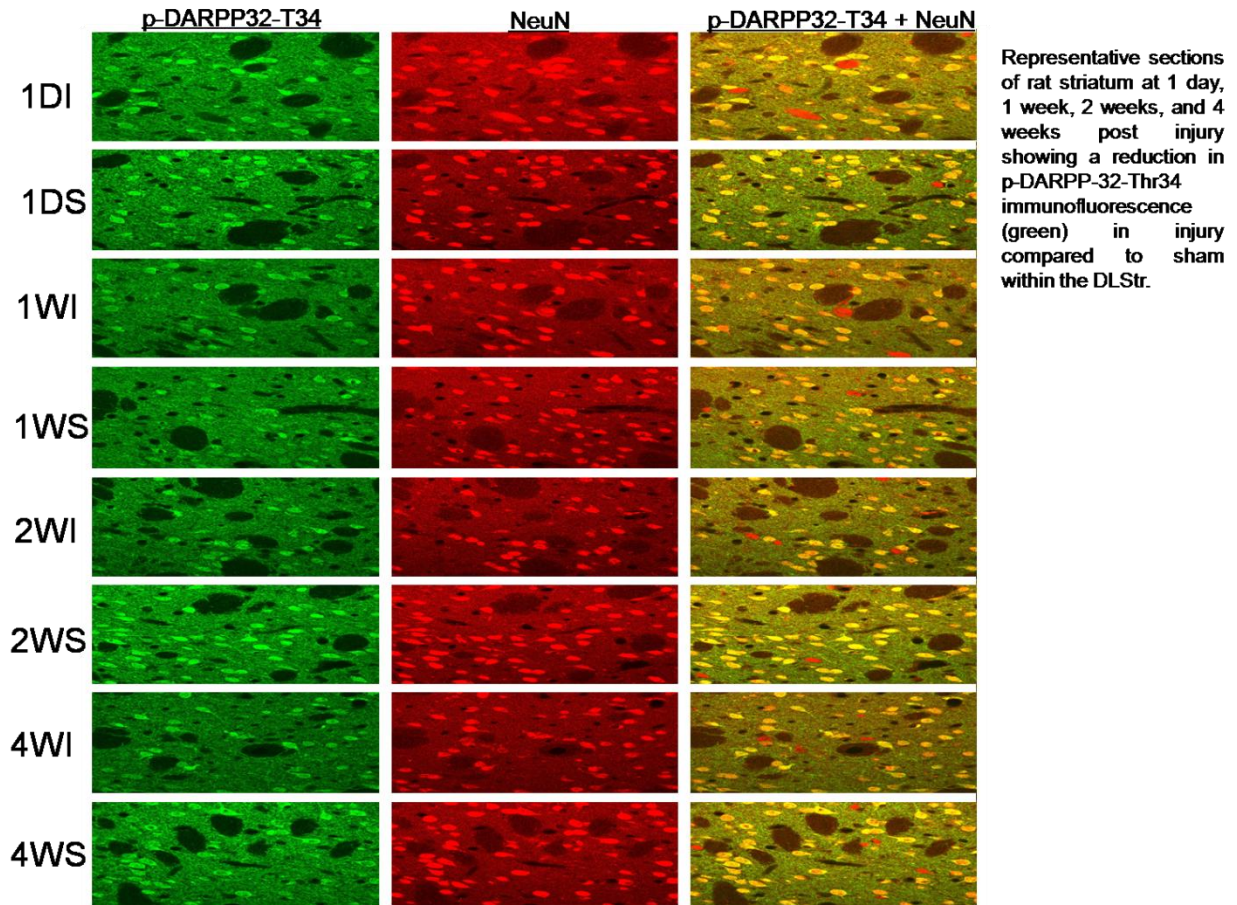


Figure 19. Timecourse of TBI effect on p-DARPP-32-T34 immunofluorescence in rat striatum (N=4 per group at each timepoint). Representative sections of rat striatum at 1 day, 1 week, 2 weeks, and 4 weeks post injury showing a reduction in p-DARPP-32-Thr34 immunofluorescence (green) in injury compared to sham within the DLStr. There does not appear to be a subset of MSN (NeuN red marker) in injured tissue that show a greater reduction in p-DARPP-32-Thr34 cell soma fluorescence. *Abbreviations: D = Day; W = Week; I = injured; S = Sham; MSN = Medium Spiny Neurons; p-DARPP-32-Thr34 = Phosphorylated Dopamine and cAMP regulated phosphoprotein 32 at Threonine-34; TBI = Traumatic Brain Injury.*

3.3.4 Protein Phosphatase-1 Activity is Increased Following TBI

The phosphorylation state of DARPP-32 directly regulates protein phosphatase-1 (PP-1) activity (Hemmings & Greengard 1986). Increases in p-DARPP-32-Thr34 lead to a decrease PP-1 activity while decreases in p-DARPP-32-Thr34 lead to an increase PP-1 activity (Hemmings et al., 1990). Given the decrease in p-DARPP-32-Thr34 seen following injury we analyzed PP-1 activity utilizing a modified protein phosphatase 2A activity assay (**Appendix A: Supplemental Figure 2**) to determine the effect of decreased p-DARPP-32-Thr34 on PP-1 activity following TBI (**Figure 20**). There was a significant increase in PP-1 activity in injured ipsilateral striatum compared to sham at both 1 day ($p < 0.001$) and 2 weeks ($p < 0.01$) following injury (**Figure 20**). At 1 day following injury there was also a significant increase in PP-1 activity in the contralateral injured striatum compared to sham ($p < 0.01$) that was not seen at 2 weeks post injury (**Figure 20**). Increases in PP-1 activity are consistent with the loss of p-DARPP-32-Thr34 seen following TBI indicating that decreases in p-DARPP-32-Thr34 after TBI are part of a persistent dysfunction in intracellular signaling cascades.

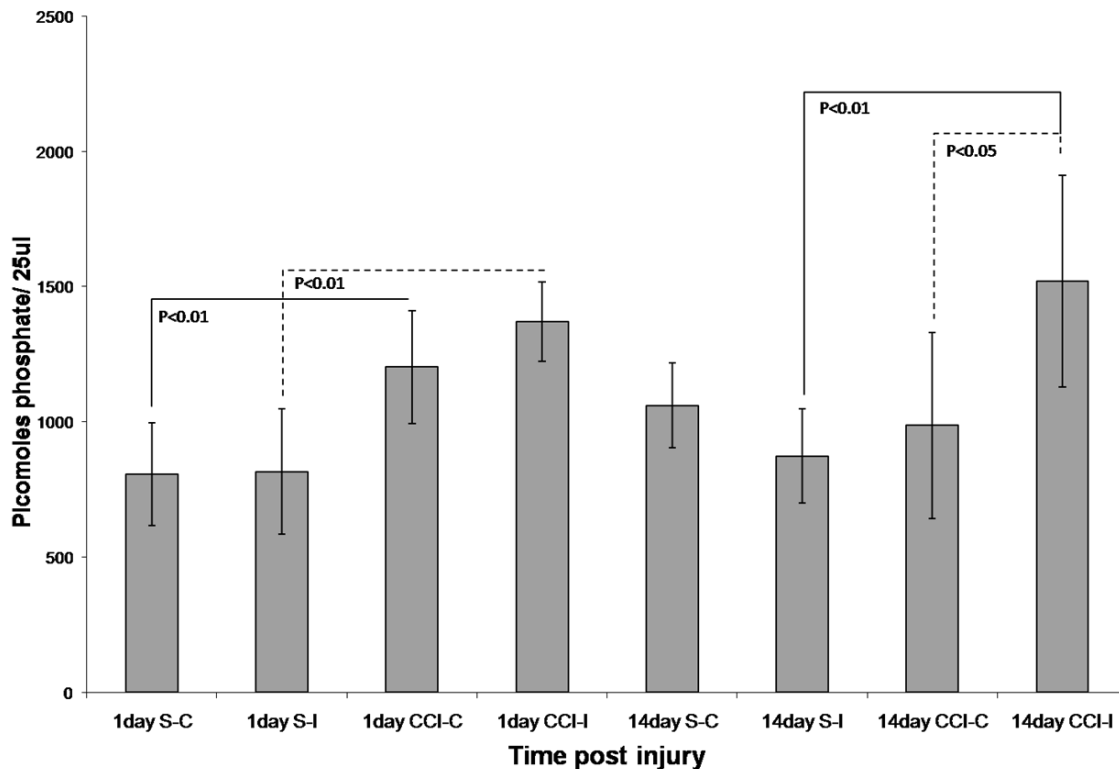


Figure 20. Analysis of protein phosphatase-1 (PP-1) activity in homogenized rat striatum. There is a significant increase in PP-1 activity in both the contralateral (N=6/group; $p<0.01$; ANOVA) and ipsilateral (N=6/group; $p<0.01$; ANOVA) striatum compared to sham at 1 day post injury. By 2 weeks post injury there remains a significant increase in PP-1 activity in the ipsilateral (N=6/group; $p<0.01$; ANOVA) striatum compared to sham. Increases in PP-1 activity are consistent with decreases in p-DARPP-32-T34. Data represents means \pm standard deviation. *Abbreviations: S = Sham, C = Contralateral, I = Ipsilateral; CCI = Controlled Cortical Impact.*

3.3.5 Acute decrease followed by a chronic increase in PKA activity following a TBI

Protein kinase A (PKA) is a key modulator of DARPP-32 signaling, but also plays an intrinsic role in downstream signaling events independent of DARPP-32 action. This includes PKA mediated activation of ERK through the mitogen activated protein kinase pathway and PP1

through a rho kinase pathway (Waltereit et al., 2003; Nairn et al., 2004). Alterations in PKA activity could potentially explain changes in p-DARPP-32/Thr34 levels and could play a synergistic role in downstream dysfunction of nuclear signaling pathways. We utilized a commercially available PKA activity kit to determine PKA activity at 1 day and 2 weeks post injury (n = 6 animals per group). There was a significant decrease in PKA activity at 1 day post TBI in ipsilateral striatum ($p < 0.05$) compared to sham ipsilateral striatum. There was also a significant decrease at 1 day in contralateral striatum PKA activity post TBI in injured ($p < 0.01$) versus sham. At 2 weeks post injury PKA activity in injured striatum both contralateral ($p < 0.01$) and ipsilateral ($p < 0.01$) was increased significantly compared to the corresponding sham striatal PKA activity levels (**Figure 21**). An acute decrease in PKA activity post TBI is consistent with decreases in both p-DARPP-32/Thr34 and increases in PP1 activity. However, a chronic increase in PKA activity moves counter to the observed changes in DARPP-32 phosphorylation and PP-1 activity. A chronic increase in PKA activity may represent an attempt by the striatal system to normalize downstream dysfunction or ongoing upstream pathology.

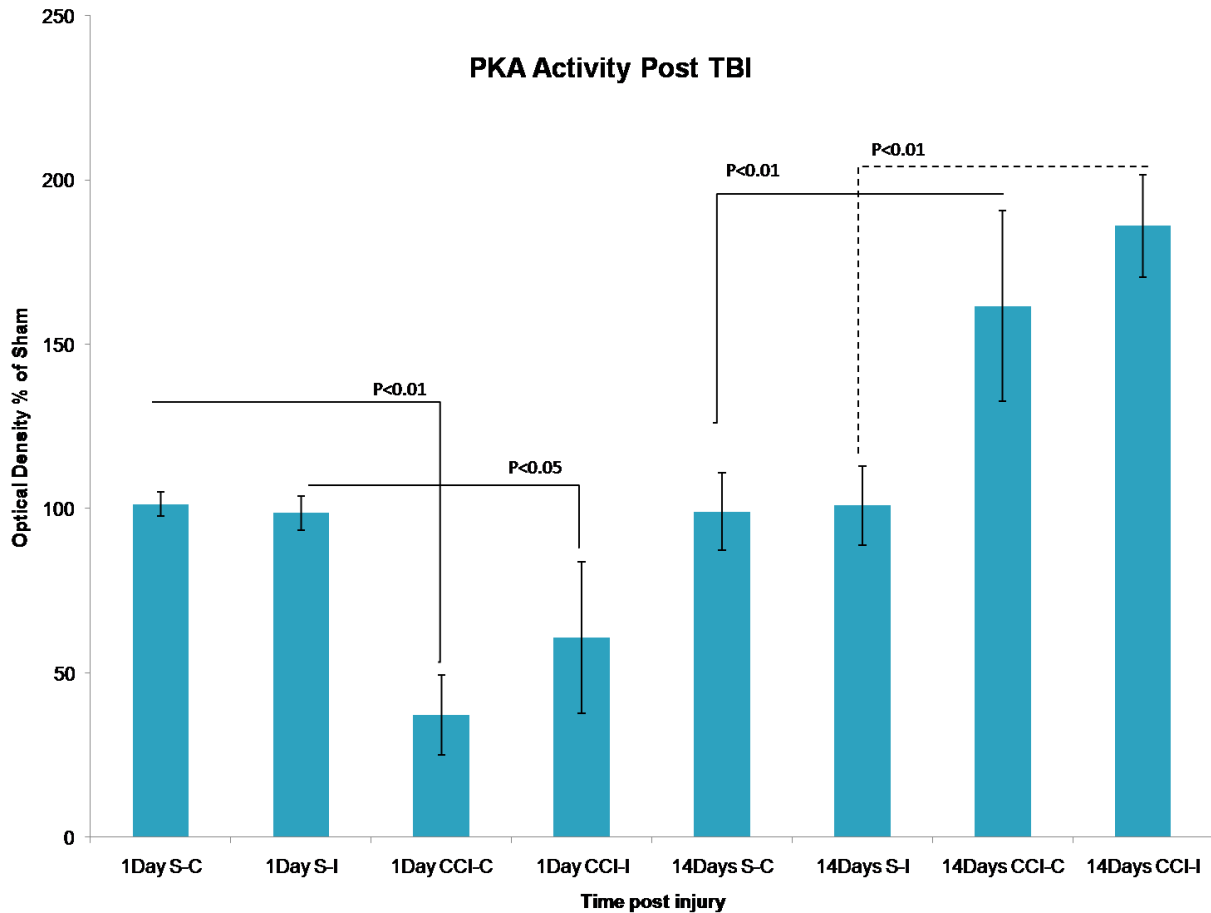


Figure 21. Analysis of protein kinase A (PKA) activity in homogenized rat striatum. There is a significant decrease in PKA activity in both the contralateral (N=6/group; $p < 0.01$; ANOVA) and ipsilateral (N=6/group; $p < 0.05$; ANOVA) striatum compared to sham at 1 day post injury. By 2 weeks post injury there is a significant increase in PKA activity in both the contralateral and ipsilateral (N=6/group; $p < 0.01$; ANOVA) striatum compared to sham. Data represents means \pm standard deviation. *Abbreviations: S = Sham, C = Contralateral, I = Ipsilateral; CCI = CCI.*

3.3.6 Significant decrease in ERK phosphorylation in the nuclear fraction of striatal homogenates chronically post TBI.

ERK 1/2 and CREB phosphorylation are downstream targets of both PKA and PP-1 activity (Greengard et al., 1999; Waltereit et al., 2003). By assessing these two important cell signaling regulators we will be able to get a better idea of what signaling pathologies are associated with the observed changes in PKA activity and DARPP-32 phosphorylation alterations. Furthermore, changes in ERK 1/2 and CREB phosphorylation are important to nuclear transcription and synaptic plasticity. Western blot analysis of p-ERK 1/2 to ERK 1/2 and p-CREB (Ser133) to CREB levels in injured animals versus sham (n= 6 per group) demonstrated no significant differences between p-ERK or p-CREB levels at either 1 day or 2 weeks post injury. This may indicate that dysfunction in PKA and DARPP-32 signaling may be affecting a different set of downstream targets, may not be severe enough to overcome the multitude of other signaling events that regulate ERK and CREB, or we may not be able to detect alterations in p-ERK 1/2 and p-CREB in whole cell lysates.

Whole cell lysates demonstrated no difference in ERK 1/2 or CREB phosphorylation. Phosphorylated ERK 1/2 and CREB are predominantly within the nucleus so we prepared nuclear extracts in order to further examine potential alterations in phosphorylation state. An increase in PP-1 activity should theoretically lead to a reduction in ERK 1/2 phosphorylation and subsequent reduction in CREB phosphorylation. Western blot analysis of striatal nuclear extracts demonstrated a significant decrease in ipsilateral (relative to injury) ERK 1/2 Thr202/Tyr204 (pERK 1/2) phosphorylation ($p < 0.05$) in TBI animals versus sham (n=6 per group) at 3 weeks post injury (**Figure 22**). There was no significant decrease in CREB phosphorylation in injured versus sham animals (**Figure 22**).

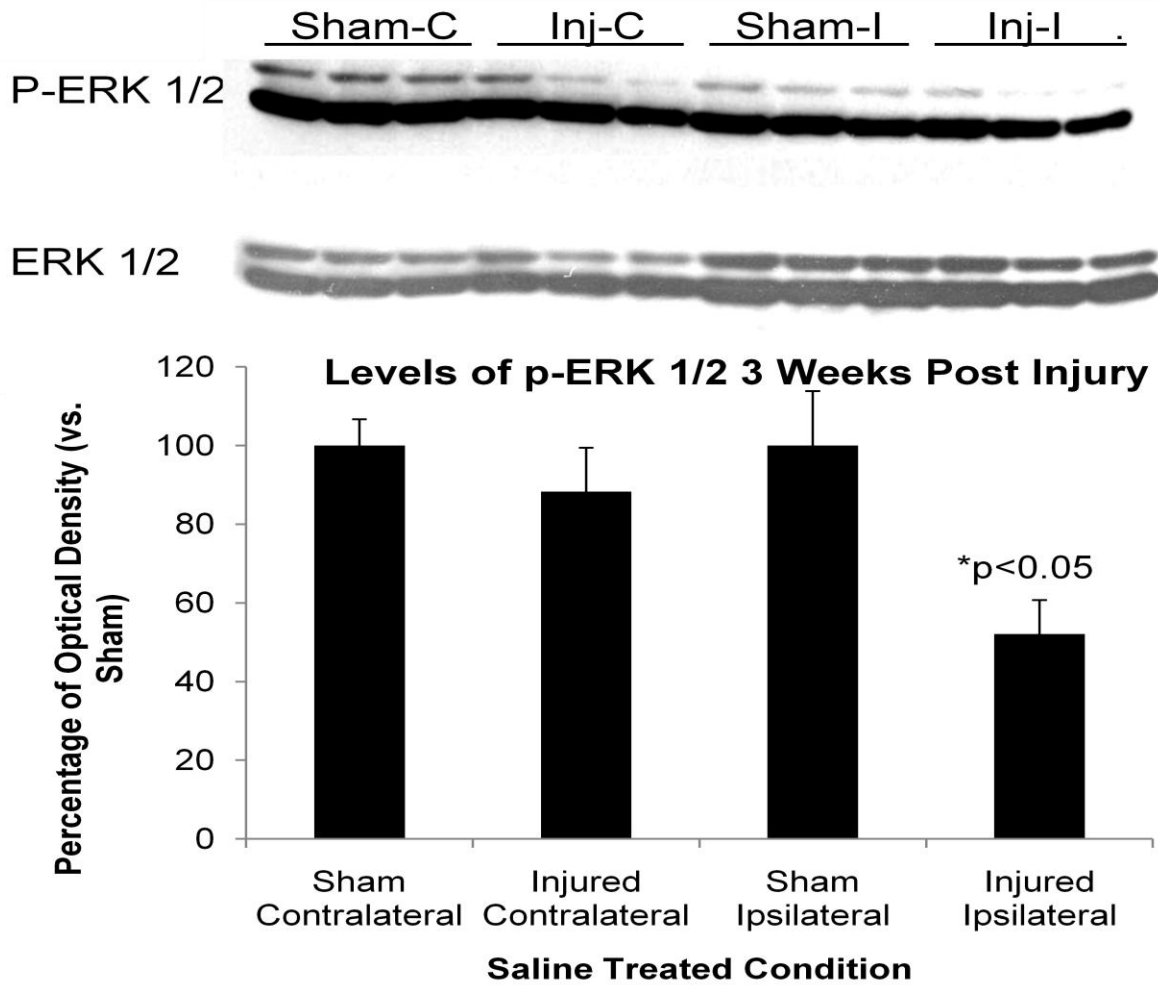


Figure 22: Analysis of nuclear fraction ERK 1/2 expression and Thr202/Tyr204 (p-ERK 1/2) phosphorylation in the striatum at 3 weeks post experimental TBI. Densitometry of p-ERK 1/2 normalized to total ERK 1/2 expression shown below with a representative western blot above. There was a significant decrease in p-ERK 1/2 expression in the injured ipsilateral striatum compared to sham (ANOVA; $p < 0.05$). The decrease appears to be predominantly within the Thr202 form (p-ERK 1). Data represents mean + SEM; $n = 6$ per group.

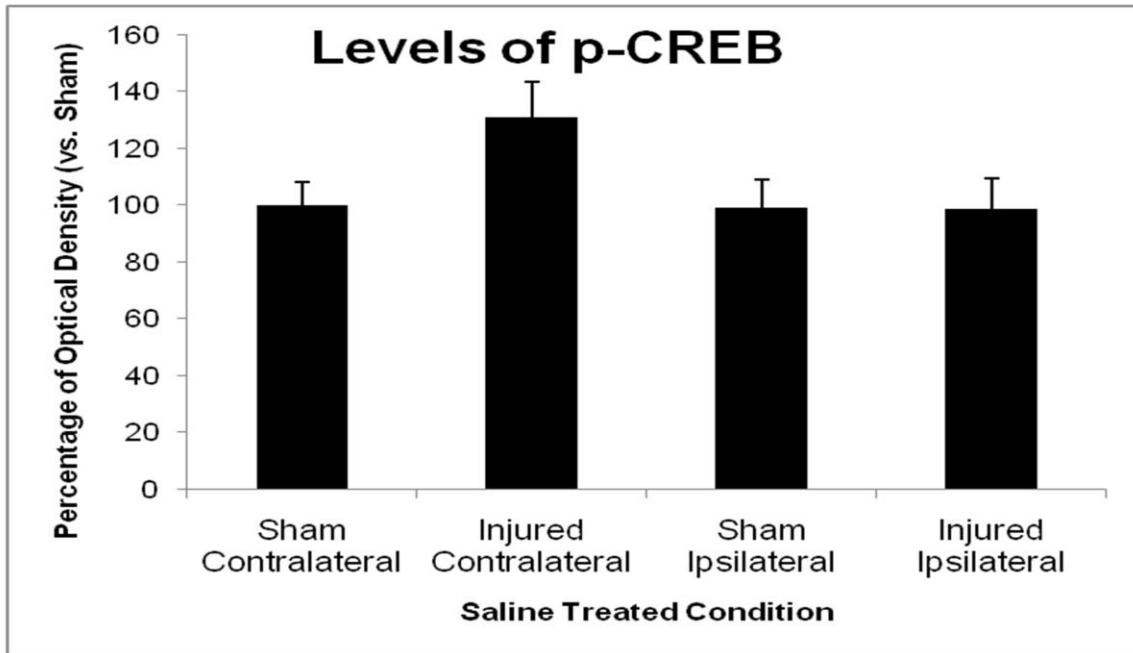
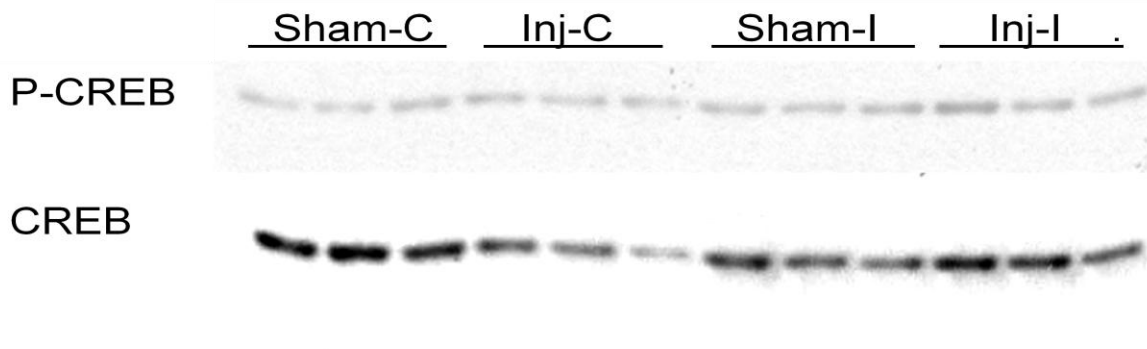


Figure 23: Analysis of nuclear fraction CREB expression and p-CREB (Ser133) phosphorylation in the striatum at 3 weeks post experimental TBI. Densitometry of p-CREB normalized to total CREB expression shown below with a representative western blot above. There was no significant alteration in p-CREB expression after injury. Data represents mean + SEM; n = 6 per group.

3.4 DISCUSSION

TBI remains a leading cause of disability in industrial countries and has few effective therapies. Identification of novel, effective therapeutic targets that can be quickly translated into clinical

practice is necessary. The aims of the present study were to evaluate the striatally enriched phosphatase DARPP-32 as a novel target for TBI induced dysfunction. We tested the hypothesis that TBI induces an acute, persistent dysfunction in DARPP-32 signaling in the striatum.

TBI induces a decrease in the phosphorylation of DARPP-32 at Thr34 within striatal MSN that occurs acutely and persists for all examined timepoints. The decrease in phosphorylation at Thr34 is not due to downregulation or loss of DARPP-32 protein expression (**Figure 17**), nor is it indicative of non-specific dephosphorylation of DARPP-32 as indicated by the lack of change in Thr75 phosphorylation (**Figure 17**). While loss of Thr34 phosphorylation is primarily observed in the striatum, it does become apparent in the FC at later timepoints (**Appendix A: Supplemental Figure 3**) indicating that the affect of TBI on the phosphorylation state of DARPP-32 is ubiquitous, with the striatum being the most profoundly affected.

The loss of p-DARPP-32-Thr34 seen following a TBI is not restricted to a single area of the striatum (**Figure 18**). There is a noticeable decrease in p-DARPP-32-Thr34 immunoreactivity within both the DLStr and the NAcc (**Figure 18**). Interestingly cell count analysis (**Figure 18**) indicates that in the DLStr the reduction in immunoreactivity is predominantly within the neuropil suggesting that p-DARPP-32-Thr34 decreases in the DLStr are within the dendritic trees of the MSNs. However, within the NAcc, cell count analysis shows a persistent loss of cell soma immunoreactivity for p-DARPP-32-Thr34 (**Figure 18**) indicating that these two regions may be differentially affected by TBI. Analysis of DARPP-32 positive cells (**Figure 18**) shows that the loss of immunoreactivity is not due to TBI causing a loss of DARPP-32 positive neurons within the DLStr or NAcc.

The MSNs within the striatum are composed of populations that have distinct signaling pathways and function (Fink et al., 1992; Gerfen et al., 1990). Prior research has shown that drugs of abuse have predominant effects on one subset of neurons as opposed to the other. For example, both cocaine and haloperidol increase the phosphorylation of DARPP-32 at Thr34, however these two drugs have very different consequences on the ERK signaling pathways in the distinct MSN populations (Bertran-Gonzalez et al., 2008). Confocal fluorescent images suggest that in TBI the neuronal subpopulations are equally affected, indicating a global dysfunction in DARPP-32 phosphorylation which would be consistent with glutamatergic input to both sets of neurons (**Figure 19**). MSN decreases in p-DARPP-32-Thr34 within the DA receptor D2 predominant neurons could explain TBI literature indicating that while amphetamines are beneficial after TBI, haloperidol, a potent D2 inhibitor, is detrimental (Kline et al., 2008; Hoffman et al., 2008). This suggests that TBI induced alterations in DARPP-32 signaling may affect all striatal functions, both motor and cognitive, and further examination may help predict drugs with detrimental effects similar to haloperidol.

Consistent with the alteration in DARPP-32 phosphorylation there is a significant increase in PP1 activity at examined timepoints (**Figure 20**), indicating that the loss of Thr34 phosphorylation causes an alteration in relevant downstream DARPP-32 signaling and is not merely an isolated dephosphorylation event without effect on the signaling pathway. A persistent increase in PP-1 activity following injury has significant implications for MSN function. Increased PP-1 activity leads to a decrease in p-CREB and subsequent reduction in nuclear transcription of numerous proteins including neurotrophic factors and cell survival signals (Genoux et al., 2002; Choe et al., 2005). Furthermore, PP-1 activity is important to the regulation of glutamatergic signaling and synaptic plasticity (Centonze, et al., 2001; Kopnisky et al., 2003). We examined ERK 1/2 and CREB phosphorylation at both 1 day and 2 weeks post injury and found no significant difference in phosphorylation within injured animals compared to

sham in whole cell lysates. However, in a separate experiment we examined the nuclear fraction of striatal homogenates at 3 weeks and found significantly reduced ERK 1/2 phosphorylation within the ipsilateral injured striatum compared to sham (**Figure 22**). Whole cell lysates may have hidden the alterations in CREB and ERK 1/2 phosphorylation after injury and the utilization of a nuclear extraction allowed us to examine potential phosphorylation alterations more carefully. Future examination of nuclear extracts at other timepoints may demonstrate CREB and ERK1/2 alterations acutely. A chronic decrease in ERK 1/2 phosphorylation indicates that there is ongoing cell signaling pathology that is critically relevant for synaptic plasticity and nuclear transcription.

This is the first time that DARPP-32 and PP-1 have been examined following a TBI. Given the importance of the DARPP-32/PP-1 signaling pathway within the striatum and frontal cortex and its role in behavior, an alteration in function has significant implications for prolonged motor and cognitive dysfunction in TBI patients.

Multiple lines of evidence have previously implicated DA as an important player in persistent cognitive dysfunction following TBI. Animal studies have consistently demonstrated benefits in post-TBI cognitive tasks including learning and memory following the administration of DAergic agonists (Dixon et al., 1999; Kline et al., 2004; Wagner et al., 2005).

In this study we have shown an intrinsic alteration in signaling pathway of striatal MSN. The alteration in DARPP-32 phosphorylation causes the predicted alteration in the downstream signaling target PP1. Persistent loss of DARPP-32 Thr34 phosphorylation in the striatum and subsequent alterations in PP1 activity have widespread implications to striatal MSN function. After hypoxia-ischemia injury, DARPP-32 phosphorylation states were shown to be important to membrane potential, glutamate receptor activity, and oxidative stress (Yang et al., 2007).

DARPP-32 acts as a signal transduction molecule with multiple neurotransmitter groups modifying DARPP-32 phosphorylation and relevant downstream signaling. DA, glutamate, acetylcholine, and calcium signaling all play a role in modulating DARPP-32 within the striatum (Hamada et al., 2005; Valjent et al., 2005; Ahn et al., 2007). Following TBI there is well documented increases in glutamate release and calcium influx that occur almost immediately following injury and contribute to persistent cellular dysfunction (Hovda et al., 1990; Shapira et al., 1989; Fineman et al., 1993). Examination of DARPP-32 allows for the analysis of a central signaling molecule that is responsive to nearly all of the acute and secondary injuries that persist in TBI. The pathology of DARPP-32 alterations that persist following TBI allow for a better understanding of ongoing cellular pathology. We have demonstrated a specific decrease in the phosphorylation of DARPP-32-Thr34 and corollary increase in PP-1 activity (**Figure 17 and Figure 20**) within the striatum. A number of possible mechanisms exist that could explain the alteration in DARPP-32 signaling including specific changes in neurotransmitter systems, such as a decrease in DA signaling or increases in glutamatergic release. There could also be neurotransmitter independent intracellular events such as calcium release or changes in phosphatase and kinase activity.

Alterations in PKA activity could also lead to a change in DARPP-32 phosphorylation and could independently affect PP-1 activity (Waltereit et al., 2003). To evaluate this possibility we examined PKA activity at both 1 day and 2 weeks post TBI. Unlike DARPP-32 phosphorylation which stayed consistently depressed over the timecourse evaluated and PP-1 activity which stayed consistently increased at 1 day and 2 weeks, PKA demonstrated a timepoint dependent alteration in activity. At 1 day post injury PKA was significantly decreased in both the ipsilateral and contralateral striatum (**Figure 21**) which is consistent with both the decrease in p-DARPP-32-Thr34 and an increase in PP-1 activity (Greengard et al., 1999; Nairn et al., 2004). However,

at the more chronic timepoint of 2 weeks PKA is significantly increased in both the ipsilateral and contralateral injured striatum compared to sham. This may explain why PP-1 activity within the contralateral striatum is not significantly increased at 2 weeks given that an increase in PKA activity would theoretically lead to decreased PP-1 activity. The increase in PKA activity in the ipsilateral striatum may be unable to overcome more significant pathology within this side of the injured striatum. Further evidence of ongoing pathology within the ipsilateral striatum is confirmed by a significant reduction in ERK phosphorylation within the ipsilateral nuclear fraction at a 3 week timepoint (**Figure 22**). This suggests that the ongoing dysfunction in DARPP-32/PP-1 pathway signaling continues to play an important role in chronic cell signaling even with events that should act to increase downstream activity.

Given the complexities of TBI, the identification of acute and persistent DARPP-32 alterations after TBI is most likely indicative of multiple ongoing disease processes. Thus, treatments aimed at molecular targets that are integrative, such as DARPP-32, allow for targeted therapeutics that can potentially correct signaling deficiencies through multiple mechanisms.

3.5 CONCLUSION

This study provides the first evidence of a TBI induced alteration in DARPP-32 signaling in the striatum of injured animals. The data presented indicates that the decrease in p-DARPP-32-Thr34 following TBI leads to a functionally relevant alteration in downstream PP-1 activity and is relatively striatal specific. Furthermore, there appears to be a difference in the response of striatal subregions (DLStr vs. NAcc). The present results further implicate persistent striatal cellular dysfunction in prolonged cognitive difficulties seen following TBI.

4. TARGETING STRIATAL DYSFUNCTION: THERAPEUTIC INTERVENTIONS

4.1 INTRODUCTION

In the prior chapter we established that following TBI there is a significant, persistent dysfunction in striatal DARPP-32 signaling. While the neurological and cognitive consequences of TBI are numerous and complex, the most common include memory impairment and difficulties with attention and concentration, functions that are mediated by multiple brain structures. The striatum is functionally important for both cognitive and motor behaviors (Divac et al., 1967; Ding et al., 2001). Focal damage to the striatum in non-human primates and rats results in difficulties with spatial working memory (Miyoshi et al., 2002). Studies investigating dementia in Parkinson's (PD) and Huntington's disease (HD) indicate the striatum is part of a neuronal network mediating prefrontal executive functions (Backman et al., 1997; Duchesne et al., 2002). The striatum is also particularly vulnerable to damage from TBI including axonal degeneration, neuronal cell loss, and ischemia (Dietrich et al., 1998). Functionally, the striatum acts as a convergence point for signals from multiple systems. Glutamatergic projections from the frontal lobes of the cortex and DAergic projections from the substantia nigra pars compacta both synapse upon the predominant neuronal cell type within the striatum - medium-sized, spiny, GABA-ergic neurons (Nishi et al., 2002; Nairn et al., 2004).

DA induced phosphorylation of ionotropic glutamate and GABA receptors is attenuated in DARPP-32 KO mice suggesting that DARPP-32 plays an important role in regulating excitotoxicity (Flores-Hernandez et al., 2002; Borgkvist et al., 2007). Ethanol reinforcement, which is known to act through regulation of NMDA receptors, is also reduced in DARPP-32 KO models

(Risinger et al., 2001). Studies assessing the behavioral affects of other drugs of abuse, including cocaine and morphine, have shown that DARPP-32 KO mice demonstrate lower levels of psychomotor activation following drug administration compared to wild-type mice (Borgkvist et al., 2007). Further evidence using KO mice indicates that DARPP-32 has an important role in long-term depression (LTD) and long-term potentiation (LTP), both important processes in models of memory acquisition and consolidation which represents areas of dysfunction common to TBI patients.

This study represents the first to examine DARPP-32 phosphorylation as a therapeutic target following a TBI. DA targeted therapies assist in cognitive recovery, but remain unable to completely restore function and patient response can be variable. A therapeutic approach utilizing a combined drug strategy to affect multiple components of the DARPP-32 system as well as other brain regions may overcome current therapeutic limitations.

DARPP-32 has previously been proposed as a therapeutic target in Parkinson's Disease (PD) (Coccorello et al., 2004), given its importance to both motor and cognitive functions of the striatum. In addition to DA agonists, NMDA antagonists, such as Amantadine Hydrochloride (AMH), are potential mediators of DARPP-32 signaling in PD (Greenamyre and O'Brien, 1992).

AMH has known DAergic action that has shown both experimental and clinical benefits in cognitive recovery following TBI (**Table 2**). The experimental administration of FK-506 has been shown to improve cognition following TBI through decreases in white matter damage with FK-506 administration (Butcher et al., 1997; Reeves et al., 2007). Furthermore FK-506 is a potent calcineurin inhibitor and calcineurin is directly involved in regulating the phosphorylation state of DARPP-32. By utilizing a combined treatment approach with FK-506 acutely and AMH chronically we hypothesize that we will be able to produce a synergistic effect within the striatum

providing improvements in DARPP-32 phosphorylation state and cognitive outcome above either drug alone. **Figure 24** demonstrates the theoretical affect of a combined treatment strategy of FK-506 and AMH on DARPP-32 within the striatum.

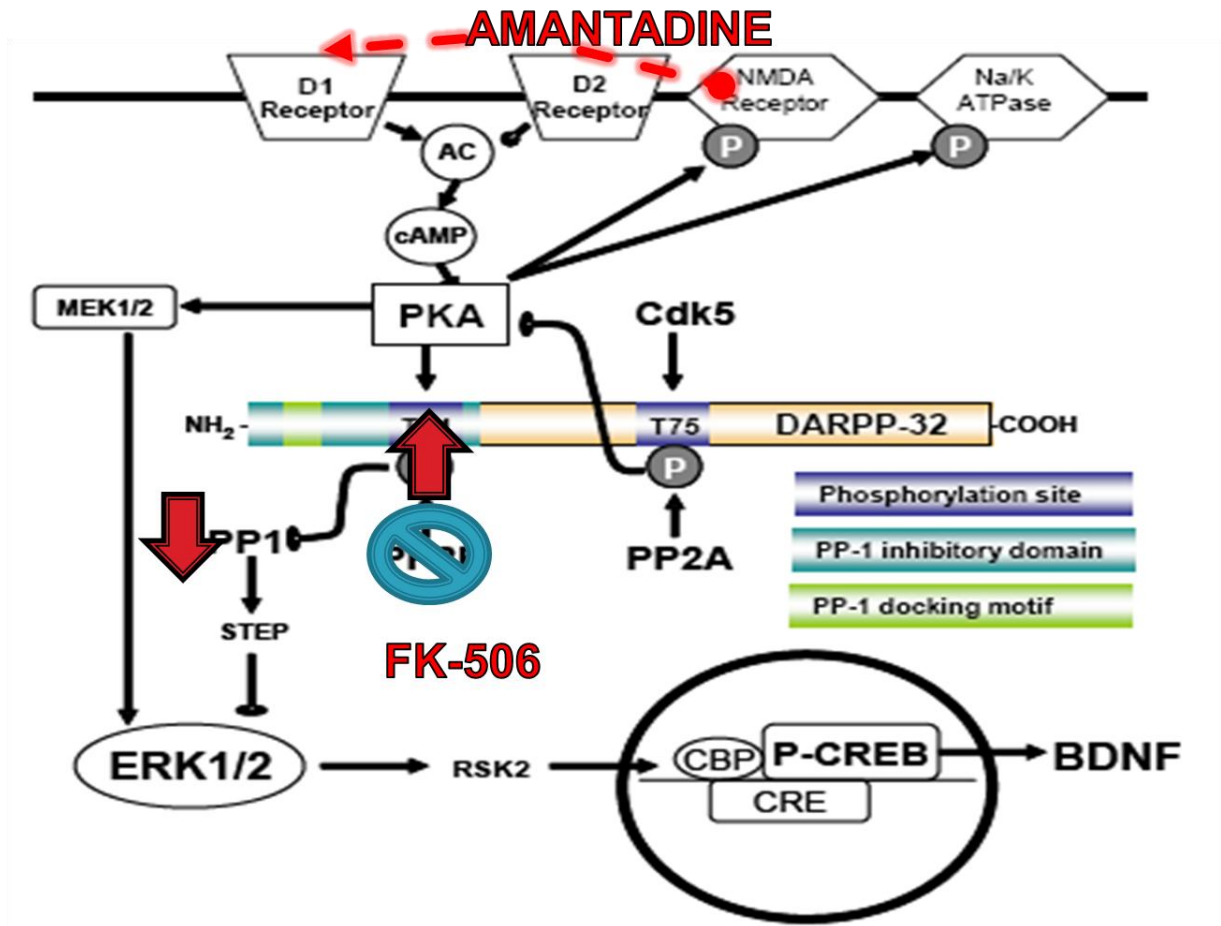


Figure 24: The combination therapy of Amantadine (AMH) and FK-506 should additively act to increase DARPP-32-Thr34 phosphorylation and decrease protein phosphatase-1 (PP-1) activity. The inhibition of NMDA receptors and activation of D1 receptors is a theoretical mechanism for AMH action that coupled with the known inhibition of calcineurin by FK-506, would theoretically increase DARPP-32-Thr34 phosphorylation above the use of either drug alone.

We hypothesize that chronic administration of AMH (10 mg/kg i.p.) will attenuate TBI induced alterations in DARPP-32 phosphorylation. We hypothesize that a single (at 5 mins post injury) administration of FK-506 (2 mg/kg i.p.) will reverse TBI induced alterations in DARPP-32 phosphorylation. We hypothesize that the DARPP-32 phosphorylation effects of FK-506 and AMH will be dose dependent. We hypothesize that a combined treatment of chronic AMH with acute FK-506 will further reverse TBI induced alterations in DARPP-32 phosphorylation and its targets and show a synergistic improvement of cognitive recovery as assessed using the Morris water maze task. This represents the first study to analyze a combination therapy of FK-506 and Amantadine to address long term cognitive dysfunction following TBI.

4.2 METHODS AND MATERIALS

4.2.1 Animals

Adult male Sprague-Dawley rats ($N = 152$) were used in the study. Rats (275-325 g) were purchased from Hilltop Laboratories (Scottsdale, PA, USA) and housed in pairs under a 12:12 light/dark cycle. Rats were given food and water with *ad libitum* throughout the study. All experiments were carried out in accordance with the University of Pittsburgh's guidelines for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

4.2.2 Surgical Procedures for TBI

On the day of surgery anesthesia was initiated with 4% isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL, USA) and 2:1 N₂O/O₂. Rats were intubated and maintained on 1.5-2% isoflurane during the surgical procedure. Following intubation, rats were placed on a thermal

blanket to regulate body temperature (37°C) and the animals head placed in a stereotaxic frame. An incision was made down the midline of the skull and the soft tissues and periosteum deflected. A craniotomy was then performed over the right parietal bone to expose the dura. Controlled cortical injury (CCI, Pittsburgh Precision Instruments, Inc.) at a depth of 2.6mm at 4m/sec was carried out as previously reported. A total of 100 rats were injured, and the remaining 52 rats were shams. Righting reflex was monitored in the immediate post-surgical period to assess acute recovery.

4.2.3 Drug Treatment Protocols

AMH Treated Animals for dosage study and protein phosphatase-1 assay: For the 6 hour AMH timepoint: 30 mins post CCI surgery animals received an i.p. injection of AMH (10 mg/kg; Sigma, St. Louis, MO) dissolved in 0.9% physiological saline (n = 6) or saline alone (n = 6); sham animals received only saline (n = 6). For the 2 week AMH timepoint: beginning one day post CCI surgery animals received daily i.p. injections of AMH (10 mg/kg or 5 mg/kg or 1 mg/kg; Sigma, St. Louis, MO) dissolved in physiological saline (n = 18) or saline alone (n = 6); sham animals received only saline (n = 6). AMH was made fresh weekly.

FK-506 Treated Animals for dosage study: For the 6 hour FK-506 timepoint: 5 mins post CCI surgery animals received an i.p. injection of FK-506 (2.0 mg/kg) dissolved in 0.9% physiologic saline (n = 6) or saline alone (n = 6); sham animals received only saline (n = 6). For the 2 week FK-506 timepoint: 5 mins post CCI surgery animals received an i.p. injection of FK-506 (2.0 mg/kg or 1.0 mg/kg or 0.5 mg/kg) dissolved in 0.9% physiologic saline (n = 18) or saline alone (n = 6); sham animals received only saline (n = 6). FK-506 was diluted from stock on day of use.

Eighty rats (n = 20/treatment; 10 sham and 10 injured as described in **Table 6**) underwent CCI.

At 5 minutes post injury FK-506 (1.0 mg/kg i.p.) was administered to the FK-506 alone and combined groups (n = 40 total). Beginning at 24 hours post injury AMH (5 mg/kg i.p.) was administered to the AMH alone and combined group rats (n = 40 total) daily for 18 days. Combination therapy included the single injection of FK-506 with chronic AMH. Saline treated animals were used for controls. At days 1-5 post injury animals underwent motor testing at days 14-19 post injury all animals underwent Morris water maze testing.

TABLE 6: Protocol for combination therapy following TBI	Treatment:				
	Saline (daily i.p.)	FK-506 (1.0 mg/kg i.p. once 5 mins post injury)	AMH (5.0 mg/kg i.p. daily)	Combined (Single dose FK-506 plus daily AMH)	
Analysis:					
Histology	N=4	N=4	N=4	N=4	
Excised tissue for western blot analysis of DARPP-32, PP1, PKA	N=6	N=6	N=6	N=6	
Total N for motor and cognitive tasks:	10	10	10	10	80 total

4.2.4 Motor Performance

Established beam-balance and beam-walk tasks were used to assess motor function. Briefly, the beam-balance task consists of placing the rat on an elevated (90 cm) narrow beam (1.5 cm wide) and recording the time it remains on up to a maximum of 60 sec. The beam-walk task, originally devised by Feeney and colleagues (1982), consists of training/assessing rats using a negative-reinforcement paradigm to escape a bright light and white noise by traversing a narrow elevated beam (2.5 × 100 cm), and entering a darkened goal box situated at the opposite end. When the rat entered the goal box the adverse stimuli (light and noise) were terminated, thus serving as negative reinforcement (reward) for completing the task. Performance was assessed by recording the time it took for the rat to traverse the beam. The rats were trained on both

motor tasks 1 day prior to surgery, and baseline performance was assessed on the day of surgery. Post-surgery motor function was assessed on postoperative days 1–5. Balance and traversal times were recorded. Each rat was given three trials (60 sec allotted time with an inter-trial interval of 30sec) per day on each task. The average daily scores for each subject were used in the statistical analyses.

4.2.5 Cognitive Function

Spatial learning was assessed for five consecutive days (postoperative days 14–18) in a Morris water maze (MWM) task established as a sensitive measure of cognitive function after TBI (Hamm et al., 1992; Hoffman et al., 2008; Kline et al., 2001, 2002; Scheff et al., 1997). Briefly, the maze consisted of a plastic pool (180 cm diameter and 60 cm high) filled with tap water ($26 \pm 1^\circ\text{C}$) to a depth of 28 cm that was situated in a room with salient visual cues that remained constant throughout the study. The platform was a clear acrylic glass stand (10 cm diameter and 26 cm high) that was positioned 26 cm from the maze wall in the southwest quadrant and held constant for each rat. Each rat was given a block of four daily trials (120 sec allotted time with an inter-trial interval of 4 min) to locate the platform when it was submerged 2 cm below the water's surface (i.e., invisible to the rat). To control for the contributions of non-spatial factors (e.g., sensorimotor performance, motivation, and visual acuity) on maze performance, each rat was provided with two additional day (postoperative days 19 and 20) of testing to locate the platform when it was raised 2 cm above the water's surface (i.e., visible to the rat). For each daily block of trials the rats were placed in the pool facing the wall at each of the four possible start locations (north, east, south, and west) in a randomized manner. Each trial lasted until the rat climbed onto the platform or until 120 sec had elapsed, whichever occurred first. Rats that failed to locate the platform within the allotted time were manually guided to it by the experimenter. All rats remained on the platform for 30 sec before being placed in a heated incubator between trials. The average time of the four daily trials for each rat were used in the statistical analyses.

To measure retention of spatial learning all rats were given a single probe trial on postoperative day 19, which was 1 day after the final acquisition training session. Briefly, the platform was removed from the pool and the rats were placed in the maze at a location most distant from the quadrant where the platform was previously situated (i.e., the target quadrant) and allowed to freely explore the pool for 30 sec. In principle, rats that have learned the specific location of the hidden escape platform will exhibit a spatial bias and thus spend significantly more time in the target quadrant. All the data, which included time to locate the platform, distance to the platform, time in the target quadrant, and swim speed (assessed during the visible platform test), were obtained using anymaze tracking software and system.

4.2.6 Cortical Lesion Volume

The area of the lesion (mm^2) was calculated in a subset ($n=4$) of TBI animals from each treatment condition by outlining the cortical lesion for each section taken at 0.7-mm intervals through the extent of the lesion (ImageJ; NIH). The volume (mm^3) of the lesion was determined by summing the areas of the lesion obtained from each section, as previously reported (Cheng et al., 2008; Hoffman et al., 2008; Kline et al., 2004).

4.2.7 Western Blot Analysis

Animals ($n = 6$ in each timepoint for each group for a total $n = 48$ for animals used in behavioral analysis; $n = 3-6$ per group for AMH and FK-506 studies not part of behavioral studies). After deeply anesthetized with pentobarbital (Nembutal, 80-100 mg/kg; Abbott Laboratories, North Chicago, IL), animals were decapitated and the brains quickly removed and chilled on ice. Tissue from the striatum was excised and frozen in liquid nitrogen. Tissue was stored at -70°C until used for analysis.

Preparation of nuclear extracts used in western blot analysis. Nuclear fractions were prepared utilizing a nuclear extraction kit (Active motif, Carlsbad, CA). Briefly, frozen tissue was homogenized on ice with 250 μ l of 1x Hypotonic buffer with DTT and detergent. Homogenized tissue was centrifuged for 10 minutes at 850 x g at 4°C. Cells were resuspended in 250 μ l of 1x Hypotonic buffer by pipetting up and down and incubated on ice for 15 minutes. 15 μ l of detergent was added and the suspension was vortexed for 10 seconds. Suspension was then centrifuged for 30 seconds at 14,000 x g at 4°C. Supernatant was collected (cytoplasmic fraction). The remaining pellet (nuclear fraction) was resuspended in 100 μ l of complete lysis buffer and vortexed for 10 seconds. Suspension was incubated for 30 minutes on ice on a rocking platform. Suspension was then vortexed for 30 seconds and centrifuged for 10 minutes at 14,000 x g at 4°C. Supernatant was collected (nuclear fraction). Fractions were used for western blot analysis as described below.

Protein concentrations were determined using a BCA protein Assay Kit (PIERCE, Rockford, IL). Samples containing 20-100 μ g of protein were subjected to SDS-polyacrylamide gel electrophoresis through a 10% acrylamide gel, and after were transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (Sigma, St. Louis, MO) in tris-buffered saline (TBS-T). Following block membranes were washed 3 x 10 minutes in TBS-T and immunolabeled with antibodies (1:10,000 DARPP-32; 1:2000 p-DARPP-32-Thr34; 1:2000 p-DARPP-32-Thr75; Cell Signaling, Danvers, MA) in 5% BSA in TBST for 18-24 hours. Following primary incubation membranes were washed 3 x 10 minutes in TBS-T and incubated for 2 hours in by goat anti-rabbit immunoglobulin G conjugated to peroxidase (1:10,000; PIERCE, Rockford, IL). Proteins were visualized with a chemiluminescence detection system (SuperSignal, PIERCE, Rockford, IL). To assure equal loading, all membranes were striped and re-blotted with rabbit anti-actin antibody (1:20,000, Sigma, St. Louis, MO). Blots were exposed to autoradiographic X-ray film for 10 s to 2 min and bands were semi-quantified using ImageJ

(NIH, USA) software. Values are given as a ratio (percentage change) of optical density of injured samples versus sham control within individual blots. All optical densities were normalized to β -actin.

4.2.8 Statistical Analysis

Statistical analyses were performed on data collected using SPSS 12.0 (Abacus Concepts, Inc., Berkeley, CA). The motor and cognitive data were analyzed by repeated-measures analysis of variance (ANOVA). The data for acute neurological assessments, histology, probe trial, and swim speed were analyzed by one-factor ANOVAs. When the overall ANOVA revealed a significant effect, the data were further analyzed with the Bonferroni/Dunn *post-hoc* test to determine specific group differences. The data are presented as the mean \pm standard error of the mean (SEM), and are considered significant when corresponding p values are ≤ 0.05 , or as determined by the Bonferroni/Dunn statistic after adjusting for multiple comparisons. Optical density of western blot bands from each group were compared according to a two-factor ANOVA (time versus injury) followed by Bonferroni post-hoc comparison with a significance level of $p \leq 0.05$. Western blot data are expressed as the group means \pm standard error (S.E.) of the mean. Phosphatase activity data are expressed as the group means \pm standard deviation. Statistical evaluations were performed according to ANOVA with a significance level of $p \leq 0.05$ used for all tests.

4.3 RESULTS

4.3.1 Amantadine Treatment Post Traumatic Brain Injury Attenuates the Alteration in the Striatal DARPP-32 Signaling Cascade

Clinically AMH has shown benefit in both Parkinson's Disease (Brenner et al., 1989) and TBI (Kraus et al., 2005). AMH has also shown beneficial effects in the CCI model of TBI (Dixon et al., 1999), showing an improvement in Morris water maze following 2 weeks of treatment. However, it remains unclear what the exact mechanisms of AMH benefit in TBI is. Given AMH's effects on DA and glutamate DARPP-32 represents a potential signaling molecule for AMH action. We therefore analyzed the effect of AMH on DARPP-32 following TBI (**Figure 25**). 6 hours following injury a single dose of AMH (10 mg/kg i.p.) given at 30 mins post injury demonstrated a significant attenuation of the loss in p-DARPP-32-Thr34 ($p < 0.05$) seen with TBI ($p < 0.001$) (**Figure 25**). 2 weeks of AMH given daily (10 mg/kg i.p.), as described previously (Dixon et al., 1999), demonstrated an increase in p-DARPP-32-Thr34 over the level in sham animals in both the ipsilateral ($p < 0.001$) and contralateral ($p < 0.01$) striatum (**Figure 25**). There was also a significant reduction in p-DARPP-32-Thr75 with chronic AMH treatment compared to sham striatum (Contralateral $p < 0.05$; ipsilateral $p < 0.05$) (**Figure 25**). AMH effects on DARPP-32 phosphorylation are consistent with the proposed mechanisms of AMH activity as both a partial DAergic agonist and partial NMDA receptor antagonist. In particular partial inhibition of NMDA receptors should reduce Ca^{2+} signaling within the MSN thus leading to an increase in DARPP-32 phosphorylation at Thr34 while leading to a decrease in DARPP-32 phosphorylation at Thr75. To determine whether the attenuation of p-DARPP-32-Thr34 decreases was functionally significant the effect of daily AMH treatment for 2 weeks post injury on PP-1 activity was assessed. Rats treated with AMH daily (10 mg/kg i.p.) for 2 weeks showed significantly reduced

PP-1 activity compared to animals receiving vehicle ($p < 0.001$) (saline injection) (**Figure 25**).

There was no effect of AMH treatment on DARPP-32 protein expression

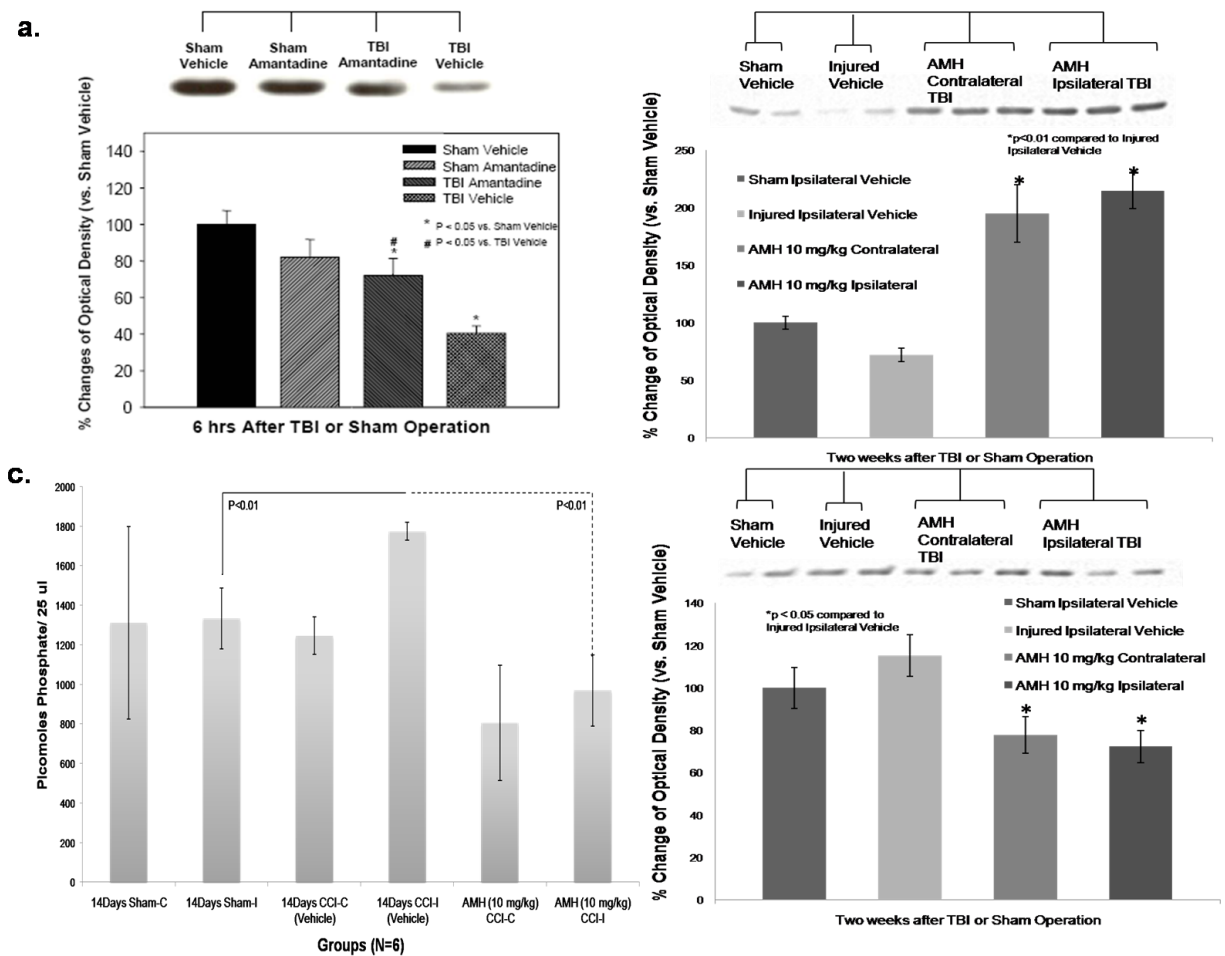


Figure 25. Analysis of AMH effects on DARPP-32 signaling post TBI. *A*, Single dose of AMH (10 mg/kg i.p.) given 30 mins post injury. Striatal DARPP-32 phosphorylation at 6 hours post injury. AMH effectively reduces the level of p-DARPP-32-Thr34 loss following TBI. Representative western blot of ipsilateral striatum (above) and optical density (below) of p-DARPP-32-T34. (N=6/group; $p < 0.05$ normalization to β -actin compared to TBI with vehicle; ANOVA). Data represented as percentage of sham following normalization to β -actin \pm S.E. *B*, Chronic AMH (10 mg/kg i.p. daily) on striatal DARPP-32 phosphorylation at 2 weeks post injury. Chronic AMH administration reverses the loss of p-DARPP-32-Thr34 seen with a TBI and

increases p-DARPP-32-Thr34 above sham levels. (N=6/group; $p < 0.01$ normalization to β -actin compared to TBI with vehicle; ANOVA). Data represented as percentage of sham following normalization to β -actin \pm SEM. *C*, Chronic AMH (10 mg/kg i.p. daily) on striatal PP-1 activity. Following chronic AMH administration there is a reduction in PP-1 activity compared to TBI with vehicle. (N=6/group; $p < 0.01$; ANOVA). Data represents means \pm standard deviation. *D*, Chronic AMH (10 mg/kg i.p. daily) on striatal p-DARPP-32-Thr75 at 2 weeks post injury. Chronic AMH decreases the levels of p-DARPP-32-Thr75 compared to injury.. (N=6/group; $p < 0.01$ normalization to β -actin compared to TBI with vehicle; ANOVA). Data represented as percentage of sham following normalization to β -actin \pm SEM. *Abbreviations: AMH = Amantadine hydrochloride, DARPP-32 = Dopamine and cAMP regulated phosphoprotein 32; TBI = Traumatic Brain Injury.*

4.3.2 The level of DARPP-32 phosphorylation at Thr34 is dose dependent with respect to AMH.

We administered 1 mg/kg i.p., 5 mg/kg i.p., and 10 mg/kg i.p. of AMH to rats (n=3 per group for 1 and 5 mg/kg groups) for 2 weeks to evaluate the dose dependent affect of AMH on DARPP-32 phosphorylation post TBI. There was no significant effect of AMH 1 mg/kg i.p. on p-DARPP-32-Thr34 levels post injury compared to injured vehicle. There appears to be a dosage dependent effect of 1 and 5 mg/kg i.p. on p-DARPP-32-Thr34 levels post injury compared to injured vehicle (**Figure 26**), however the effect was not as impressive as that seen with 10 mg/kg i.p. (**Figure 25**).

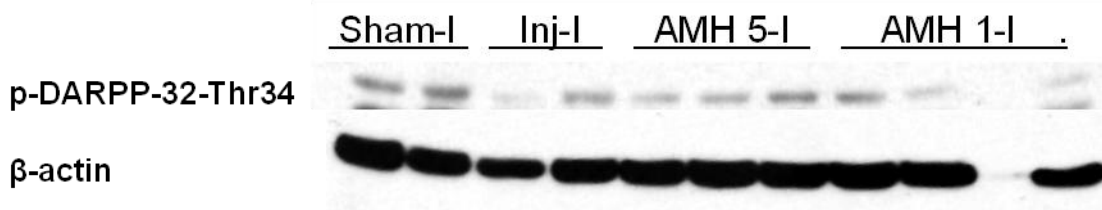


Figure 26: Qualitative western blot demonstrating a dosage response of p-DARPP-32-Thr34 to AMH at 5.0 and 1.0 mg/kg i.p. given daily for 2 weeks (n=3 for AMH groups).

4.3.3 FK-506 Given acutely post-TBI Attenuates the Alteration in DARPP-32-Thr34 phosphorylation.

FK-506 is a potent inhibitor of calcineurin that has demonstrated significant neuroprotection in experimental TBI (Butcher et al., 1997). Calcineurin is a key regulator of DARPP-32 phosphorylation. Increases in calcineurin activity cause a dephosphorylation of DARPP-32 at Thr34. Therefore inhibition of calcineurin activity should reduce a loss of DARPP-32/Thr34 phosphorylation if calcineurin is activated post TBI in the striatum. A single acute administration of FK-506 (2.0 mg/kg i.p.) given at 5 mins post TBI demonstrates a significant increase in p-DARPP-32-Thr34 over TBI animals receiving vehicle (n=6 per group) at 6 hours post injury. This benefit of FK-506 administration extends into chronic timepoints with a demonstrated attenuation of p-DARPP-32/Thr34 loss at 2 weeks post TBI compared to vehicle treated animals (**Figure 27**).

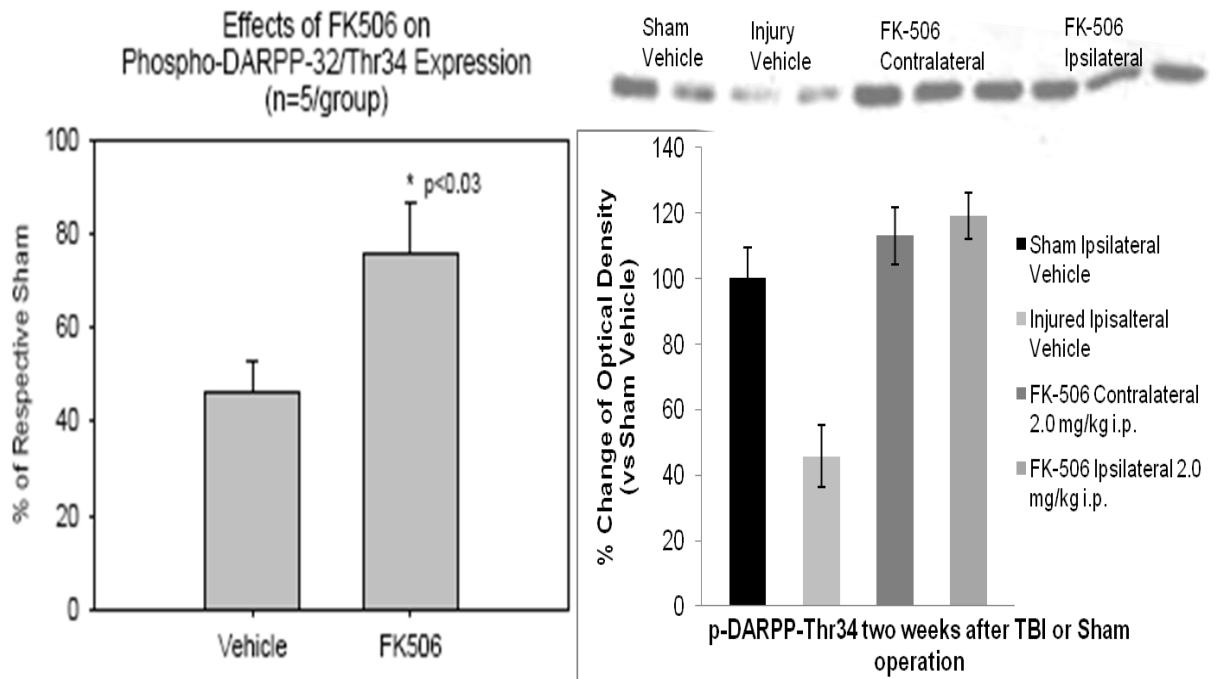


Figure 27: Analysis of FK-506 effects on DARPP-32 signaling post TBI. **Left,** Single dose of FK-506 (2.0 mg/kg i.p.) given 5 mins post injury. Striatal DARPP-32 phosphorylation at 6 hours post injury. FK-506 effectively reduces the level of p-DARPP-32-Thr34 loss following TBI. Optical density of ipsilateral striatum p-DARPP-32-T34. (N=5/group; $p < 0.05$ normalization to β -actin compared to TBI with vehicle; ANOVA). Data represented as a percentage of sham following normalization to β -actin \pm SEM. **Right,** Single dose of FK-506 (2.0 mg/kg i.p. 5mins post injury) on striatal DARPP-32 phosphorylation at 2 weeks post injury. Acute FK-506 administration reverses the loss of p-DARPP-32-Thr34 seen with a TBI. (N=6/group; $p < 0.01$ normalization to β -actin compared to TBI with vehicle; ANOVA). Data represented as percentage of sham following normalization to β -actin \pm SEM.

4.3.4 The level of DARPP-32 phosphorylation at Thr34 is dose dependent with respect to FK-506.

We administered 0.5 mg/kg i.p., 1 mg/kg i.p., and 2 mg/kg i.p. of FK-506 to rats (n=3 for 0.5 and 1 mg/kg groups) at 5 mins post TBI to evaluate the dose dependent affect of AMH on DARPP-32 phosphorylation post TBI. There appears to be a dose response to FK-506 at 0.5 mg/kg i.p. and 1.0 mg/kg i.p. (**Figure 28**) which was more modest than the effect seen with FK-506 2.0 mg/kg i.p. (**Figure 27**).

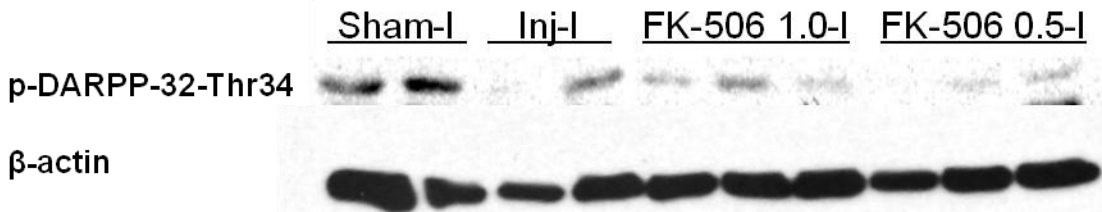


Figure 28: Qualitative western blot demonstrating a dosage response of p-DARPP-32-Thr34 to a single dose FK-506 5 mins post injury at 1.0 and 0.5 mg/kg i.p. (n=3 for FK-506 groups).

4.3.5 Combination therapy of FK-506 and AMH is able to restore DARPP-32-Thr34 phosphorylation levels.

To evaluate the effect of a combination therapy of FK-506 and AMH on striatal DARPP-32 dysfunction after TBI we chose to utilize doses of both drugs that demonstrated minimal increases in DARPP-32-Thr34 phosphorylation independently to maximize the potential for synergistic evaluation. A combined therapy of a single acute administration of FK-506 given 5 mins post injury (1.0 mg/kg i.p.) and AMH (5 mg/kg i.p.) given once daily for 2 weeks was utilized (n = 6 animals per group). Combination therapy demonstrated significant increases in p-DARPP-32-Thr34 over vehicle treated animals (**Figure 29**).

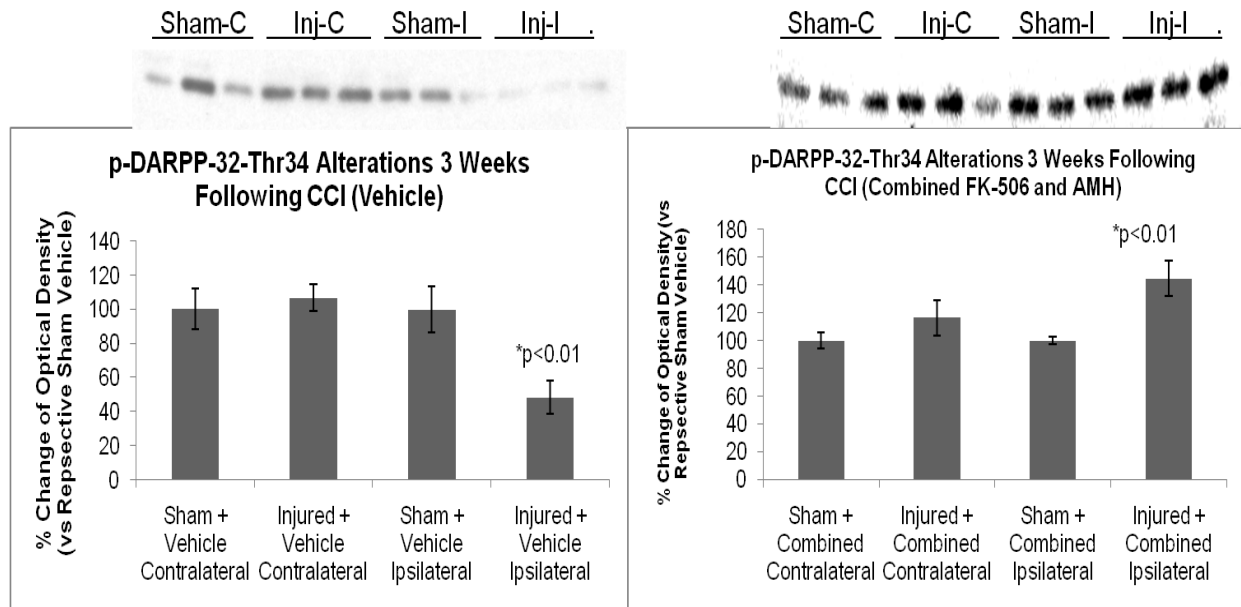


Figure 29: Analysis of combined FK-506 (1.0 mg/kg i.p. once 5 mins post injury) plus AMH (5 mg/kg i.p. daily) effects on DARPP-32 signaling post TBI. **Left**, p-DARPP-32-Thr34 levels 3 weeks post injury with vehicle treatment. There is a significant decrease in p-DARPP-32-Thr34 expression in the ipsilateral injured striatum compared to sham ($p<0.01$). Optical density of ipsilateral striatum p-DARPP-32-T34. (N=6/group; $p<0.01$ normalization to β -actin compared to sham with vehicle; ANOVA). Data represented as a percentage of sham following normalization to β -actin \pm SEM. **Right**, Combined treatment of a single dose of FK-506 (1.0 mg/kg i.p. 5mins post injury) plus daily AMH (5 mg/kg i.p.) on striatal DARPP-32 phosphorylation at 3 weeks post injury. Combined treatment reverses the loss of p-DARPP-32-Thr34 seen with a TBI above sham levels. (N=6/group; $p<0.01$ normalization to β -actin compared to TBI with combination therapy; ANOVA). Data represented as percentage of sham following normalization to β -actin \pm SEM.

4.3.6 Combination therapy of FK-506 and AMH attenuates ERK phosphorylation decreases in striatal nuclear fractions post TBI.

Prior research examining drug therapies on DARPP-32 phosphorylation have shown significant increases in ERK 1/2 and CREB (Ser133) phosphorylation. Increases in the phosphorylation of these two nuclear signaling molecules are associated with increases in nuclear transcription associated with synaptic plasticity and learning. Prior evaluation in whole cell lysates discussed in the previous chapter demonstrated no effect of TBI on ERK or CREB phosphorylation. However, analysis of nuclear fractions demonstrated a significant decrease in ERK 1/2 phosphorylation at 3 weeks following injury, consistent with increases in PP-1 activity seen at 2 weeks following TBI. Utilizing nuclear extraction we evaluated the effect of the combination therapy on ERK 1/2 and CREB phosphorylation following TBI. There was no significant difference between sham and injured animals with combination drug therapy in either ERK 1/2 (**Figure 30**) or CREB (**Figure 31**) phosphorylation indicating that combination therapy effectively attenuated the loss of ERK 1/2 phosphorylation seen with TBI.

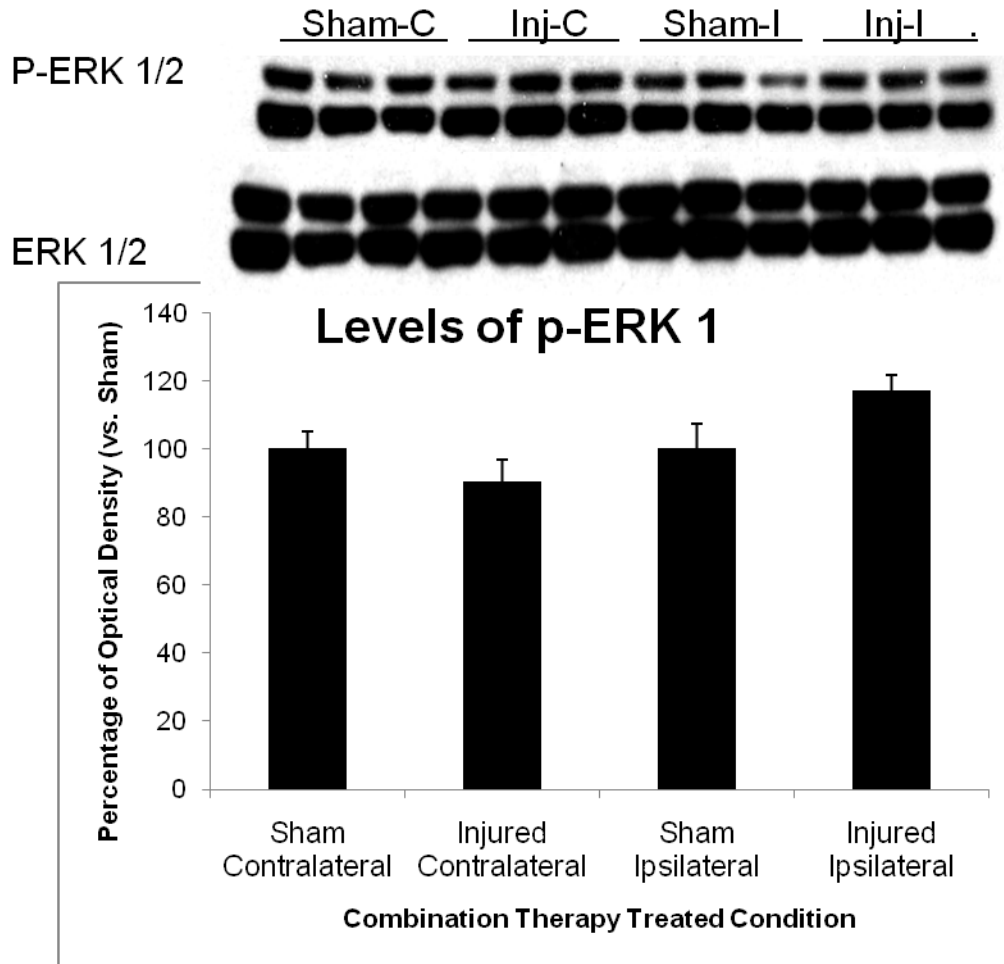


Figure 30: Analysis of nuclear fraction ERK 1/2 expression and Thr202/Tyr204 (p-ERK 1/2) phosphorylation in the striatum at 3 weeks post experimental TBI with a combined treatment of FK-506 (1.0 mg/kg i.p. given 5 mins post injury) and AMH (5 mg/kg i.p. given daily). Densitometry of p-ERK 1/2 normalized to total ERK 1/2 expression shown below with a representative western blot above. There was no significant difference in p-ERK 1/2 expression in the injured ipsilateral striatum compared to sham. Data represents mean + SEM; n = 6 per group.

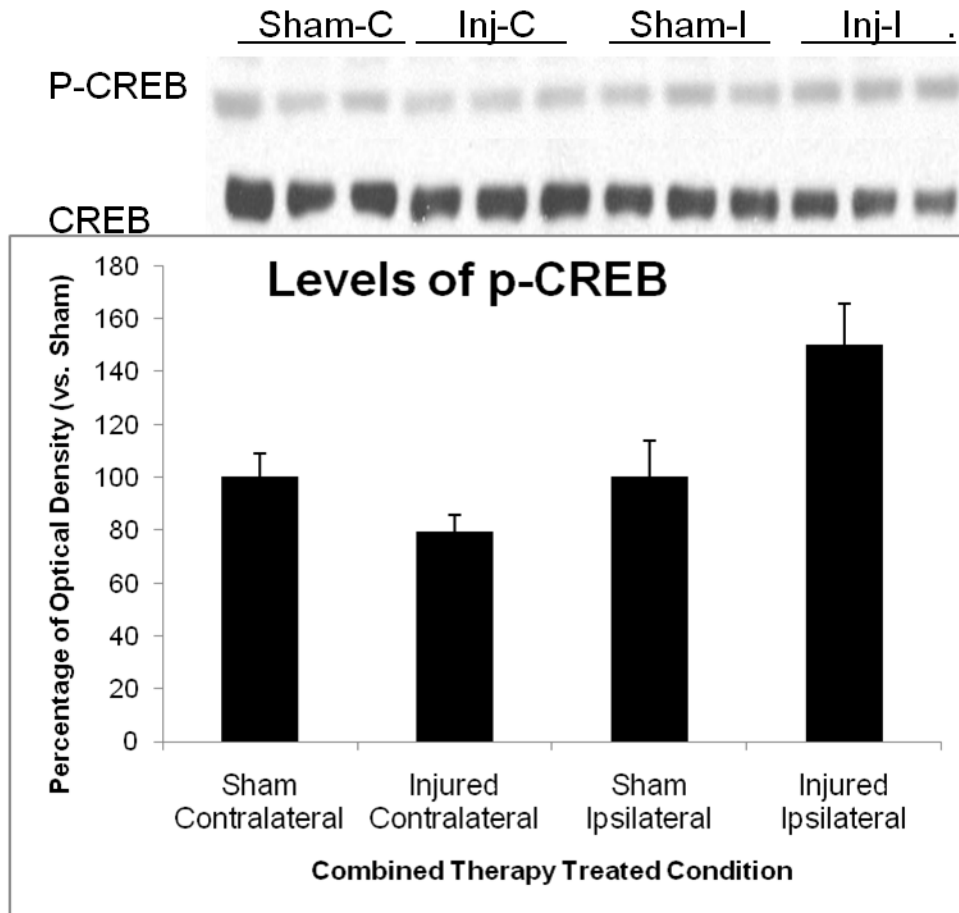


Figure 31: Analysis of nuclear fraction CREB expression and p-CREB (Ser133) phosphorylation in the striatum at 3 weeks post experimental TBI with combined FK-506 (1.0 mg/kg i.p. given 5 mins post injury) and AMH (5 mg/kg i.p. given daily). Densitometry of p-CREB normalized to total CREB expression shown below with a representative western blot above. There was no significant decrease in p-CREB expression after injury. However, there was a significant increase in p-CREB expression in the ipsilateral injured side compared to sham (ANOVA $p < 0.05$). Data represents mean + SEM; $n = 6$ per group.

4.3.7 Single therapy of FK-506 and combination therapy of FK-506 and AMH reduces lesion volume post TBI.

To evaluate potential neuroprotective properties of our therapeutic approach we examined lesion volumes following CCI in vehicle versus combined therapy animals (n=4 per group). There was a significant difference ($p<0.05$) in the lesion volumes between TBI plus FK-506 (27.9 ± 1.53 mm³) versus vehicle and combination therapy (25.9 ± 2.2 mm³) compared to TBI plus vehicle (33.4 ± 1.5 mm³) (**Figure 32**). There was no difference between TBI plus AMH alone and vehicle groups. This suggests that FK-506 alone or in combination is able to provide a level of neuronal protection in CCI consistent with previous reports in FP injury (sample images in **Appendix A: Supplemental Figure 4**).

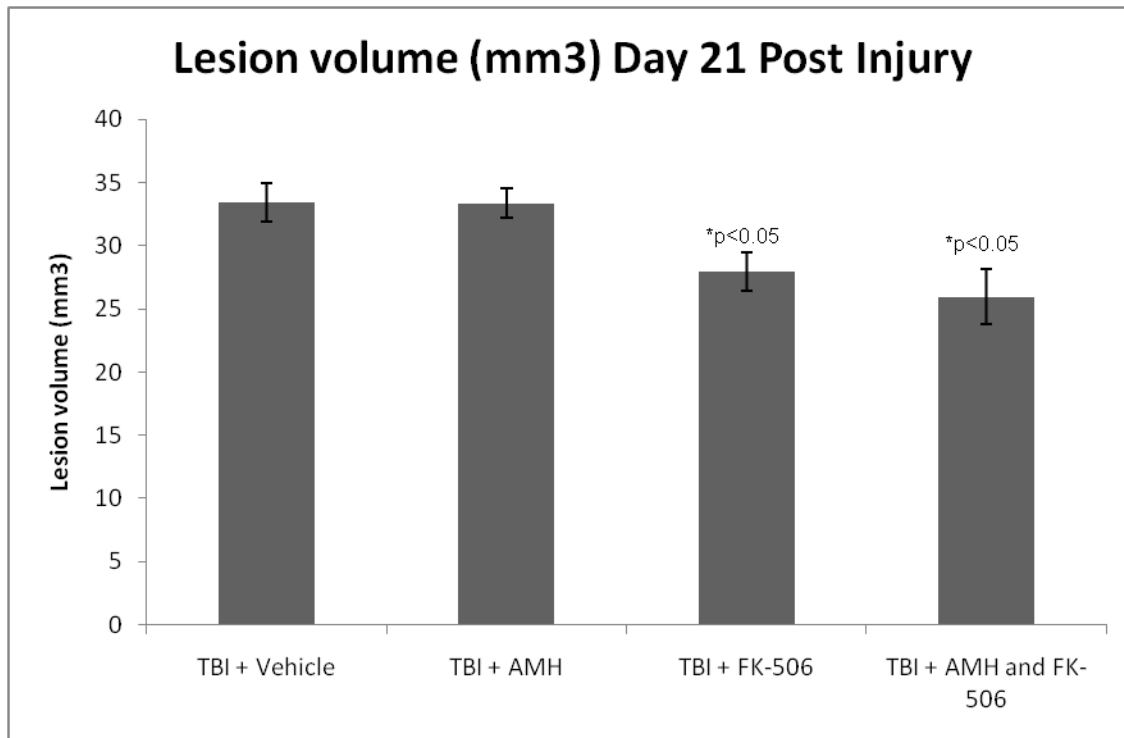


Figure 32: Mean \pm SEM of cortical lesion volume (mm³) 3 weeks after cortical impact injury.

* $p<0.05$ versus TBI + vehicle.

4.3.8 No difference in acute neurological function amongst groups.

No significant differences were observed amongst the TBI groups in latency to elicit a withdrawal reflex in response to a brief paw pinch of the left limb (range 365.25 ± 20.5 sec to 378 ± 21.5), or for the return of righting ability (range 462 ± 8.5 sec to 519 ± 12.6 sec) after the cessation of anesthesia. The lack of significant group differences in these acute neurological indices suggests that all rats experienced an equivalent level of brain injury and anesthesia.

4.3.9 Combination therapy of FK-506 and AMH reduces motor deficits following a TBI.

No pre-surgical beam-balance differences were observed among groups, all rats were capable of balancing for the allotted 60 sec (**Figure 33**). However, a repeated-measures ANOVA on post-TBI data demonstrated significant group ($p < 0.001$) and day ($p < 0.001$) differences, as well as a significant group x day interaction ($p < 0.001$). Bonferroni post-hoc analyses revealed that AMH alone ($p < 0.05$), FK-506 alone ($p < 0.001$), and vehicle treated ($p < 0.001$) groups were able to balance for significantly shorter time periods over the testing period than sham controls, while the combined AMH and FK-506 treated group was not statistically different from sham ($p > 0.05$). No other group comparisons were significant. Similar to the beam-balance task, there were no pre-surgical differences in beam-walking among groups (**Figure 34**). Following TBI a repeated-measures ANOVA demonstrated significant group ($p < 0.001$) and day ($p < 0.001$) differences, as well as a significant group x day interaction ($p < 0.001$). Bonferroni post-hoc analyses revealed that all injured groups had significantly longer latencies over the testing period than sham controls ($p < 0.001$).

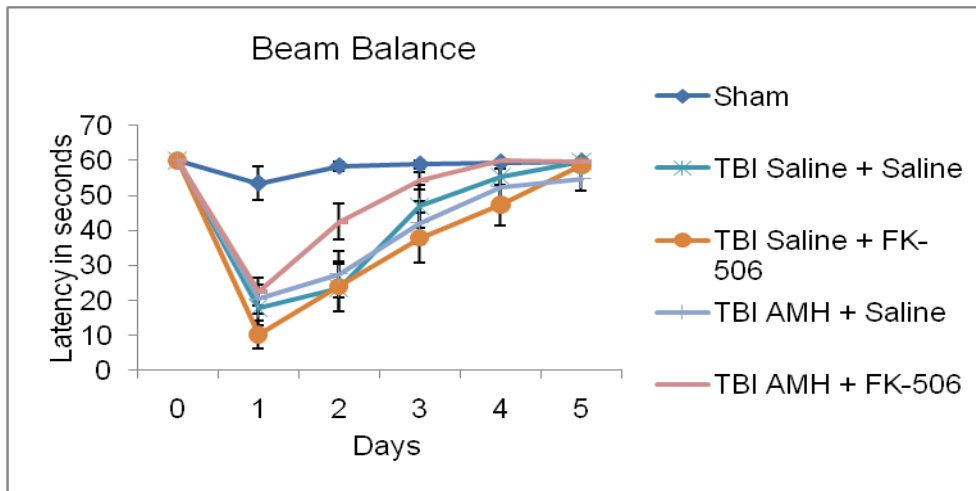


Figure 33: Beam balance task assessed at days 1-5 post injury. All sham groups compiled into a single line to clarify data due to significant differences between sham treatments. For repeated measures ANOVA analysis TBI groups were compared to their sham controls. All animals were pre-assessed on day 0 at the same level. Day 1 indicates 1 day post injury or sham surgery. The combined group demonstrated no significant difference from sham over time compared to other injury groups which demonstrated significance compared to sham ($p < 0.001$). Data represents mean time on beam \pm SEM.

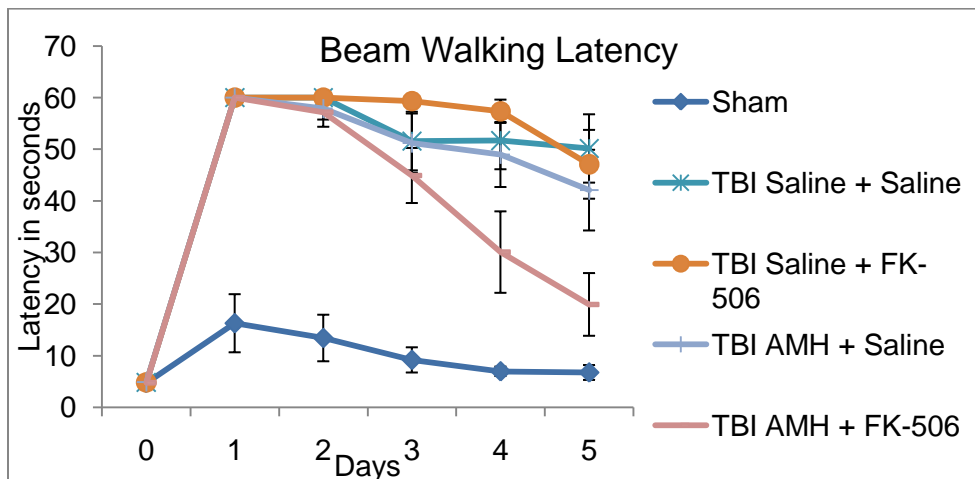


Figure 34: Beam walk latency on days 1-5 post injury. Sham groups were compiled into one to simplify the data presentation. All injured groups were compared utilizing a repeated measures

ANOVA with Bonferroni post-hoc analysis against their respective sham group. All injured groups took significantly longer to traverse the beam over the recovery period than sham ($p < 0.001$). Data presented as mean latency \pm SEM.

4.3.10 Combination therapy of FK-506 and AMH improves spatial learning post TBI.

Analysis of spatial learning revealed significant group ($p < 0.001$) and day ($p < 0.001$) effects. Bonferroni post-hoc analyses demonstrated the combination therapy group (AMH plus FK-506) was not statistically different from sham controls concerning the time to locate the escape platform over the testing period. All other injury groups (Vehicle, AMH alone, FK-506 alone) were significantly different (all $p < 0.001$) from sham controls. No injury group was significantly different than any other (**Figure 35**). A repeated-measures ANOVA performed on the visible platform data revealed significant group ($p < 0.001$) and day ($p = 0.001$) effects, as well as a significant group \times day interaction ($p < 0.001$). Bonferroni post-hoc analyses demonstrated significantly increased time to find escape platform latencies for both the vehicle treated ($p < 0.001$), AMH alone treated ($p < 0.001$) groups compared to sham controls. There was no significant difference in the time to find platform for the FK-506 alone or combination of AMH and FK-506 groups compared to shams (**Figure 35**). No significant differences in swim speed (range = 27.8 ± 0.88 cm/sec to 33.8 ± 1.63 cm/sec) were observed between groups.

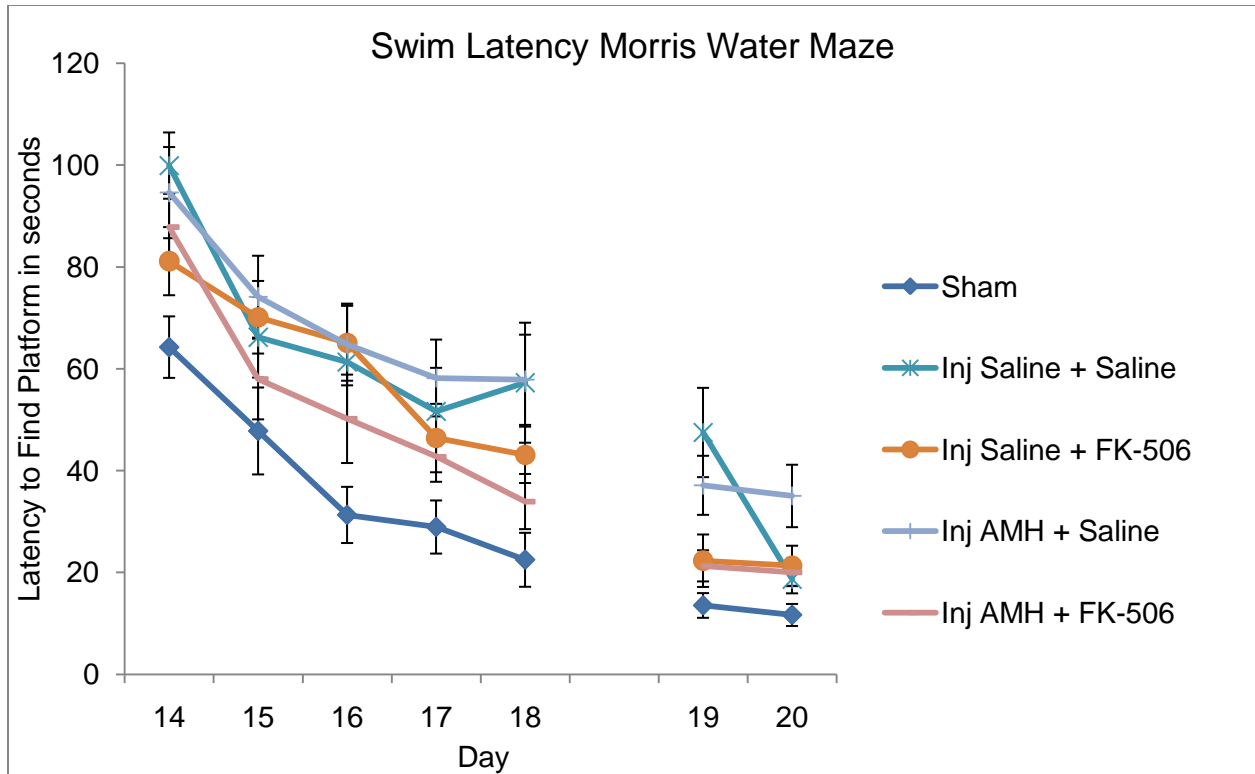


Figure 35: Morris water maze data assessed on days 14-18 for submerged (hidden) platform and days 19-20 for visible platform. All injury groups were significantly different from sham ($p < 0.001$) except the combined treatment group for latency to find submerged platform. Sham groups were combined for ease of visualization but were not combined for analysis. Data represents mean latency to find the platform \pm SEM.

Analysis of probe trial data revealed no significant difference between any of the groups on percentage of time spent within the target quadrant (**Figure 36**).

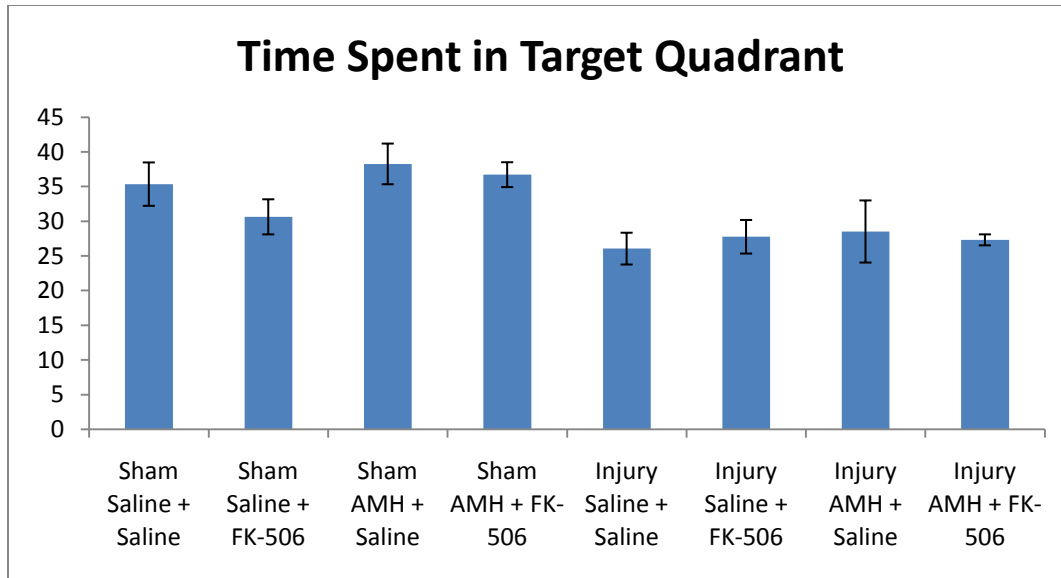


Figure 36: Analysis of probe trial on day 19. Mean time spent in target quadrant as a percentage of total time \pm SEM. No significant differences between any groups as analyzed with ANOVA.

4.4 DISCUSSION

Cellular death remains the most prominent target in post-TBI therapeutic research. However, often the ability to protect cells from death is not associated with significant improvements in behavioral function. In addition, many therapeutic strategies that have shown benefit demonstrate little to no neuroprotection. To address these concerns two areas of ongoing TBI research have arisen. One, identify novel therapeutic targets that may be more pertinent to behavioral dysfunction than neuronal death and two, utilize combination drug treatments in order to increase the level of neuroprotection and potentially treat other cellular pathology. In this study we have addressed both of these avenues of research. We evaluated DARPP-32 as a potential therapeutic target following TBI and we utilized a combined drug therapy approach to assess DARPP-32's viability.

We have shown that AMH hydrochloride, a partial NMDA antagonist with catecholaminergic effects, can effectively restore sham levels of p-DARPP-32-Thr34 in injured animals (**Figure 25**). AMH improves cognitive function in CCI injured animals (Dixon et al.,1999). The exact mechanism of AMH's action following a TBI is unclear. Here we demonstrate that both acutely (**Figure 25**) and chronically (**Figure 25**), AMH is able to prevent the loss of p-DARPP-32-Thr34 following a TBI. Furthermore, the increase in p-DARPP-32-Thr34 over vehicle treated animals, with 2 weeks of (10 mg/kg i.p.) AMH corresponds with significantly less PP-1 activity compared to injury (**Figure 25**). Chronic AMH treatment also showed significantly less p-DARPP-32-Thr75, compared to vehicle treated sham and injured animals (**Figure 25**). There is no effect of AMH treatment on DARPP-32 protein levels. Our results suggest that the effect of AMH on striatal signaling post TBI at 10 mg/kg is most likely a combination of DAergic effects and NMDA antagonism as the shift of p-DARPP-32 towards Thr34 phosphorylation and subsequent decrease in Thr75 phosphorylation is consistent with reduced Ca^{2+} signaling due to NMDA antagonism and activation of adenylyl cyclase via DA receptor D1 (Valjent et al., 2005; Nishi et al., 2005). Given the known effectiveness of AMH following a TBI this also provides the first evidence for DARPP-32 being a promising therapeutic target for the treatment of post-TBI cognitive dysfunction. Furthermore, we have demonstrated that reversing the TBI induced p-DARPP-32-Thr34 decrease is associated with a reversal of the increase in PP-1 activity as well, suggesting that we can effectively treat dysfunctions in downstream signaling by targeting DARPP-32 phosphorylation.

FK-506, a potent inhibitor of calcineurin, given once at 5 minutes post injury also demonstrated both acute and chronic (**Figure 27**) attenuation of p-DARPP-32-Thr34 decreases post TBI. The ability of a single dose of FK-506 to attenuate even chronic decreases in DARPP-32-Thr34 phosphorylation suggests that immediately post TBI there is an increase in calcineurin activity within the striatum similar to what was shown in the hippocampus at 2 hours post injury in

chapter 2 of this thesis. This is consistent with evidence that the striatum is exposed to acute increases in glutamatergic tone that would theoretically act to upregulate calcium influx, to which calcineurin is extremely sensitive. By inhibiting the acute activation of calcineurin, FK-506 is able to prevent the loss of DARPP-32-Thr34 phosphorylation chronically as well suggesting that TBI induced DARPP-32 dysfunction is an acute event compounded by the inability of the post-TBI striatum to correct the deficit.

Both AMH and FK-506 showed a dose dependent effect on DARPP-32-Thr34 phosphorylation (**Figures 26 and 28**) further indicating that these drugs have a direct effect upon DARPP-32-Thr34 phosphorylation following a TBI. Utilizing these dose dependent effects we designed a combined drug therapy of acute single dose administration of FK-506 plus chronic daily administration of AMH. The doses chosen for the combined therapy examination were below the maximal dose given in the dose response study in order to examine the combined therapeutic effect of the two drugs given together.

There was a concern that employing the maximal drug doses would produce a ceiling effect on either DARPP-32 phosphorylation or behavior and limit the evaluation of single drug therapy versus combined drug therapy. A second concern was that high doses of FK-506 can have detrimental effects including seizure and immune suppression that may not be beneficial post TBI. Future examinations of this combination regimen will need to adjust drug dosages to determine maximal effectiveness.

We showed that a combination therapy of a single acute administration of FK-506 post TBI (1.0 mg/kg i.p.) combined with a daily chronic treatment with AMH (5 mg/kg i.p.) prevented the loss of DARPP-32-Thr34 phosphorylation at 3 weeks post injury compared to vehicle treated animals indicating that the combination therapy was able to effectively treat TBI induced DARPP-32

dysfunction (**Figure 29**). Furthermore, examination of ERK 1/2 phosphorylation with combination therapy demonstrated that the combined therapy was able to attenuate ERK 1/2 phosphorylation decreases seen in the striatum post TBI at 3 weeks as well (**Figure 30**). This demonstrates that the combination therapy was able to effectively treat alterations in cell signaling pathways within the striatum associated with TBI. Interestingly as compared to our earlier results (**Figure 17**), there was no decrease in p-DARPP-32-Thr34 in contralateral striatum (**Figure 29**) at 3 weeks post injury. This discrepancy is most likely due to the behavioral analysis that the rats underwent for this experiment. DARPP-32 is extremely responsive and it is possible that the behavioral analysis induced some level of benefit in striatal signaling, which is intriguing when one considers paradigms examining environmental enrichment.

In addition to striatal effects the combination of FK-506 and AMH reduced the lesion volume in TBI animals compared to vehicle (**Figure 32**). The same effect in the reduction of lesion volume was seen with FK-506 alone, but not with AMH alone indicating that FK-506 at 1.0 mg/kg i.p. has some neuroprotective effects. This is particularly interesting when coupled with the behavioral data which demonstrates that there is no benefit to behavior with this dose of FK-506. This strongly suggests that the synergistic benefit of FK-506 and AMH post injury is related to non-neuroprotective events.

It is important to note that while studies have shown the neuroprotective effect of BDNF administration and ERK 1/2 activation, there is also research demonstrating an ERK 1/2 signaling pathway that promotes caspase activation and neuronal death. In brain injury research it has been shown that oxidative stress can induce aberrant activation of ERK 1/2 and CREB which corresponds with mitochondrial dysfunction and cell death (Enomoto et al., 2005). Therefore it is possible that by activating the DARPP-32-Thr34 site and inducing an increase in ERK 1/2 activation we could have facilitated further cellular dysfunction. However, our research

would suggest that, at least chronically, ERK 1/2 activation plays a positive role in outcomes. Future evaluations will need to examine ERK 1/2 activation temporally within the striatum to better elucidate its role in striatal dysfunction.

Combination drug therapies represent a promising new area in TBI research as does the targeting of subcortical brain structures. We demonstrate that the combination of FK-506 and AMH was able to attenuate striatal dysfunction post-TBI. Furthermore the combination therapy demonstrated no significant difference from sham for recovery of beam-balance performance (**Figure 33**) or in spatial learning (**Figure 35**). While single drug treatment groups demonstrated significant differences in both spatial learning and on motor tasks compared to sham. The potential mechanisms of benefit for these two drugs are many. FK-506 improves cell survival, axonal protection, and decreases inflammatory signaling in TBI paradigms. AMH is known to improve outcomes in both experimental and clinical TBI.

4.5 CONCLUSION

We present here a common mechanism shared between both FK-506 and AMH within the striatum. The attenuation of striatal DARPP-32 dysfunction after TBI is a novel therapeutic target after TBI and we have demonstrated that it represents a viable synergistic target for combined treatment strategies. Furthermore, we have provided an initial lesion volume analysis indicating that neuroprotection does not play a key role in these synergistic benefits.

5. POTENTIAL UPSTREAM SIGNALING DEFICITS INVOLVED IN DARPP-32 MODULATION

5.1 INTRODUCTION

Alterations in DARPP-32 signaling post TBI could be caused by a number of upstream signaling events. Following the acute primary injury, which often consists of a focal contusion and more diffuse structural damage, there are a series of subsequent secondary responses, which include, but are not limited to, excitotoxicity, ischemia, oxidative stress, and ongoing structural and chemical alterations. Both glutamate and DA play key roles in modulating DARPP-32 signaling, thus dysfunction in either signaling molecule could present a potential mechanism for ongoing DARPP-32 dysfunction. However, therapies targeting glutamatergic excitotoxicity have shown limited benefit and unfortunate toxicity. Given that we see DARPP-32 dysfunction chronically as well as acutely it is logical to assume that striatal alterations consist of both early and late or persistent events. DA, within the striatum, acts at both D1 and D2 receptors. Given that prior research has demonstrated that D2 antagonists can negatively affect recovery from a TBI (Kline et al., 2007; Hoffman et al., 2008) it may be that there is a dysfunction in D2 signaling that has not been elucidated post injury.

One area in particular that has not been well studied in TBI and has just begun to be elucidated in other disease states including Parkinson's disease (PD) is the role that non-DA receptors play in DA signaling. Of particular interest is the role of adenosine A2a receptors and the control they exert over DA D2 receptors. Changes in A2a signaling have been shown to be important to D2 signaling within the striatum and play a crucial role in cell signaling events that affect learning and memory, including long term potentiation (LTP) and long term depression (LTD).

D2 receptors can act through inhibitory G-proteins that lead to an inhibition of AC and cAMP (Weiss et al., 1985; Onali et al., 1985) or independently of cAMP pathways (Memo et al., 1986). D2 receptors have also been shown to inhibit PI hydrolysis and subsequent Ca mobilization (Vallar et al., 1989; Picetti et al., 1997). Furthermore activity at D2 receptors is coupled to alterations in DARPP-32 phosphorylation and function (Greengard et al., 1999). These signaling pathways have been shown to be important for striatal goal directed learning and memory. Changes in D2 receptor activation, protein, and second messenger signaling systems have been implicated in psychotic disorders and are a prime target for therapeutic drugs used to treat both PD and HD (Fuxe et al., 2005; Fuxe et al., 2007).

An interesting caveat of D2 receptor activation is that in vitro studies utilizing co-immunoprecipitation methods and in-vivo studies utilizing fluorescent and bioluminescent energy transfer (FRET and BRET) analyses have shown that many D2 receptor systems exist in heterodimerized pairs (Canals et al., 2003; Fuxe et al., 2005). D2 receptors have been shown to form heterodimers with adenosine A2a receptors and metabotropic glutamate-5 (Mglut-5) receptors (Ferre et al., 2008). A2a and Mglut-5 heterodimers are of particular interest for TBI research due to their role in striatal long term potentiation (LTP) and long term depression (LTD) and subsequent plasticity of striatal connections (Fuxe et al., 1998). The identification of heterodimer systems within the striatum indicate that D1 and D2 have control over CREB signaling within striatal neurons. Further, they modulate LTP and LTD dependent glutamatergic and adenosine transmitter systems (Centonze et al., 2003). Changes in the function of these heterodimers can drastically alter DAergic signaling through the D2 receptor and have been implicated in numerous disease states including schizophrenia, diseases of addiction, and Parkinson's disease (PD).

In PD, the contribution of neurotransmitters beyond DA is important to therapeutic manipulation as utilization of these other receptor systems provides an alternative way to manipulate DAergic signaling. D2-A2a interactions proposed by Fuxe et al., (2005) allow for precise alterations in intracellular signaling pathways that have been associated with cognitive function and striatal output. Furthermore, radioligand binding studies have identified a tight antagonistic relationship between A2a and D2 receptors that is acutely affected by DA concentration.

In patient populations, adenosine A2a antagonists combined with traditional PD therapies including levodopa have decreased the incidence of negative side effects, increased DA drug effectiveness, and reduced the dosage of DAergic medications necessary for therapeutic benefits (Antonelli et al., 2006). Natural antagonists of A2a receptors have also been associated with decreased incidence of PD and other neuroprotective effects. In A2a knockout mice models there is noticeably less neuronal death and dysfunction when animals are exposed to toxic compounds such as MPTP and rotenone typically used to induce PD in mice.

Alterations in A2a receptors relevant to striatal DAergic D2 receptor signaling represent a possible mechanism of cognitive dysfunction after TBI. A2a receptors can control D2 receptor signaling via two independent mechanisms; either through a direct heterodimeric system or through second messenger systems (**Figure 37**). In either situation adenosine through action at the A2a receptor acts as an inhibitor of D2 signaling (Fuxe et al., 1998; Fuxe et al., 2005). Changes in the behavior of A2a and D2 receptors can have dramatic effects on DA effects in the striatum and represent a potential mechanism of DARPP-32 dysfunction following TBI.

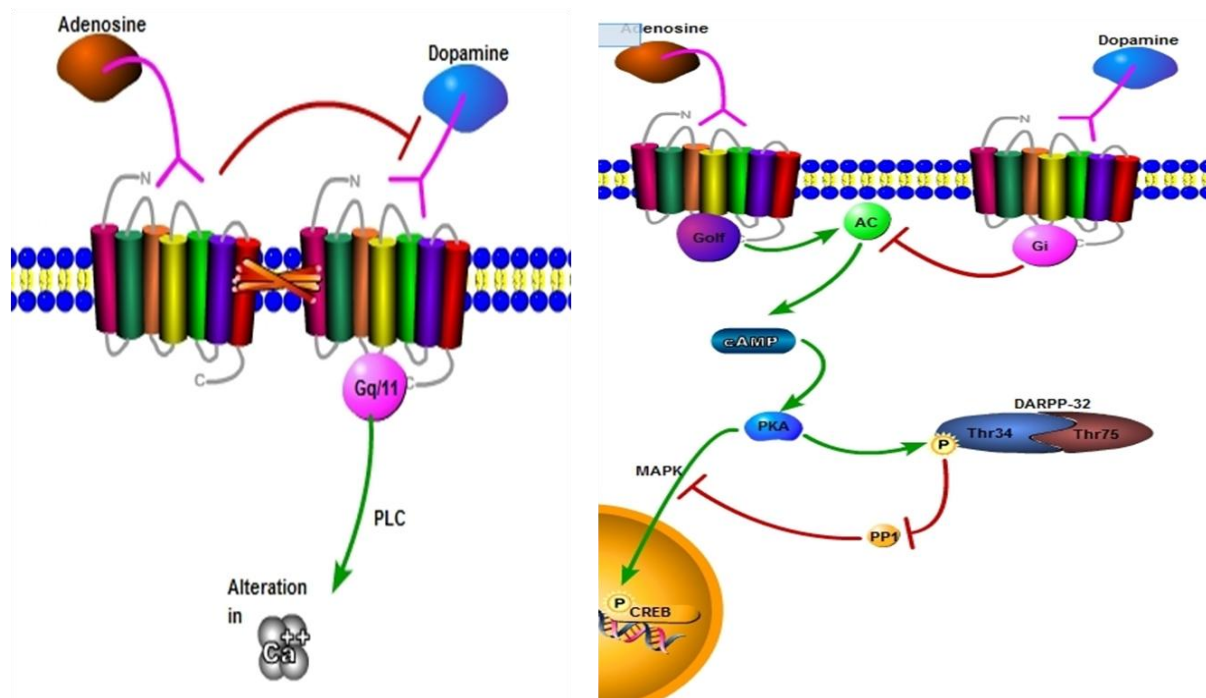


Figure 37: A2a and D2 receptors interact in the striatum in two different ways. Either through a PKA independent heteroreceptor system that induced intracellular alterations of calcium or through a PKA dependent second messenger system that affects MAPK and DARPP-32 signaling pathways.

5.2 METHODS AND MATERIALS

5.2.1 Animals and Surgery

CCI. Sprague Dawley rats (300-325 g) were used for these experiments (n = 36). As described previously (Cernak, 2005; Dixon et al., 1991). A craniotomy is performed by one trained technician and a pneumatic cylinder with a 4 to 5 cm stroke, mounted on a cross bar to adjust position of impact, is adjusted to an impact velocity of .5 to 10 m/s, cortical deformation between 1 and 3 mm, and duration of impact between 25 and 250 ms. Each animal was injured with an impact velocity of 4m/s and 2.7 mm cortical deformation over the right cerebral cortex. The

animals were sacrificed at 1 day, 2 weeks, or 4 weeks following TBI. The brain was taken from each animal and striatal tissue excised and placed into -70 Celsius until use.

5.2.2 Western Blot Analysis

After deeply anesthetized with pentobarbital (Nembutal, 80-100 mg/kg; Abbott Laboratories, North Chicago, IL), animals were decapitated and the brains quickly removed and chilled on ice. Tissue from the striatum was excised. Tissues was homogenized in Lysis buffer (suspension buffer) which contains 0.1M NaCl, 0.01M Tris-Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), and protein concentrations were determined using a BCA protein Assay Kit (PIERCE, Rockford, IL). Samples containing 20 µg of protein were subjected to SDS-polyacrylamide gel electrophoresis through a 10% acrylamine gel, and after transferred to nitrocellulose membranes and immunolabeled with antibodies specific for adenosine A2a or DA D2 receptors (mouse monoclonal 1:500; Santa Cruz) followed by goat antimouse immunoglobulin G conjugated to peroxidase (1:10,000; PIERCE, Rockford, IL). Proteins were visualized with a chemiluminescence detection system (SuperSignal, PIERCE, Rockford, IL). To assure equal loading, the membrane were restriped and reblotted with rabbit anti-actin antibody (1:15,000, Sigma, St. Louis, MO).

5.2.3 Co-Immunoprecipitation

Following homogenization and protein determination as detailed above 100 µg of protein was incubated with rabbit polyclonal adenosine A2a receptor antibody and 20 ml of strepavidin beads (PIERCE, Rockford, IL) diluted in ethanol. Following overnight incubation the bead-antibody-protein complexes were boiled and the supernatant subjected to the western blot

analysis as detailed above. For co-immunoprecipitation all primary antibodies used following running and transfer were mouse monoclonal to reduce the detection of rabbit IgG attached to the streptavidin bead complex.

5.2.4 Immunohistochemical Methods

Tissue Preparation: Animals were be deeply anesthetized with pentobarbital (Nembutal, 80-100 mg/kg; Abbott Laboratories, North Chicago, IL) at the appropriate interval after CCI and sham procedure. The rats were transcardially perfused with 100 ml 0.1 M phosphate buffered saline (PBS) with 5 U/ml heparin pH 7.4, followed by 500 ml 4% paraformaldehyde with 15% saturated picric acid in 0.1 M phosphate buffer pH 7.4. After perfusion, the brain was removed and placed into the same fixative for 30 minutes, then immersed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C overnight. The brain was transferred to 15% sucrose in 0.1 M phosphate buffer pH 7.4 at 4°C for 24 hours, then to 30% sucrose in 0.1 M phosphate buffer pH 7.4 at 4°C until the brain sunk.

Section of Tissue: The cryoprotected rat brain was frozen and used for cryostat sectioning. Coronal sections were cut for the striatum in 35 µm thickness in a cryostat (Jung CM 1800; Brodersen Instrument, Valencia, PA) and collected in 24-well culture plates containing 0.1 M phosphate buffered saline (PBS).

Confocal Fluorescent Immunohistochemistry: Immunohistochemistry for D2 and A2a was conducted in 24-well culture plates by free floating technique. Sections were pre-blocked with 10% normal rabbit serum (NRS) and 0.1% Triton X-100 in 0.1 M PBS. Sections were incubated with primary antibodies (Chemicon, Inc.) with 5% NRS and 0.1% Triton X-100 in 0.1 M PBS at 4°C for 16-24 hours, respectively. Affinity-purified rabbit anti-rat IgG and rabbit anti-goat IgG

labeled with fluorescent tags (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) were incubated as secondary antibodies with 5% NRS and 0.1% Triton X-100 in 0.1 M PBS at 4°C for 2 hours on a shaker. Tissue was rinsed between all steps with 0.1% Triton X-100 in 0.1 M PBS three times for at least 10 min each time. Sections were rinsed several times in 0.1 M Tris-buffered saline (TBS), mounted on subbed slides, dehydrated in alcohols, defatted in xylenes, and coverslipped for confocal microscopic analysis. At least three sections of brain tissue through striatum were processed for each antibody immunoreactivity. Control experiments were run in parallel to confirm specificity. Primary antibodies were either omitted or preabsorbed with peptide at 4°C for 16 hours before the incubation.

5.3 RESULTS

5.3.1 Alterations in non-dimerized A_{2A}-D₂ receptor protein following TBI

To investigate the levels of A_{2A} receptor protein in rat striatum following CCI model of TBI we homogenized rat striatal sections excised from fresh rat brain and stored them at -70 degrees Celsius. Membranes with rat striatal protein were probed with a primary antibody to A_{2A} receptors to determine protein amount at 1 day, 2 weeks, and 4 weeks following TBI. B-actin was run on all membranes to verify equal protein loading in all samples. Membranes were also probed with D₂ receptor antibodies to compare D₂ protein levels to A_{2A} protein levels.

Densitometry on A_{2A} receptor western blots (**Figure 38**) comparing sham surgery on the ipsilateral side (sham right) to CCI ipsilateral side (injured right) showed no significant difference at 1 day or at 2 weeks. Nor was there any significant difference in D₂ receptor levels at 1 day or 2 weeks. However analysis of 4 week western blots showed a significant difference for both A_{2A} receptors (p=0.05) and D₂ receptors (p<0.05).

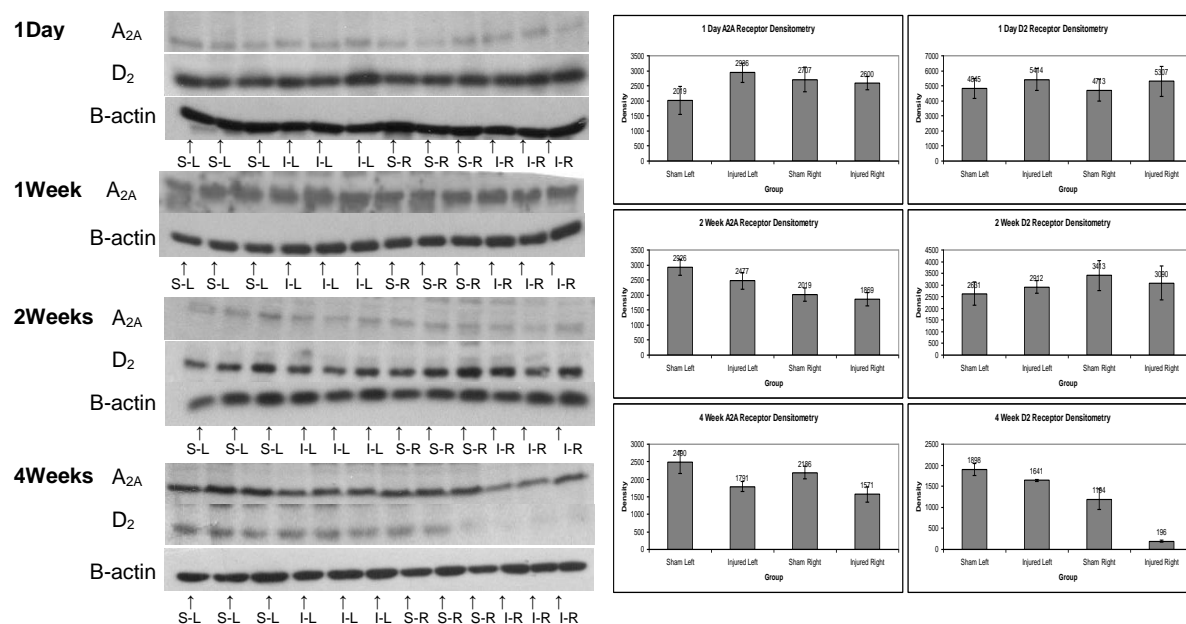


Figure 38: A_{2A} and D₂ receptor protein levels as determined by western blot in 1 day, 1 week, 2 week, and 4 week striatal sections. Densitometry analysis performed using ImageJ on 1 day, 2 week, and 4 week samples. 4 week striatal sections showed significantly reduced A_{2A} and D₂ receptor protein levels in injured right side compared to sham right side. S-L (sham left side); S-R (sham right side); I-L (injured left side); I-R (injured right side). * $P \leq 0.05$

There is also a significant difference in the ratio of A_{2A}-D₂ receptor protein ($p < 0.05$) in TBI compared to sham ipsilateral at 2 weeks post injury suggesting a possible alteration in A_{2A}-D₂ tonic function (**Figure 39**).

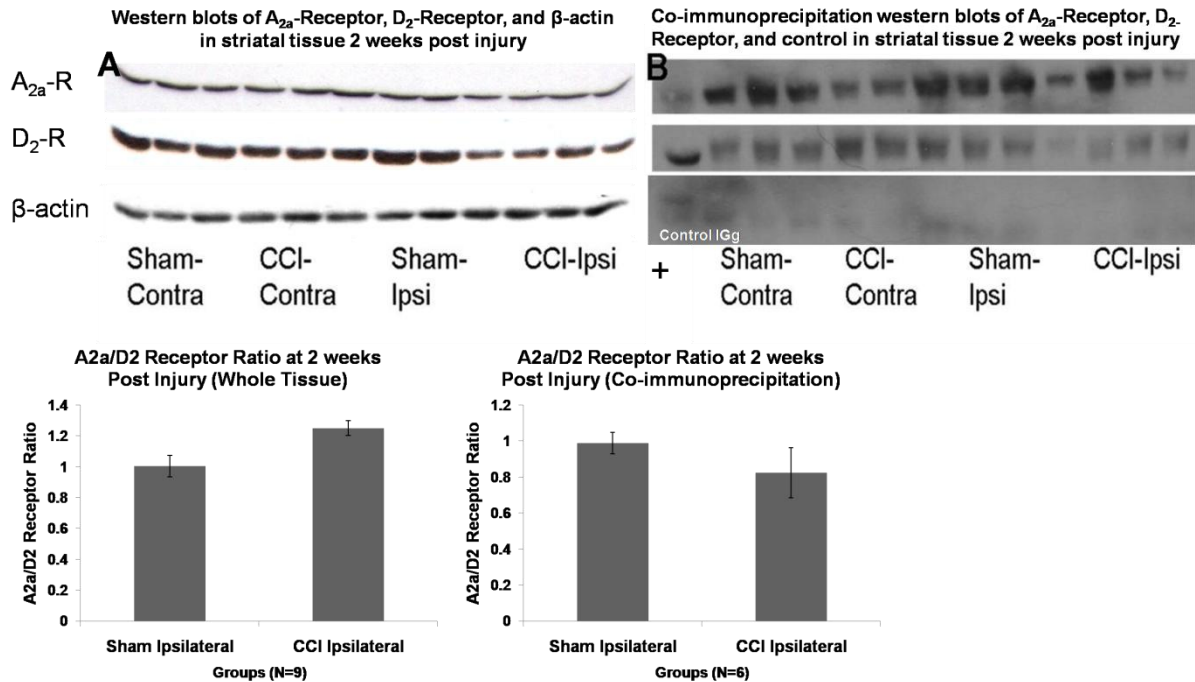


Figure 39: Example western blots and densitometry analysis of A_{2a}/D₂ receptor levels in standard and co-ip preparations. Standard A_{2a}/D₂ receptor ratios demonstrate a significant increase in the number of A_{2a} to D₂ receptors in ipsilateral injured striatum compared to sham ($p < 0.05$) while there is a trend for significance in a dimerized co-ip preparation ($p = 0.06$). Data presented represents mean A_{2a}/D₂ ratio standardized to actin \pm SD.

5.3.2 Alterations in A_{2a}-D₂ receptor heterodimer protein following TBI

To determine if there was a change in A_{2a} and D₂ receptor heterodimer expression post TBI in the striatum, we examined co-immunoprecipitation of A_{2a}-D₂ protein expression and confocal co-localization at 1 day and 2 wks post TBI. Confocal co-localization demonstrates a decrease in the co-localization of A_{2a}-D₂ receptors in striatal neurons (**Figure 40**). Co-immunoprecipitation analysis showed a trend towards alterations in A_{2a}-D₂ heterodimer levels following TBI ($p = 0.06$) (**Figure 39**).

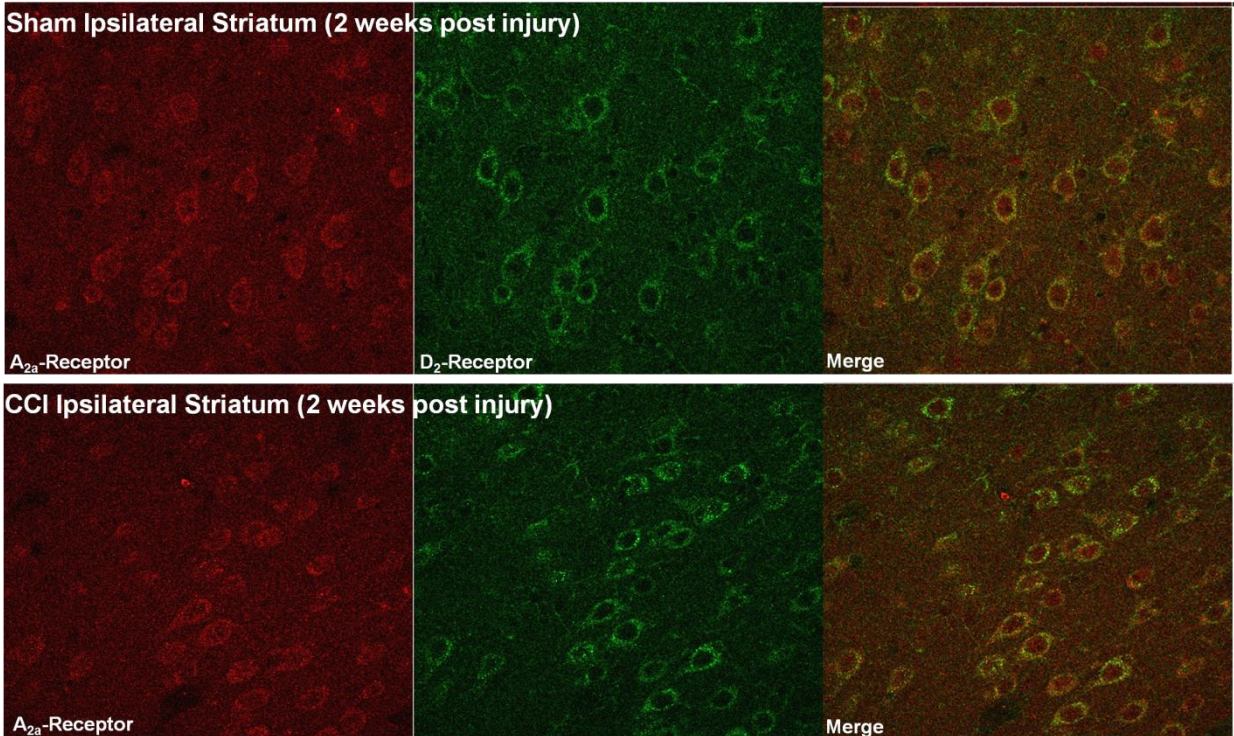


Figure 40: Confocal imaging shown co-localization of A_{2a} and D_2 receptors in (top) ipsilateral sham striatum and (bottom) ipsilateral CCI striatum. Confocal examination demonstrates a decrease in A_{2a}/D_2 co-localization due to an alteration in D_2 receptor staining pattern in CCI striatum. D_2 receptors in injured striatum demonstrate an increase in punctate staining that may be related to receptor aggregation.

5.4 DISCUSSION

The striatum is composed of two separate but morphologically indistinguishable GABAergic medium spiny neuron populations. D1 predominant and D2 predominant cell populations have unique projection patterns that dictate different mechanism both within the striatal network and to other brain regions. Our analysis of DARPP-32 signaling deficits post TBI does not indicate a predominant effect on either cell population, though future studies need to fully characterize each cell population with respect to DARPP-32 and ERK 1/2 phosphorylation alterations.

However, prior research has demonstrated a significant level of behavioral dysfunction in D2 antagonist treated animals with a TBI (Kline et al., 2007; Hoffman et al., 2008). Because of this we investigated the direct consequence of a TBI on D2 receptor expression and found that there was a significant alteration post TBI at chronic timepoints (**Figure 38**). We also found an alteration in both the level of D2/A2a receptor expression within the striatum and a trend towards a specific decrease in the level of D2/A2a heterodimer expression as determined using co-immunoprecipitation and confocal co-localization (**Figures 39 and 40**). This indirectly suggests that post TBI there is a significant alteration in D2 binding characteristics as prior studies have shown that A2a activity can directly affect DA binding to D2 receptors.

A persistent decrease in the tonic inhibition that A2a receptors have on coupled D2 receptors would cause an ongoing increase in intracellular Ca²⁺ release associated with D2 action (**Figure 41**). This would have the effect of increasing calcineurin activity within the striatum and subsequently decreasing DARPP-32-Thr34 phosphorylation. The ability of FK-506 to block p-DARPP-32-Thr34 loss after TBI (see prior chapter) is consistent with increases in calcineurin activity. This represents a potential cause of persistent DARPP-32 dysfunction in the striatum post TBI.

Interaction of A_{2a} and D₂ receptors post TBI: Theoretical Model

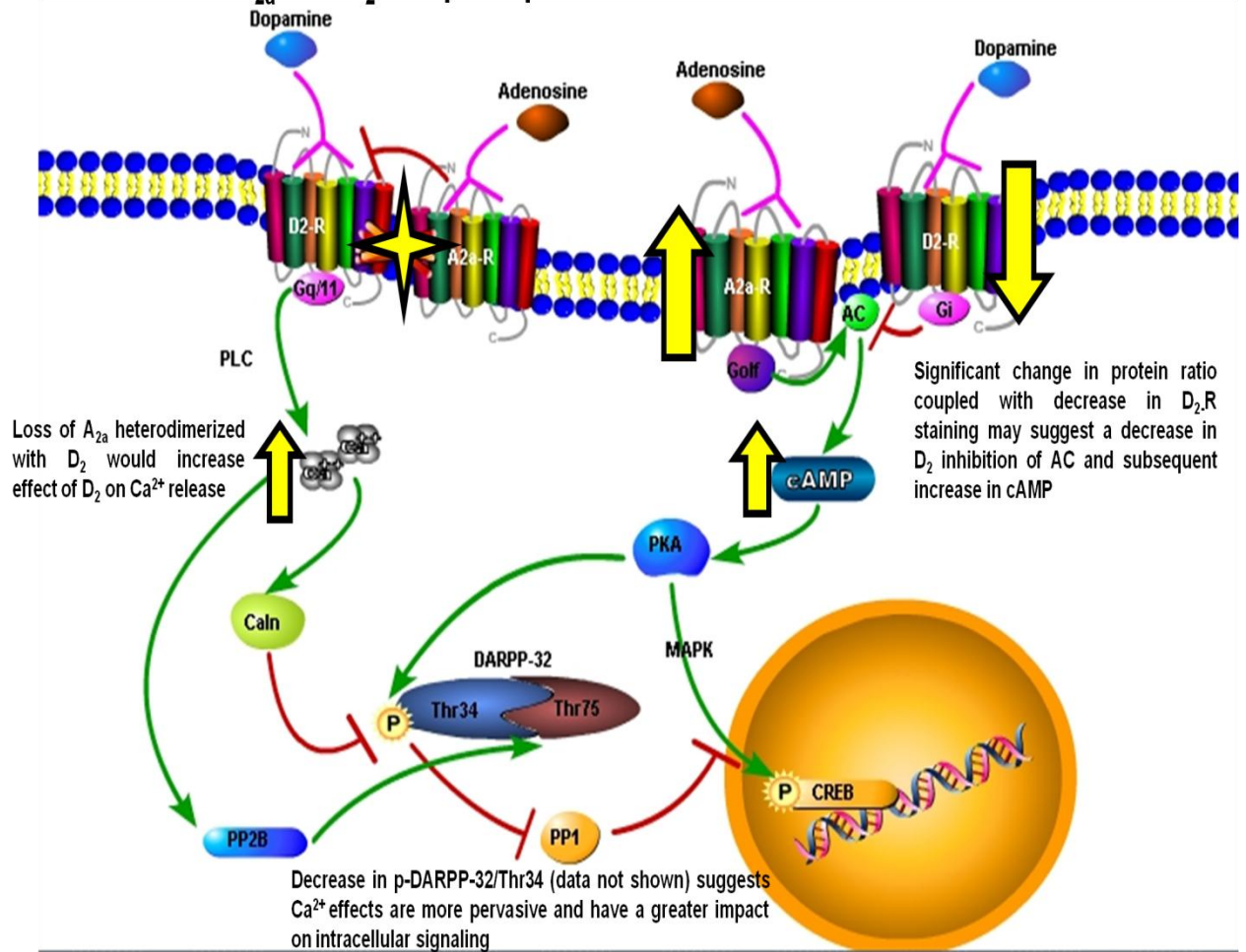


Figure 41: Schematic representation of the theoretical effects of A_{2a}/D₂ alterations in both heterodimer and non heterodimer interactions on intracellular signaling.

6. SUMMARY AND CONCLUSIONS: PERSISTENT DYSFUNCTION FOLLOWING A TBI AND THE POTENTIAL ROLE OF DARPP-32

6.1 HISTORICAL PERSPECTIVE

Examination of neurologic function following an experimental TBI has been integral to TBI research since suppressed reflex testing first described by Denny-Brown and Russell (1940). Early paradigms limited themselves to the assessment of unconsciousness as a marker for neurologic function following injury (Ommaya et al., 1966; Ommaya and Gennarelli, 1974; Yarnell and Ommaya, 1969). Most criteria for establishing the level or severity of injury was based predominantly upon a calculation of the forces involved or a histologic analysis of traumatic damage (Brown et al., 1972; Saunders et al., 1979). While important to evaluating the neuronal, axonal, and vascular damage that occurred due to a TBI, the necessity of dissection made these assessments difficult to translate to clinical outcomes of surviving patients. In the early 1980's more detailed cardiovascular monitoring (heart rate, respiratory rate, blood pressure, perfusion pressure) coupled with tests of neurologic deficit (consciousness, corneal reflex) were used in monkeys to enhance the clinical applicability of injury models (Brown et al., 1980; Gennarelli et al., 1981). In 1974 Teasdale and Jennett introduced the Glasgow Coma Scale (GCS) to provide a reproducible, consistent scoring system for TBI. Based upon the GCS a battery, of acute neurological tests was developed to examine the immediate severity and scope of experimental TBI in felines (Hayes, et al., 1984) and rats (Dixon, et al., 1987). To evaluate gross neurological motor function over a period of recovery McIntosh and colleagues (1989) developed a composite neuroscore to evaluate the chronic neurobehavioral state of

animals following a TBI. The composite neuroscore utilizes a series of tests designed to evaluate motor and somatosensory components of rodent behavior.

Both the acute examination of reflex suppression (Dixon et al., 1987) and the chronic evaluation of motor neurologic function (McIntosh et al., 1989) are utilized to evaluate and track the status of experimental TBI. This allows researchers to determine injury severity, recovery, and outcome without relying purely upon pathology and histology. Furthermore, reflex suppression and the composite neuroscore are more direct measures of neurologic damage than physiologic monitoring that involves cardiodynamic processes.

TBI causes persistent dizziness and disturbances in motor function and balance (Rutherford, 1977). Rutherford (1977) also noted disturbances in fine motor skills in even mild levels of TBI. The examination of complex motor tasks is an important part of the evaluation of experimental injury. The combination of beam-balance, to examine dizziness and balance, and beam-walking, to examine components of fine motor coordination, have been used extensively in rodent models of TBI (Dixon et al., 1987; Dixon et al., 1991; Kline et al., 2002).

The utilization of a beam-walking task in experimental brain injury was first described by Feeney and colleagues in 1982, while the beam balance task was described by Dixon et al., 1987. Both beam tasks (beam balance and beam walking) were used to characterize the FP (Dixon et al., 1987) and cortical impact (Dixon et al., 1991) models of injury. In addition to the beam tasks, the rotarod task was modified by Hamm and colleagues to evaluate deficits following injury in 1994. The rotarod task was first developed by Dunham and Miya in (1957) to evaluate the neurological effects of drugs and is the equivalent of a forced walking task often employed in patient recovery. Vestibulomotor testing in rodent models of TBI shows excellent sensitivity and

reliability in predicting severity and monitoring outcomes (Hamm, 2001). The rotarod and beam tests are also valuable in the evaluation of therapeutic interventions.

The combination of vestibular and motor examinations following experimental TBI in rodents is particularly useful given that the parietal cortex, the location of the primary motor and sensory cortices, is often the cortex of greatest impact. The examination of complex vestibulomotor testing following experimental brain injury allows for a clinically relevant paradigm of recovery, one which is both reliable and sensitive to therapeutic manipulation.

TBI patients also report significant cognitive disorders that present immediately after the initial injury or evolve during the subsequent months to years. These include deficits in memory, learning, and executive function (Binder et al., 1986; Arciniegas et al., 2000). The evolution of experimental TBI towards rodent models has allowed a much more in depth characterization of cognitive deficits following a TBI. Examination of cognitive deficits post TBI has been integral to understanding TBI deficits in patients. One of the most commonly employed methods of cognitive assessment post TBI is the Morris water maze apparatus (Dixon et al., 1997; Adelson et al., 1997; Kline et al., 2008). As discussed in the introduction, while the Morris water maze has limitations, it allows for a much more in depth analysis of cognitive dysfunction after TBI than simple neurological scores or motor behavior. This is particularly important considering that cognitive processing generally involves a wider spectrum of brain regions and is potentially less likely to recover than motor deficits. Furthermore, treatment strategies aimed at attenuating cell loss within cortical regions may be effective in dealing with motor deficits without affecting cognition.

The Morris water maze task is particularly useful for examining spatial learning and memory. This makes it an excellent tool for evaluating hippocampal dysfunction. Both FP and CCI

models of TBI have demonstrated significant cell loss in hippocampal CA1, CA3, and DG regions (Graybiel et al., 1990; Fox et al., 1998; Bye et al., 2007). There are well documented deficits in learning and memory associated with hippocampal lesions (Aggleton et al., 1999). This has made efforts to reduce cell death in the hippocampus of prime importance to TBI research. However, clinical and experimental failures of neuroprotective strategies to produce meaningful cognitive benefit necessitate a new direction for TBI research.

6.2 MOVING BEYOND THE HIPPOCAMPUS

TBI is a heterogenous and complex injury composed of acute, sub-acute, and chronic injuries (Kochanek, 1993; Park et al., 2008). Animal models including CCI (Lighthall, 1988; Dixon et al., 1991) and FP injury (FP) (Dixon et al., 1987) have provided insight into the cellular and mechanical mechanisms of central nervous system (CNS) dysfunction and cell death in TBI, allowing for the examination of valuable treatment strategies (Kline et al., 2007) and a better understanding of persistent deficits (Fox et al., 1998; Wagner et al., 2002; Wagner et al., 2004). Unfortunately, many of the neuroprotective strategies employed in TBI research have not translated well into clinical settings (Gualtieri, 1988; Tolia & Bullock, 2004). Potential reasons for poor translation of animal results include, but are not limited to, 1) the complexities of multi-system traumas typically seen in clinical settings (Ladanyi & Elliot, 2008; Capone-Neto & Rizoli, 2009), 2) a compromised blood brain barrier (Whalen et al., 1998; Folkersma et al., 2009), 3) potential drug toxicities and side effects (Muir, 2006), and incomplete preclinical evaluations. Strategies that target glutamatergic excitotoxicity acutely to provide neuronal sparing have proven particularly difficult given the important function of glutamate signaling in learning, memory and cellular potentiation (Parsons et al., 2007; Christoffersen et al., 2008; Gil-Sanz et al., 2008). To address these issues, multiple studies have utilized paradigms designed to inhibit cell death pathways with the intent to reduce the level of acute neuronal loss following injury

(Faden et al., 1989; Jennings et al., 2008). This, however, has often met with varied success due to persistent cellular dysfunction even with significant cell sparing (Muir, 2006; Bye et al., 2007).

We examined the hippocampus at a moderate injury level in chapter 2 and found persistent dysfunction in calcineurin signaling at up to 2 weeks post injury. This included nearly two-fold increases in calcineurin activity and regional alterations in hippocampal expression of both the calcineurin regulatory and catalytic subunits. Of particular interest is the fact that the alterations in calcineurin subunit distribution within CA1 and CA3 hippocampal regions do not appear to be movement towards the nucleus which is associated with cell death signaling pathways. Rather the most significant events appear to be the loss of catalytic expression within dendritic fields and the impressive upregulation of both subunits within the dentate gyrus. This suggests that calcineurin pathology post TBI is not exclusively related to neuronal death and there may be more insidious deficits that we have yet to fully understand.

At a more severe injury level as used in chapters 3 and 4 there remains little to no ipsilateral hippocampus in injured animals making the analysis of hippocampal pathology difficult at chronic timepoints. However what we can conclude from our studies is that even in the face of lesser cell death there remains significant alterations in key cell signaling molecules within the hippocampus post TBI. The dysfunction in calcineurin signaling has been well reported in FP injury (Kurz et al., 2005) and treatment with the calcineurin inhibitor FK-506 demonstrated a restoration of axonal conduction deficits seen post FPI (Reeves et al., 2007). Calcineurin is not limited to the hippocampus, however, and is widely expressed throughout the brain. For this reason FK-506 has the potential to play a significant role beyond hippocampal protection, which makes it a promising of choice for TBI induced deficits. There are some concerns over the use of FK-506 as it has significant toxicities associated with higher drug concentrations.

Ongoing, significant, hippocampal signaling pathologies not associated with widespread hippocampal loss, the benefits of FK-506, a drug that potentially acts on multiple brain regions, and the nature of a diffuse injury such as TBI all strongly indicate that it is critically important to begin looking beyond the hippocampus into other areas of potential dysfunction in experimental TBI.

6.3 DIVING INTO THE STRIATUM

6.3.1 Complexities

The striatum is complex. It is an interconnected network of excitatory and inhibitory signaling with predominantly GABAergic inhibitory outputs. **Figure 42** from Albin et al., 1989 demonstrates a simplified schematic of the basal ganglia with the more realistic schematic determined from experimental preparations.

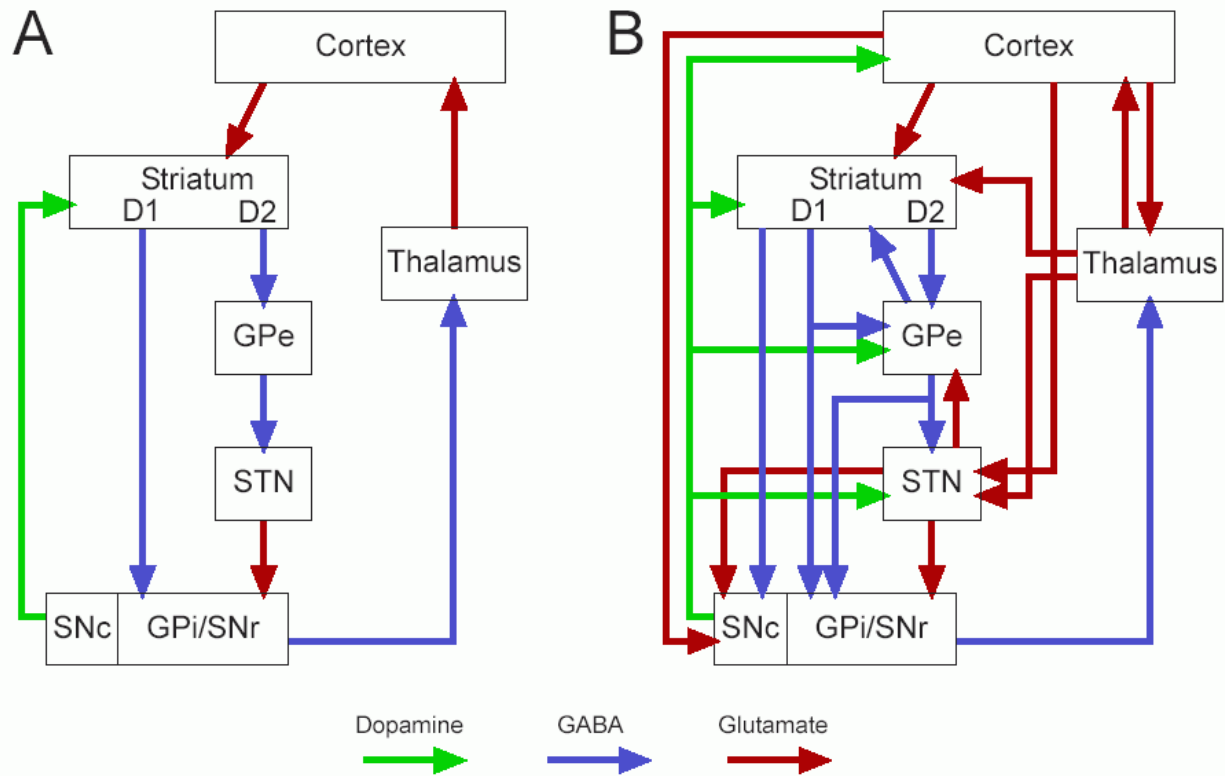


Figure 42: A) Simplified schematic of striatal signaling relevant to thalamic and cortical activity. B) The more realistic schematic demonstrated by electrophysiology and binding studies involving striatal outputs (From Albin et al., 1989).

Compared to the relatively simple architecture of the hippocampus, the striatum is a nightmare of signaling events, all of which could potentially be altered in TBI. There are significant alterations in DAergic tone within striatal tissue following a TBI (Massuci et al., 2004). Alterations in either DA or glutamate have significant implications for striatal function. In general, when considering striatal function it is easiest to think of the striatum as a modulator. It essentially dictates the strength of signaling within both the thalamus and cortex through tonic involvement of medium spiny neuron outputs (Mega et al., 1994; Calabresi et al., 2007). In doing so striatal signaling essentially works to assist the other brain areas in appropriate functional control.

This is apparent with regards to movement disorders, especially Parkinson's Disease, where striatal dysfunction essentially removes the control that patients have over the initiation of movements and maintenance of fine motor control. What is less clear is how this striatal function acts in cognition. The predominant theory involves a similar mechanism to that proposed in movement control. Essentially striatal output modulates cognitive processing allowing other brain regions to properly function. A dysfunction in striatal output therefore has the effect of making it difficult for the brain to properly choose the appropriate signal. Thus, reduced striatal output essentially leads to attention difficulties, and learning and memory deficits. Increased striatal output can have a similar effect where it overly dampens other neuronal systems leading to addictive behavior and psychosis (Goldman-Rakic, 1995; Wickens et al., 2007). In TBI this may actually be temporally related with an upswing in DA and striatal activity early followed by chronic dysfunction (**Figure 43**).

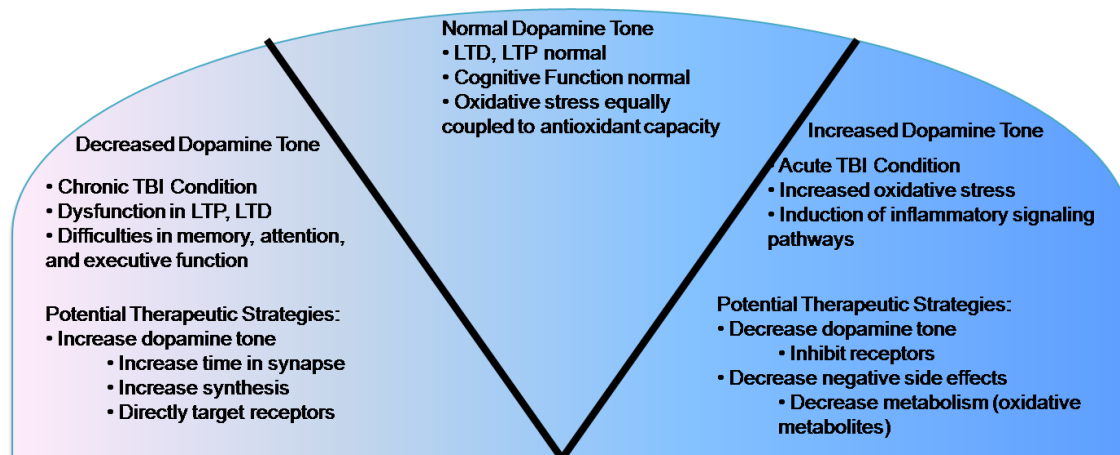


Figure 43: Dopamine is a tightly regulated system that has potential negative consequences with increased or decreased dopaminergic tone. Currently studies indicate that immediately following TBI there is an increase in dopamine while chronically there is a decrease. Therapeutic strategies used in TBI should consider the implications of this bi-phasic response in dopamine systems following TBI.

Given these complexities, treatments aimed at striatal dysfunction are difficult to evaluate. Is cell death or axonal damage important in a system where in Parkinson's Disease you require an 80% loss of DAergic inputs to even see behavioral alterations? Probably not, but then how do you evaluate possible dysfunction if it exists?

6.3.2 Benefit of Striatal Examination

While examining the striatum may be difficult there remains one significantly important aspect of striatal function that makes it almost a perfect system to examine in the complex pathology of TBI. Striatal outflow signaling is almost exclusively done by the GABAergic medium spiny neurons. While other neuronal populations exist within the striatal architecture, they are internally modulatory and do not directly involve themselves in outflow signaling (Snyder et al., 1998). This essentially creates a system with innumerable inputs, but only one output. Any alteration in upstream signaling events should be represented within the medium spiny neurons. Thus, to examine neuronal pathology within the striatum one need only examine one group of neurons. This sounds overly simple, and it does represent a simplification of the problem. There still exists glial elements and the potential for inflammatory processes which may cause pathology not directly linked to neuronal signaling, but one could argue that when determining the function of the striatum it is the neuronal population that is the most important to consider.

Within the striatum the GABAergic medium spiny neurons comprise about 95% of the neuronal architecture making them an abundant neuronal type within striatal samples. They receive glutamatergic, DAergic, GABAergic, adenosine, noradrenergic, and serotonergic inputs from multiple brain regions including the cortex, substantia nigra, and ventral tegmental area (Snyder et al., 1998; Calabresi et al., 2000; Azdad et al., 2009). More importantly nearly all of the

function of the medium spiny neurons is dictated by the key regulatory molecule that we examined in chapters 3 and 4, DARPP-32.

DARPP-32 is essentially the molecular equivalent of the striatal medium spiny neuron. It is expressed within more than 90% of the medium spiny neuron population, is only expressed within medium spiny neurons, and responds to nearly every signaling event within the neuronal body. It regulates nuclear transcription, synaptic plasticity, and upstream signaling. As explained in the introduction and further in chapter 3, DARPP-32's effects are determined by the phosphorylation state of its Thr34 and Thr75 sites. By examining the phosphorylation sites of DARPP-32 you can essentially determine if your manipulation is having an effect on striatal signaling. This effectively turns DARPP-32 into a molecular screening tool for potential therapeutic effects on striatal pathology. Furthermore, since DARPP-32 is important to the essential function of striatal outflow neurons manipulating it is a powerful tool in altering striatal signaling and potentially providing neurologic benefit. So that is what we did.

6.3.3 DARPP-32 in TBI

TBI causes a significant, prolonged dysfunction in striatal DARPP-32 phosphorylation and downstream signaling events including PP-1 activity and ERK 1/2 phosphorylation (**Figures 17,20,22**). DARPP-32 dephosphorylation occurs acutely at Thr34 following a TBI and persists up to 4 weeks after injury (**Figure 17**). An increase in PP-1 activity is seen at both 1 day and 2 weeks post injury (**Figure 20**) consistent with the loss of phosphorylation at DARPP-32-Thr34. ERK 1/2 phosphorylation in nuclear extracts is decreased at 3 weeks post injury relative to sham levels (**Figure 22**). Each of these events demonstrates individual dysfunction at points within the cell signaling pathway of striatal medium spiny neurons, taken together they indicate

a breakdown of striatal cellular signaling (**Figure 43**) that has significant implications for the function of the frontal cortex, amygdala, limbic cortices, and a number of other brain regions.

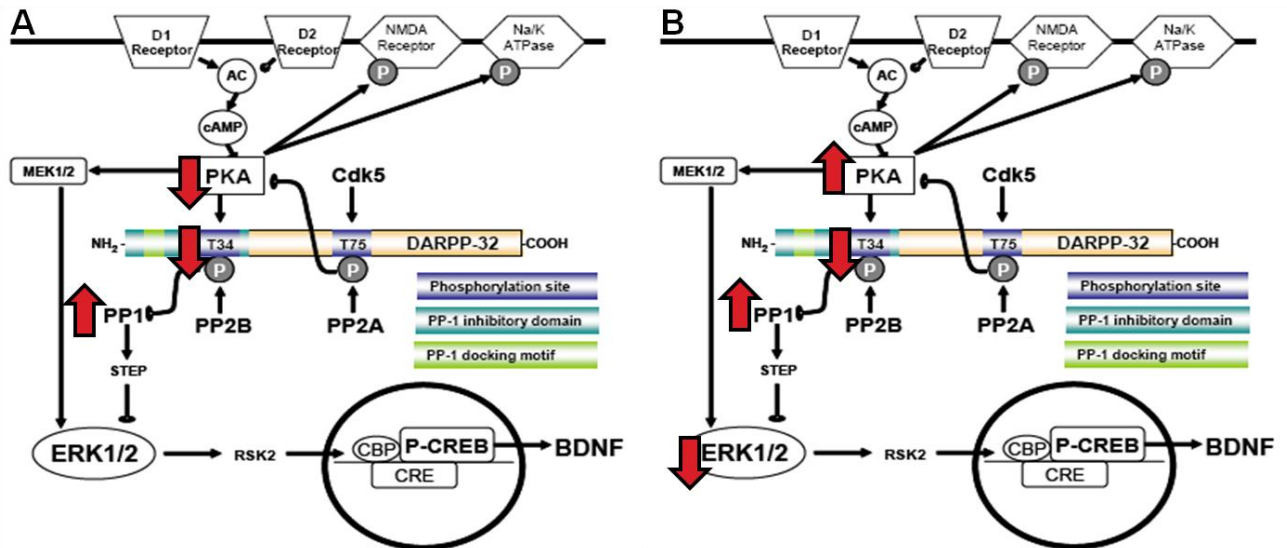


Figure 44: A) Early alterations (up to 1 day post injury) seen in DARPP-32 signaling post TBI. B) Chronic alterations (past 2 weeks) in DARPP-32 signaling post TBI. The only system that appears to correct itself overtime is PKA activity, however as is evident from the figure even the increase in PKA activity seen at later timepoints is not enough to overcome DARPP-32 dephosphorylation effects. A persistent increase in PP-1 activity and decrease in ERK 1/2 phosphorylation can alter neuronal plasticity, nuclear transcription, and cell survival signaling.

This chronic alteration in DARPP-32 signaling is consistent with data demonstrating decreases in DA within the striatum overtime (Massucci et al., 2004). Furthermore the change in DARPP-32 signaling confirms that there is a dysfunction in striatal neurons following a TBI regardless of the upstream mechanism. This is critically important because it indicates that the striatum is not only a viable target for post TBI deficits, but is potentially a necessary one in addition to the hippocampus and cortex.

6.4 DEVELOPING NEW THERAPIES FOR TBI

There are two approaches to the development of new therapeutic strategies for TBI and both have seen success. First, there is the examination of new molecular targets in multiple brain regions or within other systems that may affect brain function, including inflammation. The majority of these examinations remain focused on neuronal survival, however they are beginning to address cellular pathology in addition to neuroprotection. Recent studies involving steroids have shown promise theoretically because in addition to neuroprotection they also involve the inhibition of inflammation and the potentiation of beneficial neuronal cell signaling (Gibson et al., 2008). In addition multiple drugs which target mitochondrial oxidative stress signaling have shown experimental promise, again predominantly through a cell death mechanism, but oxidative stress is also important in synaptic plasticity (Friberg et al., 1998; Mbye et al., 2009).

The second approach to new therapeutic interventions post TBI is to combine two current therapies with the hope that they will provide a synergistic or complimentary benefit in post TBI pathology. Again the focus of many of these manipulations is on targeting multiple aspects of neuroprotection, however it is generally recognized that by targeting a number of different cellular pathologies benefits are more likely to appear in behavioral measures (Kline et al., 2007; Griesbach et al., 2008). Unfortunately there is an inherent difficulty in combining existing TBI therapies in experimental trials. Many treatments developed for use in TBI rely upon the inhibition of one negative event with the caveat that they may potentially cause another. This is true in studies involving hypothermia where the inhibition of cell death is the positive event which needs to be weighed against the potential for hemodynamic consequences that could lead to worsened outcomes (Yan et al., 2000; Kline et al., 2002). This is also true of NMDA antagonists designed to inhibit glutamatergic toxicity, but at the potential consequence of

limiting normal neuronal plasticity (McIntosh et al., 1989; Izquierdo et al., 1997). Thus by combining a number of therapies with different mechanisms of action it becomes difficult to manage appropriate levels when the combined therapies may potentially cause the negative events associated with a single therapy to be compounded.

We chose to take both approaches with the hope that we would be able to design a combination therapy that provided a synergistic benefit and limited adverse outcomes by targeting a specific molecular event, DARPP-32 phosphorylation alterations after TBI.

6.5 CONCLUSION

Striatal DARPP-32 pathology post TBI represents a very promising therapeutic target that responds to multiple signaling events, is an excellent readout of striatal cellular dysfunction, and has the potential to affect function in multiple other brain regions.

In chapter 4 we discussed how we proceeded to target DARPP-32 phosphorylation deficits post TBI. **Figure 24** outlines the theoretical model of combining FK-506 and Amantadine in post-TBI treatment. However, the benefits of utilizing FK-506 and AMH extend beyond a synergistic role in DARPP-32 function. FK-506 has shown benefit in both hippocampal pathology and on white matter damage post TBI (Reeves et al., 2007). AMH has known clinical benefits on cognitive and behavioral processing post TBI (**Table 2**). Our hope was that by using DARPP-32 as a synergistic target we could gain the benefits associated with both of these drugs without enhancing any negative side-effects and in addition provide significant improvements in striatal function.

We showed that by using dosages of both drugs that did not provide benefits in rodent behavioral examinations (**Figures 33-35**) and combining them into a single treatment strategy we were able to modestly improve behavioral outcomes post TBI (**Figures 33 and 35**). By utilizing DARPP-32 as our molecular target we effectively developed a synergistic drug therapy that demonstrated benefits in striatal molecular signaling (**Figures 29 and 30**) and outcomes.

Furthermore, our benefits were not directly related to neuroprotection as our lesion volume analysis shows that there is no added benefit to lesion volume (**Figure 32**) with the addition of chronic AMH administration over a single dose of FK-506. A significant reduction in lesion volume suggests that neuroprotection was not important for the combination therapy. Behavior itself may also play an important role in recovery post TBI. We saw an attenuation of DARPP-32 phosphorylation loss in the contralateral striatum of rats undergoing behavioral analysis in **Figure 29** compared to what was previously seen in **Figure 17**. Given DARPP-32's important role in motor and memory functions of the striatum this may indicate that a relatively intact contralateral striatum was able to respond to simple behavioral interventions.

It remains to be seen if the reversal of DARPP-32 pathology played a significant role in behavioral outcomes or if it was simply a read-out of the benefits provided by the combination therapy. We believe it represents both, and future studies utilizing DARPP-32 knockout mice will likely demonstrate that this combined drug therapy is less able to provide functional benefits in the absence of DARPP-32 signaling.

6.6 LIMITATIONS AND FUTURE DIRECTIONS

We recognize that there are limitations in the analysis of this system. While DARPP-32 is specific to the striatal medium spiny neurons in our cell homogenates, PKA, ERK, CREB, and

PP-1 are not. Thus, while the trend in PKA and PP-1 activity is potentially within the neuronal population there is also the possibility of influences from other interneurons, glia, and infiltrating inflammatory cells. What is interesting, however, is that even with these considerations our drugs have an effect on the system of interest. Future examinations of fluorescent co-localization studies will help to elucidate the striatal compartment where we are getting the majority of our effects.

There are also limitations in the analysis of the immunohistochemistry data for both the calcineurin subunit analysis and p-DARPP-32 cell expression. We chose to use semi-quantitative methods in both instances over the more quantitative stereologic methods for a number of reasons. One was due to the technical challenge of performing unbiased stereology in each of these studies. The second was dependent upon how we approached the examination of our DAB staining. We were interested in the expression patterns of the DAB stain not only within cell bodies or dendritic branches, but also within the neuropil. A stereologic approach does not allow us to comment on the level of neuropil staining since there is nothing to count. We chose to use a blinded scoring method to analyze the calcineurin data in the hopes that we could capture multiple aspects of any alterations and were thus limited to the utilization of non-parametric analyses to determine statistical significance. With regards to the p-DARPP-32 semi-quantitative cell count method, this method was utilized because it was not felt that an unbiased approach could be employed to identify “positive” neuronal bodies. We wanted to determine if there was a reduction in cell body expression of p-DARPP-32-Thr34 and setting a threshold for what was considered a positive neuron (based solely on its level of p-DARPP-32-Thr34 staining) seemed to be a logical way to approach the question. Another potential approach would be to use densitometry on cell bodies to determine overall decreases in cell staining, however the difficulty with a densitometry approach is that differences in background staining can affect the outcome. Future studies utilizing fluorescent staining may be able to overcome

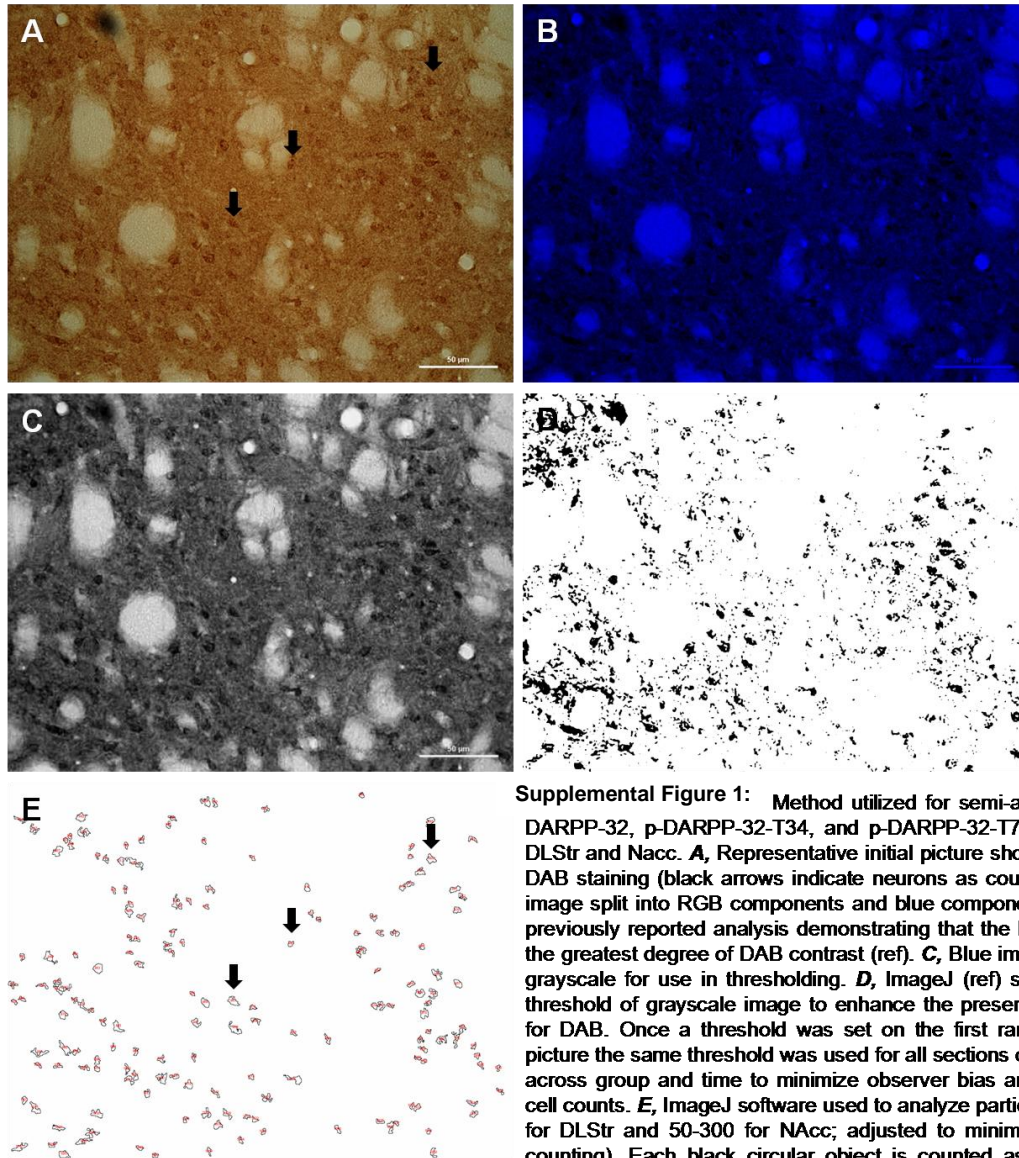
these limitations by employing a measure of the level of fluorescence of cell bodies in injury versus sham.

For these studies we utilized the classic spatial learning version of the morris water maze, which has been well published in TBI literature. However, there are modifications which are better able to examine working memory which may be more relevant to a striatal injury. There are also a number of other potential behavioral tasks that should be examined that have stronger striatal components including novelty seeking, reward learning, motor learning, and executive function tasks. By developing a more widespread analysis of behavioral deficits we would be able to potentially tease out individual brain region dysfunctions in a TBI paradigm.

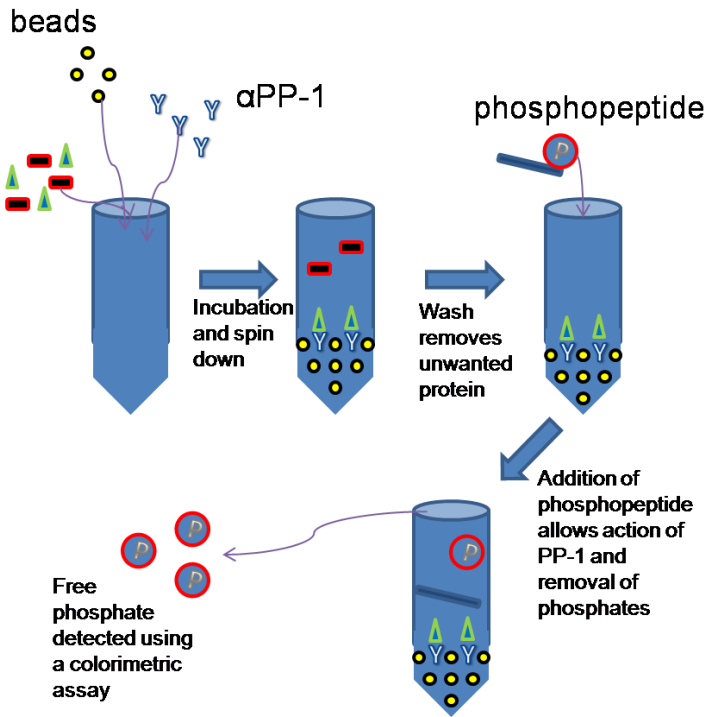
Future studies will also need to employ a variety of injury severities and locations which may cause variations in striatal damage that would help elucidate the responsiveness of DARPP-32 to injury. Also a more rostral injury could cause more significant striatal dysfunction given that it would have a greater affect on cortical neurons that send projections into the striatal compartment than the more caudal injury utilized for these studies.

Finally there remains a significant concern over the specificity of our drug treatments. While we observed alterations in our system of interest, this does not mean that these drugs mechanisms of actions are limited to DARPP-32. In fact both FK-506 and Amantadine have multiple potential targets within the brain including other brain regions, inflammatory response, and a number of potential neurotransmitter targets. This makes detailing the exact effect of drug therapy difficult to determine, but may actually provide a more consistent benefit in the complex injury caused by a TBI. The future utilization of a DARPP-32 knockout model will help elucidate the particular role of DARPP-32 in this combination therapy and other potential therapeutics in a TBI.

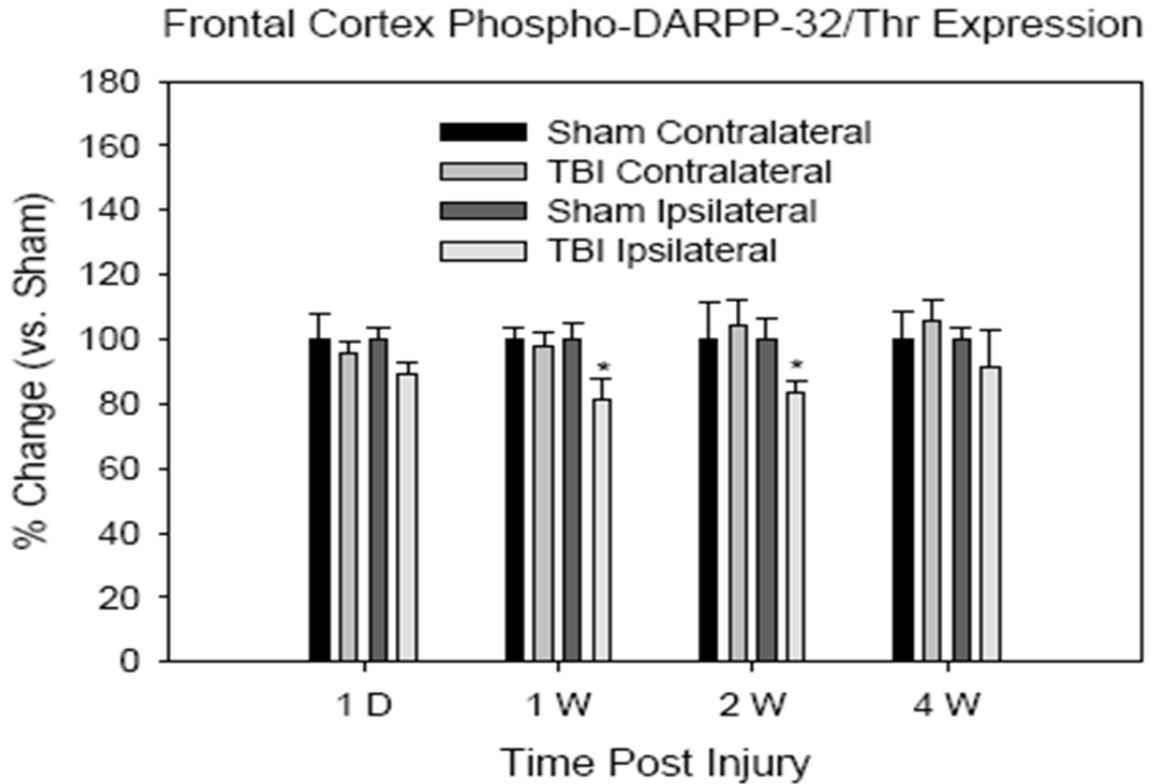
APPENDIX A: SUPPLEMENTAL MATERIAL FOR CHAPTERS 3 AND 4



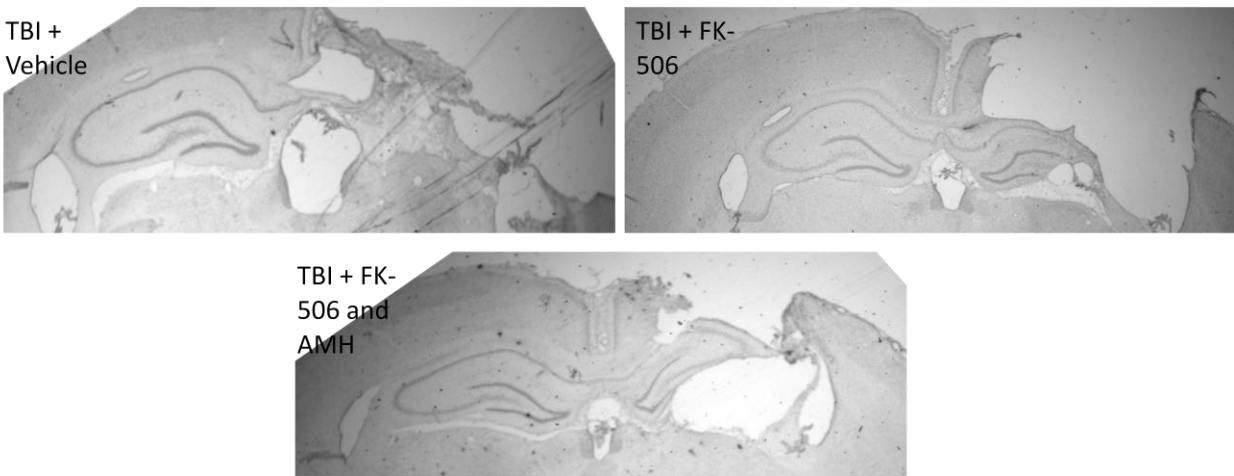
Supplemental Figure 1: Method utilized for semi-automated cell count of DARPP-32, p-DARPP-32-T34, and p-DARPP-32-T75 positive MSN in the DLStr and Nacc. *A*, Representative initial picture showing DLStr with brown DAB staining (black arrows indicate neurons as counted in *E*). *B*, Initial tiff image split into RGB components and blue component selected out due to previously reported analysis demonstrating that the blue component shows the greatest degree of DAB contrast (ref). *C*, Blue image converted to 16 bit grayscale for use in thresholding. *D*, ImageJ (ref) software used to adjust threshold of grayscale image to enhance the presence of neurons positive for DAB. Once a threshold was set on the first randomly selected tissue picture the same threshold was used for all sections of that primary antibody across group and time to minimize observer bias and standardize positive cell counts. *E*, ImageJ software used to analyze particles (pixel size 100-500 for DLStr and 50-300 for NAcc; adjusted to minimize non-neuronal stain counting). Each black circular object is counted as one positive staining neuron (black arrows).



Supplemental Figure 2: Depiction of PP-1 assay to detect specific PP-1 activity in homogenized brain tissue. 100 μ g of protein, determined via BCA assay, is added into a eppendorf tube with 25 μ l of agarose A/G beads and 6 μ l of anti-PP-1 δ rabbit antibody. Following an overnight incubation in 4°C and brief spin down at 10,000 g, beads are washed 3 times with ice cold TBS and once with Ser/Thr phosphopeptide buffer. Following final wash 30 μ l of phosphopeptide and 20 μ l of Ser/Thr phosphopeptide buffer is added to the eppendorf tube. The eppendorf tube is then incubated for 10 minutes in a water bath at 30°C. 25 μ l of supernatant is placed in a 96 well plate (in duplicates) and malachite green detection assay is utilized to visualize free phosphates. Visualized plate is read on a plate-reader at 650 nm. A standard curve is calculated and the level of phosphatase activity is reported in picomoles of phosphate released per 25 μ l of supernatant. 10 μ l of TBS and 10 μ l of 2x loading buffer is added to the eppendorf. The eppendorf tube is then heated to 95°C for 5 minutes and western blots are run to determine the relative amount of PP-1 protein in each tube. Final values are normalized to western data to ensure equal protein for each sample.



Supplemental Figure 3: Timecourse of TBI effect on p-DARPP-32-T34 in rat frontal cortex (N=6 per group at each timepoint). Following TBI there was a delayed decrease in only ipsilateral cortex, relative to injury, p-DARPP-32-T34 that did not persist past 4 weeks. * $p \leq 0.05$ normalized to β -actin and compared to sham; ANOVA. Data represented as a percentage of sham following normalization to β -actin \pm S.E.



Supplemental Figure 4: Sample images of lesion post controlled cortical impact.

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