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Restoration of Experience-Dependent Plasticity through Enhancement of Glutamatergic

Neurotransmission after Developmental Traumatic Brain Injury

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biomedical Engineering

by

Naomi Sulit Sta Maria

2012

ABSTRACT OF THE DISSERTATION

Restoration of experience-dependent plasticity through enhancement of glutamatergic

neurotransmission after traumatic brain injury in the developing brain

by

Naomi Sulit Sta Maria

Doctor of Philosophy in Biomedical Engineering

University of California, Los Angeles, 2012

Professor Christopher C. Giza, Chair

Traumatic brain injury (TBI) in children can cause persistent cognitive and behavioral dysfunction and inevitably raises concerns about lost potential or the inability to return to "normalcy" in these injured youths. After a diffuse model of traumatic brain injury, lateral fluid percussion injury (FPI), there is evidence of pathological activation of major ionotropic glutamate receptors, N-methyl-D-aspartate receptors (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate receptors (AMPAR), and an NMDAR-mediated downstream effector (calcium-calmodulin dependent protein kinase II – CAMKII) in the hippocampus.

These injury-induced molecular alterations have been shown to last longer in the developing brain compared to the mature brain. Moreover, these molecules are crucial mediators of experience-dependent plasticity and normal cerebral development, which prime the synaptic milieu for pro-plasticity circuitry formation. FPI administered to weanling rats has further been shown to impair enriched environment (EE)-induced experience-dependent plasticity that persists into adulthood.

Glutamate neurotransmission is essential in normal physiology, maturation and plasticity, and is greatly perturbed by TBI. While spontaneous recovery still occurs, the young injured brain is less receptive to the benefits of behavioral experience. Injured subjects may still recover to pre-injury baseline, depending on the severity of injury; however, they could demonstrate altered development and never perform at the same level as their non-injured peers. This is translated into lost potential. Clinically, however, glutamate blockade as a therapeutic intervention has failed to show neuroprotection or promote functional recovery clinically. Recently there is a shift in focus to agents that target the glutamatergic system positively. NMDAR agonists have facilitated recovery of learning and memory after experimental TBI in the mature brain. Particularly, when treated with NMDA or D-cycloserine (DCS), a partial agonist at the NMDAR glycine-binding site, brain injured animals have shown rapid functional recovery.

The central hypothesis of this dissertation is that the administration of an NMDAR agonist (DCS) during the post-injury period of diminished glutamatergic neurotransmission would do the following two things: 1) restore hippocampal glutamate-mediated markers of plasticity in the post-subacute time point (one week post-injury); and 2) reinstate hippocampal-

dependent, EE-induced experience-dependent plasticity. This translates into the restoration of lost plasticity after developmental FPI.

Twenty-four to seventy-two hours post-injury, DCS was administered intra-peritoneally, once every 12 hours, to postnatal day 19 rats that received either sham or FPI. In Chapter 1, the effects of DCS treatment on glutamate-mediated pro-plasticity molecules (NMDAR, AMPAR, and CAMKII) were investigated on post-injury day four (PID4) using western blotting. At the same time point, the effects of DCS were assessed using novel object recognition (NOR), a hippocampally-mediated, NMDAR-dependent working memory task. Not only was NOR used as a functional assay of NMDAR-mediated neurotransmission in the subacute PID4 time point, but also as measure of capacity for EE-induced plasticity. The ability to distinguish novelty would be essential in benefiting from the EE experience.

In Chapter 2, following either an FPI or a sham procedure, postnatal day 19 rat pups were differentially housed in standard cages or an EE for 17 days. The extent of EE-induced experience-dependent plasticity was measured by testing the animals' performance in a spatial learning paradigm (Morris water maze) thirty days after injury when they have matured. How early DCS treatment after developmental FPI influences experience-dependent plasticity was investigated. From the results of Chapter 1 and 2, enhancing NMDAR function during the period of reduced glutamatergic transmission following traumatic brain injury in young rats can reinstate molecular and behavioral responses. This subsequently manifests as rescued potential and experience-dependent plasticity later with long-lasting beneficial consequences.

Furthermore, glutamatergic neural activity can now be indirectly quantified by mapping the coupled change in relative cerebral blood volume (rCBV) using pharmacological magnetic

resonance imaging (phMRI). The noninvasive and translatable potential of this measure allows for a powerful within subject experimental design. In Chapter 3, a stimulation dose of an NMDAR agonist (DCS) was used to induce increases of rCBV specific to the hippocampus in intact developing rats. This evoked change in the NMDAR-mediated ΔrCBV was explored as a physiological biomarker for FPI. Clinical translational potential for this type of noninvasive diagnostic measure include the ability to assess the extent of diminished NMDAR-mediated glutamatergic activity and the efficacy of a glutamate-mediated treatment after TBI.

Using converging operations, this dissertation demonstrates that NMDAR-mediated glutamatergic dysfunction is an underlying mechanism of long-lasting impaired plasticity after developmental FPI. NMDAR agonist treatment after FPI during a critical period of development can reinstate the pro-plasticity milieu, which is crucial for acquiring beneficial experience-dependent plasticity.

The dissertation of Naomi Sulit Sta Maria is approved.

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2012

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INTRODUCTION

EPIDEMIOLOGY OF TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a major health problem in the United States. Annually, 1.7 million people are hospitalized or receive medical care due to TBI (Faul et al., 2010). This estimate can reach to approximately 4.5 million when taking into account mild and sports-related TBI for which medical care may not be sought (Langlois et al., 2006). Even then, this estimate may still be low because mild TBI is often under-diagnosed or under-recognized. There are an estimated 275,000 (16.3%) hospitalizations for nonfatal TBI, 1.365 million (80.7%) emergency department visits, and 52,000 (3%) TBI fatalities each year (Langlois et al., 2006; Faul et al., 2010). 124,000 (43%) of those who were acutely hospitalized develop TBI-related long-term disability (Selassie et al., 2008).

TBI has been referred to as the 'silent epidemic' due to its high incidence but concomitant frequent under-recognition. However, more and more people are becoming cognizant of its devastating and sometime insidious effects. TBI is recognized as the "signature wound" of the current military conflicts in Iraq and Afghanistan and the increased awareness of TBI has resulted in significant recent changes to the military's policies regarding casualty care and management (Meyer et al., 2010). There has also been substantial media attention on the dangers of sport concussion and growing concern for increased vulnerability to repeat injuries in both the young and adult populations. California has recently passed a bill mandating immediate removal from play of athletes who are suspected of having a concussion in school districts that sanction athletic events. These student athletes will not be allowed to return to play until they

are cleared by certified health care provider (Bill number: AB 25, Hayashi. School districts: athletics: concussion and head injuries, signed October 4, 2011).

The major risk factors for TBI include age (both oldest and youngest), male gender, and lower socioeconomic status. Children 0 to 4 years and 15 to 19 years are more likely to experience TBI than any other age group for all hospitalization, emergency department visits, and deaths combined (Langlois et al., 2006). For hospitalizations only, those older than 65 years have the highest incidence of TBI (Faul et al., 2010). Males are twice as likely to sustain TBI as females. In general, those with low economic status have a higher propensity for injury. People with no health insurance are estimated to be twice at risk of acquiring TBI than persons with private insurance (Langlois et al., 2006). A retrospective review of the National Trauma Data Bank (from 2000 to 2005) showed that insured patients with the most severe TBI had significantly lower mortality rates compared to uninsured patients (Alban et al., 2010).

TBI is caused mainly by falls (35.2%), motor vehicle incidents (17.3%), struck by or against events (which include colliding with a moving or stationary object) (16.5%), and assaults (10%) (Faul et al., 2010). Among active military personnel in war zones, improvised explosive device (IED) blasts are a leading cause of TBI. Sports and recreational activities are also a major source of TBI. Up to 3.8 million sports-related TBI injuries are estimated to occur each year (Langlois et al., 2006). These numbers for incidence and prevalence of TBI, and mild TBI in particular, may be severe underestimates as they do not take into account those who sought care outside hospitalization, or those who went unrecognized.

Not only can TBI lead to lasting disability due to neurobehavioral deficits, including impairments in cognition, mood and/or behavior, but TBI can also lead to increased risk for other

health conditions. Estimates of 5.3 million people in the U.S. live with TBI-related disabilities

(Selassie et al., 2008). There are reported presentations of sleep disturbances (Orff et al., 2009),

chronic pain (Manpiaparampil, 2008), anxiety, mood disorders, and depression (Horner et al.,

2008; Vaishnavi et al., 2009). One to three years post-injury, people with TBI are 1.8 times as

likely to report binge drinking, 11 times as likely to develop epilepsy, and 7.5 times as likely to

die (Langlois et al., 2006). With advancing age, those with TBI are 1.5 at risk of depression, and

have 2.3 and 4.5 times increased risk of Alzheimer disease for moderate and severe injury,

respectively (Langlois et al., 2006). TBI poses as a major public health concern with significant

economic burden. In the United States, TBI is estimated to cost $76.5 billion per year (Coronado

et al., 2012). This estimate reflects the cost of acute care, lifetime medical costs, and societal

costs or value of lost productivity (defined by short- and long-term wage loss costs). On

average, the direct medical and rehabilitation cost of severe TBI is approximately $65,000 per

patient. This estimate ranges from $36,000 for patients with moderate disability to good

outcomes to $92,600 for severely head injured patients or in a vegetative state (Faul et al., 2007).

Overall estimated societal cost per person is $330,000. Per deceased person, it is approximately

$1,000,000. For non-fatal TBI, the average societal cost is $4,103 for patients with moderate

disability to good outcomes and $74,673 for patients with more severe TBI outcomes. When

taking into account the long-term neuropsychiatric sequelae of TBI, the presence of psychiatric

illness has been associated with more than doubling the costs of non-mental health care in

patients with moderate to severe TBI (Rockhill et al., 2011).

TBI Severity

The Centers for Disease Control (CDC) have defined TBI as "craniocerebral trauma, specifically an occurrence of injury to the head (arising from blunt or penetrating trauma or from acceleration-deceleration forces) that is associated with any occurrences attributable to the injury: decreased level of consciousness, amnesia, other neurologic or neuropsychological abnormalities, skull fracture, diagnosed intracranial lesions, or death." The classification of injury severity in TBI is pertinent to both clinicians and researchers. It is important in assessing proper acute medical care for patients with acquired brain trauma, as well as in prognosticating recovery and outcome. Injury severity indices currently used in classifying TBI include describing the intensity of structural neuroanatomical alterations and the person's acute mental status. Most commonly used severity indices are the Glasgow Coma Scale (GCS) and the Abbreviated Injury Scale (AIS). These scales are cumulative scores assigned by trained personnel to assess the injured person's level of injury and responsiveness (usually administered within 48 hours post injury). The recorded GCS is a composite score from three tests: eye, verbal and motor responses. Scores from each test and their sum is considered and used to assess the level of consciousness after injury. The AIS is an anatomically-based score that classifies the severity of every injury in a body region (e.g. Head) according to a six point ordinal scale (1 Minor, 2 Moderate, 3 Serious, 4 Severe, 5 Critical, 6 Maximal – untreatable). Other measures of severity used are occurrence and duration of loss of consciousness (LOC) or of posttraumatic amnesia. All of these measures are categorized into *mild*, *moderate*, and *severe* and are usually used for patient triage management and to predict outcome (Corrigan et al., 2010). The CDC defines the severity of a TBI may range from "mild" (a brief change in mental status or consciousness) to "severe" (an extended period of unconsciousness or amnesia after the injury).

Table 1. Comparative Grouping of Indices of Severity
From Corrigan et al., 2010, with permission.

Index of Severity	Measurement Approach	Severity Category		
		Mild	Moderate	Severe
Abbreviated Injury Scale	Anatomic/structural damage	1-2	3	4-6
Glasgow Coma Scale	Neurologic deficits/mental status	13-15	9-12	3-8
Loss of Consciousness	Mental status/alertness	<30 min	30 min – 24 hr	> 24 hr
Posttraumatic Amnesia	Mental status/memory and recall	0-1 day	> 1 to ≥ 7 days	> 7 days

PATHOPHYSIOLOGY OF TBI

TBI is the result of an external mechanical force applied to the head, causing a rapid displacement of the brain within the skull that can lead to transient or lasting impairments in cognition and behavior. There are two major pathophysiological processes that contribute to brain injury after trauma: primary and secondary injuries. The primary injury is the damage that results directly from the mechanical force on cerebral tissue or vasculature at the moment of insult. Typically, the primary injury is irreversible and the extent of the induced damage depends on the load applied to the brain. The secondary injury begins immediately following after the trauma and can exacerbate the primary injury damage through activation of pathological cellular sequelae, which can persist over a period of hours or days from the initial injury. The underlying mechanisms for the secondary injury are potentially reversible processes because they need time to evolve and may be intervened upon. Concussions and mild TBI cases, however, result from the biomechanical activation of the secondary injury sequelae without the presence of primary injuries.

Primary Injury

The physical forces involved at the moment of trauma can dictate the type of primary injuries, which can lead to lasting dysfunction after TBI. Margulies and Coats (2010) describe that large impact forces to the head can result in one or a combination of skull fractures, epidural, subdural or subarachnoid hemorrhages, cerebral contusions and lacerations. High angular accelerations are more correlated with subdural hematomas and traumatic axonal injury (commonly known as diffuse axonal injury – DAI) than linear accelerations (Gennarelli et al., 1982; Raghupathi and Margulies, 2002). DAI involves shearing of the axonal cytoskeleton and the damage occurs not only at the time of injury but can last up to years due to subsequent deleterious biochemical cascades and Wallerian-type degeneration[1]. DAI has been widely accepted as the main pathological substrate for TBI, especially for mild TBI.

Rapid angular accelerations can occur without head impact, such as in blast injuries in military personnel or shaken-baby syndrome in children, but are usually associated with impact to the cranium, for instance in motor vehicle accidents, falls and assaults (2010). Furthermore, if there were no evidence of contact injury, as in the case of abusive head trauma, it does not mean that impact did not occur. Prange, et al, showed that a forceful impact of an infant to a 4-inch thick soft foam could dissipate the force to the head and leave no external evidence of impact but delivers head angular acceleration three times greater than shaking (Prange et al., 2003).

The site of injuries can also occur in other regions of the head other than the site of impact and can usually have a coup-contrecoup pattern. A coup injury can occur at the site of an impact or initial direction of the angular acceleration, which usually takes the form of cerebral

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[1] Wallerian degeneration: degeneration of nerve fibers that occurs following injury or disease and that from the place of injury along the axon away from the cell body while the part between the place of injury and the cell body remains intact (Merriam-Webster).

contusion or local hemorrhage. Injury to the opposite site of the impact or initial direction (contrecoup) also occurs, can be focal and/or diffuse in nature and can be of greater severity that coup injuries. Precise mechanisms of the contrecoup contusions need further investigation. It is proposed that the buoyant brain in CSF is displaced towards the opposite side of impact (Allen, 1896; Ommaya et al., 1971; Ommaya et al., 2002; Drew and Drew, 2004).

Using radiological imaging, primary injuries may be visualized and prognosis and plan of action can be further identified. Traditional computed tomography (CT) scanning is inexpensive and can be easily executed in the clinical setting. CT quickly and accurately identifies conditions that require immediate medical attention (e.g. mass lesions - hemorrhages, depressed skull fractures and cerebral edema). CT findings have also been correlated with long term outcome. In adults with TBI, a midline shift of greater than 5 mm or subcortical contusions evident in CT one week post injury have been associated with greater need for assistance (Englander, 2003).

However DAI is incredibly difficult to diagnose by CT scan. Less than 10% of the DAI patients demonstrate the classical CT primary injury manifestations of petechial hemorrhages at the gray-white junction of the cerebral hemispheres, corpus callosum, or brainstem (Kim and Gean, 2011). Since more than 80% of DAI is non-hemorrhagic, it is not evident in most CT scans. Magnetic resonance imaging (MRI) is a better diagnostic tool to more effectively detect both hemorrhagic and nonhemorrhagic lesions. Also, advanced MRI techniques, such as diffusion tensor imaging (DTI), have been used to examine potential DAI in white matter of living adult and adolescent TBI patients (Arfanakis et al., 2002; Levin, 2003; Huisman et al., 2004; Wilde et al., 2008) and measures of white matter integrity such as fractional anisotropy have been correlated with outcome (Benson et al., 2007; Suskauer and Huisman, 2009).

Assessment of primary injuries allows for identification of the magnitude and duration of neural unresponsiveness after TBI and would further help prognosticate the timeline of recovery.

Secondary Injury

Secondary injuries result indirectly from the biomechanical insult and develop from minutes to days after the primary trauma to the brain. These injuries consists of complex biochemical cascade of events that results in cerebral swelling as well as lasting cognitive deficits if no intervention is set in place. Secondary injuries echo that TBI is not merely a single event, but instead a progressive condition.

In severe cases of TBI, critical care management of clinically accessible secondary injuries such as intracranial pressure (ICP) (< 20 mm Hg) and cerebral perfusion pressure (CPP) (between 50 – 70 mm Hg) and cerebral oxygenation have been shown to maximize recovery and avert compounding the injury in adults (Nangunoori et al., 2011) and children (Stiefel et al., 2006). CPP is the difference between mean arterial pressure (MAP) and ICP and is used as an index for cerebral blood flow (Kim and Gean, 2011) (Bratton et al., 2007). CPP drives oxygen and nutrient delivery to the brain. Increased ICP is one of the hallmark consequences after TBI. When ICP increases due to TBI, or systemic blood pressure falls, CPP decreases. However, for instance, after a very severe injury, normal autoregulation (i.e. capacity of the brain to maintain appropriate blood pressure and blood flow to affected regions) is compromised. In this case, the brain loses the compensatory ability to maintain adequate blood flow and becomes ischemic, which leads to infarction and damage (Chesnut et al., 1993). Children and young adults are more susceptible to post-traumatic dysautoregulation that can lead to vasodilation, hyperemia,

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and cerebral swelling. Children are twice as likely to sustain diffuse cerebral swelling after TBI than adults (Marmarou et al., 2006). Prins et al has shown experimentally that postnatal day 17 (P17) rats demonstrated significantly different ICP and MAP response after a diffuse TBI (Prins et al., 1996). However, reduced oxygenation of brain tissue has been independently associated with poor outcome in adults and children and can occur with adequate control of ICP and CPP (Bohman et al., 2011; Rohlwink et al., 2011). The relationship of ICP, CPP and brain oxygenation needs further investigation for optimal patient management and facilitation of recovery.

With targeted attempts to reduce secondary injuries, such as increased intracranial pressure (ICP) due to cerebral edema after TBI, many systemic interventions have been shown to lead to improved outcomes. For example, a decompressive craniotomy has been used to alleviate severe increases of ICP and allows for the brain to expand during edema. Removal of hematomas could also relieve upsurges in pressure. Lastly, osmotherapy using hypertonic saline treatment have been shown to reduce cerebral edema while still allowing for the regulation of CPP to homeostatic levels (Noppens et al., 2006; Ziai et al., 2007).

Neurochemical and Metabolic Consequences in Secondary Injuries

Following TBI, cells experience neurochemical and metabolic changes that alter the extra- and intra- cellular environments. Depending on the time courses and interaction of these events, functional outcomes may be further exacerbated by the resulting secondary sub-cellular processes. The cascade of events includes an indiscriminate release of glutamate, which corresponds to TBI-induced ionic flux across the cell membrane and triggers the subsequent subcellular sequelae. While glutamate is normally in the mM concentrations physiologically,

clinical and experimental studies utilizing microdialysis have shown high levels of extracellular levels of glutamate (Katayama et al., 1990; Brown et al., 1998; Reinert et al., 2000) that can increase more than 100% within minutes after TBI. Excessive presence of glutamate triggers the phenomenon known as *excitotoxicity*, which is a pathological process due to excessive stimulation by neurotransmitters, such as glutamate. This is common pathway of necrotic cell death in TBI, in other brain insults and neurodegenerative diseases (Choi, 1992).

The acute TBI-induced glutamate release binds to corresponding receptors, such as the N-methyl-D-aspartate receptor (NMDAR), which in turn results in massive efflux of potassium ions and sustained intracellular increases in calcium levels (measured by $^{45}Ca^{2+}$ autoradiography) (Fineman et al., 1993; Osteen et al., 2001; Osteen et al., 2004). Consequently, there is a demand for the sodium-potassium (Na^+-K^+) pump action to return the cell from the ionic imbalance to a homeostatic level. The Na^+-K^+ pump requires large amounts of adenosine triphosphate (ATP) and its activation results in a drastic increase in glucose metabolism. Following experimental TBI, [^{14}C]-2-deoxy-D-glucose autoradiography has shown marked increases in glucose metabolism, lasting from 30 to 60 minutes after injury (Yoshino et al., 1991). Contribution of excitatory amino acid receptor activation in this hyperglycolysis following TBI has already been explored, wherein lesion of regions with high levels of excitatory amino acid receptors, like the hippocampal CA3, or use of excitatory amino acid antagonists after concussive brain injury has been shown to block this rise in glucose utilization (Kawamata et al., 1992; Yoshino et al., 1992). Additionally, accelerated glucose utilization has been shown to result in increased levels of lactate, which is a metabolic byproduct of anaerobic glycolysis (Kawamata et al., 1995). Lactate accumulation in the brain has been associated with "energy failure," (i.e. ATP hydrolysis exceeds ATP synthesis) and worsened neuronal damage in pathological states, such as ischemia

(Rehncrona et al., 1981; Siesjo, 1988) and TBI (Vink et al., 1988). During these metabolic

derangements, cerebral blood flow is reduced (Hovda et al., 1995). Therefore, the resulting ionic

imbalance drives the cell into a "metabolic crisis"; wherein the cell is not able generate energy

ATP to restore ionic homeostasis concurrent with high energy demand from increased glycolysis

(Hovda et al., 1995; Giza and Hovda, 2001).

As early as six hours after the injury, the initial hyperglycolysis is followed by depressed

glucose and oxidative metabolism (Yoshino et al., 1991). Figure 1 shows a summary of the time

course of the neurochemical and metabolic cascade after TBI.

The acute pathophysiology that occurs following TBI includes excitotoxicity, excessive

calcium influx into cells, mitochondrial dysfunction, cell swelling, inflammation, free radical

formation and cell death. Many of these processes may interfere with neural development,

plasticity and have significant impact in subsequent cognitive and behavioral outcomes after TBI

(Giza and Hovda, 2001; Giza and Prins, 2006).
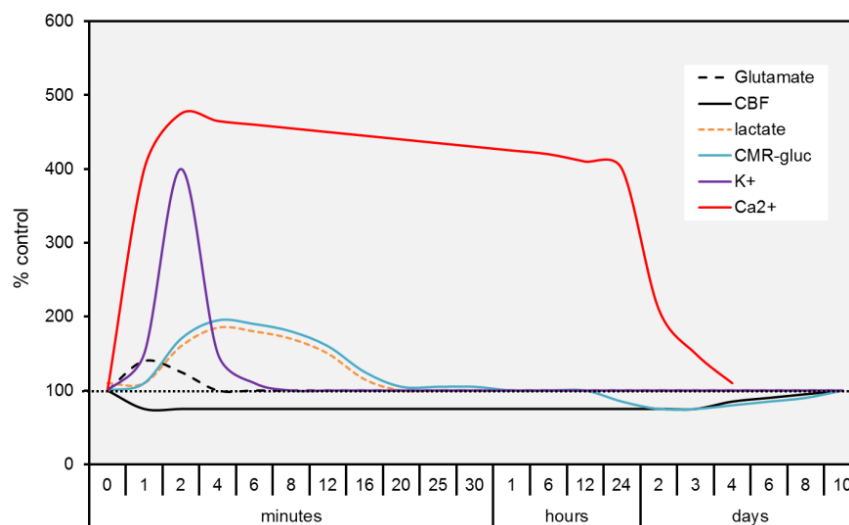
Figure 1. Neurochemical and Metabolic Cascade after TBI
Adapted from Hovda et al.,1995.

PEDIATRIC TRAUMATIC BRAIN INJURY AND NEUROPLASTICITY

TBI is the leading cause of death and disability among youths (NCIPC, 2000) and is a major public concern in the U.S. and worldwide. Out of the 1.7 million people who are hospitalized for TBI each year, 45% are children (Langlois et al., 2006). Children aged 0 to 5 years has the highest rate of TBI-related emergency department visits (1,256 per 100,000 population), followed by older adolescents ages 15-19 years (757 per 100,000 population) (Faul et al., 2010). Pediatric TBI is mainly caused by falls (50.2%) and struck by/against events (24.8%). Motor vehicle-traffic incidences have amounted only 6.8% of external causes in children, but have become the predominant cause of TBI cases in older adolescents and young adults (Faul et al., 2010).

Proponents of neuroplasticity argue that young children sustain less severe damage from focal brain injury compared to older children and adults. This is because brain function and structure is highly modifiable early in life, wherein intact healthy regions could compensate and assume functions of absent or damaged tissues. This enhanced plasticity in the developing brain has been elegantly described in detail by Margaret Kennard in monkeys after unilateral focal brain injury (Kennard, 1938; Kennard and Fulton, 1942). In rats, those that received hemicortication early in life exhibited better behavioral outcomes than adults (Kolb and Tomie, 1988). While adult rats demonstrated significant deficits in the Morris water maze (MWM) after lateral fluid percussion injury (FPI) (Prins and Hovda, 1998), injured weanling (postnatal day 17 – P17) rats showed no deficits in MWM acquisition and recall when compared to shams. The view of "younger is better" has been described as the "Kennard Principle." However, in some cases of brain injury, the immature brain may show devastating developmental outcomes after injury compared to adults with similar injury severity. Hebb has shown that children with frontal

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lobe injuries had more severe functional loss than adults with similar sized injuries (Hebb, 1942). In TBI, there are higher reported incidences of posttraumatic epilepsy in children with TBI than in adults (Annegers and Coan, 2000). Moreover, infants and toddlers, arguably the most plastic, can suffer the most severe consequences of TBI (Ewing-Cobbs et al., 1997; Anderson et al., 2005; Anderson et al., 2011), with persistent deficits in intellectual ability, memory, and processing speed.

What is it about children that can make their functional outcomes worse than adults? In contrast to focal lesion injuries, TBI forces are applied generally throughout the brain; therefore, reorganization may be diminished because there is little unaffected tissue. Structural factors among children can increase the likelihood for more generalized insults and more affected brain regions, which may contribute to more severe end results. Early in development, the cranial bones are not yet fully-fused and have greater flexibility and compliance to absorb traumatic forces. The relatively larger size of the head among children supported by a smaller neck (the mass of the head of a small child is 25% of the body mass) also increases the risk for more diffuse injuries. Furthermore, many brain regions are still undergoing myelination during the early stages of brain development. Unmyelinated fibers have been shown to be more vulnerable to injury impacts (Reeves et al., 2005). Lastly, frontal and subcortical regions, which are centers for capacities such as executive function, attention and memory, are more susceptible loci for impact rotational forces after diffuse TBI in the young brain. Thus, the younger brain may be experiencing the traumatic impact more severely compared to a mature brain that experiences the same injury.

Alternatively, with such high incidence rates, it is apparent that TBI is a common interruption of the course of normal development of children. Although most of TBI cases are mild and result in little to no long-term deficits, those who do sustain severe head trauma demonstrate lasting impairments in a myriad of capacities, such as attention, memory, learning, social function, and behavior (Anderson et al., 2005). These deficits in particular can affect the way children interact with their environment which then result in delayed acquisition of skills necessary for normal cognitive and behavioral development (Anderson et al., 2011). Children who suffer significant TBI have persistent deficits in general intellectual functioning and executive functions (Babikian and Asarnow, 2009) and even quality of life in adulthood (Anderson et al., 2011). Despite some recovery, children with severe TBI not only fall behind in neurocognitive performance to non-injured peers but also fall farther over time (Ewing-Cobbs et al., 1997; Babikian and Asarnow, 2009), neither recovering back to their premorbid levels nor achieving their potential. This disruption in development, in addition to the structural factors, may be attributed to the worsened outcomes in pediatric TBI compared to injury outcomes in adults. Interference with existing neural networks and the ability to form new ones through coordinated synaptic activity (Hebbian learning) could lead to missed critical periods of development. Throughout maturation, TBI early in life would then be expected to result in cumulative deficits because of the limited or altered skill set that injured children would acquire, which makes it difficult for subsequent consolidation of new skills and knowledge. TBI may be sending the immature brain into an altered developmental trajectory, wherein the children "grow into" their cognitive deficits and exhibit a developmental lag from non-injured peers.

In his delivery of the Ingersoll Lecture in 1898, William James, an American psychologist and philosopher, remarked: "Everyone knows that arrest of brain development

occasion imbecility, that blows on the head abolish memory or consciousness, and that brain –

stimulants and poisons change the quality of our ideas." Contemporary scientists have echoed

this developmental approach to injury of the brain, which was first declared by James, and argue

that the recovery of function is necessary after pediatric brain injury (Gil, 2003). Functional

recovery needs to be assessed not only by the age at injury, but also through evaluation of the

developmental stage of the child before the injury, what skills were impaired or lost, and at what

time course do skills in normal development are acquired, if any, by the injured child. Other

predictors for TBI and recovery are the mechanism of injury, premorbid cognitive and learning

abilities, family function, and access to rehabilitation (Schwartz, 2003). The interaction of

cognitive and biological recovery processes must be considered in order to provide optimal

treatment following injury.

EXPERIMENTAL TBI

Experimental models of TBI have been developed to gain a better understanding of this complex clinical problem. Although the primary biomechanical injuries sustained in various models of TBI may differ, the cascade of secondary cellular and physiological damage that follows have been shown to occur under similar conditions in both humans and animals.

The fluid percussion injury (FPI, Figure 2) is a highly reproducible and well characterized diffuse model of TBI (Dixon et al., 1987; McIntosh et al., 1989; Prins et al., 1996; Thompson et al., 2005) that simulates many clinically relevant sequelae of human closed head injury. FPI has been shown to result in acute compromise of the blood brain barrier (BBB) (Cortez et al., 1989; Soares et al., 1992; Schmidt and Grady, 1993) or progressive edema formation without continued BBB permeability (Beaumont et al., 2006a), alterations in cerebral blood flow and metabolism (Hovda et al., 1991; Yoshino et al., 1991), and chronic neurological motor and cognitive dysfunction (McIntosh et al., 1989; Fineman et al., 2000; Giza et al., 2005; Reger et al., 2005; Whiting and Hamm, 2006).

Briefly, force is delivered onto the dura mater within a closed fluid system. The force is generated by a pendulum that impacts the piston of a fluid filled (0.9% saline) cylinder creating a 21-23 ms fluid pulse through a craniotomy into the closed cranial cavity. The fluid pulse in turn causes movement and brief brain deformation that can trigger a series of pathophysiological responses. Adjustment of the pendulum hammer drop angle can change the magnitude of the administered force (Prins et al., 1996; Thompson et al., 2005). The location of the craniotomy is chosen depending on the scientific question in focus. When placed between the coronal and

lambdoid sutures in the antero-posterior direction and laterally between longitudinal and lateral fissures, this model is called the *lateral fluid percussion injury*.

Experimental animal models have also allowed further elucidation of the sub-cellular pathophysiology of TBI. Immediately after a biomechanical event, there is an indiscriminate release of excitatory amino acid (EAA) neurotransmitters, such as glutamate. Pathologic activation of EAA membrane receptors, particularly glutamate receptors such N-methyl-D-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate receptors (AMPAR) and kainate receptors, ensues and leads to massive ionic perturbations characterized by increased K^+ and Mg^{2+} efflux and Na^+ and Ca^{2+} influx (Katayama et al., 1990; Osteen et al., 2001). The excess intracellular Ca^{2+} may be sequestered into the mitochondria causing further energy failure (hypometabolism). Additionally, increased levels of Ca^{2+} may trigger cell death mechanisms that can lead to free radical overproduction and apoptosis (Raghupathi, 2004). These mechanisms have been attributed as the driving force for subsequent increased cerebral glucose uptake (hyperglycolysis ~30 min) followed by a prolonged period of metabolic depression (7-10 days) after FPI in adult rats (Yoshino et al., 1991).

A progressive, diffuse pattern of cell death and axonal injury have been observed throughout the brain after experimental brain injury, particularly in the parietooccipital cortex and in hippocampal pyramidal cell layer CA3 (Povlishock and Christman, 1995; Hicks et al., 1996; Conti et al., 1998). In the young rat, a diffuse model of FPI has been shown to result in little to no cell loss (Prins et al., 1996; Gurkoff et al., 2006). However, there are brain regions that remain structurally intact but become pathologically altered. Neuronal dysfunction caused by FPI even without significant cell death can evolve into lasting impairments in cognition,

behavior and experience-dependent plasticity (Lyeth et al., 1990; Fineman et al., 2000; Giza et al., 2005; Reger et al., 2005).
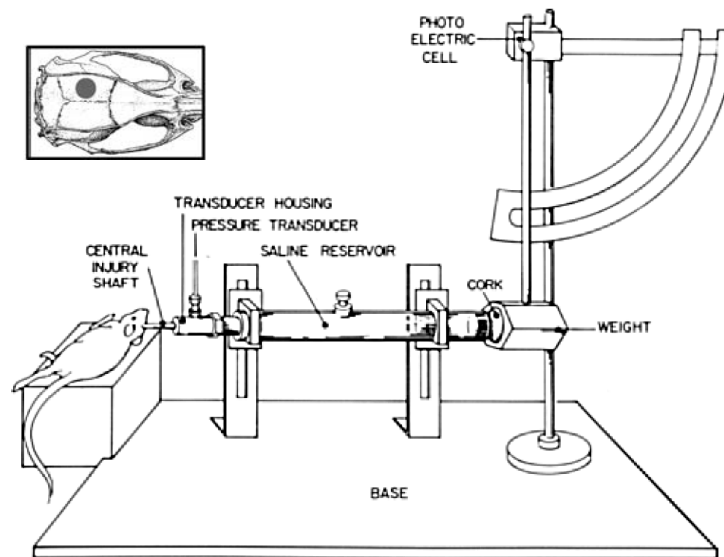
Figure 2. The Lateral Fluid Percussion Injury (FPI) Device
The insert shows the craniotomy location. An injury cap is cemented over the craniotomy and the rat is attached to the injury device via the injury cap. From Dixon et al, 1987, with permission.

HIPPOCAMPAL VULNERABILITY

The most common cognitive impairments reported following TBI are in the areas of learning and memory. The hippocampus, which is integral in learning and essential in declarative memory formation, is particularly vulnerable to damage after TBI. Frequent posttraumatic nonconvulsive seizures that occur acutely after TBI has been associated with hippocampal atrophy measured at a chronic time point (Vespa et al., 2010). In vivo MRI has revealed significant hippocampal atrophy in moderate to severe TBI patients (Tate, 2000; Tomaiuolo, 2004) and have been shown to be highly associated with subsequent long-term cognitive and behavioral deficits after TBI (Bigler et al., 1997). However, persistent deficits in hippocampal function can still occur without overt pathology following TBI, especially in concussive or mild TBI. Concussive or mild TBI makes up to about 75% to 90% of all reported cases of human TBI (Kraus and Nourjah, 1988; Kraus et al., 1994) and resulting impairments generally resolve spontaneously over several weeks, but some can last up to one year after the head injury in adults (McAllister, 2008). In children, significant deficits from moderate to severe TBI involve perceptual coordination and processing speed, both of which recruit the hippocampus (Anderson et al., 2005; Anderson et al., 2011). Impairments in these two domains have been shown to be more severe in younger children compared to adolescents (Anderson et al., 2005). Focusing on dysfunction in the absence of gross histological damage, this dissertation will investigate FPI-induced impairments in the hippocampus in the young rat.

DEVELOPMENT AND PLASTICITY

Development

It is important to understand what occurs in normal cerebral development as a basis for comprehending injury effects, as well as for developing treatments for maturing brain injured patients. In this dissertation, the rat model will be used to investigate the effects of TBI on developmental plasticity and recovery. A myriad of behavioral measures and resultant functional outcomes after normal development and after injury have also been demonstrated in rats.

Figure 3 shows the timeline of cerebral development in rats and a comparable timeline in humans (Kolb et al., 2000). Like many mammals, the rat brain originates from a neural tube, which is the embryo's precursor to the central nervous system. Cells are that eventually form the cortex begin to be generated on embryonic day 12 (E12) until E21. Rats are born on day 22 (postnatal day 0 – P0), with neurogenesis already completed. Generated cells migrate to appropriate locations, from the first week after birth until about 7 to 10 days of age, and begin to differentiate. Cell differentiation is almost complete by eye opening (P15), but neuronal maturation still continues and peaks for another 2 to 3 weeks then declines.

Identifying critical developmental events, such as neurogenesis, completion of cell migration, maximum dendritic growth, and maximum synaptic density, can be used to compare this animal rat model to humans and other species. Note that the rat cerebrum is less developed at birth compared to the human brain, and that the first week of life is approximately equivalent to the third trimester in humans. Maximum dendritic growth occurs around 2 weeks from birth in rats, which corresponds to around 8 months in humans; whereas synaptogenesis peaks around

P35 in rats and 7-8 years in humans. Injury at various time points during these developmental

stages has been shown to result in different functional outcomes by disrupting the trajectory of

neural network formation and maturation (Kolb and Tomie, 1988).

Plasticity

Having plasticity has been generally viewed as being beneficial, especially in terms of

recovery from injury or disease. To be able to adapt to changing external cues can increase

chances for survival in demanding circumstances. The younger brain has been shown to possess

higher levels of plasticity, exhibiting resilience to insults and rapid recovery after injury. For

instance, young rats that received hemicortication exhibited better behavioral outcomes than

adults (Kolb and Tomie, 1988). While adult rats demonstrated significant deficits in the Morris

water maze (MWM) after lateral fluid percussion injury (FPI) (Prins and Hovda, 1998), injured

weanling (postnatal day 17 – P17) rats showed no deficits in MWM acquisition and recall when

compared to shams. This enhanced plasticity in the developing brain has been first described in

detail by Margaret Kennard in 1942 in monkeys after unilateral focal brain injury. The view of

"younger is better" has been described as the "Kennard Principle."

Cerebral plasticity is described as the capacity of the brain to alter its structure and

function in reaction to environmental diversity (Kolb et al., 2011), a concept which can be traced

to the Hebbian postulate. For instance, housing animals in complex, enriched environments (EE)

has been shown to alter neuronal morphology and enhance cognition and behavior (Rosenzweig

et al., 1972, Greenough, 1976, Rosenzweig and Bennett, 1996). In much of his work, Kolb has

investigated many aspects of neural plasticity using EE as an assay for plasticity that will be

discussed here (Kolb et al, 2011). EE-induced changes are age-dependent. When Kolb and

colleagues placed weanling, adult, or senescent rats in EE for 90 days, all groups manifested

enhanced synaptic changes with increased dendritic arbors. But some changes were different for the different age groups. The increases in dendritic arbors were highest in the weanling compared to the adult and senescent rats. In contrast, while adult and senescent rats showed increased spine density after EE exposure, the weanling rats showed a decrease in spine density (Kolb et al., 2003). Newborn rats given early tactile stimulation on with a soft brush, three times a day for 10 days, have also displayed similar decrease in spine density (Kolb and Gibb, 2010). Additionally, there are sex differences in spine density responses after EE. While adult females showed increases in spine density in the parietal cortex after EE exposure, weanling females showed decreases in parietal spine density (Kolb et al., 2003).

Plastic changes can be time- and region-dependent. When placed in EE in varying intervals of 4, 8, or 16 days, Comeau and colleagues have demonstrated that there is a transient dendritic length increase in Golgi-stained neurons in the prefrontal cortex after 4 days of EE but was not observed after 16 days of EE. However, in the sensory cortex, increased dendritic arborization seemed evident in all EE intervals (Comeau et al., 2010). Moreover, experience-dependent changes accumulate and interact, which is also known as metaplasticity. When methylphenidate and amphetamine were administered to weanling and adult rats, respectively, then exposed to EE, the drug blocked EE-induced changes in dendritic arbors. Although the drugs themselves did not show any disturbance in physiology or morphology, it is the interaction with the pharmacology and EE that resulted in impaired experience-dependent plasticity (Kolb et al., 2003). In many cases of experimental TBI in adult rats, EE exposure itself has shown expected enhancements in neuroanatomy and improved outcomes. In several cases, only combined effects of EE and drug treatment or task-specific neurobehavioral experience have been demonstrated functional improvement after injury (Hoffman et al., 2008). Motor ability in

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TBI adult rats and acquisition of spatial learning in the MWM significantly improved when animals were also exposed to EE during the behavioral tests (Matter et al., 2011). However, EE after developmental TBI has failed to induce gains in cortical and behavioral plasticity (Fineman et al., 2000; Giza et al., 2005).

Lastly, since the benefits of plasticity are contingent upon the nature of the stimuli or experience, not all plasticity is good. For instance, when rats have increased play, there is increased number of synapses in the medial prefrontal cortex that correlates with enhanced cognition. However, when rats are given repeated doses of nicotine, a psychomotor stimulant, lasting cognitive and behavioral dysfunctions arise, but increases in synapses in the medial prefrontal cortex are also observed (Kolb et al., 2011). Therefore, the response of increased synapse number can have a dichotomous nature and may not always be good.

Children affected with TBI are known to develop long lasting cognitive and behavioral impairments (Babikian and Asarnow, 2009), and they are more susceptible to posttraumatic epilepsy (Annegers and Coan, 2000). Furthermore, infants and toddlers, arguably having the most neuroplasticity, seem to have the worst developmental outcomes after receiving significant TBI as compared to adults (Ewing-Cobbs et al., 1997; Anderson et al., 2005). Our current understanding of pediatric traumatic brain injury (TBI) suggests that 'younger is not always better.' Understanding the interplay between the developmental stages of the brain and plasticity can better elucidate underlying mechanisms and outcomes in TBI.
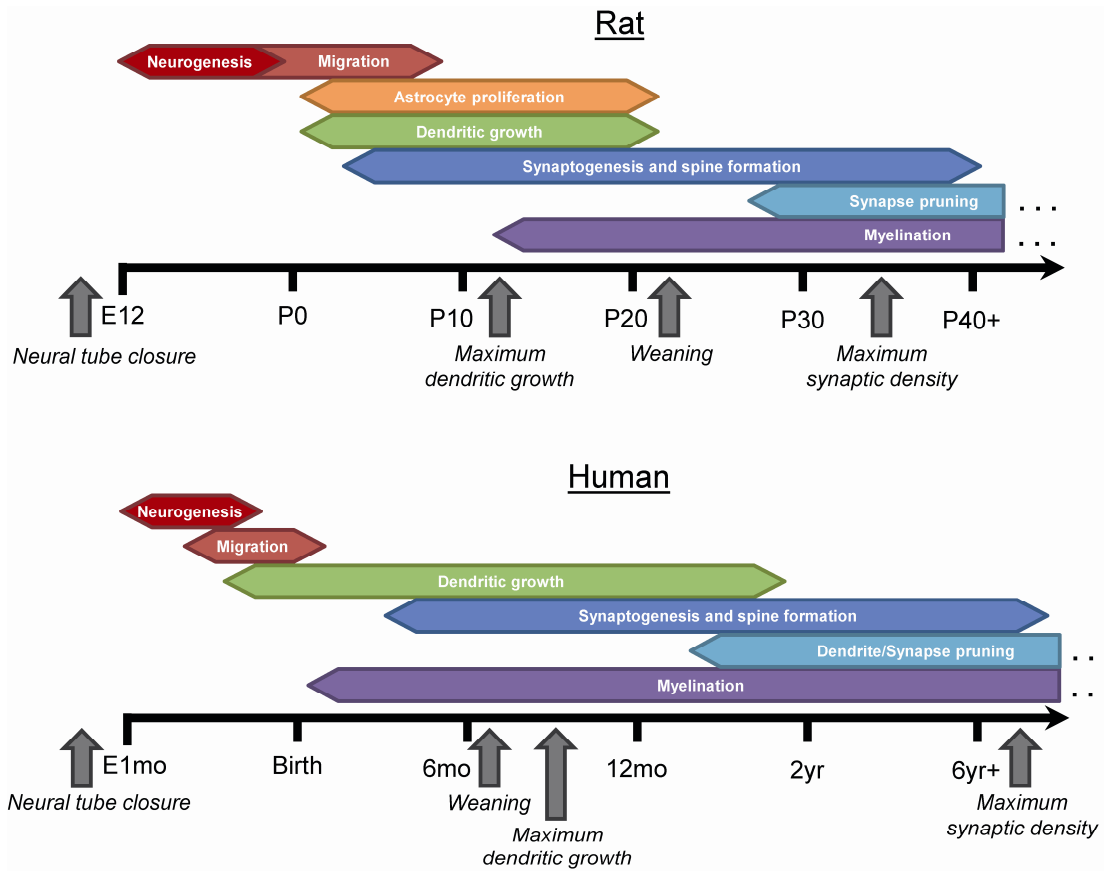
Figure 3. Schematic Illustration of Brain Development in Rat and in Human
Abbreviation: E = embryonic day; P = postnatal day; mo = month; yr = year. Hexagonal bars indicate main cellular events. Adapted from Kolb et al., 2000, with permission.

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GLUTAMATERGIC NEUROTRANSMISSION

In 1949, Hebb hypothesized that neural circuitry can be formed and strengthened by repeated coordinated activity of the presynaptic and postsynaptic cells. It is the signals generated by synaptic response associated with experience that initiate the formation of neural circuits. These signals that allow neurons to communicate with each other include the release of chemical messengers called neurotransmitters. In response to stimuli, an electrical impulse generated in one cell causes influx of Ca^{2+} ions and subsequent release of these neurotransmitters. The neurotransmitter diffuses across the synaptic cleft and stimulates or inhibits the postsynaptic cell through its interaction with the receptor proteins. These neurotransmitters bind to proteins called receptors to evoke electrical responses through opening and closing of postsynaptic ion channels.

Postsynaptic receptors could be from either of two families: ionotropic or metabotropic. Ionotropic receptors have two functional domains: an extracellular site for neurotransmitter binding, and a transmembrane domain that forms the ion channel. Their structure consists of multimers that have four or five subunits, each of which contribute to the pore of the ion channel. They are also called ligand-gated ion channels. The second family is the metabotropic receptors that do not have ion channel domains in their structure. Among many things, metabotropic receptors affect channels by activating intermediate molecules, called G-proteins. These receptors are monomeric proteins with an extracellular ligand binding site and an intracellular domain that binds G-proteins. Neurotransmitter binding to metabotropic receptors can activate G-proteins that dissociate from the receptor and interact directly with the ion channel or affect ion channel action indirectly through other effector proteins, such as enzymes. Metabotropic receptors can also have effects independent of ion channels. For instance, they can activate

kinases or lipases that alter intracellular molecules by phosphorylation, dephosphorylation, and cleaving lipids.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Its action on large families of metabotropic and ionotropic receptors is crucial in many forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), which mediate learning and memory formation. Ionotropic glutamate receptors have been identified based on the synthetic agonists that activate them: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate (AMPA), and kainate. Therefore, these receptors have been candidate targets for treating cognitive impairments that result from injury or disease. This dissertation examines the response of glutamate receptors NMDA and AMPA, as well as the secondary effectors that are associated with NMDAR or AMPAR after diffuse TBI and their roles in mediating experience-dependent plasticity.

NMDAR

Composition

The NMDAR is a cation channel permeable to Na^+, K^+, and more so to Ca^{2+}. Full activation requires binding of 2 glutamate molecules to NR2 subunits and 2 glycine molecules to NR1 subunits. Under normal conditions, the resting (polarized) NMDAR can be opened by glutamate but is voltage-gated. The presence of Mg^{2+} causes an open channel block, wherein the pore is permeated but there is no passage of ions across. When depolarization occurs, this removes the Mg^{2+} block and allows full ionic flow.

The NMDAR is a heteromeric assembly containing 4 subunits, usually composed of 2 NR1 and 2 NR2 or NR3 subtypes. NMDARs may consist of the following subunit complexes: NR1/NR2, NR1/NR3, or NR1/NR2/NR3. The NR1 transcript can be spliced to produce 8 distinct isoforms at three sites in the subunit protein, one in the N-terminus and 2 in the C-terminus. Four separate genes make up NR2 (A-D) and 2 genes produce NR3 subtypes (A-B). The NR1 subunit is a necessary constituent for a functional receptor, whereas the NR2 or NR3 subunit confer modulatory properties that regulate glutamate sensitivity and channel opening (Ishii, 1993; Cull-Candy et al., 2001; Low and Wee, 2010; Benarroch, 2011). For instance, when NR3 complexes with NR1, it forms a glycine-responsive NMDA receptor that does not require glutamate. Table 2 summarizes the NMDAR subunit function and distribution. For instance, predominantly NR2B-containing NMDARs are more sensitive to glutamate ($EC_{50} = 0.3 \ \mu M$), conduct larger currents (peak current = 667 nA) and have a longer deactivation time constant (τ = 300 ms) than NR2A-containing NMDARs ($EC_{50} = 2.1 \ \mu M$; peak current = 364 nA; τ = 50 ms). NR1/NR2C NMDARs have similar deactivation constant to NR2B (τ = 280 ms), while NR1/NR2D receptors are open much longer than the rest (τ = 1.7 s) (Scheetz and Constantine-Paton, 1994; Cull-Candy et al., 2001). The diversity of NMDARs depends on the RNA splicing of NR1, the type of NR2 subunit, and the presence or absence of the NR3 subunit.

The developmental stage of the brain, how the NMDARs are cellularly localized, and what environmental cues are being experienced can dictate NMDAR subunit number and composition, and therefore NMDAR function. During normal development, NR2B subunit-containing NMDARs predominate. Over time there is a decreasing, but significant, contribution from NR2B to the synaptic current, which is associated with the increase in NR2A (Monyer et al., 1994; Flint et al., 1997; Laurie et al., 1997; Roberts and Ramoa, 1999; Cull-Candy et al.,

2001).   Depending on the brain region, NR2A begins to be expressed during the second and third postnatal weeks (Monyer et al., 1994; Sheng et al., 1994; Wenzel, 1997) and goes on to exceed NR2B expression in adulthood. NR2C is not detected in the whole rat brain until around weanling age (postnatal day 20) up to adulthood, during which is predominantly expressed in cerebellar granule cells. Lastly, similarly to NR2B, NR2D is highly expressed early in development in the diencephalon and brain stem and diminishes during maturation (Laurie et al., 1997; Cull-Candy et al., 2001).

As for the recently cloned NR3 subunit (Low and Wee, 2010), NR3A is only detected in the fetal rodent brain until two weeks postnatally, whereas NR3B gradually increases over development. There are very low levels of NR3A in adult rodent cerebrum and NR3A-containing NMDARs are only localized in sparse thalamic and pontine nuclei and the amygdala. Higher NR3A levels can be detected in the rat spinal cord (Eriksson et al., 2007). When NR3A co-assembles with NR2A, the NR3A subunits reduces channel conductance (Cull-Candy et al., 2001). NR3B protein is widely expressed in the brain and spinal cord, particularly in the hippocampus, cortex, projection neurons and interneurons of the striatum, and the cerebellum (Low and Wee, 2010). NR3B is detected in both neuronal and non-neuronal cells. Interestingly, NR3 subunits are quite similar to NR1 subunits that can bind glycine rather than glutamate. Therefore, NR3-only containing NMDARs form excitatory glycine activated receptors independent of glutamate action.

Experience and environmental cues can also induce the NR2B-toNR2A shift. In adult rats, genes that regulate NR2A and NR2B expression in the hippocampus increase with acute and chronic voluntary periods of exercise (Molteni et al., 2002). Eye opening and visual stimuli can induce increases in NR2A. But this enhanced NR2A expression can be delayed or reversed

by dark-rearing (Quinlan et al., 1999a; Quinlan et al., 1999b). Nurturing maternal behaviors, such as arch-back nursing and high frequency of licking, correlate with increased NR2A levels in rats (Liu et al., 2000). NR2A-containing NMDARs are primarily found in the synapse, whereas NR2B is expressed both synaptically and extrasynaptically. Activation of these respective NMDAR pools can induce very different cellular responses. Activation of synaptic NMDARs has been demonstrated to promote neuroprotection by triggering pro-survival signal pathways that include cAMP response element binding protein (CREB) dependent gene expression (Papadia et al., 2005) and brain-derived neurotrophic factor protein (BDNF) expression. Extrasynaptic NMDA activity inhibits pro-survival pathways and promotes CREB inactivation as well as blockade of BDNF transcription, when concurrently activated with synaptic NMDA (Hardingham et al., 2002).

Structure

The NMDAR structure consists of two large extracellular amino (NH_2) N-terminal (NTD) and agonist-binding domains (ABD), a pore membrane domain with three transmembrane segments (M1, 3 and 4) and a re-entrant loop (M2), and an intracellular carboxyl (COOH) C-terminal domain (CTD) that contains phosphorylation sites (Figure 4A). The NTD contains allosteric modulatory sites for hydrogen ions and polyamines in the NR1, zinc (Zn^{2+}) in the NR2A subunit, and ligands such as ifenprodil, an NR2B-selective antagonist, in the NR2B subunit. The pairing of two discrete segments (S1 and S2) forms the bilobed ABD. The resulting cavity between the lobes constitutes the binding sites for glutamate or glycine. The M2 segment lines the pore membrane domain and contains an asparagine (N) residue that is crucial for ion selectivity, particularly for Ca^{2+} permeability and Mg^{2+} sensitivity. This asparagine residue is also a binding site for several NMDAR blockers, such as memantine, ketamine,

MK801, and phencyclidine (PCP). The extracellular Mg^{2+} localized in the channel pore voltage-gates the NMDAR. Lastly, the CTD contains phosphorylation sites for various kinases that influence activity-dependent modulation of NMDARs (Furukawa et al., 2005; Paoletti and Neyton, 2007; Chaffey and Chazot, 2008; Benarroch, 2011). In the NR1subunit, the CTD has 2 sites for gene splicing that can result in variant NR1 forms (C1 and C2 segments).

The NMDAR have been shown to assemble as a *dimer of dimers* depending on the interactions between different domains. The arrangement NR1/NR1/NR2/NR2 allows for formation of either homo- or heterodimers. Functional dimerization depends on the type of NR2 or NR3 present in the receptor. Figure 4B shows the top view of an NMDAR with the quaternary arrangement.

Allosteric modulation

There are several modulatory sites in the NMDAR that affect the channel function, which include polyamine site, Zn^{2+} site, proton-sensitive site, and a redox modulatory site (Figure 4C). Depending on the NR1 and NR2 subunits present, endogenous polyamines like spermidine and spermine, can either stimulate or inhibit NMDAR currents. Zinc inhibits NR1/NR2A in a voltage-dependent manner. Zinc, however, is released with glutamate by Ca^{2+}-dependent exocytosis and may play a physiological role in NMDAR inhibition. Protons that bind to the proton-sensitive site on the NTD of the NR1 subunit also inhibit NMDARs. The redox modulatory site is located in the NTD of NR2 subunits. Nitric oxide (NO) reacts with the cysteine residues, which also inhibits the NMDAR. (Paoletti and Neyton, 2007; Chaffey and Chazot, 2008; Benarroch, 2011).

Trafficking

Synaptic NMDARs are localized in the postsynaptic density (PSD) [2] and are part of a

large macromolecular signaling complex This complex includes scaffolding and adaptor

proteins that connect the NMDAR to the cytoskeleton and downstream effector proteins and to

metabotropic receptors. PSD scaffolding protein 95 kDa in size (PSD-95) links the NR1/NR2

receptors to signaling effectors, such as calcium-calmodulin kainase II (CAMKII), protein kinase

A (PKA), protein kinase C (PKC), and NO synthase, in mature synapses.

Functional receptors are assembled in the endoplasmic reticulum and are transported to

then synapse via microtubules. The number and subunit composition at the cell surface depends

on the balance of subunit insertion and clathrin-mediated endocytosis based on the

developmental stage and neuronal activity. Furthermore, the activity-dependent synaptic or

extrasynaptic targeting, insertion, and retrieval of NMDARs depend upon the trafficking signals

intrinsic to the subunits that are present. NR1 phosphorylation by PKA and PKC promotes

NMDAR trafficking to the plasma membrane. On the other hand, NR2 phosphorylation by

protein tyrosine kinase Fyn prevents clathrin-mediated endocytosis. Overexposure by glutamate

dephosphorylates this tyrosine site and promotes rapid receptor internalization. Activity-

dependent protein degradation by ubiquitin protomease cascade can also affect receptor number

and composition. Increased synaptic activity enhances NR1 and NR2B turn over, resulting in

higher levels of NR2A and PSD95 at the synapse. Please see Lau et al. (2007) for a review of

the molecular mechanisms of NMDAR trafficking (Lau and Zukin, 2007; Benarroch, 2011).

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[2] PSD is a protein dense region that concentrates many neurotransmitter receptors, kinases and phosphatases in close apposition to the presynaptic neurotransmitter release zone in the synaptic cleft. This structure is involved in signal transduction, wherein kinases and phosphatases are influence protein expression in the dendritic spine or are transported in the nucleus (Purves et al., 2004).

Synaptic plasticity

The NMDAR functions as a coincidence detector due to the voltage-gating of the Mg^{2+} block. This is crucial for experience-dependent synaptic activity like long-term potentiation (LTP) or depression (LTD). Activation of NMDARs increases intracellular Ca^{2+}, which activates kinases, such as CAMKII, and phosphatases, such as calcineurin. CAMKII-mediated phosphorylation of GluR1 AMPARs promote their synaptic insertion, resulting in increased AMPAR conductance and LTP. Calcineurin, on the other hand, can in turn dephosphorylate AMPAR and promote receptor internalization, which results in LTD. An optimal NMDAR:AMPAR ratio is typically maintained depending on neuronal activity (Cull-Candy et al., 2001; Lau and Zukin, 2007; Paoletti and Neyton, 2007; Benarroch, 2011).

Table 2. NMDAR Subunit Function and Distribution
From Benarroch, 2011, with permission.

Subunit Type	NR1	NR2	NR3
Variants	8 splice variants	NR2A-D	NR3A-B
Function in NMDAR	Obligatory	NR2A or NR2B are the most common	Activated by binding of glycine only
	Binds glycine	Bind glutamate	
		Dictates receptor kinetics	Inhibits Ca^{2+}
Binding site	Glycine	Glutamate	Glycine
Modulatory sites	Polyamines	Zn^{2+}	?
	Protons	Redox site	
		NO (nitrosylation) site	
Predominant location	Postsynaptic density (PSD) and extrasynaptic	NR2A: PSD	Dendritic spines during development
		NR2B: Extrasynaptic (NR2A/NR2B ratio increases in adult brain	Oligodendrocytes
Predominant distribution	Throughout the CNS	Hippocampus (NR2A, NR2B)	Neurons
		Neocortex (NR2A, NR2B)	Oligodendrocytes
		Cerebellum in Purkinje cell (NR2A, NR2C), molecular (NR2B) and granule cell (NR2C) layers Cortical interneuron (NR2C) Brainstem (NR2C)	
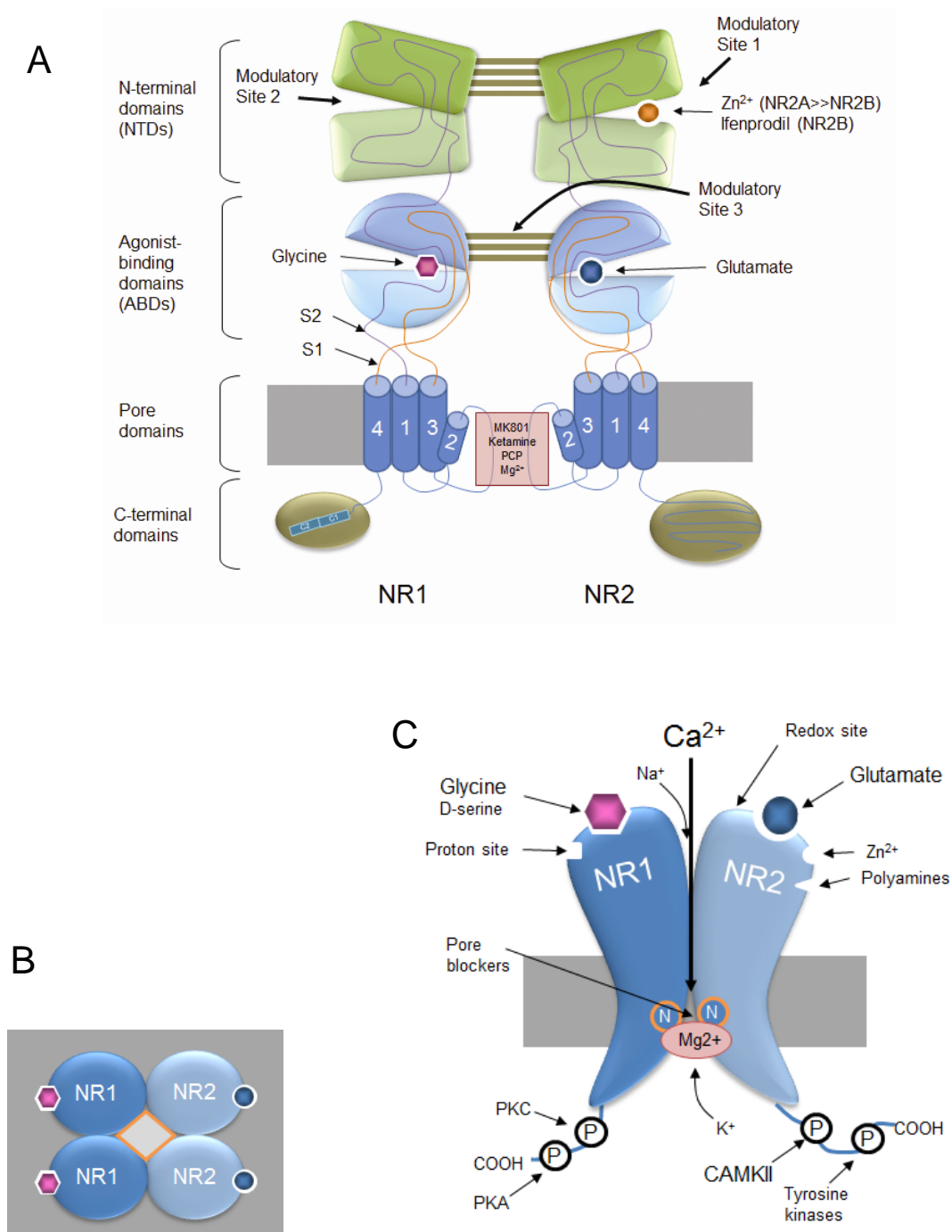
Figure 4. NMDAR Structure
Schematic diagrams of NMDAR heterodimer structure (A), tetramer assembly (top view) (B), and heterodimer sites of modulation (C). Each NMDAR subunit consists of extracellular N-terminal domain (NTD), agonist-binding domain (ABD), pore membrane domain, and a C-terminal domain (CTD). Adapted from Paoletti et al., 2007 and Benarroch, 2011, both with permission. Gray bar indicates the plasma membrane.

AMPAR

Composition

The AMPAR is also a cation channel permeable to Ca^{2+}, Na^+, and K^+. The functional properties of AMPARs also depend on the subunits that assemble as homo- or heterotetramers (GluR1-4). Most AMPARs are heteromeric receptors that assemble as dimer of dimers containing 2 GluR2 and 2 GluR1 or GluR3 or GluR4. AMPARs are found in both neurons and glia as heteromers containing GluR2. GluR1 and GluR2 subunits are highly expressed in the hippocampus and cortex, with only low levels of GluR3 and GluR4 detected in these regions. Mature pyramidal cells primarily express heterotetramers of GluR1 and GluR2 (Isaac et al., 2007).

Development, synaptic activity, and injury can influence the Ca^{2+}-permeability and subunit composition of AMPARs, via the regulation of the GluR2 subunit expression. During the first postnatal weeks of neocortical development, during maximal synaptogenesis and increased expression of spiny neurons, GluR1 levels are higher than GluR2, which increases rapidly during the first week after birth. Additionally, GABAergic interneurons also express primarily GluR2-lacking AMPARs during developmental stages. GluR2-containing AMPAs are impermeable to Ca^{2+}, and this suggests that AMPARs play a crucial role in developmental synaptic function (Pickard et al., 2000; Kumar et al., 2002a; Cull-Candy et al., 2006; Isaac et al., 2007). Synaptic inactivity due to tetrodotoxin (TTX), high K^+ or AMPA-blockers have been shown to increase the expression of Ca^{2+}-permeable (GluR2-lacking) AMPARs in the cerebellum, visual cortex and hippocampus. Synaptic potentiation, on the other hand, increases

the proportion of GluR2-containing AMPARs in hippocampal slice cultures (Cull-Candy et al., 2006).

Structure

AMPAR subunits consist of an extracellular N-terminal domain (NTD), an agonist-binding domain (ABD), a pore membrane domain that includes three transmembrane proteins (M1, M3, and M4) and a re-entrant loop (M2) that lines the channel pore, and a cytoplasmic C-terminal domain (CTD), similar to NMDARs (Figure 5). The pairing of two discrete segments (S1 and S2) also forms the bilobed ABD, which contains the glutamate binding site. Each AMPAR subunit can vary due to alternative splicing and RNA editing. Particularly, all AMPAR subunits exist as two splice variants, known as flip and flop. The flip/flop splice cassette is found between M3 and M4, and it determines the speed of desensitization and resensitization of the receptor, as well as rate of channel closing. GluR2-containing AMPARs become impermeable to Ca^{2+} due to editing of the GluR2 mRNA glutamine (Q) to arginine (R). This Q/R editing site is located at the top of M2 (Cull-Candy et al., 2006).

Trafficking

Translation and dimerization of AMPAR subunits occur in the endoplasmic reticulum (ER). AMPARs are continuously internalized (endocytosed) and inserted in the plasma membrane. Surface AMPARs are moved to the synapse via lateral diffusion and stabilized by postsynaptic density scaffolding proteins. Depending on the subunits present, receptors are trafficked to synaptic or extrasynaptic sites. The C-terminal domain has many binding sites of protein interactions and phosphorylation sites that influence trafficking of the receptor. The PDZ binding site on GluR2 binds PICK1 (protein interacting with C kinase) and GRIP (glutamate

receptor interacting protein). GRIP keeps the receptor in place, whereas PIK1 allows movement of the receptor in and out of the plasma membrane. Phosphorylation of GluR1 by CAMKII and PKC during LTP promotes translocation of GluR1-containing AMPARs to the synapse. GluR1 has 4 phosphorylation sites (serine 818, S831, threonine 840 and S245).

Polyamine modulation

Polyamine and polyamine derivatives, which are either extracellularly applied or are intracelluarly endogenous, can block GluR2-lacking AMPARs. Development and pathological conditions changes polyamine availability in the cell, which affect AMPAR function (Isaac et al., 2007). The polyamine spermine is greatest in immature neuron during the developmental stage when cells contain mostly Ca^{2+}-permeable AMPARs.

Synaptic plasticity

AMPA receptors mediate fast excitatory neurotransmission and have been implicated in synaptic plasticity and learning and memory (Cull-Candy et al., 2006). Ca^{2+}-permeable AMPARs play a crucial role in the maintenance of NMDAR-dependent LTP at synapses. In basal conditions, most AMPARs in the synapses contain GluR2 subunits. After induction of LTP, GluR2-lacking AMPARs (primarily GluR1-containing) are transiently inserted into the postsynaptic membrane, which promotes Ca^{2+} influx and signal propagation. Additionally, Ca^{2+} influx through these receptors is important in the subsequent regulatory insertion of Ca^{2+}-impermeable AMPARs (Kauer and Malenka, 2006). Activation of Ca^{2+}-permeable AMPARs has also been shown to lead to NMDAR-independent LTP and LTD in inhibitory interneurons in the basolateral amygdala and hippocampus. Mossy fiber- interneuron synapses in CA3 can contain populations of either GluR2-lacking or GluR2-containing AMPARs. Although Ca^{2+}

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entry via AMPARs is modest compared to that through NMDARs, AMPAR-mediated

transmission still plays a crucial role in development, neuroplasticity and disease.
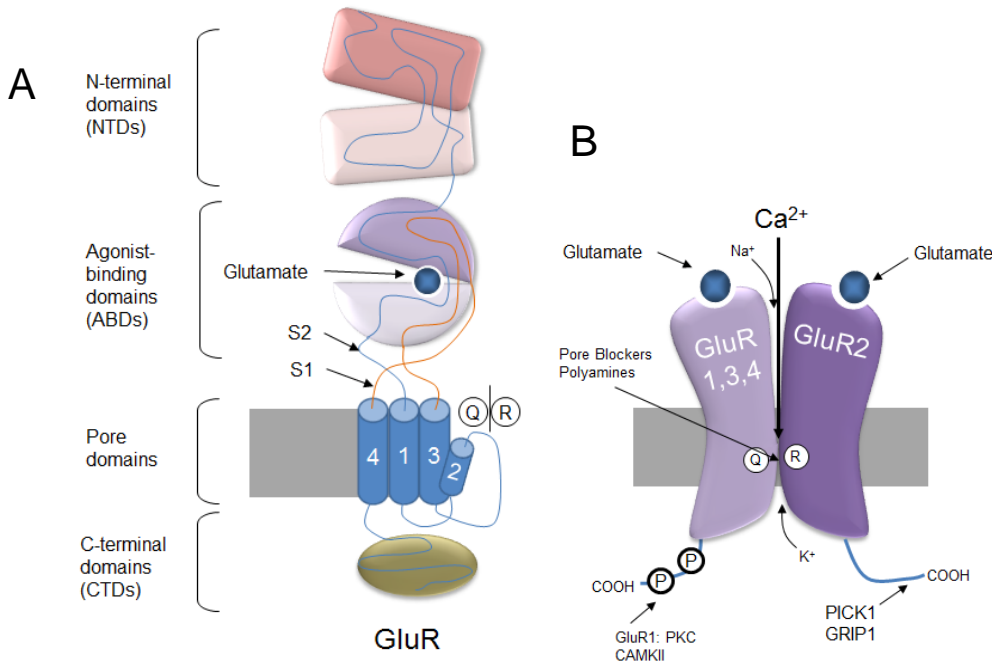
Figure 5. AMPAR Structure
Schematic diagram of the AMPAR. (A) Each subunit consists of extracellular N-terminal
domain (NTD), agonist-binding domain (ABD), a pore membrane domain, and a C-terminal
domain (CTD). (B) A heterodimer with Glur2 and Glu1 or Glur4 or Glur4 subunits and CTD
sites of phosphorylation and protein interaction. Gray bar indicates the plasma membrane.
Adapted from Fleming and England, 2010, and Gouaux, 2003, both with permission.

Second Messenger Targets and Effectors

Hippocampal-dependent learning requires the activation of NMDARs and AMPARs which allows the influx of Ca^{2+} into the postsynaptic neuron. Intracellular calcium in turn activates various protein kinases that facilitate memory formation, such as calcium-calmodulin protein kinase (CAMK) and extracellular signal regulated kinases (ERK). ERKs are also known as mitogen-activated kinases (MAPK). These kinases, as well as a downstream effector of ERK (cAMP response element binding protein – CREB) are implicated in long-lasting changes in brain activity, such as long-term synaptic plasticity and spatial learning (Purves et al., 2004). Figure 6 shows NMDAR-mediated action on these secondary messenger targets and effectors.

CAMKII is a calcium activated enzyme that is highly abundant (1- 2% of total protein) in the brain. This enzyme constitutes about 30-40% of the protein in the PSD, where it is optimally located to function as a functional protein at the synapse (Lisman et al., 2002). CAMKII, once activated, it autophosphorylates and then translocates to the PSD and directly binds to the NMDAR. Upon arrival in the PSD, CAMKII can continue to be active without further calcium/calmodulin activation and this is considered to be a mechanism in strengthening synapses and forming memories (Lisman et al., 2002; Purves et al., 2004). Autophosphorylation of CAMKII leads to strengthened binding of CAMKII to NMDAR subunit NR1 or NR2B, which regulates kinase activity. After CAMKII binds to NR2B, it remains active even after CAMKII dissociates from its phosphate group. This type of activation propagates autophosphorylation of CAMKII.   Lee et al. have demonstrated that CAMKII a necessary mediator in LTP, as well as in synapse specificity[3] in living neurons (Lee et al., 2009). Additionally, CAMKII has been shown

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[3] Synapse specificity entails that "only synapses that are activated become strengthened, and neighboring synapses located on a micrometer or two away on the same neuron remain unaffected" Thompson SM, Mattison HA (2009) Secret of Synapse Specificity. Nature 458:296-297.

to have a role in the trafficking of AMPARs in the PSD. CAMKII phosphorylates AMPARs at the serine 831 site, which increases the channel conductance and synaptic strength. CAMKII can also facilitate membrane AMPAR integration through the exocytosis of endosomes containing AMPARs. This results in the increase of AMPARs in the synapse to promote LTP (Lisman et al., 2002) and confer synapse specificity during synaptic plasticity.

The ERK pathway, synonymous with the MAPK pathway, are involved in differentiation, proliferation or apoptosis in mammalian cells and have been shown to be necessary in associative learning (Atkins et al., 1998; Purves et al., 2004; Thomas and Huganir, 2004). In the ERK pathway, Ras activates c-Raf, followed by ERK 1/2 regulate cell survival and axonal growth. This pathway is important in spatial learning and hippocampus-dependent memory formation. Ras and raf are part of protein families found inside cells and are involved in signal transduction. On the other hand, regulation of MAPK c-Jun N terminal kinase (JNK) has been shown to mediate cell stress or death. Activated JNK is evident in apoptotic neurons and glia. Elevated levels of JNK precede neuronal cell death after global cerebral ischemia (Raghupathi, 2003).

The cAMP response element binding protein (CREB) is a ubiquitous transcriptional activator that is normally bound to its binding site on DNA, called cAMP response element (CRE). It is usually found as a homodimer or bound to another, closely related transcription factor. Only when CREB is phosphorylated that it has transcriptional activity. PKA and the Ras pathway phosphorylates CREB. Increased intracellular Ca^{2+} can also phosphorylate CREB, wherein CRE site is activated. Here CRE is also called the CaRE (calcium response element) site. Ca^{2+}-mediated phosphorylation of CREB is mainly due to CAMKIV and ERK. The CREB

phosphorylation must be long enough in duration for transcription to occur, even if only increases in Ca^{2+} levels are transient (Purves et al., 2004).

Hippocampal synaptic plasticity can be perturbed by disturbances in these secondary messenger targets and effectors. CAMKII is necessary for LTP induction. Inhibition of ERK activation blocks LTP maintenance in the hippocampus (English and Sweatt, 1997; Atkins et al., 1998). Inability to activate ERK may prevent CREB-mediated gene expression of brain derived neurotrophic factor (BDNF). Sustained phosphorylation of CREB is involved in the activation of genes that promote enhanced cognition, such as BDNF (Finkbeiner, 2000), and neural protection and recovery from injury (Griesbach, 2004).
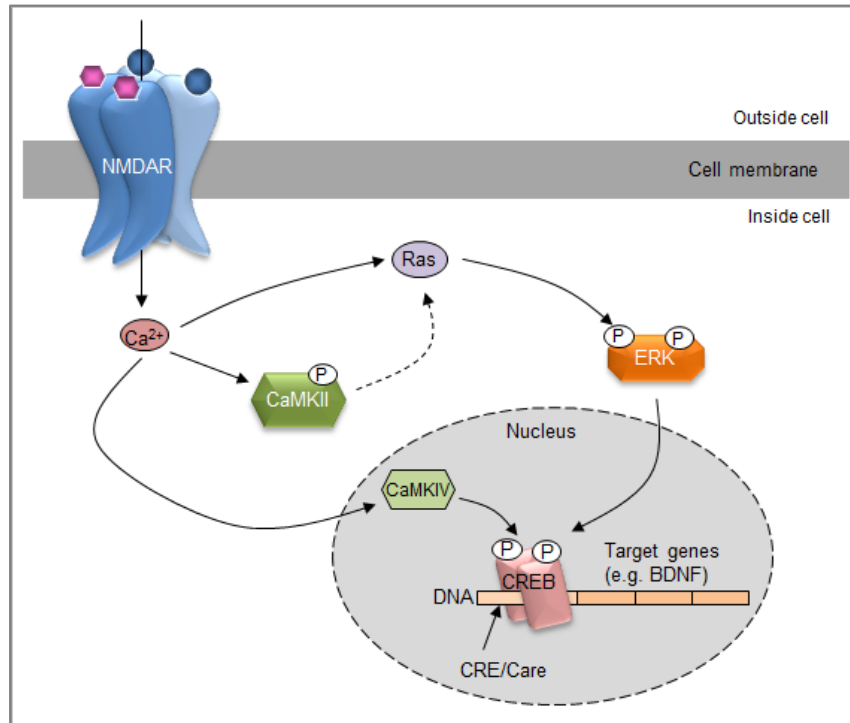
Figure 6. NMDAR-Mediated Secondary Effectors
Simplified schematic of NMDAR activated secondary effectors crucial in synaptic plasticity. Once activated, NMDAR allows Ca^{2+} to enter the cell. Ca^{2+} can activate calcium-dependent kinases, such as CAMKII and IV, which in turn can phosphorylate (circled P) downstream targets (e.g. Ras, ERK, and CREB). CREB activation leads to transcription of key target genes, like BDNF that is important in cell survival and synaptic plasticity. Adapted from Thomas and Huganir, 2004, with permission.

Glutamatergic Neurotransmission and Developmental TBI

Following TBI, the increase in intracellular calcium has been attributed to the activation of NMDARs and AMPARs, which has been shown to change as a function of time post-injury, brain region and age. NMDAR activation dramatically increases 15 minutes after experimental TBI in adult mice (Biegon et al., 2004; Schumann et al., 2008), followed by diminished NMDAR expression (hours to days) in cortex and hippocampus (Biegon et al., 2004). A decrease in NMDAR binding has been reported three hours post-injury in the hippocampus and neocortex (Miller et al., 1990), and reduced protein expression occurs as early as 6 hours after TBI (Kumar et al., 2002a; Osteen et al., 2004). This downregulation lasts even longer in the developing brain. In postnatal day 19 (P19) rats that sustained a lateral FPI, the NR2A subunit protein is reduced by 40% in the ipsilateral hippocampus during the first post-injury week (Giza et al., 2006). This molecular response has correlated with reduced electrophysiological activation through hippocampal NMDAR. FPI in young rats showed reduced amplitude of NMDA-mediated excitatory post-synaptic currents (EPSCs) (down 40-50%) in hippocampal CA1 neurons. Also, application of subunit specific inhibitors isolating NR2A currents showed that the reduced NMDA current is due primarily to a 50-60% loss of NR2A-mediated currents (Li et al., 2005). Furthermore, the post-TBI alteration of NMDARs has been shown to functionally manifest as a loss of plasticity in the young rat. Rats that received FPI at P19 failed to show EE-induced anatomical (increased cortical thickness, expanded dendritic arborization) and cognitive enhancements (improved spatial learning in the Morris water maze), when tested as young adults (Fineman et al., 2000; Ip et al., 2002; Giza et al., 2005). The period of diminished neural plasticity within one week post-FPI in the developing brain coincides with impaired working

memory (Reger et al., 2005), as well as with the critical period of neural responsiveness to EE-rearing (Giza et al., 2005).

In post-kindled and post-ischemic pyramidal cells, Ca^{2+}-permeable AMPARs are re-expressed when spines are withdrawn. Brain injury due to ischemia has demonstrated downregulation of GluR2, as well as suppression of enzyme, ADAR2, normally responsible for the mRNA GluR2 glutamine (Q) to arginine (R) editing at the nucleus (Cull-Candy et al., 2006). After FPI in adult rats, there is an increase in Ca^{2+}-permeable AMPARs, as well as a decrease in GluR2 levels (Bell et al., 2007; Bell et al., 2009). In a closed head injury model of TBI, GluR1 levels increased in the hippocampus ipsilateral to the injury 15 minutes after closed head injury (weight drop) (Schumann J. et al., 2008).

The global changes in calcium signaling following TBI suggest that downstream signaling molecules would also be affected. TBI induces change in CAMKII levels, first with an initial increase followed by a long lasting reduction, that is associated with impaired cognition and experience-dependent plasticity (Glazewski et al., 2000; Schwarzbach et al., 2006; Wu et al., 2009, 2011). FPI induces transient increases in phosphorylated CAMKII (Atkins et al., 2006; Folkerts et al., 2007) and total CAMKII in the hippocampus and cortex ipsilateral to the injury site (30 minutes) (Atkins et al., 2006; Folkerts et al., 2007). Additionally, downstream effectors of CAMKII (GluR1 and cytoplasmic polyadenylation element-binding protein – CPEB) also increase phosphorylation states in synaptic samples post-injury. CPEB regulates the translation of dendritic mRNAs during hippocampal LTP 30 minutes after FPI and returns to sham levels by 4 hours (Atkins et al., 2006). This suggests that the aberrant increase of autophosphorylated CAMKII can result in loss of synapse specificity of neuronal activation and play a role in deficits long term memory formation. In CNS injury, ERKs have a different pathophysiological

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response. Following TBI, ERK1/2 is activated in injured cortical neurons, as well as non-neuronal cells in cortex, hippocampus and thalamus, as early as 3hrs lasting up to 3 days (Raghupathi, 2003). Pre-injury treatment of phosphorylated ERK inhibitor was shown to reduce ERK activation and the extent of cell death, but worsened cognitive and motor deficits in TBI animals (Dash et al., 2002). Long lasting deficits in ERK activation have been shown after FPI up to 12 weeks after injury (Atkins et al., 2009). JNK activation, in contrast, was not observed in injured regions nor correlated with trauma-induced cell death (Raghupathi, 2004). Lastly, after FPI in adult rats, phosphorylated CREB is decreased from one week after injury (Griesbach, 2004). This reduction have been shown to persist up to 12 weeks (Atkins et al., 2009). Table 3 briefly summarizes glutamate-mediated responses following TBI.

Table 3. Summary of TBI-Induced Glutamate-Mediated Responses

Target	Post TBI response
NMDAR	Transient increase in activity followed by lasting reduction in activation (up to 1 week)
AMPAR	Adult rats: Increase in Ca^{2+}-permeable AMPARs (GluR1 increases); and decrease in Ca^{2+}-permeable AMPARs (GluR2 decreases)
CAMKII	Transient increase followed by long lasting reduction (up to 1 week)
ERK	Lost lasting reduction in activation (up to 12 weeks)
CREB	Long lasting reduction in phosphorylated CREB (up to 12 weeks)

NMDAR AS TARGET FOR THERAPEUTIC INTERVENTION

To protect the brain from the acute excitotoxic effects of indiscriminate activation of glutamate receptors, NMDAR inhibitors have been used as a therapeutic intervention in the TBI setting. Although this approach has been implemented successfully in experimental models of TBI (Rao et al., 2001; Schumann et al., 2008; Han et al., 2009), NMDAR blockade has failed to show neuroprotection or promote functional recovery clinically, and in some cases even led to worsened outcome (Morris et al., 1999; Albers et al., 2001; Ikonomidou and Turski, 2002; Naranyan et al., 2002; Muir, 2006; Maas et al., 2010). Due to the dynamic response of NMDAR activity post-injury, NMDAR antagonists may be been delivered during periods of already down-regulated NMDAR function that missed the critical window of receptor hyperactivity. Furthermore, during the recovery phase, excitatory synaptic activity may be necessary to facilitate optimal outcome (Ikonomidou and Turski, 2002). This follows from emerging evidence that NMDAR inhibition at critical developmental time points can increase apoptosis and promote pathological processes detrimental for cell survival, such as increased oxidative stress and formation of abnormal cortical neural connections. (Bittigau et al., 1999; Ikonomidou, 1999; Pohl et al., 1999; Kaindl et al., 2008). Glutamatergic blockade has also been shown to impair neuroplasticity during development by interfering with LTP and reducing expression of pro-plasticity molecules, such as BDNF and CREB (Hardingham et al., 2002; Hardingham and Bading, 2003; Hardingham, 2006; Hardingham, 2009). NMDAR blockade in adult CNS may not kill cells right away but can worsen cell death in response to TBI.

More recently, the use of NMDAR agonists has shown to promote neuroprotection and has facilitated rapid recovery after TBI (Temple and Hamm, 1996; Biegon et al., 2004; Yaka et al., 2007), with a potentially wide therapeutic window (Adeleye et al., 2009). Low doses of

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NMDA have been shown to preferentially activate synaptic NMDA receptors due to significant increases in action potential firing and activation of a pro-survival molecular cascade. Toxic levels of NMDA suppress firing rates below baseline and extrasynaptic NMDA signaling dominates (Soriano et al., 2006). Global NMDA antagonism may therefore be inducing a pro-apoptotic state, resulting in even worsened outcome (Ikonomidou and Turski, 2002).

The opening of the NMDAR channel requires both the binding of glutamate and the activation of its co-agonist glycine-binding site by glycine or D-serine (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Evidence of glycine's interaction with the NMDAR has been shown biochemically through the enhancement by glycine of the binding of radiolabelled ligands (N-(1-[2-thienyl]cyclohexyl)3,4-piperidine ([^{3}H]TCP) and [^{3}H]MK-801) to the phencyclidine (PCP) recognition site of the NMDAR (Bonhaus et al., 1987; Johnson and Ascher, 1987; Thomas et al., 1988; Hood et al., 1989). [^{3}H]TCP is a derivative of PCP and binds with high affinity in brain regions highly correlated with that of NMDAR sites. MK-801 is a non-competitive antagonist of the NMDAR that binds to the PCP site in the channel pore. The binding of [^{3}H]glycine in rat brain also demonstrated properties of a modulatory site in the NMDAR (Monahan et al., 1989b). In the absence of glycine, the binding of glutamate alone will not activate NMDAR channel opening. Addition of glycine has been shown to potentiate NMDAR responses in cultured cortical neurons (Johnson and Ascher, 1987). Historically, this modulatory site was named for the binding of glycine and its interaction with the NMDAR complex, but the site has been recently shown to have a more natural affinity for the endogenous amino acid D-serine (Wolosker, 2007). Furthermore, this glycine-binding site has been shown to not be saturated in hippocampal neurons in culture and in slice preparation (Wilcox et al., 1996)

and therefore allows modulation of NMDAR function without posing direct risks of excitotoxicity.

D-cycloserine has also been shown to selectively bind to the [3H]glycine recognition site and possesses partial agonist characteristics on the NMDAR (Hood et al., 1989; McBain et al., 1989; Watson et al., 1990; Priestley and Kemp, 1994; Baxter and Lanthorn, 1995). Depending on the concentration of DCS, DCS can act as an agonist or an antagonist. As an agonist, DCS stimulates ([^{3}H]TCP) binding to a max of 40-50% of the stimulation induced by glycine or D-serine alone. In the presence of glycine ($\geq .1\mu M$), D-cycloserine demonstrates antagonist properties and can reduce glycine stimulation (Hood et al., 1989; Lanthorn, 1994). DCS has been described as a "cognitive enhancer" and has been utilized in different paradigms in both experimental and clinical setting in pursuit of neural repair (Monahan et al., 1989a; Pitkänen et al., 1994; Pitkänen et al., 1995; Temple and Hamm, 1996; Yaka et al., 2007; Norberg et al., 2008; Krystal et al., 2009; Bado et al., 2011). Interestingly, while DCS has partial agonist property compared to glycine (35-65% efficacy of glycine controls) when binding to NR2A or NR2B containing NMDARs, DCS has much higher efficacy than glycine controls for NR2C subunits (Sheinin et al., 2001).

GLUTAMATERGIC DYSFUNCTION HYPOTHESIS

NMDAR binding is first increased then decreased acutely after FPI injury (Miller et al., 1990; Kumar et al., 2002a). Reduced binding can persist up to one week after closed head weight drop injury (Biegon et al., 2004). There is a region specific alteration of NMDAR expression following experimental TBI in immature rats. Hippocampal NR2A is downregulated within the first post-injury week (Giza et al., 2006), and this hippocampal downregulation of NR2A has also been demonstrated electrophysiologically by measuring NMDAR excitatory post-synaptic currents, after administration of an NR2B inhibitor ifenprodil (Li et al., 2005). This period of diminished neural plasticity after FPI in the developing brain coincides with impaired NMDAR mediated hippocampal working memory (Reger et al., 2005), as well as with the critical period of neural responsiveness to enriched environment rearing (Giza et al., 2005). Lastly, experimental FPI in developing rats results in no gross histological damage (Prins et al., 1996; Gurkoff et al., 2006). Impairments in cognition and behavior are then attributed to cellular dysfunction rather than destruction. Injury-induced dysfunction includes alterations in physiological expression of glutamate receptors, particularly NMDARs.

The glutamatergic dysfunction hypothesis then holds that enhancing glutamatergic neurotransmission during the subacute phase (after the immediate 24 hours and within one week post-injury) will promote recovery, while inhibition of glutamate-mediated transmission during this period will impair recovery (Figure 7).
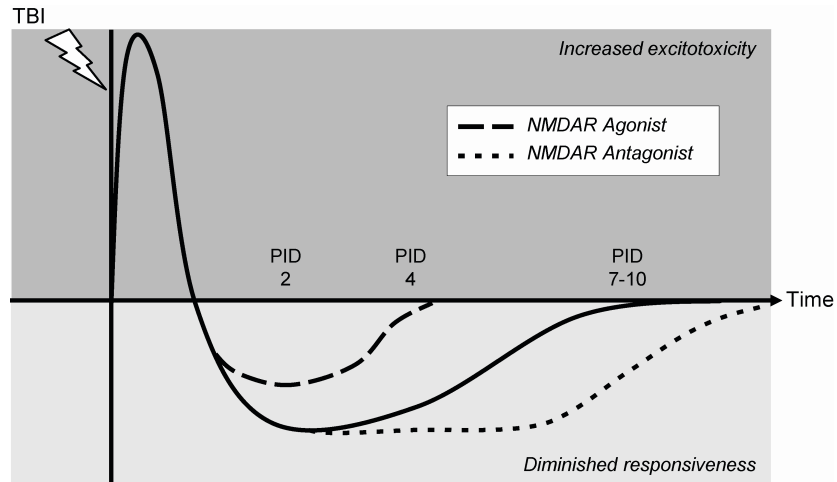
Figure 7. The Glutamatergic Dysfunction Hypothesis
Following TBI, there is a hyperacute release of glutamate, followed by a period of diminished glutamatergic neurotransmission. The hyperacute peak is posited to mediate neuronal injury and cell death through increased excitotoxicity; whereas the delayed impairment of glutamate-driven transmission is a more likely underlying mechanism of lost plasticity and a determinant of the duration and magnitude of neural recovery. Enhancing glutamatergic neurotransmission via agonist therapy during the post-subacute period of diminished responsiveness will facilitate recovery. In contrast, inhibition of glutamate-mediated neurotransmission during this time will impair recovery.

TOWARDS A PHYSIOLOGICAL BIOMARKER

One of the goals of this dissertation is to identify a viable noninvasive physiological biomarker that can be used to determine the appropriate time period after the insult to deliver a specific treatment intervention. This dissertation has targeted the NMDAR-mediated transmission as a biomarker for TBI, due to the intimate involvement of NMDARs in normal development, plasticity and the pathophysiology of TBI. Furthermore, glutamatergic neural activity can now be measured using magnetic resonance imaging (MRI) (Bonvento et al., 2002). Functional MRI signals acquired for investigating neural functions, such as blood oxygen level dependent (BOLD) signal, has been shown to be primarily driven by NMDARs. Using a somatosensory evoked potential stimulation, BOLD signal can be significantly reduced using the NMDAR antagonist MK801 (Gsell et al., 2006). In addition, pharmacological MRI (phMRI) has also been utilized to elicit increases in hippocampal NMDAR-mediated change in relative cerebral blood volume (ΔrCBV) by administering D-serine, an NMDAR agonist. This induced increase has been shown to be blocked by the co-administration of an NMDAR antagonist L-701,324 (Panizzutti et al., 2005). PhMRI rCBV signals were measured in developing sham and FPI rats during the subacute phase post-injury. The phMRI rCBV was generated using D-cycloserine (DCS), a partial NMDAR agonist, to induce the NMDAR-mediated rCBV response.

SCOPE OF THE DISSERTATION

Following TBI to the developing brain, an acute neurometabolic cascade occurs that results in pathological activation of glutamatergic synapses and leads to altered neural development and lost potential. This alteration in neural development manifests through molecular and behavioral measures of plasticity. The central hypothesis of this dissertation is that enhancing glutamatergic neurotransmission at a critical post-injury time window will facilitate recovery of impaired neural activation and will subsequently restore experience-dependent plasticity following developmental TBI.

This dissertation demonstrated three main findings: (1) TBI during a time when the brain is still developing will result in subacute reduction of hippocampal, glutamate- (NMDAR)-driven neural responsiveness that leads to impaired plasticity in young adulthood; (2) pharmacological facilitation of this NMDAR-mediated responsiveness in the appropriate post-injury time window can be used as a strategy to rescue impaired neuroplasticity; and (3) the use of phMRI can serve as a physiological biomarker that can correlate with NMDAR-mediated transmission to help assess NMDAR-mediated glutamatergic dysfunction after TBI. TBI was induced using the lateral fluid percussion injury (FPI) model in postnatal day 19 rats, which causes diffuse cerebral dysfunction without significant neuronal loss. Therefore, deficits in plasticity may be separated from the deficits due to overt tissue damage. Diminished hippocampal neural responsiveness has been measured molecularly, behaviorally, and physiologically. The following chapters will cover studies that address this central hypothesis.

In Chapter 1, the effects of FPI and DCS on molecular markers of plasticity in the hippocampus are investigated, on post-injury day four (PID4). Glutamate receptors (NMDAR

and AMPAR) and an NMDAR-mediated secondary messenger (CAMKII) were measured using western blotting techniques. Injury and drug effects on these molecular markers are then correlated with a hippocampus-mediated behavioral outcome (novel object recognition task) on the same post-injury day four.

In Chapter 2, the effects of FPI and DCS treatment on experience-dependent plasticity are assessed. To measure the effects on the capacity for plasticity, experience-dependent plasticity was induced in the developing pups by housing the animals in an enriched environment (EE). The extent of induced plasticity was measured by testing the animal's learning and memory in a spatial learning task, the Morris water maze (MWM), when they have matured into adulthood (PID 30-40).

Finally, in Chapter 3, the novel use of phMRI as a non-invasive diagnostic tool for hippocampal neural responsiveness is demonstrated. NMDAR-mediated ΔrCBV within one week and after two weeks post-FPI is shown. The ΔrCBV time course has been used to assess the magnitude and duration of NMDAR dysfunction and to target the optimal therapeutic window for an NMDAR-based treatment.

The appendix includes supplementary material that supports Chapters 1 and 3.

CHAPTER 1.

D-CYCLOSERINE RESTORES MOLECULAR AND BEHAVIORAL MARKERS OF PLASTICITY FOLLOWING LATERAL FLUID PERCUSSION INJURY IN THE DEVELOPING RAT

INTRODUCTION

Previous studies have shown that TBI can disrupt experience-dependent plasticity in the young brain through acute indiscriminate glutamate release and the pathological activation of excitatory glutamate-mediated neurotransmitter receptors (Miller et al., 1990; Kumar et al., 2002a; Biegon et al., 2004; Osteen et al., 2004), elevation of calcium levels in postsynaptic cells (Osteen et al., 2001) and eventually alterations of neural activation, connectivity, and experience-dependent plasticity (Fineman et al., 2000; Ip et al., 2002; Giza et al., 2005). In addition, the global changes in calcium signaling during TBI due to glutamatergic excitotoxicity suggest that calcium-dependent protein kinases and effectors would also be altered. Here, the effects of FPI injury on molecular markers of plasticity in the hippocampus of postnatal day 19 rats were investigated. Glutamate receptors, NMDAR and AMPAR, as well as plasticity-related secondary messenger targets and effectors, such as CAMKII and ERK, were specifically measured.

NMDARs and AMPARs are the predominant excitatory glutamate receptors in the brain and are necessary in signal transduction, learning and memory, and experience-dependent plasticity (Cull-Candy et al., 2001; Cull-Candy et al., 2006). The NMDAR is a tetramer

assembly containing two dimers (a chemical structure formed from two subunits): one NR1 and one NR2 subunit dimers. The resulting structure forms a voltage-gated channel that is activated by the binding of co-agonists glutamate and glycine. The NR1 subunit is a necessary constituent for a functional receptor, whereas the NR2A (A-D) subunits confer modulatory properties that regulate glutamate sensitivity and channel opening. NR2A and NR2B are the predominant NR2 subtypes that are extensively expressed in the hippocampus and neocortex, with differing temporal developmental profiles (Flint et al., 1997). Subunit composition determines receptor function, and changes occur during development (Sheng et al., 1994; Wenzel, 1997) or in response to physiological sensory stimuli and experience (Quinlan et al., 1999a; Quinlan et al., 1999b) or trauma.

Following experimental TBI in adult mice, there is a period of dramatic increase of NMDAR activation 15 minutes post-injury and a second period of diminished NMDAR expression hours to days later (Biegon et al., 2004). In adult rats, a decrease in NMDAR binding has also been reported three hours post-injury (Miller et al., 1990), as well as reduced NMDAR protein levels as early as 6 hours (Kumar et al., 2002a; Osteen et al., 2004). Lateral fluid percussion injury (FPI) in postnatal day 19 (P19) weanling rats results in reduced hippocampal NR2A subunit protein levels from post-injury day (PID)1-4 (Giza et al., 2006) and failure of these FPI animals to manifest enhanced cognition, behavior, and neuroanatomy after being housed in an enriched-environment (EE). When tested between PID30-50, FP-injured weanling rats reared in EE did not show enhanced spatial learning on the Morris water maze task (MWM) compared to shams (Fineman et al., 2000; Giza et al., 2005). Cortical thickness and dendritic arborization did not significantly increase in FPI animals when measured around PID50 (Fineman et al., 2000; Ip et al., 2002). Moreover, FPI in this age group did not result in overt

histological damage (Prins et al., 1996; Gurkoff et al., 2006); therefore, FPI induced deficits suggest neuronal dysfunction rather than extensive cell death.

AMPARs, much like the NMDARs, also mediate excitatory ionotropic neurotransmission and have been implicated in synaptic plasticity, learning and memory. Similar to NMDARs, AMPAR composition is as a *dimer of dimers*. A dimer is a complex with two subunits; thus, a dimer of dimers is an assembly with two dimers. The AMPAR has a tetrameric complex, which is composed of four subunits (GluR1-4). The presence of the GluR2 subunit renders the AMPAR impermeable to calcium influx. Ca^{2+}-permeable AMPARs are important in maintaining long-term potentiation (LTP) at synapses, wherein after the induction of LTP AMPARs (particularly GluR1 homo-multimers) are transiently inserted into the postsynaptic membrane (Kauer and Malenka, 2006). Changes to AMPAR subunit composition occur during synaptic activity, development and injury and can confer differential receptor function (Pickard et al., 2000; Kumar et al., 2002b; Cull-Candy et al., 2006). FPI to adult rats has been shown to reduce GluR2 expression and increase phosphorylation of GluR1. (Atkins et al., 2006; Bell et al., 2007; Bell et al., 2009).

Furthermore, the aberrant intracellular calcium influx during TBI through NMDARs and AMPARs can in turn disrupt various protein kinases that are also key players in neurotransmission, gene expression and memory formation. CAMKII, ERK, and CREB are all implicated in long-lasting changes in brain activity (Purves et al., 2004). FPI increases phosphorylated CAMKII (phos-CAMKII) in minutes (Atkins et al., 2006) to hours and decreases phosphorylated CREB (phos-CREB) one week after injury. Persistent alterations in these kinases are observed with impaired activation of phosphorylated ERK (phos-ERK) and phos-

CREB upon stimulation in hippocampal slices from FPI animals up to 12 weeks after injury (Atkins et al., 2009).

Previous work has focused on the blockade of NMDARs as an attempt to halt subsequent damage due to widespread injury-induced excitotoxicity and pathological NMDAR activation. Although this neuroprotective approach has been implemented successfully in experimental models of TBI (Rao et al., 2001; Schumann et al., 2008; Han et al., 2009), use of NMDAR antagonists has not been successful to improve outcomes in previous clinical trials (Muir, 2006). One reason NMDAR antagonists may have failed is that by the time the antagonists are administered, the NMDARs are already down-regulated following TBI, missing the critical window of hyperactivity of these receptors. Inhibition of NMDARs during this time would then interfere with normal physiological receptor function. Moreover, during the recovery phase, excitatory synaptic activity may be essential to promote pro-plasticity responses and facilitate a more favorable outcome. This idea has led to a shift of focus towards NMDAR agonists rather than antagonists in the subacute phase (greater than 24 hours, after excitotoxic period of injury-induced heightened levels of glutamate). Recently, the use of NMDA or D-cycloserine (DCS) has been shown to promote neuroprotection and has facilitated more rapid recovery after TBI in rodents (Temple and Hamm, 1996; Biegon et al., 2004; Yaka et al., 2007) with a wide therapeutic window (Adeleye et al., 2009). Specifically, the use of DCS, a partial agonist of the NMDAR glycine site, as therapy may be suitable in a post-TBI situation where partial efficacy is desired to prevent further excitotoxicity. Furthermore, DCS is an FDA-approved drug, and its pharmacokinetics and efficacy have been well characterized for safe use in humans (Baxter and Lanthorn, 1995). However, DCS not only affects NMDAR transmission directly, but also has been shown to indirectly modulate calcium signaling via AMPAR. DCS has been shown not

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only to facilitate NMDAR-mediated synaptic potentials in hippocampal slice preparations, but also to decrease AMPAR-mediated neurotransmission. (Rouaud and Billard, 2003).

The goal of this study was to investigate whether systemic DCS administration during a critical time window will modulate molecular markers of plasticity and rescue behavior subacutely following developmental TBI. This study consisted of two experiments. In Experiment 1, the effects of DCS treatment on the post-FPI molecular response of hippocampal NMDARs and AMPARs were quantified during the window of maximal NMDAR subunit change in weanling rats (PID1-4). Additionally, exploratory open field behavior was evaluated to investigate injury and drug effects on global animal behavior. In Experiment 2, we evaluated effects of FPI on the animal's novel object recognition (NOR), a test of working memory that is hippocampally mediated and NMDAR-dependent (Ennaceur and Delacour, 1988). We used this task to behaviorally assay TBI-induced deficits in hippocampal NMDAR neurotransmission. From these same subjects, pro-plasticity molecules that are glutamate-mediated downstream effectors (CAMKII and ERK) were also measured. We hypothesized FPI to the P19 rat will reduce NR2A, CAMKII and ERK levels, while increasing GluR1 levels and impairing subacute performance in the NOR task. Moreover, DCS treatment during the subacute phase will restore not only the NR2A subunit, but also NMDAR mediated downstream signaling molecules. The restoration of molecular markers by DCS will be manifest in rescued NOR behavior post-FPI.

METHODS

Experimental Design and Subjects

One hundred and two Sprague-Dawley (Charles River, Boston, MA) rat pups underwent sham or severe lateral fluid percussion (FPI) on postnatal day 19 (P19). Sixty-five and fifty-seven animals were used in Experiment 1 and Experiment 2, respectively. Animals were randomized to receive intraperitoneal (i.p.) injections every 12 hours with either saline (Sal) or DCS (Sigma Aldrich) between post-injury days one to three (PID 1-3). On P20, animals were weaned from their mothers. They were maintained in a 12 hour light-dark cycle with food and water ad libitum and 3 or more other littermates in a cage. The UCLA Chancellor's Committee for Animal Research approved all animal studies. Figure 8 illustrates the experimental design timelines for Experiment 1 and 2.

Experiment 1 consisted of five groups: Sham-Sal, Sham-highDCS (30 mg/kg), FPI-Sal, FPI-lowDCS (10mg/kg) and FPI-highDCS (30 mg/kg). Open field exploratory behavior was observed on PID 2 and 3. On PID4, 20 animals (N = 4 per group) were perfused and processed for thionine histology (see Appendix: A1). All remaining animals were then sacrificed on PID4 (N = 7-8 per group), and brains were harvested and dissected for synaptoneurosome Western blotting of NMDAR (NR1, NR2A and NR2B) and AMPAR (GluR1 and GluR2) subunits (N = 8/group).

Experiment 2 included four groups: Sham-Sal (N = 15), Sham-highDCS (N = 9), FPI-Sal (N = 15) and FPI-highDCS (N = 9). DCS treated groups were administered 30 mg/kg (i.p.) per dose. On PID4, animals were tested on the NOR task using a 1-hour retention interval. At least one hour after the NOR task, animals were sacrificed and brains were processed for

synaptoneurosomes. Total and phosphorylated levels of CAMKII and ERK1/2 were measured using Western blotting.
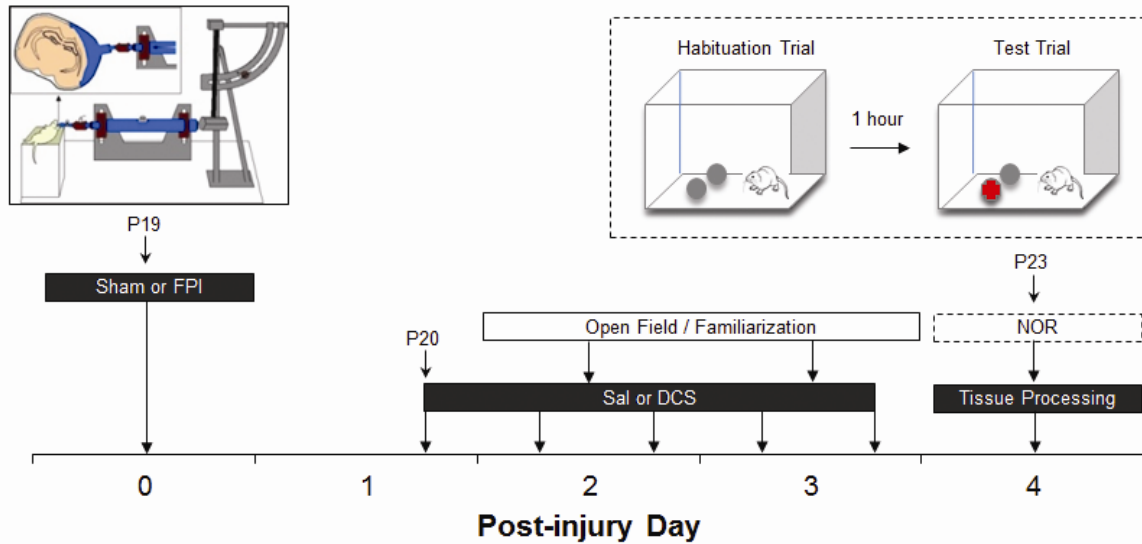
Figure 8. Experimental Design Timelines 1 and 2
Experiment 1 animals underwent sham or FPI surgery on P19 (designated as post-injury day, PID 0). After 24 hours of recovery, animals were weaned and randomized into either saline (Sal) or DCS treatment (lowDCS = 10 mg/kg, i.p.; or highDCS = 30 mg/kg, i.p., every 12 hours) from PID1-3. On PID 2-3, general exploratory open field behavior was evaluated. On PID4, hippocampal brain regions were harvested, dissected and processed for immunoblotting. Experiment 2 animals followed the same design as Experiment 1, except that open field behaviors were monitored in the post-injury period and performance in the NOR was measured on PID4.

Fluid Percussion Injury

The standard lateral FPI protocol previously described in detail (Prins et al., 1996) was followed. In brief, after general anesthesia induction using 3% isoflurane (1.0-1.5 ml/min in 100% O2) in a chamber, 1.5-2.5% isoflurane was maintained via a nose-cone in spontaneously respiring postnatal day 19 rat pups. Body temperature was kept constant (37-38°C) by a thermostatically-controlled heating pad. Following aseptic surgical preparation, the head was secured in a stereotaxic frame and a midline skin incision was made to expose the skull. A 3.0 mm diameter craniotomy was made 3.0 mm posterior to bregma and 6.0 mm lateral (left) of midline. The injury cap was secured over the craniotomy with silicone and dental cement and was later filled with 0.9% saline. Once the injury cap was secured anesthesia was discontinued and the animal was attached to the FPI device. When the animal exhibited a hind paw withdrawal reflex initiated by a toe pinch, a mild-moderate fluid pulse (~2.5 atm) was delivered. Apnea time was determined by the resumption of spontaneous respiration and the loss of consciousness time (LOC) by the return of the hind paw withdrawal reflex from toe-pinch. Positive pressure ventilation was administered through the mask with 100% O2 if the animal remained apneic for at least 30 seconds, and was maintained until spontaneous respiration returned. Only animals displaying LOC times of at least 120 seconds were included in the experiments and were considered to have sustained severe injuries.

Upon the return of the hindpaw withdrawal reflex, the animal was placed back under anesthesia for the removal of the injury cap and the cleaning and closure of the surgical wound. Intradermal injections of 0.25% bupivacaine and topical antibiotic ointment were administered at the surgical site. The animal was then removed from anesthesia and placed in a heated recovery

chamber. Sham animals underwent identical surgical procedures, but without the attachment of the injury cap or fluid pulse induction.

Drugs

D-cycloserine (DCS) was obtained from Sigma Aldrich (Boston, MA). DCS was mixed in sterile 0.9% saline with a constant injection volume of 0.25 ml/kg. DCS was frozen (-20°C) and thawed 15 min prior to each injection time.

DCS has been shown to freely cross the blood-brain barrier in humans (Baxter and Lanthorn, 1995). In mice, however, Wlaz et al showed that after subcutaneous injection of DCS at a low dose (5 mg/kg), there were only trace amounts of DCS detected in the brain. After administration of a much higher dose (320 mg/kg) of DCS, only about 14% of the DCS plasma levels were detected in mice cortex during peak plasma levels of DCS (15 minutes post-injection) .   The slower penetration into the brain was evident when higher DCS levels were detected 60 minutes after injection (Wlaz et al., 1994). The half-life of DCS in mice is 30 minutes, and 1 hour in rats, and most of the administered DCS dose is excreted in rodents unchanged (Baxter and Lanthorn, 1995).

The doses chosen in this dissertation were based on the known pharmacokinetics of DCS and on previous studies that used DCS to induce cognitive enhancement or therapeutic effects after pharmacologically- or injury-induced memory impairments. Doses levels of at least 0.3mg/kg doses were shown to enhance learning in a passive-shock avoidance task in adult rats, showing optimal improvement with 3mg/kg DCS (Monahan et al., 1989a). After a closed head injury in adult mice, a 10 mg/kg dose of DCS was demonstrated to significantly improve

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neurological outcome (Yaka et al., 2007; Adeleye et al., 2009). Following FPI in adult rats, a dose of 30mg/kg administered intraperitoneally (i.p.), and not 10 mg/kg, was found to be more effective in rescuing deficits in the Morris water maze task. Since lower DCS doses (5mg/kg) had higher rates of clearance and only resulted in small amounts in the brain during peak plasma levels of DCS (Wlaz et al., 1994), 10 mg/kg DCS was designated as the low dose in the dose-response study (Experiment 1).   30 mg/kg was used as the high dose, which is still in the range where DCS potentiates NMDAR-mediated processes and demonstrates NMDAR agonist properties (under 50 mg/kg in rats and mice; under 100 mg/kg in humans). At even higher doses (over 100 mg/kg in rodents and over 250 mg/kg in humans), DCS behaves more like an NMDAR antagonist (Baxter and Lanthorn, 1995).

In Experiment 1, DCS treatment was implemented as follows: beginning twenty-four hours following sham or FPI injury, rats were randomized into saline (Sal), lowDCS (i.p 10mg/kg), or highDCS (i.p. 30 mg/kg) treatment groups. Animals received five doses, one dose every 12 hours, from post-injury day 1 through 3 (PID 1-3). Based on the results from Experiment 1, only the highDCS (30 mg/kg) dose was used in Experiment 2 with the same treatment regimen.

Synaptoneurosome Preparation

Experiment 1 animals were sacrificed on post-injury day four. The brains were removed and sectioned into four 1mm-thick slabs and dissected on ice into regions of interest. Hippocampal regions ipsilateral and contralateral to the injury site were dissected on ice and homogenized in buffer (0.137M NaCl, 2.7mM KCl, 0.44mM KH2PO4, 4.2mM NaHCO3,

10mM HEPES, 10mM glucose, pH 7.4) with protease inhibitors (Complete[TM], Roche,

Germany). The homogenate was loaded into a 1mL syringe attached to a 13 mm diameter

Millipore syringe filter holder and forced through one 100µm nylon filter and then again through

two 5µm nitrocellulose filters. The filtered samples were centrifuged at 1000xg for 20min,

followed by 1000xg for 10min. The resulting synaptoneurosome pellet was resuspended in 150

µL homogenization buffer with protease inhibitors and stored at –70° (Johnson et al., 1997).

Western Blotting

Protein concentrations of synaptoneurosome fractions were determined using the Bio-

Rad DC Protein Assay. Protein (10µg per lane) was prepped in Laemmli sample buffer,

electrophoresed on 7.5% pre-case Tris-HCl gels (Bio-Rad, Hercules, CA) at 160V for 40minutes,

and then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) at 100V for 60

minutes. Total protein was imaged and quantified using the Bio-Rad Fluor-S MultiImager and

the Sypro® Ruby Protein Blot Stain, respectively. The nitrocellulose membranes were

subsequently blocked in 5% milk in Tris-buffered saline with 1% Tween-20 (TTBS) for 60

minutes at room temperature and probed overnight with various primary antibodies diluted in

TTBS. Primary antibodies (Chemicon, Temecula, CA) were used at the following dilutions:

NR1 (1:1000), NR2A (1:500), NR2B (1:500), GluR1 (1:1000), and GluR2 (1:1000), total-

CAMKII (1:1000), phos-CAMKII (1:1000), total-ERK1/2 (1:1000), and phos-ERK1/2 (1:1000).

Membranes were incubated in peroxidase-conjugated secondary antibodies (Santa Cruz Biotech,

Santa Cruz, CA), diluted in 1:10,000 in 1% milk in TTBS. We developed these blots using

either ECL reagents (Cell Signaling, Boston, MA) or Supersignal Chemiluminescent substrate

(PIERCE Prod#34080). Bands were detected with the Bio-Rad ChemiDoc XRS system and analyzed with the Quantity One® software (Bio-Rad).

The raw subunit density signal for each band was controlled to the total protein from the same lane, on the same blot. Protein controlled subunit values from each individual sample were normalized to the average of the Sham-Sal group, such that the mean Sham-Sal value equaled 100%. Protein levels were presented as percent change from Sham-Sal.

Open Field Exploratory Behavior

Any change in synaptic plasticity ultimately affects neural networks that control behavior. To evaluate simply the effects of FPI and DCS treatment in the subacute phase after FPI (time points more than twenty-four hours post-injury), the open field exploratory behavior of the animals was measured. The testing chamber was a standard vivarium cage (48 cm x 25 cm x 20 cm), void of the litter bedding (Figure 9A). The chamber was wiped down with 70% ethanol between subjects. Rats were allowed to freely explore the cage for three minutes. Open field behavior was observed and video recorded on PID 2 and 3. Rearing was manually scored and was averaged over PID 2 and 3. A digital tracking system was used (SMART, San Diego Instruments) to quantify locomotion (subject speed and total distance travelled) in the chamber. Occurrence of exploratory rearing was counted when both of the animal's front limbs were off the floor of the chamber and had a sniffing behavior or when the animal rears upward and places both front limbs onto the chamber walls. While the animal maintains contact with the wall even with only one front limb that would still be counted as one rearing occurrence. When the animal

displaces the front-limbs from the wall and then puts them back, this would be counted as a separate rearing instance.

Novel Object Recognition (NOR) Task

The NOR task has been previously shown to measure hippocampal, NMDA-mediated working memory (Ennaceur and Delacour, 1988; Clark et al., 2000; Hammond, 2004; Dere et al., 2007). The NOR testing chamber (Figure 9B) was a white, plexiglass arena (52 cm x 32 cm x 30 cm) that approximated the size of the home cage adjusted for the weanling rat (Reger et al., 2009). Testing objects were similar in form and color yet discernibly different (Figure 9C). The arena and objects were wiped down with 70% ethanol between subjects. The NOR task consisted of a familiarization phase followed by a testing phase.

Familiarization Phase

Animals were allowed to freely explore the testing chamber without objects for 5 minutes per session. One session per day was administered on post-injury day two and three.

Testing Phase

The testing phase was given on post-injury day four and consisted of a (1) habituation trial, followed by a (2) test trial one hour later. During the habituation trial, the animal was placed in the testing chamber for 5 minutes with two identical objects. A digital tracking system (SMART, San Diego Instruments) was used to measure the animal's locomotion and interaction with the objects. Interaction was defined as direct object contact with at least the nose or whiskers. The test trial was administered the same way as the habituation trial, but using one

object from the habituation trial and one novel object.   Positions of the novel and familiar

objects were counterbalanced in the chamber.
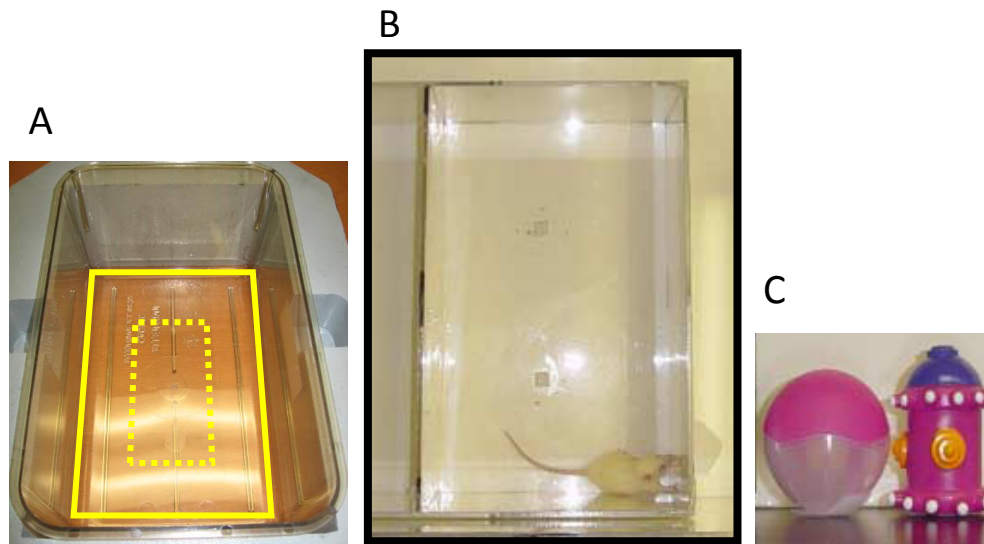
Figure 9. Exploratory Open Field Behavior and NOR Chambers and Objects
In Experiment 1, locomotion and exploratory rearing were measured a standard vivarium cage
without bedding (A). Concentric rectangular outlines define the cage perimeter (larger) and the
center zone (smaller, dotted) and were used to evaluate time spent in either outside perimeter or
center zones. In Experiment 2, the NOR chamber size used was adjusted for weanling rats (52
cm x 32 cm x 30 cm) to maximize exploration (B). NOR objects used in the habituation and test
phases are shown (C). Appropriately sized objects were used for weanling rats to maximize
interaction. NOR images (B and C) were adapted from Reger et al., 2009, with permission.

Statistics

All data are expressed as the mean $\pm$ standard error of the mean (SEM). A one-way

analysis of variance (ANOVA) was performed on the injury characteristic parameters (LOC,

apnea, atm, and PID0 weight).

Immunoblotting Analysis

All statistical analyses were conducted using SPSS version 16. Multivariate ANOVAs were implemented with injury and drug as independent variables and hippocampal western blot signal and general open field behavior as dependent variables. Post-hoc tests were Bonferroni adjusted comparisons. For all analyses, $p < 0.05$ was considered significant. Additionally, a Pearson correlation coefficient was computed to assess the relationship between the changes in NMDAR and AMPAR subunit levels in both ipsilateral and contralateral hippocampal regions.

NOR Performance Analysis

Data were analyzed for percent object interaction with the novel object (time spent with novel object divided by total object interaction time $\times$ 100%) and locomotion (subject speed and total distance travelled) in the chamber. The subject speed was computed only when the animal was on motion. Object recognition was defined as an interaction rate with the familiar object that was significantly greater than chance performance. An animal that did not recognize the object from the habituation trial was expected to split its object interaction time 50:50 between the novel and familiar object at testing. Therefore the percentage of total object interaction time spent with the novel object was tested against chance performance (50%) for each group using one-sample t-tests. Remembrance occurs when the percent time spent with the novel object is significantly higher ($p < 0.05$) than chance performance.

RESULTS

Injury Severity

All animals included in the study were administered either a sham surgery or moderate-severe FPI. Moderate-severe FPI was defined as having duration of unresponsiveness to toe pinch (loss of consciousness, LOC) of at least 120 seconds. Six and nine animals were excluded from Experiment 1 and 2, respectively, due to complications from surgery or injury or to LOC times of less than 120 seconds. Within each experiment, mean apnea, LOC, and the corresponding atmospheric pressure (atm) did not significantly differ between FPI groups (Table 4). Also, within each experiment, there were no significant differences in subject weights across groups on the surgery day (PID0) and end (PID4) weights. However, when comparing between experiments, ANOVA showed significant differences between Experiment 1 and Experiment 2 on apnea ($F_{1,60} = 5.635$, $p = 0.021$) and LOC ($F_{1,60} = 4.846$, $p = 0.032$). The corresponding atmospheric pressures during FPI induction in both experiments were comparable. Apnea and LOC times were consistently lower in Experiment 2 than those in Experiment 1. Additionally, there were significant differences between Experiment 1 and Experiment 2 subject weights on surgery day [PID0: ($F_{1,105} = 12.320$, $p = 0.001$)] and at the end [PID4: ($F_{1,105} = 11.846$, $p = 0.001$)]. Experiment 2 animals had significantly higher weights than Experiment 1 animals at both time points.

Additionally, an analysis of covariance (ANCOVA) was performed to test the hypothesis whether the difference between experiments on the injury severity parameters (apnea and LOC) still manifests itself in the data once body weight on surgery day has been accounted for. Here the independent variable again was the experiment (Experiment 1 vs. 2), the dependent variables

are apnea and LOC, and the covariate is the PID0 surgery weight on P19. The ANCOVA

resulted in significant differences between experiments on the injury parameters, apnea ($F_{1,58} = $

4.519, $p = 0.038$) and LOC ($F_{1,58} = 4.013$, $p = 0.050$). Although all animals included in both

experiments reached our criteria for moderate-severe FPI (LOC $\geq$ 120s) and had no significant

differences, Experiment 2 animals were experiencing a "more moderate" rather than a severe

injury (Table 5).

Experiment 1: FPI and DCS Effects on PID4 NMDAR & AMPAR Levels

NMDAR

In Experiment 1, a low and a high dose was used to evaluate DCS effects on hippocampal

NMDAR and AMPAR subunit levels after developmental FPI. Across all Sal and DCS samples,

ANOVA showed an overall effect of injury on NR2A subunit in the ipsilateral hippocampus

($F_{1,37} = 4.740$, $p = 0.037$). No overall effects were observed for NR1 and NR2B. Overall, the

highDCS dose showed more robust effects than the lowDCS dose. When comparing Sal and

only highDCS groups, there was a main effect of injury ($F_{1,29} = 5.148$, $p = 0.032$) and drug

treatment ($F_{1,29} = 4.384$, $p = 0.046$) on ipsilateral NR2A. FPI reduced ipsilateral hippocampal

NR2A by 20% regardless of drug treatment (Figures 10A and 12A) and DCS increased

hippocampal NR2A levels by 20% in both Sham and FPI pups (Figures10B and 12B). DCS

resulted in a dose-dependent restoration of NR2A levels in ipsilateral hippocampus. Although

no main effects were observed for ipsilateral NR2B, there was a strong positive correlation

between NR2A and NR2B in ipsilateral hippocampus (r = 0.653, N = 38, $p < 0.001$).

Representative western blots for each NMDAR subunit are shown in Figures 12 E and F.

In the contralateral hippocampus, NR2B showed trend for overall effect of injury ($F_{1,31}$ = 3.590, p = 0.068) but for DCS. There were no main effects of injury or drug for contralateral NR1 and NR2A levels. While no main effects were observed for NR1, the initial calculation of a Pearson's r correlation coefficient showed a strong positive correlation between NR1 and NR2B in the contralateral hippocampus (r = 0.559, N = 40, p < 0.001). Along with a significant reduction of NR2B (-21%) from Sham-Sal levels (Independent samples test, p = 0.05), there was a relative decrease in NR1.

AMPAR

For AMPARs, initial Pearson's r coefficient calculation revealed no correlations between GluR1 and GluR2 subunit levels in ipsilateral and contralateral hippocampus. Two-way ANOVA showed no overall effect of injury for GluR1 and GluR2 subunits in the ipsilateral hippocampus (Figure 13A). There was a significant main effect of DCS ($F_{1,39}$ = 4.772, p = 0.015) only for ipsilateral GluR2 (Figure 11B), as well as a significant injury-by-drug interaction ($F_{1,39}$ = 6.223, p = 0.017). This result suggests that DCS had a different effect at different dose levels on ipsilateral GluR2. Post-hoc testing showed FPI-highDCS animals had significantly higher levels of ipsilateral hippocampal GluR2 (+72.5%) compared to FPI-lowDCS (p = 0.006). In the contralateral hippocampus there was a significant main effect of injury on GluR1 ($F_{1,38}$ = 6.140, p = 0.018) (Figure 13B). FPI to P19 rats resulted in increased GluR1 levels by approximately +45%, compared to Sham-Sal animals, regardless of drug. No main effect of DCS was observed in GluR1. Contralateral hippocampal GluR2 levels were unaffected by injury or DCS. Representative blots for each AMPAR subunit are shown in Figure 13 (E and F).

Table 4. Experiment 1and 2 – Injury Characteristics by Group
Mean (± SEM) starting (PID0) and ending (PID4) weights in grams (g), apnea and loss of consciousness (LOC) times in seconds (s) and the corresponding atmospheric pressure after FPI induction by group for Experiment 1 and 2.

Exp	Group	N	wtPID0 (g)	wtPID4 (g)	Drop <	Apnea (s)	LOC (s)	Atm
1	Sham-Sal	12	44.5 ± 1.4	57.6 ± 2.4	NA	NA	NA	NA
	Sham-highDCS	11	43.6 ± 1.3	59.0 ± 1.8	NA	NA	NA	NA
	FPI-Sal	11	46.0 ± 1.1	55.2 ± 2.8	16	222 ± 26	247 ± 20	2.26 ± 0.01
	FPI-lowDCS	12	44.0 ± 1.0	52.6 ± 1.8	16	230 ± 30	248 ± 28	2.28 ± 0.02
	FPI-highDCS	12	44.6 ± 0.8	54.6 ± 1.1	16	230 ± 21	254 ± 22	2.29 ± 0.01
2	Sham-Sal	15	47.3 ± 1.1	61.9 ± 1.2	NA	NA	NA	NA
	Sham-highDCS	7	46.0 ± 1.4	59.5 ± 2.1	NA	NA	NA	NA
	FPI-Sal	15	46.2 ± 0.6	57.8 ± 1.3	16	178 ± 20	199 ± 21	2.29 ± 0.03
	FPI-highDCS	7	50.9 ± 2.0	60.6 ± 1.2	16	173 ± 23	214 ± 23	2.23 ± 0.01

Table 5. Experiment 1 and 2 – Injury Characteristics by Experiment
Mean (± SEM) injury characteristic values for atm, LOC, and apnea, and surgery day (PID0) and end (PID4) weights for animals in Experiment 1 and 2. The asterisk (*) indicates significant differences were found between Experiment 1 and 2 values.

|	Exp	N	Mean	SEM
Atm	1	36	2.28	0.01
	2	24	2.27	0.02
*LOC (s)	1	36	250	13
	2	24	205	15
*Apnea (s)	1	36	227	14
	2	24	176	15
*wtPID0 (g)	1	59	44.6	0.5
	2	48	47.3	0.6
*wtPID4 (g)	1	59	55.8	0.9
	2	48	59.9	0.7
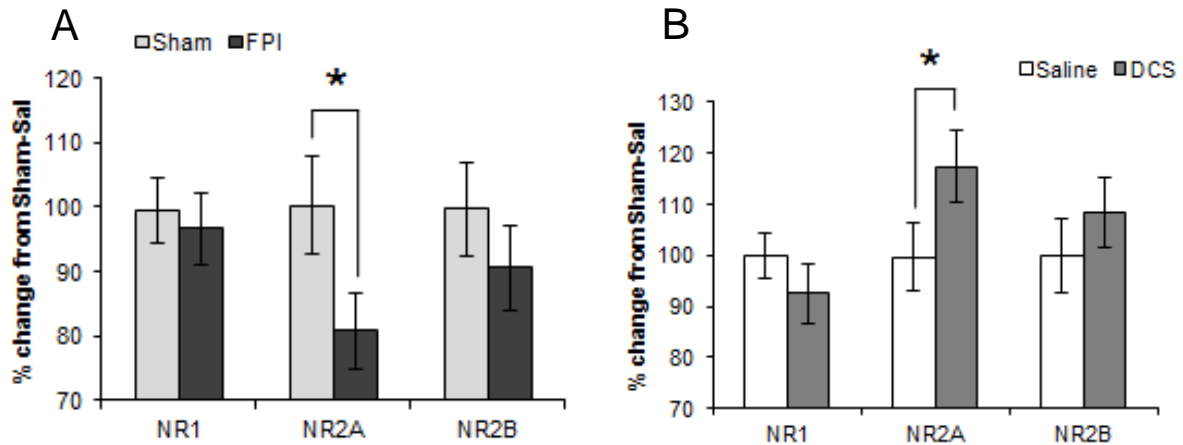
Figure 10. NMDAR Main Effects
 (Mean ± SEM) NMDAR main effects of injury (A) and DCS (B). A main effect of injury and DCS treatment was observed only for the NR2A subunit. FPI did not significantly affect NR1 and NR2B. (* $p < 0.05$).
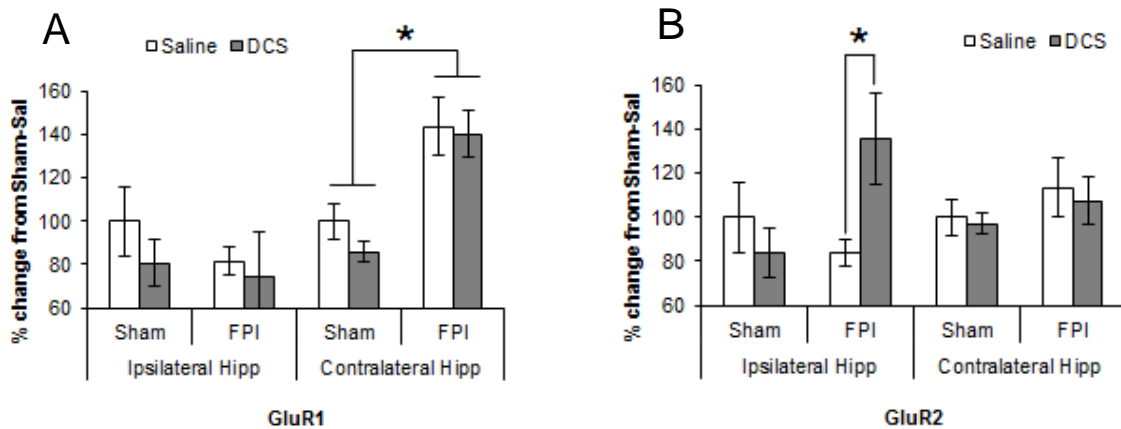

Figure 11. AMPAR Main Effects
(Mean ± SEM) (A) GluR1 main effects of injury. FPI resulted in increased levels of GluR1 in the contralateral hippocampus (Hipp). (B) GluR2 DCS main effects. DCS treatment significantly increased GluR2 levels in the ipsilateral hippocampus. (* $p < 0.05$).
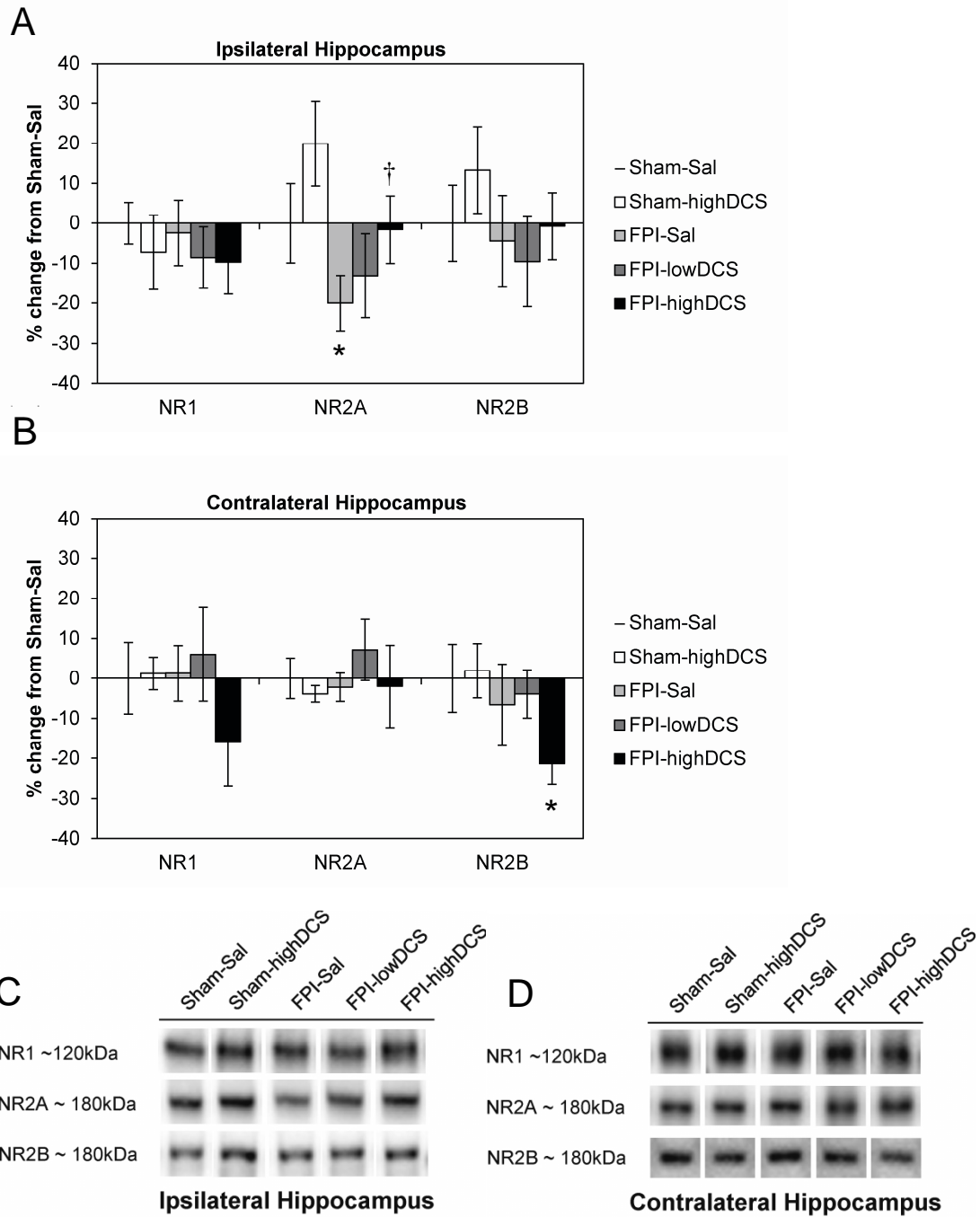
Figure 12. NMDAR Subunit Levels

(Mean ± SEM) NMDAR NR1, NR2A and NR2B subunit levels in the hippocampus, ipsilateral (A) and contralateral (B) to the FPI injury site. The asterisk (*) indicates significant difference from Sham-Sal levels ($p < 0.05$) and the dagger (†) indicates significant difference from FPI-Sal ($p < 0.05$). Representative western blots of NR1, NR2A, and NR2B subunits in ipsilateral (C) and contralateral (D) hippocampus.

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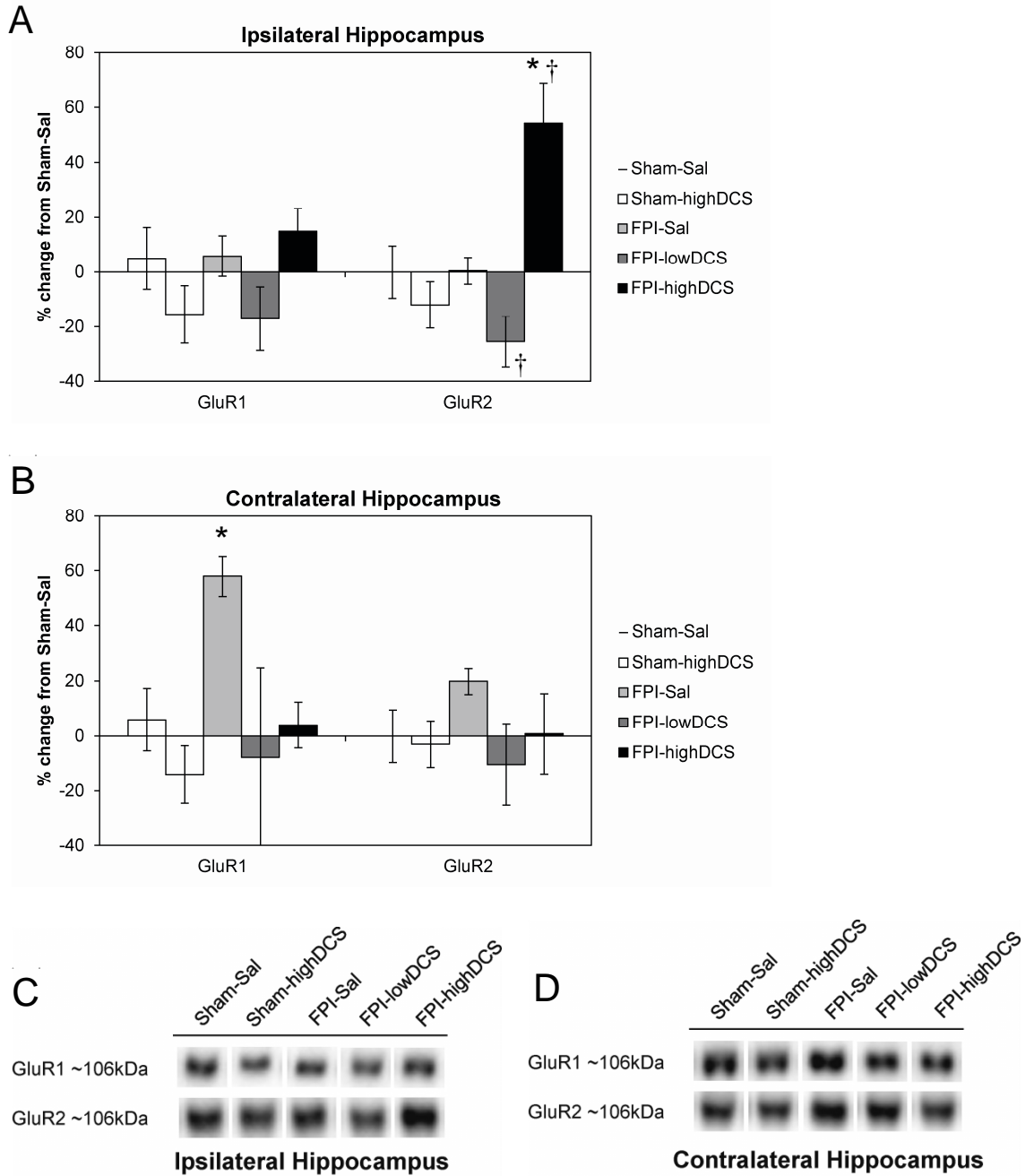
Figure 13. AMPAR Subunit Levels
(Mean ± SEM) AMPAR GluR1 and GluR2 subunit levels in the hippocampus, ipsilateral (A) and contralateral (B) to FPI injury site. The asterisk (*) indicates significant difference from Sham-Sal ($p < 0.05$) and the dagger (†) indicates significant difference from FPI-Sal ($p < 0.05$). Representative western blots of GluR1 and GluR2 subunits in ipsilateral (C) and contralateral (D) hippocampus.

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Experiment 1: FPI and DCS Effects on Open Field Behavior

Overall, ANOVA showed significant effect of DCS on rearing counts ($F_{1,57} = 4.744$, $p = 0.034$). The number of rearing increased when DCS was on board, in a dose-dependent manner (Figure 14). The highDCS dose effect on rearing only reached significance in FPI animals (*$p < 0.05$). One animal was excluded in the open field behavior test due to non-performance. Non-perfomance was defined as having no significant movements from one corner or having a total distance of less than 200 cm in 60 s. Throughout the 3 minute test on PID2 and 3, the animal had very little exploration.

Only PID3 locomotion data set was complete across all groups and was analyzed. The subject's total distance travelled and speed were quantified on two cut-off time points: 60 s and 150 s. When comparing saline and highDCS groups, independent samples test (Bonferroni corrected) showed significant reduction in the total distance travelled by injury (Sham-Sal > FPI-Sal, $p = 0.016$) and that highDCS significantly increased total distance travelled (FPI-highDCS > FPI-Sal, $p = 0.013$) to Sham-Sal levels. The same pattern in the 60 s cut-off was mirrored in the 150 s cut-off. The exploration speed results were computed at the 150 s cut-off to allot for increased acquisition of data points. The exploration speed matched the same pattern as that of the total distance travelled: FPI-Sal showed significantly reduced speed compared to Sham-Sal ($p = 0.02$), and FPI-highDCS had higher speed than FPI-Sal ($p = 0.04$), but comparable to Sham-Sal. As expected, there were strong positive correlations between these two parameters ($r^2 > 0.97$). Moreover, there was a dose-dependent graded response on distance and speed of the animals (Figure 15 A and B).
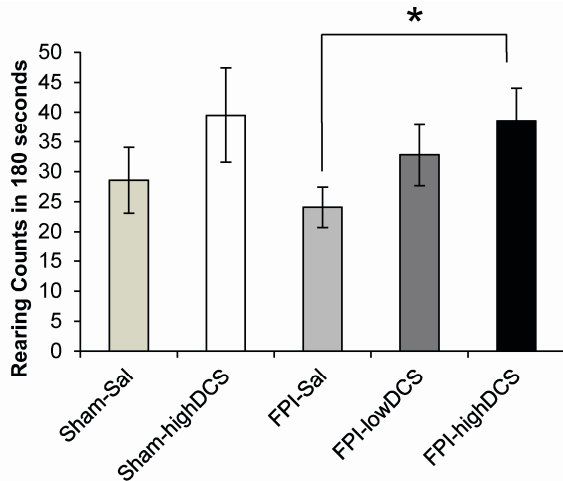
Figure 14. Open Field Exploratory Rearing Counts
Mean (± SEM) rearing counts quantified on PID 2 and PID3 during 3 minute open field exploration. No main effect of injury was observed. There was an overall significant effect of DCS across all subjects, with a DCS dose-dependent response in rearing counts. The highDCS-induced increases in rearing counts only reached significance in FPI animals (*$p < 0.05$).
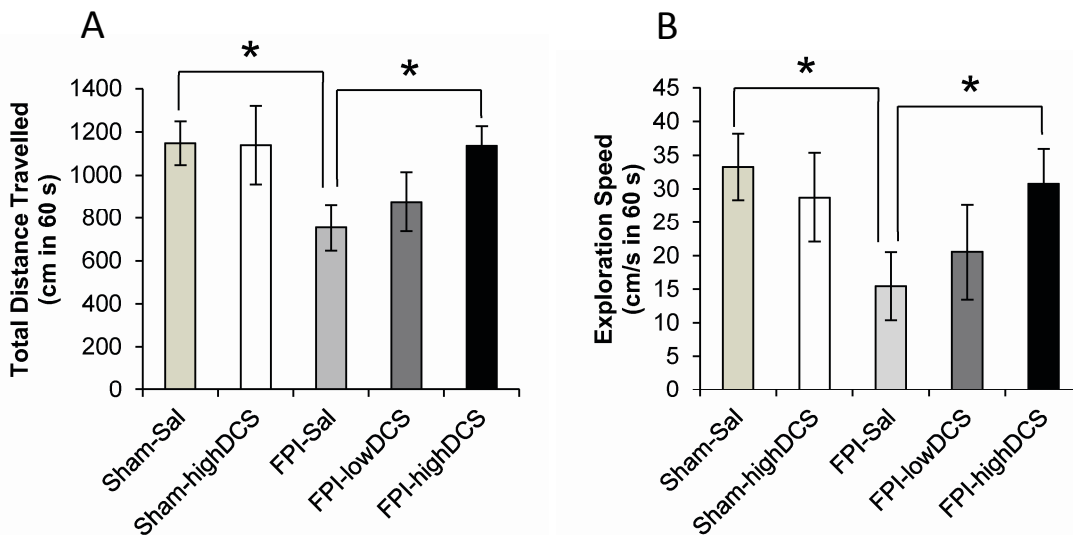
Figure 15. Open Field Locomotion
Only PID3 data set was used in the analysis. (A) Total distance travelled (cm in 60 s) and (B) total exploratory speed in arena (cm/s in 150 s) were quantified (* $p < .05$). Locomotion deficits were seen in the FPI-Sal group and highDCS treatment returned performance to sham levels, without affecting shams.

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Experiment 2: FPI and DCS Effects on NMDAR-Mediated Secondary Effectors

ERK

Initial calculation of Pearson's r correlation showed no correlations between phosphorylated-ERK (phos-ERK) and total basal ERK (total-ERK) in the ipsilateral and contralateral hippocampal regions. In ipsilateral hippocampus, two-way ANOVA showed no significant effects of injury or drug for phosphorylated-ERK (phos-ERK) and total basal ERK (total-ERK) (Figures 16 A and B).   In the contralateral side, a significant main effect of injury ($F_{1,41} = 5.723$, $p = .022$) and a significant injury*drug interaction were observed in total-ERK ($F_{1,41} = 4.241$, $p = .046$) (Figures 16 C and D).

For ERK, no significant main effects of injury or drug were observed in either phos-ERK, total-ERK, or the phos/total ERK ratio protein in the ipsilateral hippocampus. Contralateral to the FPI site, DCS significantly increases total-ERK levels only in FPI injured pups (Injury*Drug interaction: $F_{1,41} = 4.241$, $p = 0.046$). Trends for Injury*Drug interaction was also observed in the basal phos/total ERK(1/2) ratio ($p = 0.074$). Figures 16 E and F show representative western blots for phos-ERK and total-ERK in the ipsilateral and contralateral hippocampus.

CAMKII

A Pearson's r correlation calculation also showed no correlations between phosphorylated-CAMKII (phos-CAMKII) and total basal levels of CAMKII (total-CAMKII) in the ipsilateral and contralateral hippocampal regions. For CAMKII, a significant main effect of injury was detected only in basal total-CAMKII levels ($F_{1,41} = 4.314$, $p = 0.045$). Contralateral total-CAMKII showed only a significant main effect of drug ($F_{1,41} = 7.061$, $p = 0.011$).

In the ipsilateral hippocampus, post-hoc testing (Bonferroni corrected) showed FPI-Sal rats had a $53 \pm 10\%$ reduction in basal phosphorylated CAMKII ($p = 0.04$) compared to Sham-Sal rats. Although FPI significantly decreased levels of total-CAMKII in ipsilateral hippocampus (Figure 17A), the basal phos/total CAMKII ratio was restored by DCS in FPI rats (Figure 17B). By sign test, developmental FPI reduces basal levels of the phos/total CAMKII ratio ($p = 0.006$) on PID4. In the contralateral hippocampus, FPI had no overall main effect on total-CAMKII levels, whereas DCS significantly reduced levels of total-CAMKII, regardless of surgery procedure ($F_{1,41} = 7.081$, $p = 0.011$). No main effects of injury or drug were observed in phos-CAMKII or in the phos/total CAMKII ratio in contralateral hippocampus (Figures 17 C and D). Figures 17 E and F show representative western blots for phos-CAMKII and total-CAMKII in the ipsilateral and contralateral hippocampus.

NMDAR

In Experiment 2, NR1, NR2A, and NR2B subunit levels were also evaluated on PID4. ANOVA showed no main effects of injury or DCS on any of the NMDAR subunits. However, by sign test, ipsilateral hippocampal NR1 and NR2A (both, $p < 0.0001$) was shown to be significantly decreased on PID4 after FPI to a P19 rat. 14-out-of-14 ranked paired samples showed FPI-Sal was less than Sham-Sal in both NR1 and NR2A protein levels. NR2B was unchanged after FPI. This data suggest that NMDARs that were predominantly assemblies of NR1-NR2A decreased. No significant changes were detected in the contralateral hippocampus for any of the NMDAR subunits. In contrast with Experiment 1, NMDAR subunit levels seemed to be unchanged with parametric measures. However, the highly significant nonparametric test paralleled the decrease of NR2A subunits in ipsilateral hippocampus. In addition, the injury severity analysis revealed that the FPI in Experiment 2 was less severe than in Experiment 1, which may contribute to the diminished magnitude of reduction in previously observed NR2A subunit.
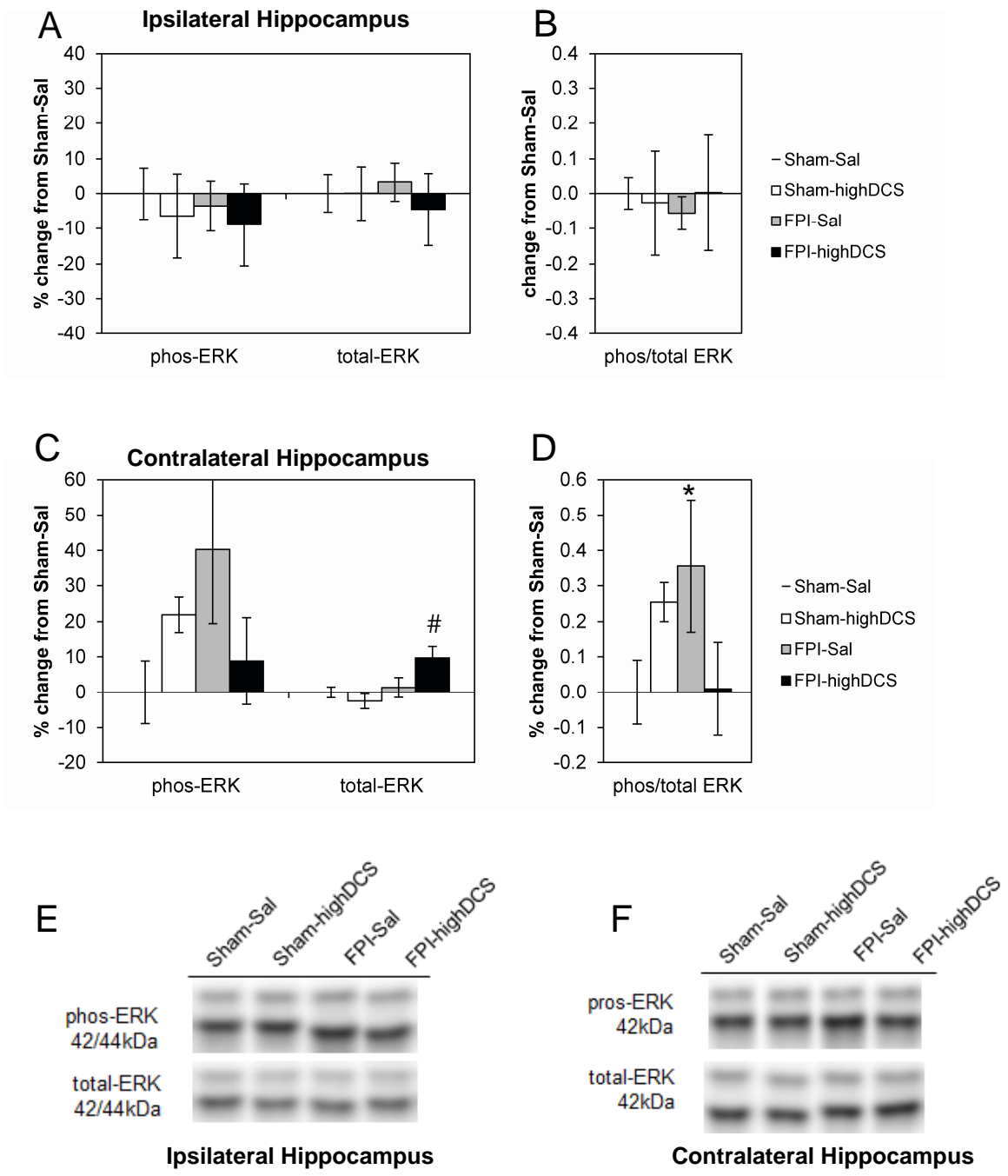
Figure 16. Phosphorylated and Total ERK Protein Levels

(Mean ± SEM) Phos- and total-ERK in the hippocampus, ipsilateral (A) and contralateral (C) to FPI. Phosphorylated-to-total protein ratio is also presented in (B) and (D) for both sides. Post-hoc testing showed FPI-DCS pups had significantly higher levels of total-ERK than the other groups (# $p < 0.05$). The asterisk (*) indicates significant difference from Sham-Sal ($p < 0.05$).
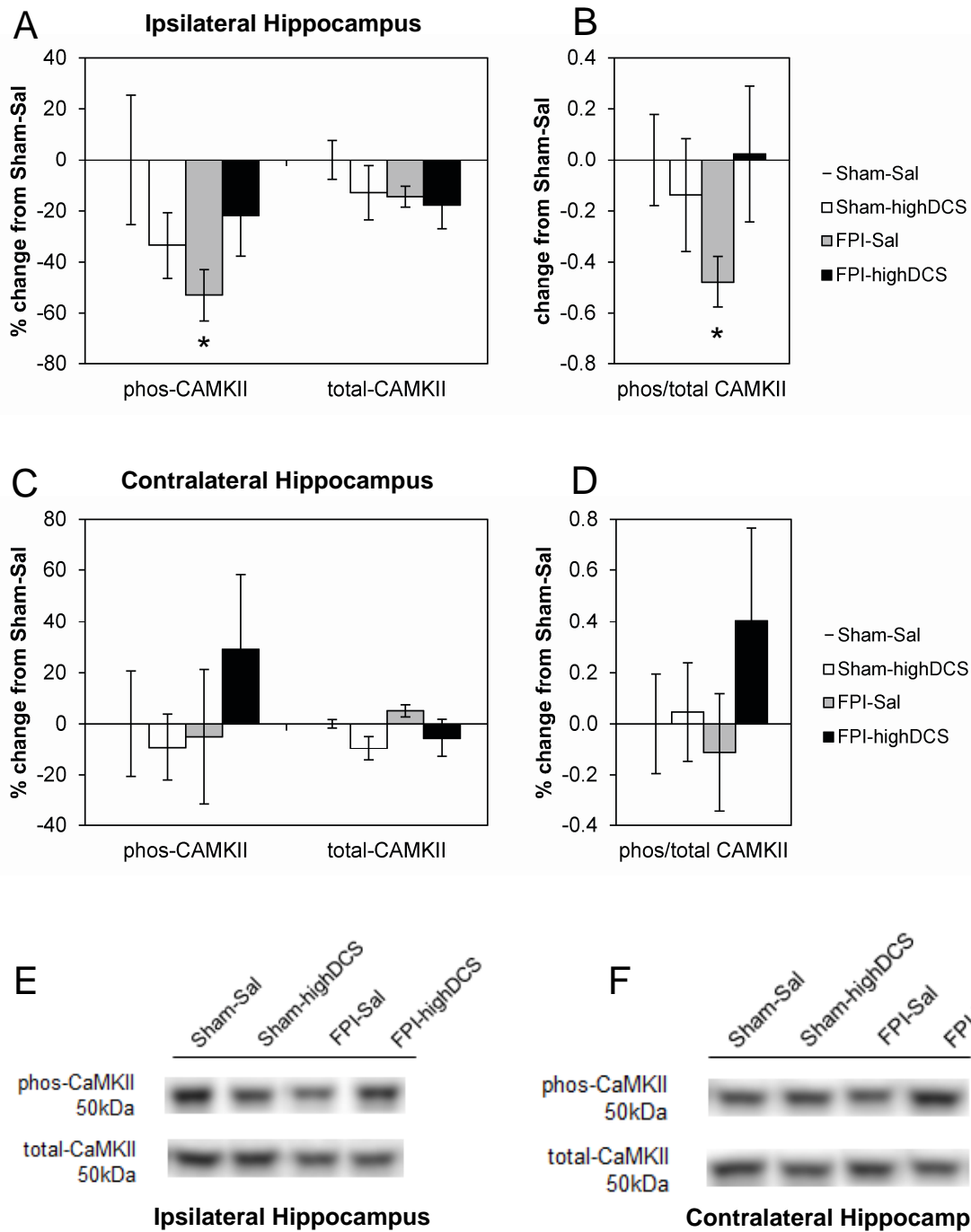
Figure 17. Phosphorylated and Total CAMKII Protein Levels
(Mean ± SEM) Phos- and total-CAMKII in the hippocampus, ipsilateral (A) and contralateral (C) to FPI. Phosphorylated-to-total protein ratio is also presented in (B) and (D) for both sides. The asterisk (*) indicates significant difference from Sham-Sal ($p < 0.05$).

Experiment 2: FPI and DCS Effects on Novel Object Recognition

      The inherent ability to distinguish novelty is hypothesized to be pertinent in development and in experience-dependent plasticity. The NOR task was used as a behavioral assay of hippocampally-mediated, NMDAR-dependent neural responsiveness after FPI and DCS treatment. The NOR task was performed on PID4, during which the NR2A subunit was found to still be significantly diminished after developmental FPI. The percentage of total object interaction time spent with the novel object after a one hour retention interval was compared to chance performance (50%) using one-sample t-tests (Figure 18A). Sham-Sal (N = 15) animals showed intact novel object recognition (66%, $p = 0.015$), whereas FPI-Sal rats (N = 15) lost the ability to distinguish between novel and familiar objects (57%, $p = 0.069$). Although Sham-highDCS animals (N = 9) showed only trends for intact novel object recognition (62%, $p = 0.077$), the FPI-highDCS group (N = 9) showed intact novel object recognition (71%, $p = 0.022$). Shams animals significantly spent more time over both object during the test trial compared to FPI pups. Interestingly, FPI-DCS animals still spent more time with the novel object compared to FPI-Sal, even though both groups had comparable amount of interaction time over both objects (Figure 18B). Lastly, locomotion was also quantified the familiarization and test phases of the NOR (Figures 19 A and B). Although no overall effect of injury or DCS was observed in speed, a significant effect of DCS was detected on the total distance travelled across all NOR phases (ANOVA, $p < 0.05$).
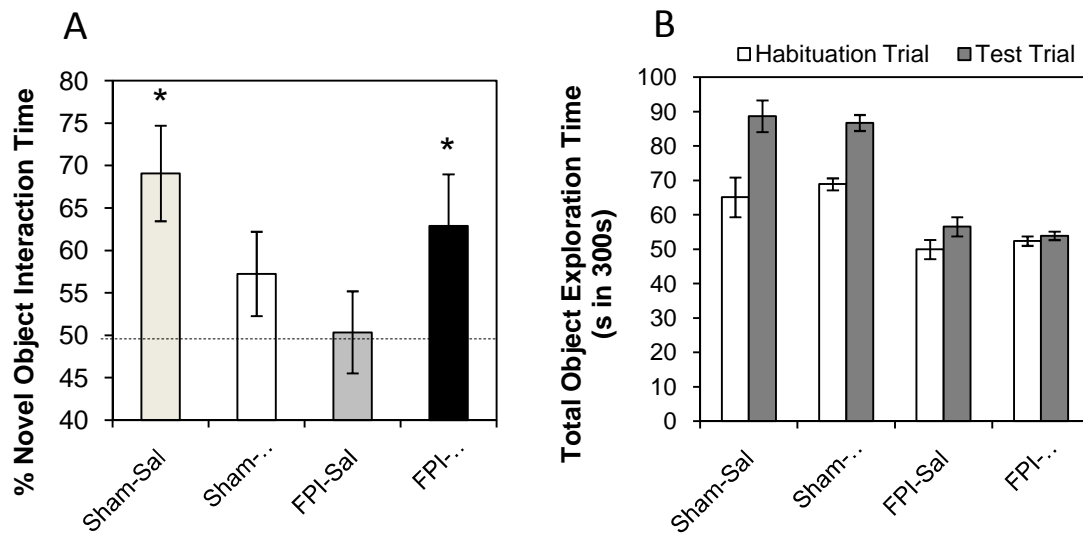
Figure 18. Novel Object Recognition Interaction Time NOR Task Locomotion
(Mean ± SEM) (A) Percent time spent with the novel object during NOR test and (B) total exploration time over both objects. Dotted horizontal line shows 50% (chance) of NOR. Sham-Sal pups displayed intact novel object recognition (* $p < 0.05$). FPI-Sal animals only exhibited chance performance. DCS treatment restored NOR in FPI animals (* $p < 0.05$).
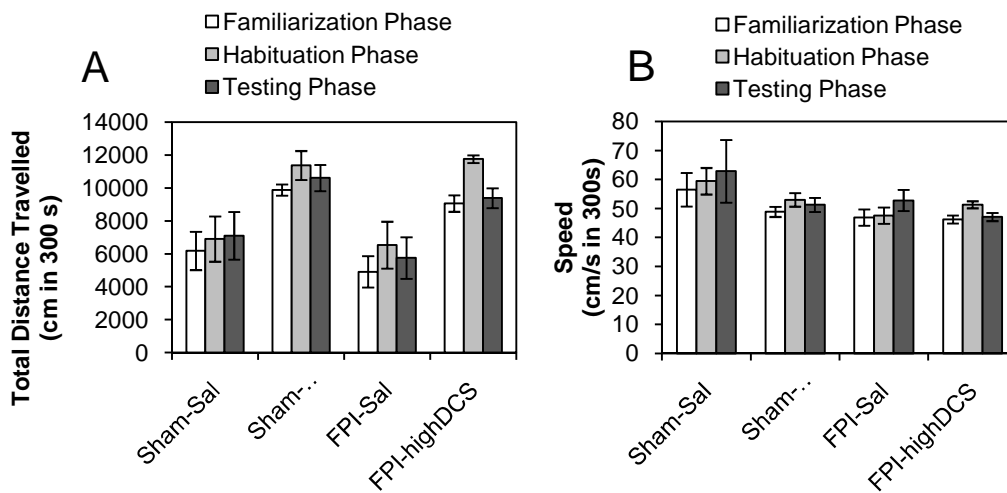
Figure 19. NOR Task Locomotion
(Mean ± SEM) Locomotion during the habituation and test trials were also recorded: (A) total distance travelled in cm, and (B) speed in cm/sec. There was a main effect of DCS observed. No overall effect of injury or drug was observed in the speed of the animals.

DISCUSSION

Neural responsiveness to a spectrum of stimuli, from physiologic to pathologic, has been closely linked with glutamatergic transmission. On one hand, physiologic stimulation of NMDAR and AMPAR glutamatergic receptors promote plasticity and cell survival and have been shown to be crucial for normal development and learning and memory (Sheng et al., 1994; Wenzel, 1997; Quinlan et al., 1999a; Quinlan et al., 1999b; Liu et al., 2000; Cull-Candy et al., 2001; Liu et al., 2004; Cull-Candy et al., 2006). On the other hand, pathological glutamatergic activation via the AMPARs and NMDARs has been demonstrated as a critical consequence of TBI that may underlie chronic deficits in behavior and cognition. FPI in adult rats has been shown to increase Ca^{2+}-permeable GluR1-containing AMPARs, as well as induce endocytosis of Ca^{2+}-impermeable GluR2-containing AMPARs that may be NMDA-mediated and may correlate with increased neuronal death (Bell et al., 2007; Bell et al., 2009). In a closed head injury model of TBI, GluR1 levels increased in the hippocampus ipsilateral to the injury (Schumann J. et al., 2008). A dramatic increase of NMDAR activation via the release of glutamate (Faden et al., 1989; Katayama et al., 1990) has been observed as early as 15 minutes after experimental TBI in rodents, followed by diminished NMDAR binding (Miller et al., 1990) and expression (from hours to days), that is long lasting in developing rats (Giza et al., 2006).

The use of NMDAR agonists has been shown to promote neuroprotection and to facilitate more rapid recovery after TBI (Temple and Hamm, 1996; Biegon et al., 2004; Yaka et al., 2007; Adeleye et al., 2009). It has been shown that there is a differential activation of NMDARs with varying concentrations of NMDAR agonists. While low doses of NMDA preferentially activate synaptic NMDA receptors and trigger a pro-survival molecular cascade, much higher toxic levels of NMDA activates extrasynaptic NMDA signaling that lead to pro-apoptotic sequelae (Soriano

et al., 2006). Failure of NMDAR blockade in clinical trials may have been attributable to mistimed delivery during periods of already down regulated NMDAR function, missing the critical window of hyperactivity of the receptors. Global antagonism of NMDARs during the period of receptor down-regulation inhibits NMDAR mediated pro-survival signals, promotes apoptosis, and results in worsened outcome (Morris et al., 1999, Albers et al., 2001, Ikonomidou and Turski, 2002, Naranyan et al., 2002, Muir, 2006, Maas et al., 2010). Both the lowDCS and highDCS treatment levels used in Chapter 1 are still considered to be in the lower ranges of the effective DCS dose (< 50 mg/kg), whereas high toxic levels would be > 50 mg/kg. Chapter 1 shows a dose-dependent restoration of hippocampal NR2A levels ipsilateral to FPI injury at using 30 mg/kg DCS.

The subacute (after 24 hours and less than one week post-injury) molecular response to developmental FPI includes reduced hippocampal NR2A ipsilateral to the injury. DCS treatment subacutely after the insult restored NR2A to sham levels. AMPAR subunits seem to be unaffected in the present developmental TBI saline treated model. Here, neither the increase in GluR1 nor the decrease in GluR2 levels was observed in ipsilateral hippocampus as compared to previous reports. However, there is an increase in GluR1 levels contralateral to injury. This could be due to differences in the investigated developmental time window (immature vs. mature rats) or to differences in injury models used (FPI vs. closed head injury – weight drop).

After DCS treatment in FPI rat pups there were increased GluR2 levels in the ipsilateral hippocampus on PID4. One possible explanation is that DCS treatment upregulated Ca^{2+}-impermeable GluR2 containing AMPARs in a neuroprotective response to counter excitotoxicity. Interestingly, the observed upregulation of GluR1 on the contralateral

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hippocampus follows previous report on TBI induced increases of GluR1 containing AMPAR in regions not directly in the injury site (Schumann et al., 2008). The data further support a contralateral enhancement of neural activation, with increased ERK(1/2) levels in addition to GluR1, as a compensatory response to depressed ipsilateral activity.

The diminished NMDAR-mediated molecular response following developmental TBI corresponded with impaired performance in the NOR task. FPI resulted in impaired novel object recognition, which may parallel the inability of injured weanlings to benefit from EE rearing reported in other studies (Fineman et al., 2000; Giza et al., 2005). Here, DCS treatment significantly affected only the FPI pups and restored novel object recognition performance to sham levels. FPI rats showed down regulation of hippocampal NMDAR (NR2A) subunits and reduced phos-CaMKII ipsilateral to the injury four days after the insult and exhibited impaired performance in NOR, a hippocampal dependent task. Brief treatment with DCS, a positive modulator of NMDARs, restored normal levels of NR2A and p-CaMKII in ipsilateral hippocampus and also resulted in preserved NOR performance by 4 days post-FPI in the immature rat.

CHAPTER 2.

RESTORATION OF EXPERIENCE-DEPENDENT PLASTICITY FOLLOWING LATERAL FLUID PERCUSSION INJURY TO THE IMMATURE RAT BRAIN

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of death and disability in the pediatric population (Faul et al., 2010). Annually, almost half a million children aged 0 to 14 years are admitted to emergency department visits due to TBI. 50.2% of TBI incidences in the young are due to falls, followed by 24.8% struck by/against injuries (injuries caused by intentional or unintentional impact by or against objects or people.) Among children, TBI is mostly mild that results in few, if any, lasting deficits. Children who sustain severe insults experience lasting impairments in a range of skills, which include information processing, attention, memory, learning, social function, and behavior, during the acute and short-term recovery phases following injury (Anderson et al., 2005; Yeates and Enrile, 2005). Children also develop worse outcomes compared to adults with similar severity of injury (Ewing-Cobbs et al., 1997; Anderson et al., 2011). Although spontaneous recovery occurs, children with TBI do not seem to return to the same level of cognitive and behavioral function as non-injured cohorts. The purported capacity for enhanced neural plasticity in the young does not seem to translate into better cognitive outcomes following TBI. From our current understanding of pediatric TBI, being young (i.e. possessing higher levels of plasticity) maybe not always be better (Anderson et

al., 2005; Giza and Prins, 2006; Babikian and Asarnow, 2009; Anderson et al., 2011). The disruption of normal NMDAR physiology during development may have lasting consequences in cognition, behavior, and experience-dependent plasticity following TBI.

Experience-dependent plasticity is the process through which coordinated patterns of stimulation from the environment help shape brain structure and function. In development, experience during a specific time in early life often times help shape the adult behavioral repertoire. These time periods are referred to as "critical periods." For instance, inducing informal learning via housing in EE can result in higher brain weights, cortical thickness, and increased dendritic arborization (Rosenzweig et al., 1962; Rosenzweig and Bennett, 1996; Fineman et al., 2000). Experience-dependent plasticity can also be induced using formal training. Adult rats housed in an impoverished environment can catch up with enriched environment housed littermates when exposed to serial behavioral testing of spatial learning and memory (Rosenzweig and Bennett, 1996). Based on Hebb's postulate, signals generated from experience at the synaptic level lead to acute or lasting changes in the brain. These signals include physiological release of neurotransmitters, like glutamate, that activate *N*-methyl-D-aspartate receptors (NMDARs) which then regulate calcium homeostasis and influence post-synaptic molecular sequelae. NMDARs are the predominant glutamate-mediated receptors and are necessary in signal transduction, learning and memory, and experience-dependent plasticity. The NMDAR is a tetramer assembly. Depending on the synaptic location, subunit composition and manner (physiologic or excitotoxic) of activation, NMDARs can have differential channel opening characteristics (Sheng et al., 1994; Flint et al., 1997; Wenzel, 1997; Cull-Candy et al., 2001), which can then trigger either pro-plasticity or pro-death mechanisms (Hardingham and Bading, 2003; Papadia and Hardingham, 2007). Furthermore, early changes in NMDAR can

result in lasting changes. FPI in the postnatal day 19 rat has resulted in NMDAR subunit

composition changes that lasted up to one week after the injury. When attempting to induce

experience-dependent plasticity in these postnatal 19 FPI pups by rearing them in an EE,

Fineman and colleagues found that the FPI pups failed to manifest EE-induced enhancements of

cortical thickness, dendritic arborization, and cognitive and behavioral performance in the Morris

water maze task (Fineman et al., 2000; Ip et al., 2002). Only when EE rearing was administered

two weeks after injury (after the period of down-regulated NMDAR function spontaneously

recovers) did the FPI pups demonstrate EE-induced improvements on MWM acquisition (Giza et

al., 2005).

It has been shown that NMDAR blockade in the young brain exacerbates neuronal death

and promotes detrimental changes in proteins associated with oxidative stress and inflammation,

cell proliferation, and neuronal circuit formation in the cerebral cortex (Bittigau et al., 1999;

Ikonomidou, 1999; Pohl et al., 1999; Papadia and Hardingham, 2007; Kaindl et al., 2008). More

recently, there is evidence that administration of NMDAR agonists, such as NMDA or D-

cycloserine (DCS), during the subacute phase (from 1-7 days post-injury) may facilitate rapid

recovery after TBI in adult rodents (Temple and Hamm, 1996; Biegon et al., 2004; Yaka et al.,

2007; Adeleye et al., 2009) .

In Chapter 1, lateral fluid percussion injury (FPI) to postnatal day 19 rats was shown to

alter pro-plasticity molecules, such as glutamate receptor (NMDAR, AMPAR) subunits and

NMDAR-mediated kinases such as CAMKII and ERK. Moreover, systemic administration of

NMDAR partial agonist (D-cycloserine – DCS) during the period of NR2A down-regulation

could restore these pro-plasticity molecules to sham levels. DCS treatment in FPI rat pups also

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rescued novel object recognition, a form of working memory that can be important for learning in an EE condition.

In this chapter, the effects of DCS administration after developmental FPI on EE-induced experience-dependent plasticity were investigated. EE was used as an assay of plasticity and the outcome measure was Morris water maze performance in early adulthood. It was hypothesized that after sustaining an early life FPI, DCS treatment could reinstate lost EE-induced potential that is measured later in life.

METHODS

Experimental Design and Subjects

Male Sprague-Dawley rat pups (Charles River, Boston, MA) underwent sham or severe lateral fluid percussion (FPI) on postnatal day 19 (P19). Animals were maintained in a 12 hour light-dark cycle with food and water ad libitum. The UCLA Chancellor's Committee for Animal Research approved all animal studies.

Rat pups were weaned and differentially housed in standard (STD) or enriched environment (EE) conditions for seventeen days after 24 hours of recovery post-surgery (P20). They were returned to standard vivarium housing until behavioral testing at thirty days post-injury (PID30) using the Morris water maze task. This study consisted of eight groups: Sham-STD-Sal (N = 9), Sham-STD-DCS (N = 8), FPI-STD-Sal (N = 8), FPI-STD-DCS (N = 8), Sham-EE-Sal (N = 8), Sham-EE-DCS (N = 9), FPI-EE-Sal (N = 9), and FPI-EE-DCS (N = 9). Figure 20 illustrates the experimental design for Chapter 2.
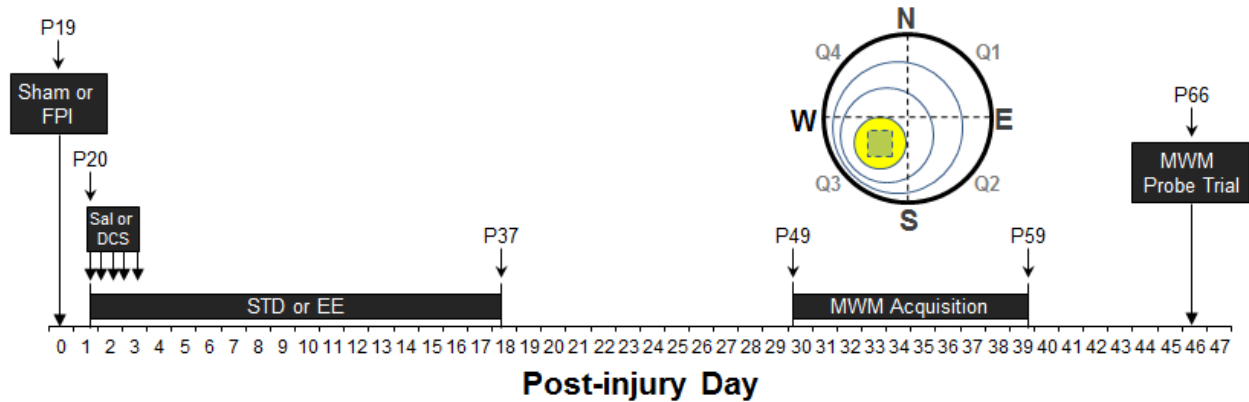
Figure 20. Experimental Design 3
P19 rats underwent sham or FPI surgery on PID0 and were given either saline (Sal) or DCS (30mg/kg, i.p.) on PID1-3.   Additionally, animals were differentially placed into standard (STD) or enriched-environment (EE) housing on PID1, lasting for 17 days. After which, all EE-housed rats were returned to standard vivarium conditions. MWM acquisition training commenced on PID30 through PID40 (P49-P59). One week later, we tested the MWM training retention using a probe trial on PID26 (P66).

Fluid Percussion Injury

Same as in Chapter 1.

Enriched Environment

The enriched environment (EE) chamber consisted of various toys, tunnels, and ladders placed in a two level cage measuring 78 cm x 36 cm x 48 cm (Figure 21). Batches of sixteen animals were housed together in the chamber for 17 days and were then returned to standard vivarium conditions until the start of the MWM training on P50. Every day the animals were removed from the EE cage and placed back after the toys and objects were changed and rearranged. A light-dark cycle of 12 hours was maintained and food and water were available ad libitum.

Figure 21. Enriched Environment (EE)
The EE chamber has two levels that are filled with toys, tunnels, and various objects. Beginning on PID1, 12-16 animals are housed in EE for 17 days. Animals were moved from one level to the other each day. All objects were also changed and rearranged daily. These rats received either a sham or FPI procedure and saline or DCS treatment. All animals were returned to standard vivarium housing conditions until the start of the Morris water maze (MWM) training on PID30.

Drugs

D-cycloserine (DCS) was obtained from Sigma Aldrich (Boston, MA). DCS was mixed in sterile 0.9% saline with a constant injection volume of 0.25 ml/kg. DCS was frozen (-20°C) and thawed 15 min prior to each injection time. The DCS treatment regimen was implemented the same as in Chapter 1, except here all DCS treated groups received the high DCS dose (30 mg/kg, i.p.). Beginning twenty-four hours following sham or FPI injury, rats were randomized into saline (Sal), DCS (30 mg/kg, i.p.) treatment groups. Animals received five doses, one dose every 12 hours, from post-injury day 1 through 3 (PID 1-3).

Morris Water Maze Training

Acquisition Training

Experiment 3 animals began the Morris water maze (MWM) acquisition training thirty days after surgery. Animals were trained for ten consecutive days. The MWM was a blue circular tank (1.5 m in diameter, 0.6 m in height) filled with water maintained between 18-20ºC. Each animal underwent two blocks of training per day (28 minutes between blocks). Each block consisted of four trials wherein the animal was released from the four cardinal directions (N, S, E, W) in random order. For each trial, the animal was given 45 seconds to locate the hidden platform. If the animal did not find the platform in the allotted time, it was then gently guided to the platform. A 15 cm x 15 cm platform submerged 2 cm below water level was placed in the SW quadrant of the tank. The animal remained on the platform for 60 seconds in between trials. The time it took the animal to reach the platform was recorded. The swim paths and velocity were recorded using a digital tracking system (SMART, San Diego Instruments).

The goal was to discover whether enriched environment rearing, injury and drug treatment are important predictors for latency in MWM acquisition. Additionally, 'trials to criterion' and slope of learning were also determined to measure learning of the MWM task. The 'trials to criterion' was defined as the number of trials achieved to reach the hidden platform in five seconds or less in one block (four consecutive trials). Slope of learning was determined from the slope of the linear regression of the mean latencies between the first block and the block when the animal reached 'trials to criterion'.

Probe Trial

Retention of the location of the hidden platform location was tested 7 days later. The hidden platform was removed and each animal was released at the center of the tank facing N for a single 60 second trial. Animals that were able to recall the learned location of the platform were expected to spend a greater amount of time swimming in the quadrant that had contained the platform. Swim path and velocity were also quantified. The first 15 seconds of the probe trial were used for analysis of recall. It has been shown that the animal has peak activity within the first 15 seconds in the MWM probe trial (Maei, 2009).

Statistical Analysis

A one-way Analysis of Variance (ANOVA) was performed on the injury characteristics (LOC, apnea, atm, and PID0 weight) by group. A two-way repeated measures ANOVA was performed on the within-subject PID weights (PID 0, 1, 2, 3, 5, and 14) with injury (Sham or FPI), housing (STD or EE), and drug (Sal or DCS) as between-subject factors. Latencies to reach the platform were analyzed using a linear mixed-effects model fit by restricted maximum likelihood in R (R Development Core Team, 2010). MWM acquisition parameters, trials to criterion and slope of learning, were analyzed using ANOVA.

RESULTS

Injury Severity

All animals included in the study were given severe FPI, defined as having duration of unresponsiveness to toe pinch (loss of consciousness, LOC) of at least 120 seconds. Twenty-six percent (26/99) of the animals were excluded from the study due to complications from surgery or injury or to LOC times of less than 120 seconds. Mean LOC, apnea times, and the corresponding atmospheric pressure did not significantly differ between FPI groups (Table 6). There were no significant differences in subject weights across groups on surgery day (PID0). A repeated measures ANOVA shows a significant main effect for post-injury day (PID) ($F_{2,92} = 1.963E3$, $p = 0.000$) and a significant PID*group interaction effect ($F_{14,92} = 7.482$, $p = 0.000$) across the post-injury day weights from PID 0-14. Between subjects, there is a significant main effect of housing ($F_{1, 46} = 18.345$, $p = 0.000$) across the PID weights. By PID14, EE-reared animals had significantly lower weights compared to standard housed animals, regardless of injury or drug treatment (Figure 22).

FPI and DCS Effects on Experience-Dependent Plasticity

Experience-dependent plasticity was induced by rearing animals in an enriched environment (EE). FPI and DCS effects were evaluated on EE-induced gains on spatial learning and memory using the Morris water maze (MWM) task between PID30-50.

Morris Water Maze Acquisition: Latency

Linear mixed effects model showed that there is a main effect of housing on MWM latency (Figure 23 A and B). Animals housed in an enriched environment have shorter latencies

in finding the hidden platform, regardless of injury or drug treatment. Over the ten days of acquisition training, all animals improved their latencies. Using group as a fixed effect (latency by group), FPI-EE-Sal group did not significantly improve their latency compared to Sham-EE-Sal animals. Only when treated with DCS, FPI-EE-DCS pups significantly improved their acquisition latencies (Sham-STD-Sal > FPI-EE-DCS, $p < 0.05$).

Morris Water Maze Acquisition: Trials to Criterion

The 'trials to criterion' was defined as the mastery of learning the task. Specifically, it is the number of trials required to reach the hidden platform in five seconds or less in one block (four consecutive trials from four different cardinal location starting points). As expected, EE rearing significantly improved the 'trials to criterion' in shams ($F_{1,33} = 20.102$, p = 0.000), but not in FPI animals ($F_{1,33} = 0.489$, $p = 0.490$) (Figure 24A). Additionally, DCS treatment only significantly affected FPI animals ($F_{1,33} = 8.869$, $p = 0.006$) and not the sham groups ($F_{1,33} = 0.110$, $p = 0.742$). While FPI-STD-DCS animals only showed a trend for reduced 'trials to criterion' (FPI-STD-DCS, 41.5±6.6 < FPI-STD-Sal, 59.6±8.1, $p = 0.106$), FPI-EE-DCS pups demonstrated significantly improved 'trials to criterion' (FPI-EE-DCS, 34.7±4.0 < FPI-STD-Sal, 59.6±8.1, $p = 0.012$).

Morris Water Maze Acquisition: Slope of Learning

The slope of learning was defined as the slope from the linear regression of the mean latencies between the first block and the block when the animal reached 'trials to criterion'. Similarly to 'trials to criterion' enriched environment rearing significantly enhanced slopes of learning only in shams ($F_{1,33} = 8.005$, $p = 0.008$) and DCS treatment only significantly affected the slope of learning in FPI pups ($F_{1,33} = 5.032$, $p = 0.032$) (Figure 24B).

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Morris Water Maze Retention: Probe Trial

       The probe trial was administered after a one week hiatus following the MWM training. The percent time spent in the target quadrant (SW) was significantly greater than any of the other quadrants ($p < 0.001$) across all groups (Figure 25A). ANOVA showed a three-way significant interaction effect of injury-by-housing-by-drug ($F_{1,67} = 6.530$, $p = 0.013$) in the number of target entries. Figure 25B shows the number of target entries per group. Independent post-hoc tests showed no significant difference between the number of target entries of Sham-STD-Sal and FPI-STD-Sal groups. EE rearing trended to significantly increase the number of entries to the target zone in Sham-EE-Sal (Sham-STD-Sal vs. Sham-EE-Sal, $p = 0.055$), whereas EE had no effects on FPI-EE-Sal animals. DCS, however, had a differential effect in Sham and FPI animals. DCS had no significant effects on the probe trial entries of Sham-STD-Sal and FPI-STD-Sal pups. Sham-EE-DCS had significantly less entries to the target zone compared to the Sham-EE-Sal group ($p = 0.022$). FPI-EE-DCS animals reached the same number of target zone entries as Sham-EE-Sal animals, which was significantly higher than entries made by Sham-STD-Sal (FPI-EE-DCS vs. Sham-STD-Sal, p = 0.037) and FPI-STD-Sal groups (FPI-EE-DCS vs. FPI-STD-Sal, p = 0.037).

Table 6. Experiment 3 – Injury Characteristics
Mean (± SEM) apnea and loss of consciousness (LOC) times in seconds (s) and the
corresponding atmospheric pressure after FPI induction.

Group	N	wtPID0 (g)	Drop <	Apnea (s)	LOC (s)	Atm
Sham-STD-Sal	9	49.1 ± 1.3	NA	NA	NA	NA
Sham-STD-DCS	8	43.8 ± 5.7	NA	NA	NA	NA
Sham-EE-Sal	8	45.8 ± 1.2	NA	NA	NA	NA
Sham-EE-DCS	8	46.1 ± 1.8	NA	NA	NA	NA
FPI-STD-Sal	11	47.6 ± 1.4	16	210 ± 31	228 ± 40	2.54 ± 0.11
FPI-STD-DCS	10	50.8 ± 1.7	16	202 ± 42	218 ± 41	2.64 ± 0.08
FPI-EE-Sal	9	46.7 ± 2.1	16	232 ± 38	290 ± 59	2.42 ± 0.10
FPI-EE-DCS	10	44.3 ± 1.2	16	195 ± 33	219 ± 31	2.60 ± 0.07
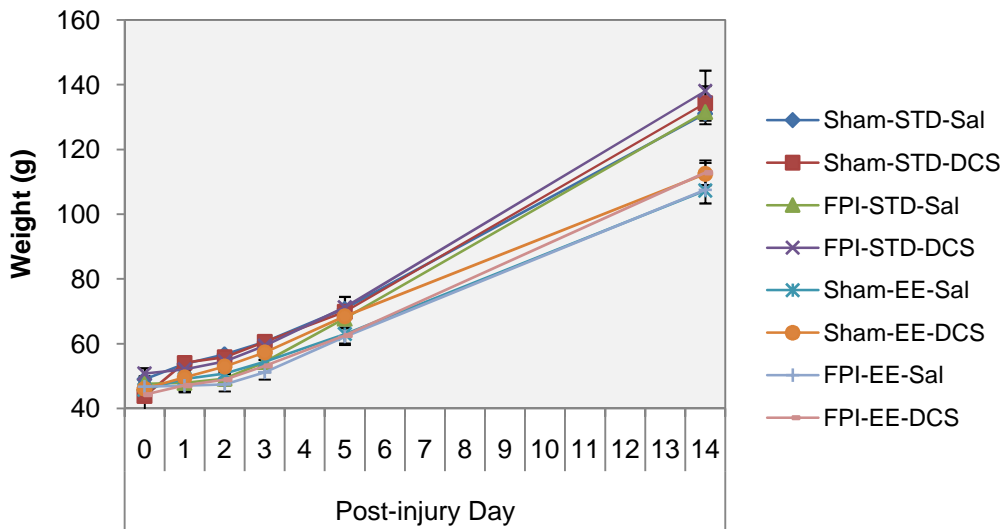
Figure 22. Experiment 3 Subject Weights Time Course
Mean (± SEM) weights in grams (g) from PID 0-14. A repeated measures ANOVA showed
significant main effects of PID (within-subject) and housing (between-subject). Animals housed
in EE had lower weights compared to animals in STD conditions.
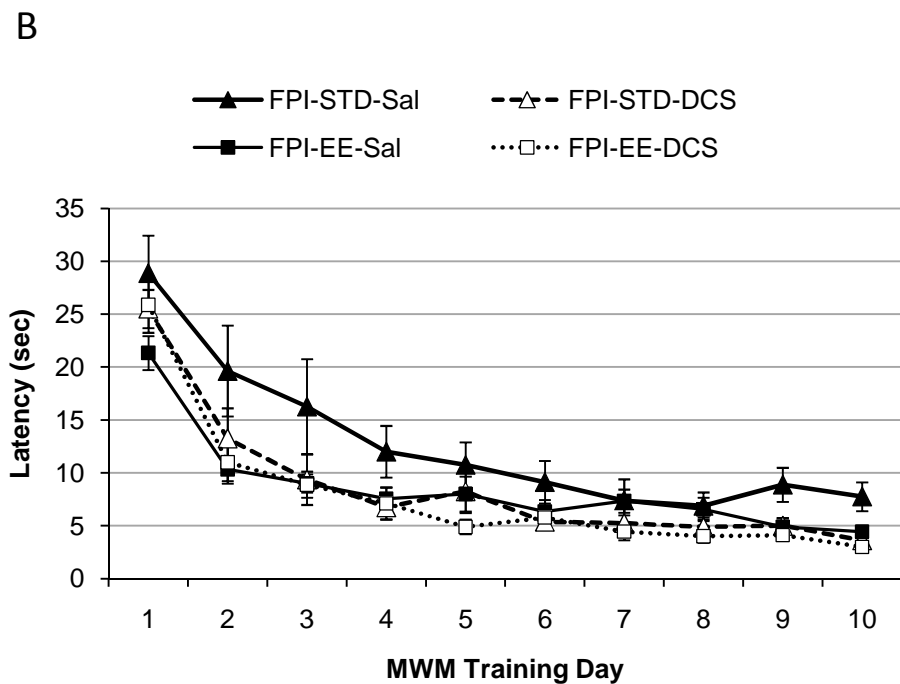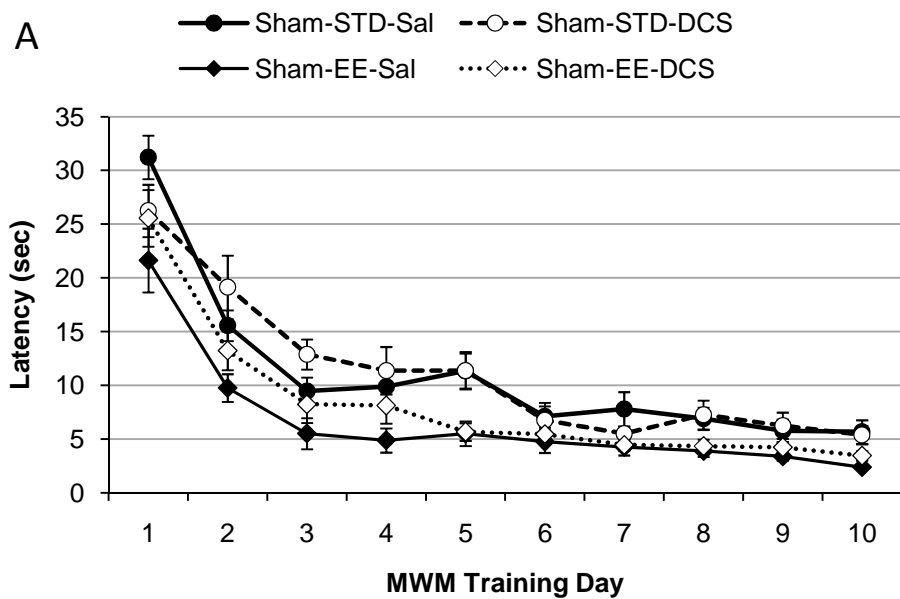
Figure 23. MWM Acquisition – Latency
(Mean ± SEM) Latency in seconds for Sham (A) and FPI (B) groups.
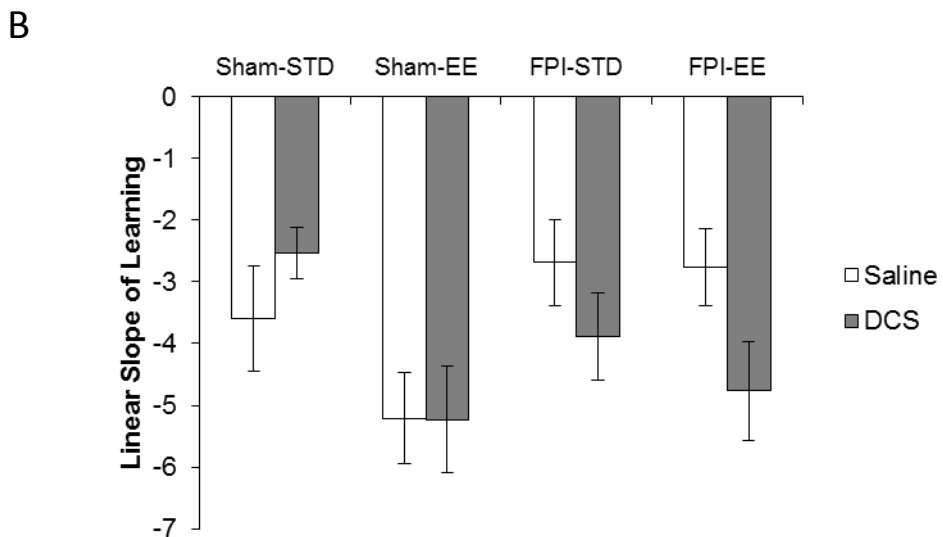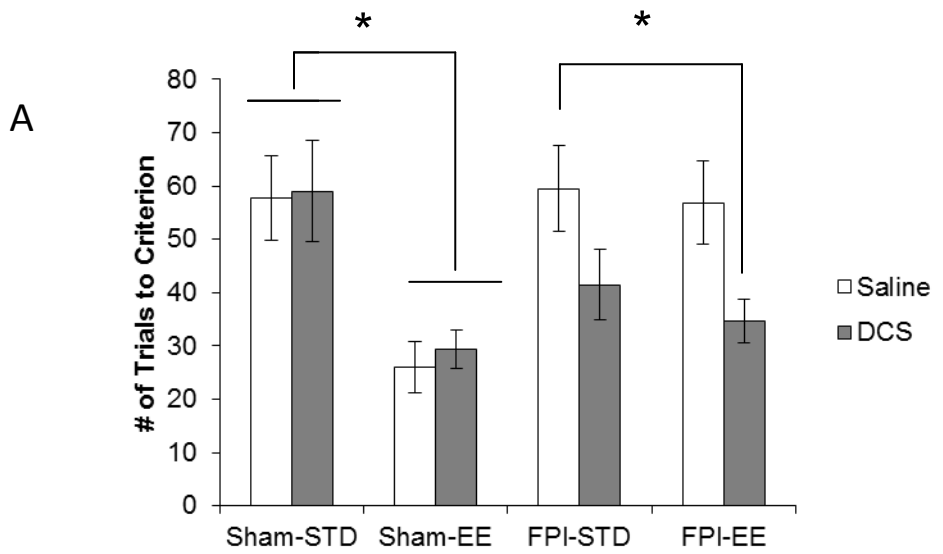
Figure 24. MWM Acquisition – Trials to Criterion and Slope of Learning
(A) Trials to criterion is a measure of MWM acquisition mastery. The trials to criterion is the number of trials the subject acquired to reach the hidden platform in 5 seconds or less in four consecutive trials. (B) Slope of learning measures the linear rate of learning the MWM task.
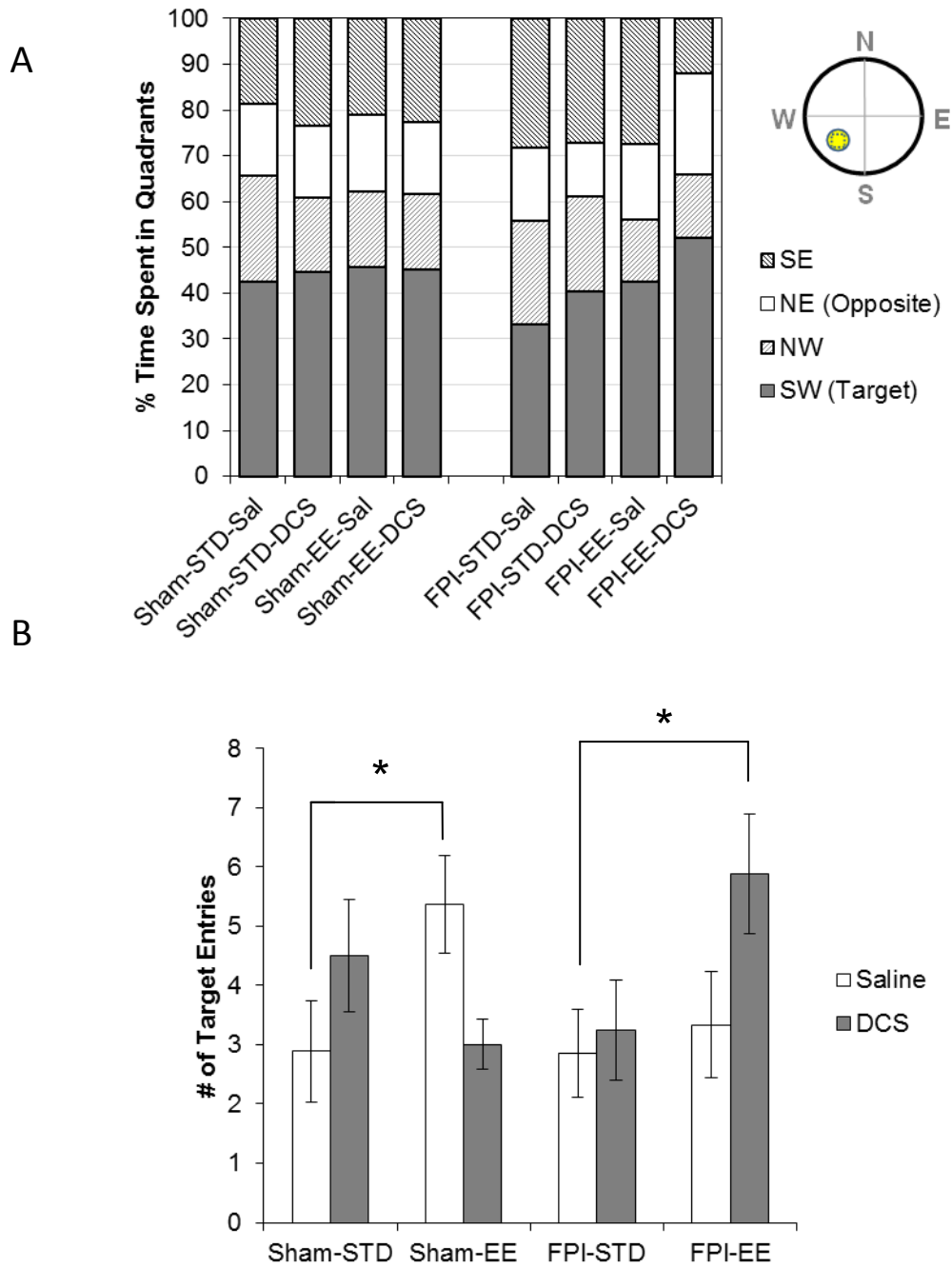
Figure 25. MWM Retention – Probe Trial
Percent time spent in MWM quadrants (A) and the (Mean ± SEM) number of entries into the
target zone (small circle shown in the SW quadrant) (B).

DISCUSSION

It is widely believed that youth promises higher levels of plasticity and increased rates of spontaneous recovery from injury or disease. For instance, cortical lesions early in life resulted in better recovery and sparing of function compared to lesions that were given to adult rats (Kolb and Tomie, 1988) and monkeys (Kennard, 1936, 1938; Kennard and Fulton, 1942).

The benefits of increased plasticity seem to be contingent upon the nature of the stimuli and the elaborate interplay of molecular, cellular and physiological responses. For instance, enriched environment rearing resulted in anatomical and cognitive enhancements in normally developing animals (Rosenzweig et al., 1972; Greenough, 1976; Rosenzweig and Bennett, 1996). In this study, Sham-EE-Sal animals significantly enhanced learning (significant reduction of the number of trials to criterion and enhanced slope of learning) and retention (increased entry to probe trial target zone) of the MWM task. However, FPI-EE-Sal animals failed to acquire the benefits of prolonged exposure to EE. Although FPI-EE-Sal pups did perform to the level of STD animals and learned and recalled the MWM task, these FPI pups did not demonstrate the same potential as their uninjured cohorts in trials to criterion and probe trial. Only when DCS was administered did the FPI-EE pups showed comparable performance in the MWM mastery and recall to the Sham-EE-Sal group.

In Sham animals, DCS had a different effect. While DCS did not alter learning of the MWM task, DCS treatment improved MWM retention in standard housed shams (Sham-STD-DCS), as demonstrated by an increase in target zone entries during the probe trial. However, Sham-EE-DCS animals had a significantly reduced number of target entries, as compared to the Sham-EE-Sal group. There are at least 2 possible explanations for this seemingly contradictory finding: 1) Sham-EE-DCS animals unlearned faster, and 2) DCS negatively modulated glutamate

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transmission in the Shams-EE-DCS group. All sham animals spent comparable times in the

target quadrant, which is greater than the amount of time spent in the other three quadrants.

Therefore, Sham-EE-DCS recalled the location of the hidden platform but did not spend

substantial time in the target zone in particular. Alternatively, it has been reported that

differential housing in EE significantly increases glycine levels in cerebral cortex (Cordoba et

al., 1984). The glycine co-agonist site on NMDARs may be more saturated in the enriched brain

and the presence of DCS may be competing with the endogenous glycine. In this situation, DCS

plays an antagonist role, reducing the NMDAR glycine-site activation efficacy (Hood et al.,

1989; Lanthorn, 1994).

Chapter 2 demonstrated that there is lasting loss of potential in diffusely injured young

animals. These injured rats were still functional and were able to learn and achieve trials to

criterion in the hippocampus- and NMDAR-mediated MWM task. Nevertheless, using the EE

experience as an assay for plasticity, there is still a developmental gap between Sham-EE-Sal

and FPI-EE-Sal pups 30 days after the insult, during their early adulthood. It is hypothesized

that worsened outcome after TBI at very young ages (infants and toddlers) may be due to altered

developmental profiles (Anderson et al., 2005). Although the FPI-EE-Sal group in our study in

the end learned the MWM trained task, they never achieved the same performance on trials to

criterion as their Sham-EE-Sal counterparts and this impairment was detectable into their early

adulthood. Giving DCS during the critical window of injury-altered glutamatergic transmission,

similar levels of mastery and recall were restored to FPI-EE-DCS pups as compared to Sham-

EE-Sal animals.

Although the persisting belief that there is better restitution of function when brain

damage occurs early in life has been ascribed as the "Kennard Principle," historically Kennard

would posit that age would not have been the sole predictor of recovery. Depending on the features of the injury, post-injury reorganization, staging of the lesion, and the timing and method of outcome measure assessment, early brain injury would be as equally devastating as damage to the mature brain (Dennis, 2010). 'Younger is not always better' in TBI. The effects of TBI can be worse and longer lasting in the immature brain. By understanding the dichotomous nature of NMDAR activation, this dissertation demonstrates that timely activation of hippocampal NMDAR during the pathological process can reinstate lost potential and experience-dependent plasticity after early life TBI.

CHAPTER 3.

GLUTAMATERGIC TRANSMISSION AS A PHYSIOLOGICAL

BIOMARKER FOR TRAUMATIC BRAIN INJURY

INTRODUCTION

Currently, there are many treatments for TBI, but there is no specific cure. This may be partly due to the heterogeneity of the injury, as well as a lack of physiological biomarkers to target and apply treatment in humans and experimental animals. One of the goals of this dissertation is to identify a viable physiological biomarker, particularly a noninvasive in vivo measure, to assess the state of the injury and target a possible treatment regimen at an appropriate time period after the insult. Due to the intimate involvement of NMDARs in normal development, plasticity and the pathophysiology of TBI, pharmacological magnetic resonance imaging (phMRI) was used not as mapping technique but rather as a non-invasive physiological biomarker for NMDAR mediated glutamatergic neurotransmission after TBI.

What is a Biomarker?

Biomarkers are important in determining which molecular processes would be crucial to target for clinical efficacy and drug development. The National Institutes of Health (NIH) Biomarker Definitions Working Group defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (Lesko and Atkinson, 2001). The NIH

defines three levels of biomarkers: type 0 can track the natural course of illnesses; type 1 examines intervention effects wherein the mechanism of action of the drug is known but its relationship with outcome is not yet known; and type 2 biomarkers are truly predictive of clinical outcome (Minzenberg, 2011). Biomarkers are generally used to measure delivery of drugs to their targets, to understand and predict pathophysiology, to elucidate how the disease state progresses or changes with therapy, and to predict clinical outcome. These are done all through monitoring parameters that have clinical relevance. Biomarkers enable proof-of-concept studies for novel agents or new drug applications, which reduces risks in safety and drug efficacy determinations early on during drug discovery for a particular condition. Not only are they useful in resolving treatment parameters, such as optimal dose and duration, but also in comparing competing treatments and exposing adverse effects of a given agent (Minzenberg, 2011).

Neuroimaging Signals as Biomarkers

Neuroimaging has been identified by the NIH Critical Path Initiative[4] (Woodcock and Woosley, 2008) to be a critical technology in elucidating underlying mechanisms of CNS diseases and disorders, as well as in drug development, The acquired imaging signals are currently argued to belong under type 0 and 1 biomarkers and can be crucial in two critical phases in drug development: 1) proof-of-biology and 2) proof-of-concept.   The noninvasive nature of neuroimaging allows early clinical testing to investigate target mechanisms hypothesized to be associated with a biological change in normal physiology or in pathology

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[4] NIH Critical Path Initiative (http://www.fda.gov/oc/initiatives/criticalpath/)

(proof of biology). In proof of concept studies, clinical experiments demonstrate that the target mechanism is linked to a significant change in a clinical endpoint. Engagement of drug on the target mechanism could change the patient condition when the treatment is administered (Wong et al., 2008; Minzenberg, 2011).

Examples of functional brain imaging include lesion-deficit models, resting brain function and activation imaging. Lesion-deficit models have been developed, wherein changes in measured signals ranging from single unit recordings, local field potentials, and structural MRI have been correlated with cognitive and behavioral testing in humans and animals. The study of aphasic patients contributed greatly to this type of neuroimaging model. Classic examples of lesion-deficit models include postmortem studies that show a patient who had impaired language articulation had damage in the third frontal convolution of the cerebrum (Broca's aphasia) and a patient with a deficit in speech comprehension had damage to the left posterior temporal cortex (Wernike's aphasia) (Toga and Mazziotta, 2000). The use of MRI to visualize structural neuroanatomy has been highly effective in identifying lesioned areas may be affected by disease or injury while the patient is still living. Another non-destructive way of performing lesion studies can be achieved using transcranial magnetic stimulation. Resting brain function can be accessed by targeting basal glucose metabolism, blood flow, resting state functional magnetic resonance imaging (fMRI), positron emission topography (PET) and resting electroencephalogram (EEG) power. Activation imaging allows measurement of evoked responses due to sensory, electrical or pharmacological stimuli administered by the experimenter. Activation studies may utilize blood flow PET, fMRI , event-related potentials (ERP), optical intrinsic signal imaging or single unit recordings (Toga and Mazziotta, 2000).

When neural activity increases in response to a sensory stimulus or a drug, oxygen consumption and glucose utilization increases. The vascular system responds by increasing blood flow and volume to the local area exceeding the increased oxygen consumption rates, which results in better oxygenation of the active region. Since deoxyhemoglobin is paramagnetic (Pauling and Coryell, 1936), changes in blood oxygenation levels lead to changes in MRI signal intensity, making deoxyhemoglobin an endogenous contrast agent. fMRI relies on intact neurovascular coupling, which translates neural activity into changes in perfusion rates. Additionally, the use of intravascular contrast agents allows for direct measurement of alteration in local perfusion and CBV (Mandeville et al., 2001). The fMRI signal using alterations in blood oxygenation is referred to as blood oxygen level-dependent contrast (BOLD) (Ogawa et al., 1990; Kwong et al., 1992) [reviewed in (Logothetis and Wandell, 2004)]. Therefore, by focusing on the various components of the complex biophysical basis of neural activity, different types of fMRI signals can be measured, such as relative cerebral blood volume (CBV), cerebral blood flow (CBF), BOLD, and oxygen extraction fraction (OEF – measures how much oxyhemoglobin is converted into deoxyhemoglobin).

fMRI has been shown to be practical for noninvasive mapping of brain and function with high spatial and temporal resolution (Belliveau et al., 1991). No exposure to radiation is required during this technique and it can be implemented for repeat imaging of individual subjects. Repeat studies allow for within-subject study designs that confer greater statistical power to evaluate normal or pathological physiology and to determine drug effects and efficacy.

MRI of NMDAR-Mediated Glutamatergic Transmission

The vascular and metabolic demands of neuronal activity underlying fMRI signals have been suggested to be mostly driven by glutamate and the effects on its receptor-mediated action on neurons and astrocytes (Bonvento et al., 2002). For instance, NMDAR activation has been shown to be the primary factor in BOLD responses. Using a somatosensory evoked potential stimulation, BOLD signal can be significantly reduced using NMDAR antagonist MK801 (Gsell et al., 2006). NMDAR mediated activation can also be elicited in the hippocampus by measuring the change in relative cerebral blood volume (ΔrCBV) elicited by pharmacological stimulus targeting the NMDAR co-agonist glycine-binding site, which is not saturated in hippocampal neurons in culture or in slice preparations (Wilcox et al., 1996). Systemic administration of D-serine, which targets the NMDAR co-agonist glycine-binding site, can increase ΔrCBV in a region-specific manner in the hippocampus using pharmacological MRI (phMRI) (Panizzutti et al., 2005). This induced increase can be blocked by the co-administration of an NMDAR antagonist L-701,324 (Panizzutti et al., 2005).

In this chapter, a proof of biology study was performed to demonstrate that the pharmacologically evoked increase in rCBV is associated with potentiated NMDAR mediated transmission. D-cycloserine (DCS) was used as the stimulating drug to evoke a change in the fMRI signal. D-cycloserine has demonstrated high specificity and partial agonist efficacy on the NMDAR glycine binding site. (Hood et al., 1989; Watson et al., 1990; Priestley and Kemp, 1994; Baxter and Lanthorn, 1995). Historically, this modulatory site was named for the binding of glycine and its interaction with the NMDAR complex (Bonhaus et al., 1987; Johnson and Ascher, 1987; Thomas et al., 1988; Hood et al., 1989; Monahan et al., 1989b), but other glycine analogs have demonstrated affinity to this site. In the absence of glycine, the binding of

glutamate alone will not activate NMDAR channel opening. The NMDAR channel opening necessitates both the binding of glutamate and the activation of its co-agonist glycine-binding site (Johnson and Ascher, 1987). Addition of glycine has been shown to potentiate NMDAR responses in cultured cortical neurons (Johnson and Ascher, 1987). Additionally, kinetic studies have shown that there is a coupling between the glutamate and glycine co-agonist recognition sites. While agonist affinity at either site is dependent upon the ligand that occupies the other site, the extent of the modulation of one site is dependent on the intrinsic activity of the type of agonist rather than its affinity (Priestley and Kemp, 1994).

NMDAR-mediated evoked rCBV response was detected by phMRI as a physiological biomarker for the diagnosis of altered glutamatergic transmission and impaired plasticity following traumatic brain injury. The immature, injured brain was hypothesized to exhibit impaired responsiveness to drug induced activation subacutely after fluid percussion injury (FPI). This subacute time point corresponds to diminished NMDAR expression (NR2A) and function, as demonstrated in Chapter 1 and by Giza et al. (2006). Additionally, we hypothesized that at a chronic time point (approximately after 2 weeks), during which NMDAR subunits have been reported to be normalized to sham levels (Li et al., 2005; Santa Maria et al., 2005), the recovered brain could then respond to drug-induced activation. Dynamic susceptibility contrast enhanced MRI (DCE-MRI) was used to measure DCS induced changes in rCBV (ΔrCBV) in weanling rats. This required two MRI scans: one scan without DCS on board and one scan with DCS. During each scan, changes in signal intensity induced by the bolus injection of a contrast agent (Gd(DTPA)-2: Magnevist) were tracked. rCBV maps were then generated from the trapezoidal integration of the signal time course. The difference between rCBV maps from pre- and post-DCS scans were calculated at different regions of interest (ROIs). In young rats,

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ΔrCBV were measured on post-injury days (PID) 3-5, during the time when NMDAR function is reduced, as well as on PIDs 13-18 when NMDARs have been reported to have normalized.

METHODS

Experimental Design and Subjects

Male Sprague-Dawley rat pups (Charles River, Boston, MA) underwent sham or severe lateral fluid percussion (FPI) on postnatal day 19. On post-injury days (PID) 3-5, animals underwent pharmacological MRI (phMRI). Figure 26 shows the experimental design for Chapter 3. Animals were maintained in a 12 hour light-dark cycle with food and water ad libitum. The UCLA Chancellor's Committee for Animal Research approved all animal studies.
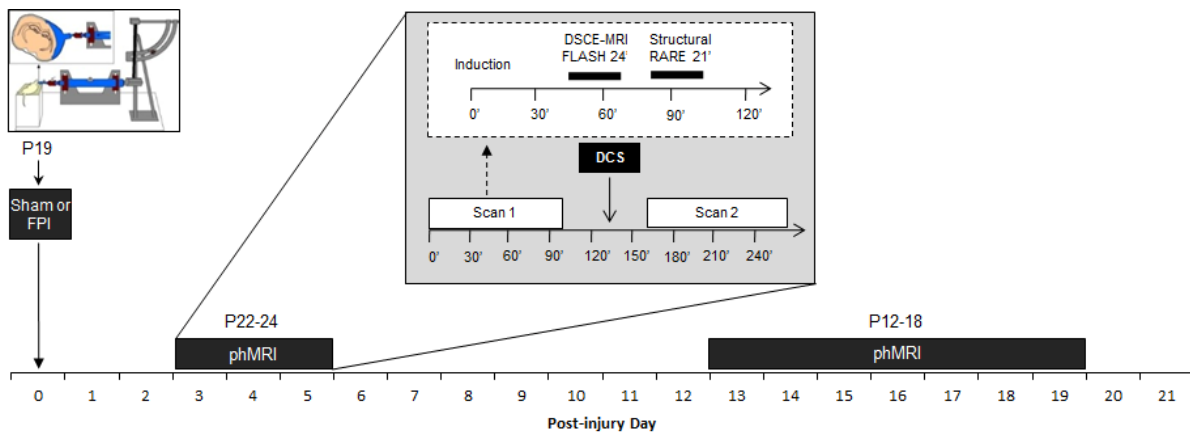
Figure 26. Experimental Design 4
T2*- weighted DSCE-MRI data were acquired in a Bruker 7 Tesla scanner from rats 3-5 days and 13-18 days following sham and FPI procedure at postnatal day 19. Data acquisition was repeated on the same day in each rat, 1.5 hour following stimulation DCS (30mg/kg, i.p.). Each subject underwent two imaging sessions, one pre- and one post-DCS. Inset shows the timeline of one imaging session.

Fluid Percussion Injury

Same as Chapter 1.

Pharmacological MRI (phMRI)

All imaging experiments were performed on a 7T (300 MHz) horizontal bore MR magnet interfaced to a Bruker MR imaging console equipped with ParaVision 5.0 software. The scanner has an actively shielded gradient system (1 T/m). Animals were scanned using a radiofrequency birdcage transmit coil decoupled from a 15 mm receive-only surface coil positioned over the head.

Two imaging sessions were performed on each rat to measure percent change in relative cerebral blood volume (ΔrCBV). The first session (pre scan) measured baseline rCBV and the second (post scan) will measure rCBV after the administration of a drug stimulus (30 mg/kg DCS, i.p.). For each session, T2*- weighted Dynamic Susceptibility Contrast Enhanced MRI (DCE-MRI) was used to acquire data that can be used to obtain rCBV. The second imaging scan was timed to capture rCBV maps approximately 90 minutes after the injection of the drug stimulus.

Animal Preparation

Animals were induced with 4.5% enflurane (in 100% O2, 1.0ml/min) via nose mask. When the animals reached a surgical level of anesthesia, the animals were maintained at 3% enflurane. The tail vein was sterilely prepped and cannulated with a 24G angiocath (BD

Science). The animals were secured into a semicircular cradle with ear bars and a bite bar to minimize movement. Rectal temperature was maintained at 37°C with a thermostatically controlled warm air and respiration rate was monitored using a balloon pressure sensor (BIOPAC). The anesthesia level was adjusted between 2-3% to maintain a respiration rate of approximately 60 breaths per minute. Rats were allowed to breathe spontaneously.

Imaging Parameters

T2*-weighted Fast Low Angle Shot (FLASH) gradient echo images were acquired. Parameters include: effective echo time (TE) = 17ms, repetition time (TR) = 250 ms, flip angle 30°, single slice thickness = 1mm, number of slices 8, field of view (FOV) = 30 mm x 30mm, and matrix size (MS) = 64 x 128, reconstructed to MS =128 x 128. After acquiring 5 minutes of baseline volumes, gadolinium (Gd(DTPA)-2 (Magnevist) , 0.5mmol/kg) was bolus injected (~20sec) via the tail vein. Total FLASH acquisition time was 24 minutes. Anatomical reference images for 20 horizontal slices corresponding to the FLASH imaging were acquired using a two-dimensional (2D) Rapid Acquisition with Relaxation Enhancement (RARE) sequence with the following parameters: TE = 60 ms, TR = 5000 ms, single slice thickness = 0.5 mm, number of slices = 20, FOV = 30 mm x 30 mm, MS = 128 x 128, number of averages (NA) = 16, RARE factor = 8. Total RARE acquisition time was 21 minutes and 20 seconds.

DCS as a pharmacological challenge

After the first imaging session (pre scan), DCS was administered i.p. approximately 20-30 min after the animal awoke and recovered from anesthesia. After 15 min following DCS injection, the animal was again induced with enflurane and prepared for the second imaging session (post scan). The same imaging parameters were used in both scans.

Analysis

The Image J plug-in, Dynamic Susceptibility Contrast MR Analysis (DSCoMAN) (https://dblab.duhs.duke.edu/modules/dscoman/ index.php? id=1) software, was used in generating rCBV maps. DSCoMAN computes $\Delta R2$ using

$$\Delta R2 = - (1/TE) \ln (S(t)/S_0),$$

where TE is the time after excitation, $S(t)$ is the dynamic signal intensity, and S_0 is the average baseline signal intensity. Figures 27A and 29B illustrate the time courses for the signal intensity and for the $\Delta R2$, respectively. The $\Delta R2$ represents the relaxivity-time curve, which is a parameter related to the concentration of gadolinium in the voxel. Relative cerebral blood volume (rCBV) maps were generated from the acquired FLASH images via trapezoidal integration of $\Delta R2$ maps from

$$rCBV = TR \, \Sigma[i=1\ldots N] \, \Delta R2i,$$

which is built in the DSCoMAN software. The rCBV and maps were normalized to the global mean rCBV to account for inter-subject and inter-session differences due to biological and experimental variation. Scaling to global mean signal accounted for vascular reactivity in response to the pharmacological stimulus and allowed detection of a drug effect (Rausch et al., 2005). Appendix section A3 further discusses the importance of normalizing to the global mean. Regions of interest (ROIs) were manually defined on the coplanar anatomical reference image and transferred to the rCBV map. ROIs included hippocampus (Hipp), thalamus (Thal), caudate/putamen (Cpu), and cortex (Ctx) (Figure 27C). The percent change in rCBV (ΔrCBV) was obtained for each ROI between sessions without and with pharmacological stimulus. Statistical analysis using the Student's t-test was performed on planned comparisons between the

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subacute Control and FPI ΔrCBV and between the subacute and chronic FPI ΔrCBV. Statistical

significance was defined as $p < 0.05$. p-values were adjusted for Type I error.

RESULTS

Injury Severity

Mean LOC, apnea times, and the corresponding atmospheric pressure (atm) did not

significantly differ between FPI groups (Table 7).   There were no significant differences in

subject weights across groups on surgery day (PID0).

Table 7. Experiment 4 – Injury Characteristics
Control subjects include naïve and sham animals.

Drug Challenge	Time point	Group	N	wtPID0 (g)	Drop <	Apnea (s)	LOC (s)	Atm
Saline	Subacute	Control	6	49.8 ± 4.5	NA	NA	NA	NA
Stim-DCS	Subacute	Control	8	50.6 ± 1.6	NA	NA	NA	NA
		FPI	6	48.1 ± 1.7	16	90 ± 26	131 ± 35	2.25 ± 0.03
	Chronic	Control	3	50.0 ± 2.2	NA	NA	NA	NA
		FPI	4	50.8 ± 1.3	16	148 ± 30	98 ± 48	2.22 ± 0.04

Sensitivity Test

Pre scan ΔrCBV values between subjects were computed to determine variability and

relative changes between pre scans across groups. Between-subject ΔrCBV signals were

computed by normalizing to their respective group means using the following relationship:

($rCBV_i$ – $rCBV_{mean}$) / $rCBV_{mean}$ × 100), where $rCBV_i$ was the raw rCBV value for a given rat and

$rCBV_{mean}$ was the mean across all subjects for the group. To determine signal variability within

a subject, a separate group of naïve and shams controls (Naïve: N = 3, Sham: N = 3) also

underwent pre and post scans following a saline (i.p.) injection instead of stimulation-DCS. Within-subject ΔrCBV values were computed between the pre and post scans. Standard errors were comparable across groups (Figure 30A). The within-subject data set was then compared to the control (Naïve: N = 4, Sham: N = 4) animals that received stimulation-DCS between pre and post imaging sessions. Region specific increases in ΔrCBV were observed in the hippocampus (Hipp) only in animals administered with the stimulation-DCS dose between the pre and post scans (Figure 28B).

DCS-Induced ΔrCBV

Figure 29A illustrates a representative rCBV map for a control and an FPI subject. The generated rCBV maps are generated in arbitrary units of 0-255 pixel intensity shown by the color calibration bar. Figure 29B shows the percent difference of the rCBV maps. Percent difference between Pre and Post-DCS scans were computed in the pre-defined regions of interests (ROI: Hipp = hippocampus, Cpu = caudate/putamen, and Ctx = cortex) visualized in Figure 29C. Since there were no significant differences between the Naïve and Sham ΔrCBV, values were collapsed together to constitute the Control ΔrCBV. DCS evoked a region-specific increase in hippocampal ΔrCBV (+65%) in control animals bilaterally. This response was absent in FPI pups that underwent phMRI subacutely on post-injury days 3-5 (Figure 30). At the chronic time point (>2weeks post-FPI), increased ΔrCBV values were observed both in Controls (+32%) and FPI animals (+100%). Further breakdown of the FPI group imaged in the subacute phase showed a trend for an injury severity response of evoked ΔrCBV (Figure 31).

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Figure 27. FLASH and RARE Images for rCBV
 (A) Sample FLASH image (left) and signal intensity tracking the bolus transit of the contrast agent (Gd(DTPA)-2: Magnevist) in a defined region of interest (ROI) generated from DSCoMAN (right). (B) Generated Delta R2 (ΔR2) map (left) using Raw Perfusion plugin from DScoMAN and the corresponding plot (right). (C) Structural anatomical RARE images with sample ROIs (Hipp = hippocampus, Ctx = cortex, and Cpu = caudate/putamen).
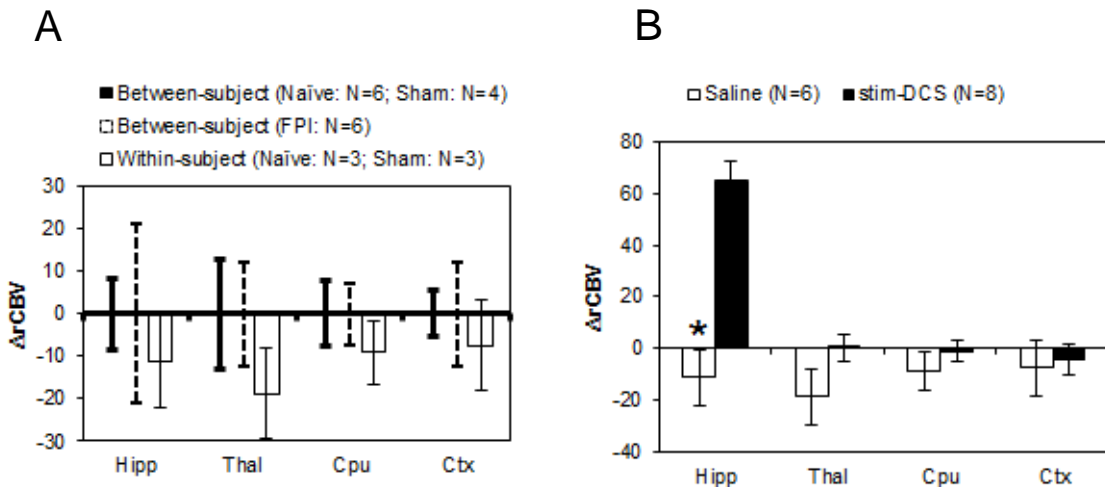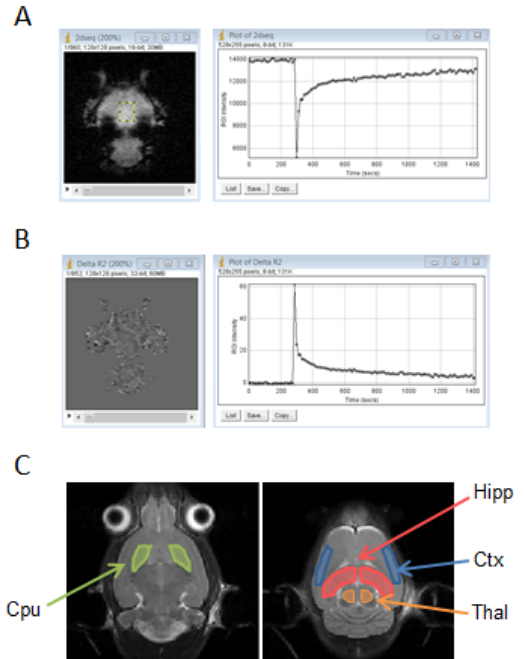
Figure 28. ΔrCBV Sensitivity
(Mean ± SEM) ΔrCBV (without DCS). (A) Between-subject pre scan ΔrCBV values were normalized to their respective mean rCBV for Control animals (bold solid error bars, Naïve: N = 6, Sham: N = 4) and for FPI rats (dashed error bars, N = 6). Bar graph with thin solid error bars show within-subject ΔrCBV (Naive: N = 3; Sham: N = 3). (B) ΔrCBV in control animals that received either saline or DCS between scans. The asterisk (*) indicates significant difference from the stim-DCS group ($p < 0.05$).

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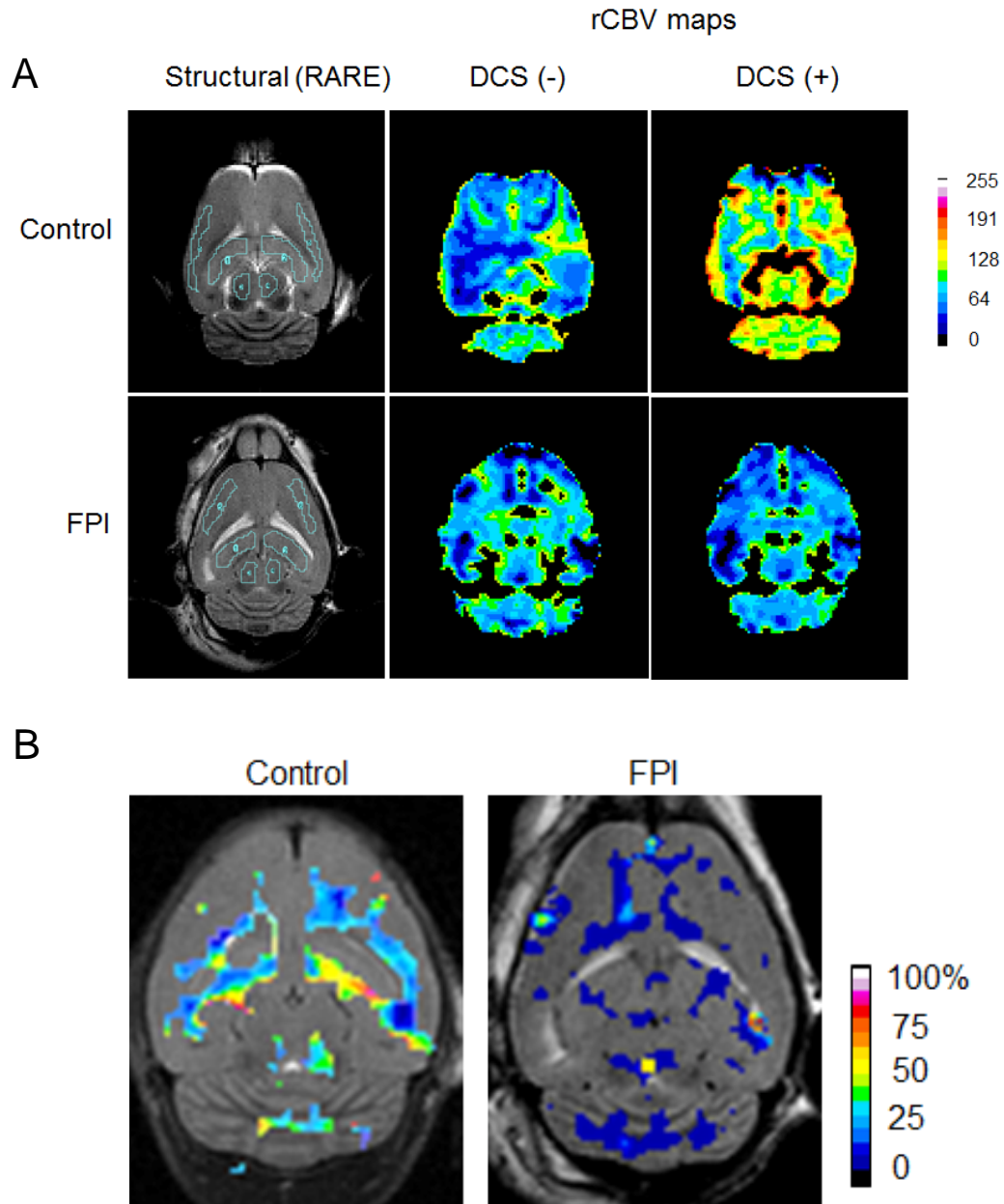
Figure 29. Representative rCBV Maps
(A) Representative Control and FPI Structural (RARE) images and colorized rCBV maps pre (DCS(-)) and post (DCS(+)) administration of DCS. (B) Percent ΔrCBV difference maps superimposed over their respective RARE images.
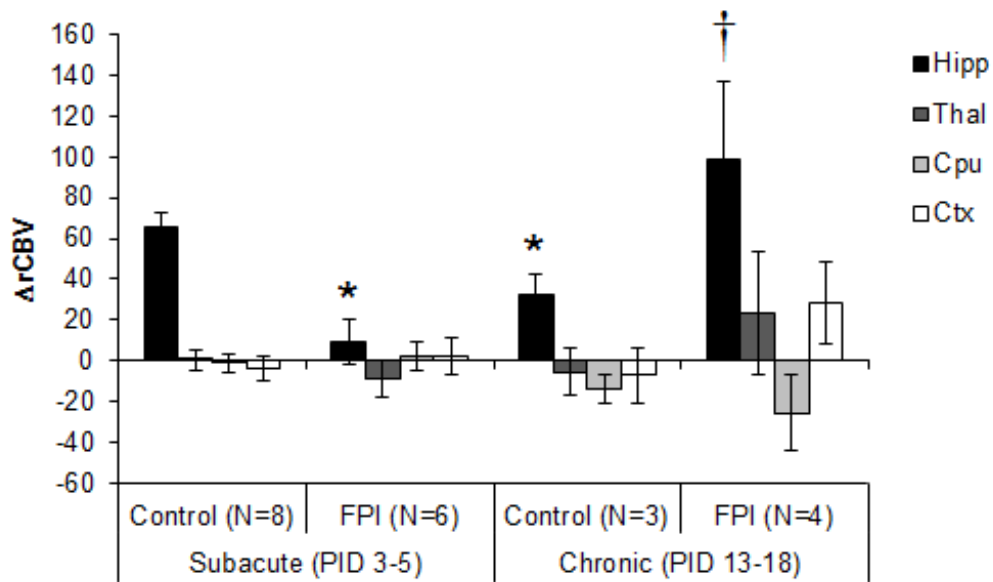
Figure 30. Subacute and Chronic ΔrCBV Post-FPI

(Mean ± SEM) DCS-induced ΔrCBV obtained from manually drawn ROIs in the hippocampus (Hipp), caudate-putamen (Cpu), and cortex (Ctx). Stimulation doses of DCS induced a hippocampal-specific increase in rCBV signal in uninjured animals (subacute, +65%; chronic, 33%) that is abolished by developmental FPI. The asterisk (*) indicates significant difference from Subacute Control group ($p < 0.05$). There is spontaneous recovery of DCS-induced ΔrCBV in the Chronic FPI group (+100%) The dagger (†) indicates significant difference from Subacute FPI group ($p < 0.05$).
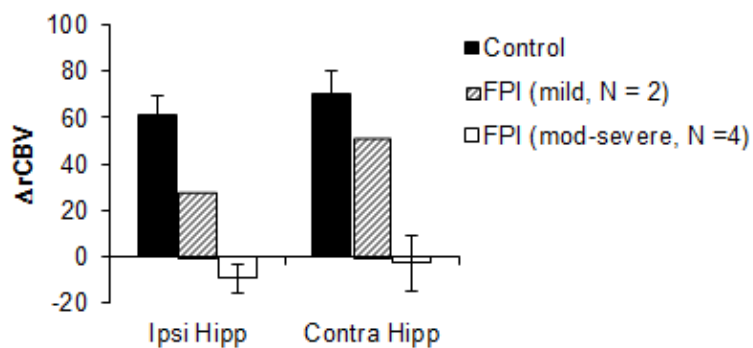
Figure 31. Subacute ΔrCBV Breakdown in FPI Animals

(Mean ± SEM) ΔrCBV in the hippocampus of FPI animals that received either mild (loss of consciousness time, LOC < 60 seconds) or severe injury (LOC > 120 seconds). Animals with mild FPI showed partial enhancement in rCBV after a DCS dose.

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DISCUSSION

Methods that are non-invasive and translatable, such as PET, SPECT, MRS and MRI, allow for the evaluation of hemodynamic parameters and are now more widely used as tools for diagnosis and drug development. In traumatic brain injury (TBI), MRI is being used, experimentally and clinically, to characterize injury effects on brain volume, intrinsic MR constants (T1 and T2) of different brain tissue, neuro-metabolism, hemodynamics, and connectivity. Injury induced changes in these parameters have also been correlated with impaired cognitive performance and behavior (Immonen et al., 2009). Due to the coupling of neuronal activity, neuronal metabolism and cerebral perfusion, rCBV response to drug can be used as a biomarker to investigate effects of pathologies, normal physiology and drug therapeutics on brain function. Additionally, an evoked change in rCBV signal can yield a measurable parameter with a high signal to noise ratio (Mandeville et al., 2001).

Glutamatergic transmission plays an inherent role in neuronal and astrocytic cerebral activity and can now be mapped using MRI (Sibson et al., 1998; Bonvento et al., 2002; Gsell et al., 2006). Pharmacological MRI (phMRI) studies have mapped neural substrates of MK-801, an NMDAR antagonist, by measuring changes in BOLD signal. MK-801 administration resulted in reduced activity in the hippocampus, anterior cingulate, retrospinal cortex, and the medial prefrontal cortex (Roberts et al., 2008). Additionally, by targeting the glycine co-agonist site of the NMDAR, administration of D-serine systemically in adult rats induced increases in local rCBV in the hippocampus (Panizzutti et al., 2005).

In this chapter, increased rCBV was elicited in hippocampus, in a region specific manner, by systemically administering an NMDAR agonist, D-cycloserine (DCS), in control subjects.

Control animals given a saline stimulation dose did not show any changes in rCBV. Moreover, FPI in P19 rats abolished the DCS-induced ΔrCBV on PIDs 3-5 (Figure 32). This is the first description of a proof of biology study using phMRI in normal and diffusely brain injured weanling rats, wherein the NMDAR system was associated with a biological change (ΔrCBV) using DCS, an NMDAR agonist. DCS has been demonstrated to have a high affinity to the glycine recognition co-agonist site in the NMDAR. The region-specificity of DCS-induced ΔrCBV, under the present experimental conditions, seems to be in accord with previous reports that indicated that the glycine-binding site in the hippocampus is not saturated compared to other brain regions (Dalkara et al., 1992; Pitkänen et al., 1994). Furthermore, ΔrCBV in cortex and caudate/putamen did not significantly differ between FPI and control animals and did not exceed the threshold of rCBV signal variability.

The time course of DCS-induced ΔrCBV parallels NMDAR-mediated neural responsiveness in the subacute and chronic time points after FPI. The absence of DCS-induced ΔrCBV in the subacute time point corresponds to diminished NMDAR function, impaired behavior and experience-dependent plasticity, in the absence of gross histological damage (Giza et al., 2005; Li et al., 2005; Reger et al., 2005; Giza et al., 2006). Four days after an FPI injury to postnatal day 19 rats, NR2A subunit protein levels are reduced by 20-40% in the ipsilateral hippocampus (Chapter 1 and Giza et al., 2006). On the same post-injury day (PID), FPI induced decreases in NMDAR-mediated EPSCs, which were attributed to diminished EPSCs from NR2A-containing NMDAR (Li et al., 2005). Weanling rats subjected to FPI also showed disturbed novel object recognition memory, a hippocampus- and NMDAR-mediated working memory task (Chapter 1, Reger et al., 2005), at the same subacute time point. Additionally, the

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loss of DCS-induced ΔrCBV in the first 7-10 days post-injury coincides with the critical time window when enriched environment rearing was not effective in inducing enhanced plasticity (Giza et al., 2005). Giza et al. showed when postnatal day 19 animals were differentially housed in an enriched environment immediately after FPI (on PID1), FPI-EE animals fail to functionally demonstrate EE-induced enhancement in MWM performance when tested in adulthood.   Sham-EE animals significantly improved their spatial learning in the MWM. However, while FPI-EE animals still were able to learn the MWM task, they only performed to the level of standard housed counterparts (Chapter 2, Fineman et al., 2000). In addition, previous studies reported that FPI-EE animals also failed to show EE-induced structural plasticity, such as increased cortical thickness and expanded dendritic arborization (Fineman et al., 2000; Ip et al., 2002). The recovery of DCS-induced ΔrCBV two weeks after FPI coincided with normalized levels of NMDAR subunits, NMDAR-mediated electrophysiology and novel object recognition memory (Li et al., 2005; Reger et al., 2005; Santa Maria et al., 2005; Gurkoff et al., 2006). Furthermore, when EE rearing was delayed until two weeks after FPI, FPI animals demonstrated responsiveness to the EE-rearing condition and significantly improved their MWM learning (Giza et al., 2005).

These results suggest that DCS-induced ΔrCBV can be used as a non-invasive surrogate biomarker of NMDAR-mediated transmission. Its clinical implications include the use of the phMRI ΔrCBV signal as a diagnostic tool to assess the magnitude and duration of impaired NMDAR-mediated glutamatergic transmission after traumatic brain injury. Moreover, Figure 33 suggests an injury severity dependence of DCS-induced ΔrCBV in the subacute FPI group. However, with a small sample size, this preliminary breakdown needs further evaluation.

DISCUSSION OF THE DISSERTATION

Traumatic brain injury (TBI) is the leading cause of death and disability in the young. Children may recover physically from the acute effects of TBI, only to manifest chronic cognitive and behavioral dysfunction during maturation. Although the developing brain demonstrates increased capacity for experience-dependent plasticity, being too adaptive and malleable can be a double-edged sword. Depending on the nature of the stimuli one experiences, the neural response may be either a physiological or pathological summation result. This dichotomous outcome is dependent on the underlying mechanisms involved in neural circuitry formation. What initiates circuitry formation are the signals generated by synaptic responses associated with the stimuli. These signals include the ionic flux or molecular alterations triggered by neurotransmitters, like glutamate. Glutamate's action on its various receptors ultimately influence intracellular calcium levels that can drive the cell into either pro-survival or pro-apoptotic states and can have lasting effects on development, learning, memory and neuroplasticity.

The N-methyl-D-aspartate receptor (NMDAR) is a subtype of ionotropic glutamate receptor that has a profound influence on calcium homeostasis and plays a key role in synaptic plasticity, learning, memory, and development, but is also involved in the pathophysiology of TBI. Modulation of this receptor could have therapeutic potential in treating conditions and disorders that are mediated by abnormal/pathological glutamatergic neurotransmission, such as in stroke (Newell et al., 1995; Dhawan et al., 2011; Lai et al., 2011), schizophrenia (Olney et al., 1999), Parkinson disease (Wang et al., 2010; Ho et al., 2011; Nandhu et al., 2011), autism

(Rinaldi et al., 2007; Moskal et al., 2011) and TBI (Biegon et al., 2004; Yaka et al., 2007; Adeleye et al., 2009).

The opening of the NMDAR channel requires the binding of both glutamate and the activation of its co-agonist glycine-recognition site, either by glycine or D-serine (Thomas et al., 1988; Monahan et al., 1989b; Kleckner and Dingledine, 1991; Wilcox et al., 1996). Depending on the subunit composition and the nature of activation, direct stimulation of the NMDARs via either the co-agonist binding sites or modulatory sites (polyamine site and channel pore) can trigger either pro- or anti- plasticity sequelae (Hardingham and Bading, 2003; Hardingham, 2006; Soriano et al., 2006; Papadia and Hardingham, 2007; Hardingham, 2009). NMDAR's intimate involvement in "good" or "bad" plasticity is currently being uncovered further.

In experimental TBI, alterations in NMDAR composition have been correlated with impairments in hippocampally-mediated learning and behavior in the developing brain, in the absence of histological pathology (Fineman et al., 2000; Giza et al., 2005; Giza et al., 2006; Gurkoff et al., 2006; Schwarzbach et al., 2006). Blockade of pathological NMDAR activation (excitotoxicity) at the different modulatory sites seemed promising in the pre-clinical setting; however, many clinical trials in acute brain injury have not been successful (Morris et al., 1999; Albers et al., 2001; Ikonomidou and Turski, 2002; Naranyan et al., 2002; Maas et al., 2010).

Alternatively, while direct pharmacological activation of the glutamate binding site can lead to a risk of excitotoxicity, NMDAR modulation via the glycine-recognition site provides a safer and more feasible way of facilitating NMDAR function. This glycine-recognition site has a high affinity for D-cycloserine, a cyclic glycine analogue, which depending on the ligand concentration can have agonist or antagonist properties (Hood et al., 1989; Watson et al., 1990;

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Lanthorn, 1994; Baxter and Lanthorn, 1995). Previous studies have demonstrated that DCS augments amygdala- (Lin et al., 2009) and hippocampus-dependent learning in rodents (Monahan et al., 1989a; Thompson et al., 1992; Pitkänen et al., 1995; Lelong et al., 2001) and in humans (Onur et al., 2010). Successful recovery of function has already been demonstrated via NMDAR activation at this co-agonist glycine site after TBI in adult rodents (Temple and Hamm, 1996; Yaka et al., 2007).

It is demonstrated in this dissertation that DCS therapy during a critical period after developmental FPI can promote neuroprotection and restoration of subacute NMDAR-mediated pro-plasticity molecules, such as the NR2A subunit and phos-CAMKII. This reinstatement to physiological levels sets the stage for a normalized pro-plasticity developmental trajectory. DCS therapy, in conjunction with EE rearing, is therefore demonstrated to restore cognitive potential lost to injury. Further investigation is necessary to optimize the needed number of doses and inter-dosage interval of DCS therapy that is translatable to humans.

SUMMARY OF FINDINGS

Fluid percussion injury causes indiscriminate glutamate release (Faden et al., 1989; Katayama et al., 1990) and pathological activation of NMDARs. The majority of the excessive calcium accumulation post-TBI has been attributed to NMDARs (Bullock, 1992; Nadler et al., 1993; Nadler et al., 1995) and altered NMDAR subunit composition. There is reduced protein expression of NR2A:NR2B ratio in within one week post-FPI in the adult and young brain (Osteen et al., 2004; Giza et al., 2006) that corresponds to the time-course of calcium accumulation (Osteen et al., 2001) post-injury across development. In P19 pups, a decrease of NR2A subunit in the ipsilateral hippocampus occurs in the first week following FPI (Giza et al., 2006).

In Chapter 1, western blotting techniques were used to measure protein levels of glutamate-mediated receptors and downstream signal transduction molecules with and without DCS treatment subacutely after FPI in postnatal day 19 rats. Receptor subunits of NMDAR and AMPAR receptors, as well as CAMKII and ERK, were also measured. DCS administration during the first few days post-injury restored ipsilateral NR2A in hippocampus to sham levels. Phos-CAMKII was also observed to be positively modulated by DCS therapy. DCS also induced increases in calcium-impermeable Glur2-containing AMPARs. This result corresponds to the indirect interaction of DCS with AMPAR-mediated transmission in rat hippocampal slices (Rouaud and Billard, 2003; Billard and Rouaud, 2007). The increase in GluR2 levels has been correlated with the developmental switch of synaptic AMPARs from Ca^{2+}-permeable to Ca^{2+}-impermeable receptors during the period of maximal synaptic growth. These results suggest that DCS induces recovery of developmental physiological AMPAR levels. Concurrently, novel object recognition, a NMDAR-mediated working memory task that involves the hippocampus, is

rescued by DCS treatment in FPI-EE pups. Novelty recognition is likely to be crucial in learning, memory and plasticity, particularly in the setting of EE rearing or perhaps other scenarios of experience-dependent plasticity.

DCS treatment sets the stage for reinstated capacity for plasticity, by normalizing pro-plasticity molecules (NR2A subunit and CAMKII levels) and inducing a milieu protected from further excitotoxicity in the injured brain (with increased GluR2 levels). This molecular restoration manifest in rescued cognitive performance using the novel object recognition task.

In Chapter 2, the results suggest that after diffuse TBI, spontaneous recovery still occurs. All animals housed in the standard condition were still successful in finding the hidden platform, reaching trials to criterion, and recalling the platform location during the probe trial when tested on the MWM on PID 30-40. These standard-housed young animals demonstrated higher learning than adult rats trained on the same task, with linear slopes of learning that were greater than 1 and exceeded the reported adult rodent MWM performance (Fineman et al., 2000).

When experience-dependence plasticity was induced, only sham animals demonstrated EE-induced enhancement in cognition. Figure 19 shows that Sham-EE-Sal and Sham-EE-DCS demonstrated significantly improved MWM acquisition and recall compared to standard-housed subjects. In contrast, FPI-EE-Sal pups showed no effects of EE-rearing, performing only to the level of standard housed subjects. This translates to lost plasticity and potential in FPI animals. While DCS had no effect in shams regardless of housing condition, DCS improved MWM acquisition (trials to criterion) in FPI animals, in particular showing a significant effect in FPI-DCS-EE pups (Figure 23). DCS treatment during a diminished period of reduced neural responsiveness after FPI resulted in rescue of experience-dependent plasticity.

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DCS, however, had a differential effect on Shams and FPI animals in the probe trial. While DCS seemed to increase the number of entries to the target zone in Shams-STD animals, Sham-EE pups had significantly reduced target entries when treated with DCS (Figure 25). DCS has been shown to facilitate declarative learning and hippocampal activity in healthy humans (Onur et al., 2010) and extinction learning in rats (Bouton et al., 2008). These data suggest that Sham-DCS reduction in probe trial target entries may be due to facilitated learning that the platform does not exist in the original location.

Alternatively, antagonist properties of DCS (Hood et al., 1989; Lanthorn, 1994; Baxter and Lanthorn, 1995) may explain this differential effect in shams. Hood et al., showed that DCS has agonist properties and stimulated ^{3}[H]TCP binding to a maximum of 40-50% that of the effect of glycine alone. DCS also has antagonist characteristics, wherein in the presence of varying concentrations of glycine, DCS reduces the maximal levels of glycine stimulation. In intact mice, EE-rearing increases levels of glycine in the brain (Cordoba et al., 1984). Increased glycine levels could facilitate NMDAR function by occupying the unsaturated glycine binding sites on NMDARs and promoting enhancement of synaptic plasticity, learning and memory. Glycine is a full agonist acting on the same binding site as DCS on NMDARs. This could arguably be an underlying mechanism for EE-induced experience-dependent plasticity. DCS administration in the presence of glycine, therefore, may be reducing the potentiated effects of elevated levels of glycine in the uninjured brain.

Based on the findings in Chapters 1 and 2, modulation of NMDAR-mediated transmission could result in either physiological or pathological plasticity. Although, effects of FPI on NMDAR subunits levels recover over one week, alteration of NMDARs during a critical

period in development has lasting consequences on experience-dependent plasticity. Diminished NMDAR-mediated transmission has been demonstrated in ex-vivo electrophysiological recordings or correlatively through cognitive and behavioral testing. It is important to be able to detect reduced NMDAR-mediated neural responsiveness non-invasively after TBI prior to translating this finding to humans. In addition, this non-invasive assessment allows for a within-subject design in the evaluation of therapies that target the NMDAR system.

In Chapter 3, pharmacological MRI was used to characterize an MRI signal that can be used as a biomarker of NMDAR-mediated transmission. It has been previously demonstrated that an NMDAR agonist, D-serine, increases hippocampal relative cerebral blood volume (rCBV) in intact adult rats. The effect is region-specific due the fact that the co-agonist glycine binding site of hippocampal NMDARs are normally unsaturated as compared to other brain areas. Co-administration of a full antagonist of the glycine site inhibited the D-serine-induced ΔrCBV indicating the specificity of this interaction (Panizzutti et al., 2005). In Chapter 3, using a partial NMDAR-agonist, DCS, increases in relative cerebral blood volume (ΔrCBV) signal were induced in a region-specific manner in developing rat. It was demonstrated that the DCS-induced increase in ΔrCBV was abolished within one week following FPI. Over time, evoked ΔrCBV recovered two to three weeks post-injury, which corresponds to recovery of NMDAR subunit protein expression, NMDAR-dependent novel object recognition, and NMDAR-mediated excitatory postsynaptic currents (Li et al., 2005; Reger et al., 2005; Santa Maria et al., 2005). Clinical implications of identifying a non-invasive physiological biomarker for TBI include the ability to assess the state of the injury (magnitude and duration), predict projected deficits, and identify potential treatment responses for NMDAR-mediated transmission targeted therapy.

Recording changes in perfusion is still a surrogate marker of neuronal activity, and pharmacological MRI carries several limitations. The stimulating drug may affect systemic and vascular physiology during scanning and could prevent acquisition of the desired biomarker. In a separate group of animals, monitoring at the bench-side validated physiological stability during the imaging sessions. Simultaneous physiological monitoring during imaging should be observed in future studies. In addition, DCS as the phMRI stimulating agent could have a potential confound when used in conjunction with DCS as treatment. Other NMDAR agonists at the different modulatory sites could be used in replacement of DCS as the stimulus to achieve the evoked ΔrCBV. Further proof of concept experiments could also be pursued through direct electrical stimulation of the hippocampus in vivo. The evoked electrophysiological response could be used as the biomarker in this setting (and would eliminate the potential confounds of using DCS for both treatment and stimulation). Within-subject effects of FPI and different drug treatments could then be evaluated through electrophysiological, molecular, and behavioral methods.

Table 8. Summary of Findings

Measure	P19 FPI		P19 FPI (+DCS)	
Molecular	↓	NR2A (PID4) Phos/total CAMKII (PID4)	↑	Restored NR2A (PID4) Restored phos/total CAMKII (PID4)
Behavioral (subacute)	↓	NOR (PID4)	↑	Restored NOR (PID4)
Behavioral (chronic)	↓	MWM Trials to Criterion MWM Probe Trial (PID30-40)	↑	Restored MWM Trials to Criterion Restored MWM Probe Trial
phMRI (subacute)	↓	ΔrCBV (evoked) (PID3-5)		-

INTERPRETATION OF DATA

Following FPI, there is a reduced NR2A:NR2B ratio during a critical period of a developmental switch to a higher NR2A:NR2B expression (Flint et al., 1997; Cull-Candy et al., 2001; Cull-Candy, 2004; Giza et al., 2006). This would yield an NMDAR assembly that is more sensitive and electrophysiologically active. Since DCS has an affinity for both NR2A- and NR2B- containing NMDARs, it would be a concern that DCS could result in potentiated NMDAR activity and pose a risk for worsened excitotoxicity, especially after FPI.

However, DCS efficacy is subunit-specific and elicited by the interaction solely through the glycine binding site of the NMDAR. DCS interacts with NR2A-only and NR2B-only containing NMDARs with 69% and 38% maximal efficacies, respectively, compared to glycine controls. In contrast, NR2C-containing NMDARs demonstrate potentiated response with DCS (192% maximal efficacy compared to glycine controls) (Sheinin et al., 2001). With NR2A and NR2B prevalently expressed and the glycine binding site unsaturated in the hippocampus, DCS demonstrates agonist properties when interacting with this NMDAR co-agonist site (with all recombinant NR1/NR assemblies) (Hood et al., 1989; Monahan et al., 1989a; Watson et al., 1990; Baxter and Lanthorn, 1995). Therefore, even with the down-regulation of NR2A after FPI to the P19 rat, it is hypothesized that DCS can still facilitate physiological activation during this developmental stage, by potentiating NR2A- more than NR2B-containing NMDARs. Weighted NR2A activation can promote fast desensitization of NMDAR activity (Cull-Candy, 2004). This action can allow necessary synaptic firing, while faster channel closing protects the postsynaptic neuron from prolonged glutamate excitotoxicity. Furthermore, activation of the more synaptically localized NR2A-containing NMDARs has been reported to promote a more pro-plasticity sequelae (Hardingham, 2006; Papadia and Hardingham, 2007). This is evidenced by

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the restoration of CAMKII to sham levels after DCS therapy and novel object recognition after FPI in the immature rat.

DCS efficacy is also pH-dependent. Brain injury decreases brain pH from 7.4 to 6.75 (Zauner et al., 1995). DCS efficacy is increased with decreasing pH levels (from 7.2 to 6.5 pH levels) for both NR2A-only and NR2B-only containing receptors (Sheinin et al., 2001). If brain pH continues to be reduced after FPI, DCS is still hypothesized to facilitate potentiated NMDAR activation in both NR2A- and NR2B-containing NMDARs in the hippocampus. While lowered pH results in inhibition of the NMDAR (Banke, 2005; Dravid et al., 2007), DCS efficacy actually increases with reduced pH levels (Sheinin et al., 2001).

The traditional approach for an injury would be to quiet down the perturbed system and allow spontaneous recovery to occur. Efficacy of this approach is evidenced in the results of Chapter 2, wherein the standard-housed FPI animals still performed to the same level as the sham group. EE-induced benefits can also still be acquired after FPI in the weanling rat without pharmacological intervention if EE rearing was started after normalization of NR2A subunit levels. FPI animals exposed to delayed-EE (two weeks post-FPI) demonstrated significantly improved trials to criterion in the MWM comparable to EE-housed shams (Giza et al., 2005). However, in the delayed-EE animals, remembrance of the hidden platform was not restored during the probe trial. In contrast, only with DCS treatment were both MWM acquisition and recall reinstated to sham levels, as shown in Chapter 2. This finding confirms the dependence on NMDAR-mediated transmission of experience-dependent plasticity.

Arguably, all humans live in a continuous complex environment that is constantly filled with new and changing stimuli. Injury to the young brain can reduce function. However, while

spontaneous recovery still occurs and development ensues, the young injured brain is not primed to fully benefit from the continuous stream of stimuli from its environment. Injured subjects could still reach their pre-injury baseline, but they would have delayed development and may yet fall behind their non-injured peers. This translates into lost potential. In Chapter 2, FPI animals housed in EE failed to manifest benefits of experience-dependent plasticity. Although the outcome in FPI-EE rats still yielded recovery of function (FPI-EE-Sal pups were still able to learn), their capacity for plasticity was impaired (FPI-EE-Sal never 'mastered' the spatial learning task).

It is demonstrated in this dissertation, using converging operations, that DCS reinstates NMDAR activation during a critical period of development that is crucial for acquiring beneficial experience-dependent plasticity. Targeting the glycine-binding site using DCS allows for NMDAR activation without the adverse effects of direct stimulation. Furthermore, NMDAR-mediated transmission can be assessed non-invasively using MRI signals. These MRI signals can be used as physiological biomarkers to ascertain the extent of the injury-induced impairment of function and the potential for enhanced therapeutic recovery.

APPENDIX

A1. HISTOLOGY

To determine whether the use of DCS as treatment following FPI affected morphology in the subacute phase (within one week, greater than twenty-four hours) following insult, four animals per group in Chapter 1, Experiment 2 (Sham-Sal, Sham-highDCS, FPI-Sal, and FPI-highDCS) were perfused on PID4. Animals were anesthetized with a lethal dose of sodium pentobarbital (100 mg/kg) and perfused via the ascending aorta with phosphate buffered saline (PBS) followed by cold 4% paraformaldehyde in PBS. Brain tissue was post-fixed in the same fixative for 24 hours in 4°C and infiltrated with 30% sucrose and stored in a – 72°C freezer. Brains were sectioned coronally on a cryostat (Leica) at 40 μm intervals, saving sections between the genu and splenium of the corpus callosum. Sections were preserved in 0.1 M Tris-buffered saline (TBS) with 0.05% sodium azide and stored at 4°C. Brains were prepared for thionine immunohistochemistry for gross evaluation of brain tissue under a brightfield microscope (Leica).

Representative sections are shown in Figure 32. No overt histological pathologies were observed following moderate-severe FPI and/or highDCS dose on PID4. Thionine stain is specific to DNA and Nissl substance, which is primarily ribosomal RNA. This type of stain does not however mark cells undergoing apoptosis. To elucidate whether DCS treatment increases apoptosis after FPI, alternative methods of histology may be pursued with the use of Fluoro Jade or Annexin V to label degenerating neurons. Furthermore, one would not be able to differentiate different types of cells (neurons vs. glia) with thionine staining alone.
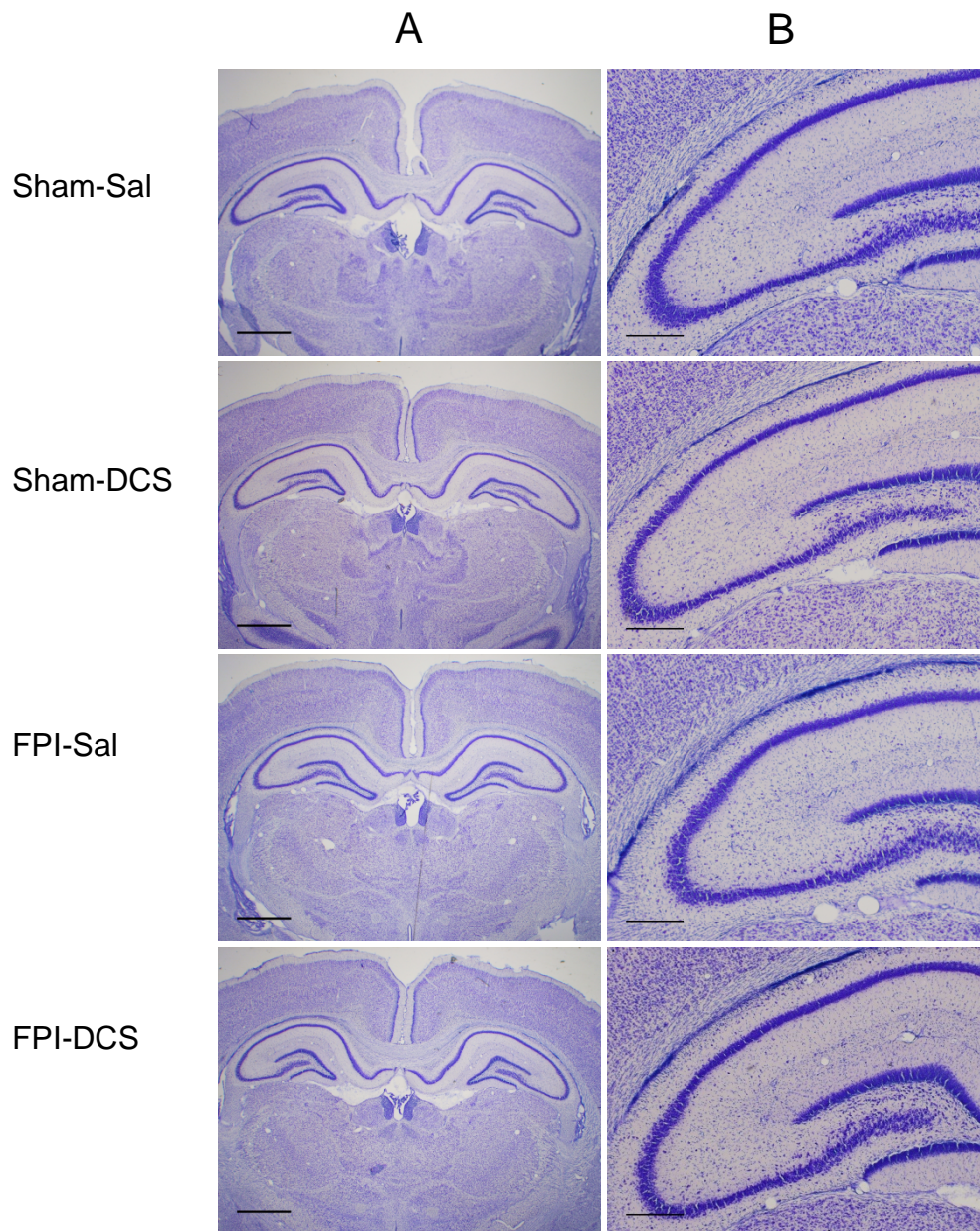
Figure 32. Experiment 1 – Thionine Histology
Coronal sections (40 μm thickness) stained with Thionine. Horizontal panels show sample
sections from the experimental groups: Sham-Sal, Sham-highDCS, FPI-Sal (3), and FPI-
highDCS (4). The columns indicate the magnification at 1x (A) and 4x (B). Column B shows
magnified regions of the hippocampus, ipsilateral to the FPI site. Sections from each group are
shown to demonstrate the lack of gross morphological damage from FPI or from DCS treatment.
Scale bars are 1 mm (A) and 500 μm (B).

A2. phMRI: DCS as a Drug Challenge – Feasibility

This preliminary experiment was designed to determine the feasibility of DCS as a pharmacological challenge during contrast enhanced pharmacological MRI (phMRI) in vivo. The early effects of DCS on MRI signal and relative cerebral blood volume was investigated. An adult naïve Sprague Dawley rat (289g) was cannulated in the right femoral vein under 2.5% isoflurane anesthesia with a 1m polyethylene (PE-50) tube. The cannula was loaded with a gadolinium bolus (Gd(DTPA)-2: Magnevist, 0.5 mmol/kg) flanked by a saline front and tail. Femoral vein cannulation was used to ensure bolus administration of the contrast and DCS. The relative cerebral blood volume ($rCBV_{calc}$) was calculated from T2-weighted gradient echo RARE images at 5 time points: baseline, 60 minutes before DCS injection (i.v.) (pre-DCS: 10 minutes after gadolinium injection), 10 minutes after DCS injection (i.v.) (post-DCS-10'), 30 minutes after DCS (post-DCS-30'), and 60 minutes after DCS (post-DCS-60') (Figure 33A). Imaging parameters were as follows: TE = 15 ms, TR = 5000 ms, slice thickness = 1 mm, number of slices = 12, FOV = 30 mm x 30 mm, MS = 128 x 128, resolution = 0.0234 cm/pixel x 0.0234 cm/pixel, NA = 16, RARE factor 8. Total RARE acquisition time was 21 minutes and 20 seconds. Regions of interest were manually drawn over the hippocampus and cortex.

Assuming a linear relationship between the tissue relaxation rate (R2 = 1/T2, T2 is the transverse relaxation time) and the total amount of contrast agent in the voxels, the signal attenuation induced by the contrast agent in T2- weighted MR images can be described by $(1/T2_{pre}) = (1/T2_{post}) + r2 \cdot cp \cdot rCBV$. $T2_{pre}$ and $T2_{post}$ are the transverse relaxation times before and after contrast agent injection, r2 is the molar relaxivity of the contrast and cp is the plasma concentration of the contrast agent. Assuming steady-state plasma levels of the contrast agent, T2 becomes proportional to the rCBV. Therefore, the $rCBV_{calc}$ values can be simply computed

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using $rCBV_{calc} = - \ln (S_{post} / S_{pre})$, where S_{pre} and S_{post} are the signal intensities prior to and after administration of the contrast agent (Panizzutti et al., 2005).

No perturbations in animal physiology (temperature and breathing rate) were observed. Calculated Pre-DCS $rCBV_{calc}$ showed comparable values to reported in literature (DCE-MRI: 0.068 ± 0.01 and positron emission topography (PET): 0.052 ± 0.012, both in mL blood per gram of tissue (ml/g), (Tofts, 2005)). Evident changes in $rCBV_{calc}$ within 60 minutes of DCS injection reflects the optimal time for maximal effect of DCS, which has a half-life of approximately 45 minutes in the rat brain (Baxter and Lanthorn, 1995) (Figure 33B).

This feasibility experiment showed that DCS can be used to induce increases in hippocampal and cortical rCBV in vivo within an imaging session, with no adverse physiological effects. This also suggests that future within-subject experiments could entail acquiring images pre- and post- DCS challenge within the same day, minimizing variability between experiment days.
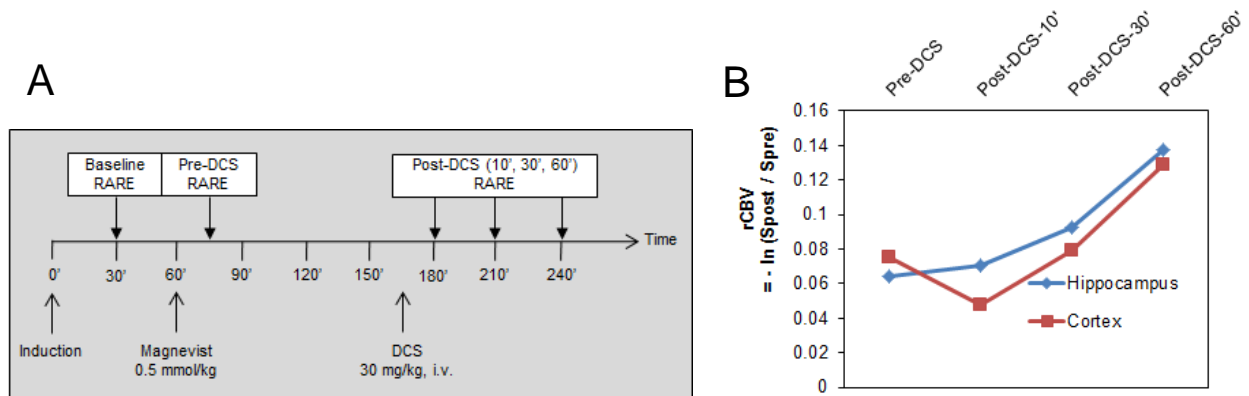

Figure 33. DCS as Pharmacological Challenge: Design and rCBV
(A) Experimental design for A2. (B) Calculated rCBV values within the same animal after DCS administration. Pre-DCS time point was obtained after injection of gadolinium.

A3. phMRI: DCS as a Drug Challenge – Physiological Concern

The feasibility experiment in A2 showed that DCS can be used as a drug challenge in phMRI in an adult control (naïve) Sprague Dawley rat. The objective of this experiment was to determine the effects of DCS on rCBV values in control (sham), Sham and FPI postnatal day P20-25 weanling rats. Two postnatal day 24 pups experienced drastic reduction in breaths per minute (BPM) (from 60-70 to 30-40 BPM) immediately following DCS tail vein injection during scanning. For weanlings, intraperitoneal (i.p.) injection of DCS was found to have less drastic physiological effects on the pup. DCS was given i.p. when the animal was awake, similar to how DCS as treatment was administered in Chapters 1 and 2. The majority (~75%) of the weanling rats that received DCS in the awake state (i.p.), however, did show transient effects when DCS was given intraperitoneally. These effects lasted approximately 10-15 minutes and included piloerection, increased breathing rate, ataxia and mild body shakes. The i.p. route in the awake state was eventually used for all subsequent experiments, to eliminate the DCS-anesthesia effects that were not yet elucidated, as well as to de-couple drug-induced effects on physiology and on neurovascular activation.

After successfully obtaining DCS-evoked rCBV responses in a cohort of animals, a few important questions were addressed to characterize the DCS-induced ΔrCBV signal. In phMRI, rCBV is taken as a surrogate marker of neural activation. In Chapter 3, animals were imaged under anesthesia. rCBV can be confounded by several factors, such as physiological measures (blood pressure, vascular CO_2, respiration rate, temperature), as well as anesthetic and drug effects on blood flow (Kalisch et al., 2001; Gozzi et al., 2007; Zaharchuk, 2007). The first question asked was how the anesthetic and the stimulation DCS dose affect physiological parameters in the weanling and juvenile rats.

During the imaging sessions, a few of the physiological monitors were not yet in place. Therefore, bench-side physiological measurements were acquired in two separate groups of naïve and sham operated rats (postnatal day 20-22 (N = 4) and postnatal day 33-34 (N = 3)) to determine the effects of anesthesia and stimulation-DCS dose during the subacute and chronic time points when performed pharmacological MRI (phMRI) was performed one and two weeks post-injury, respectively. In the subacute time point, end-tidal (exhaled) CO_2 was monitored using a micro-capnograph (Columbus Instruments, Columbus, Ohio). While end-tidal (exhaled), breathing rate (Bio-pac), and temperature (Bio-pac) were recorded in the postnatal day 20-22 pups, only breathing rate, heart rate, mean arterial pressure, and temperature were available in the postnatal day 34 animals. Enflurane anesthesia was used before and after the animals were awakened and the administered a stimulation- DCS dose (30mg/kg, i.p.).

Figures 34 and 35 show the bench-side physiological monitoring in two separate groups of animals at the two time points (subacute – PID3-5, and chronic – PID 13-21). In the anesthetized postnatal day 20-23 animals, administration of DCS resulted in a lowered breathing rate, while end-tidal CO_2 and temperature remained constant. This could mean that the maintenance of exhaled CO_2 levels and slower breathing rate with DCS on board could result from animals breathing deeper but less frequently. CO_2 is a vasodilator that causes the relaxation of smooth muscle within vessel walls, resulting in increased vessel diameter and blood flow. Blood flow would in turn result in increased blood volume. Conversely, lack of CO_2 could result in vasoconstriction and reduced blood flow and volume. However, if decreased respirations led to increased levels of CO_2, rCBV signals would be expected to increase; instead, rCBV showed no change at the early time point. Since physiological monitoring at the late time point showed no DCS-induced perturbations in any of the measured parameters, the data suggest that there

could be a DCS interaction with developmental stage of animal physiology. It is important to

consider other phMRI agents that have no physiological effects across developmental ages. In

future experiments, it is recommended that end-tidal CO_2 levels, blood pressure, breathing rate

and heart rate should be always be monitored during the imaging session.
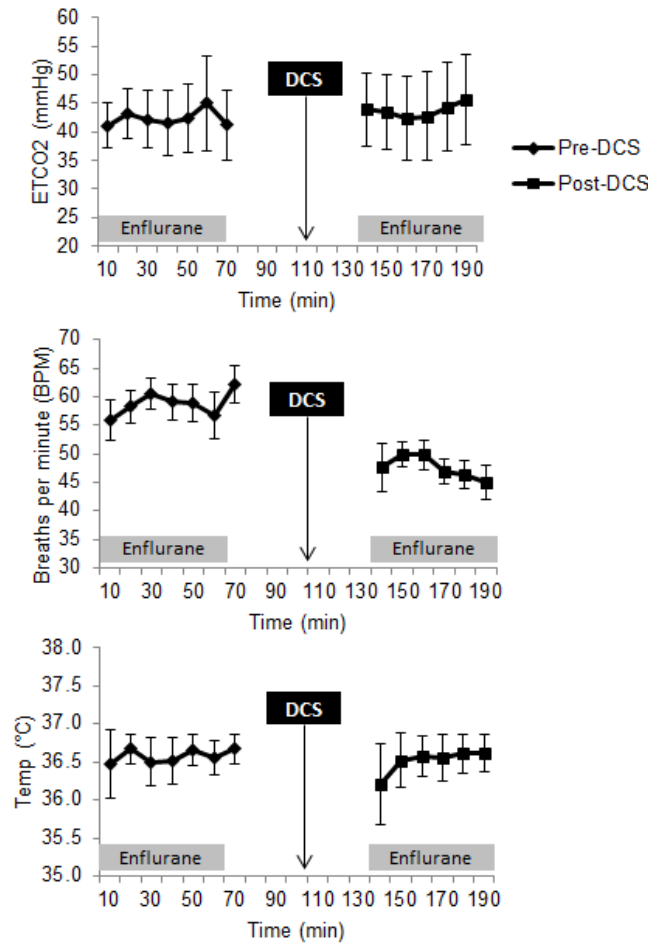
Figure 34.
Bench-Side Physiological Monitoring (Subacute)
(Mean ± SEM) End tidal (exhaled) CO_2, breathing rate, and temperature were measured in a
separate group of postnatal day 20 to 22 naïve rats (N = 4) under enflurane anesthesia before and
after the animals were awakened and administered a stimulation dose of DCS (30mg/kg, i.p.).
Although we observed decrease of breathing rate during the second scan, no significant effect of
$ETCO_2$ was detected between pre and post DCS.
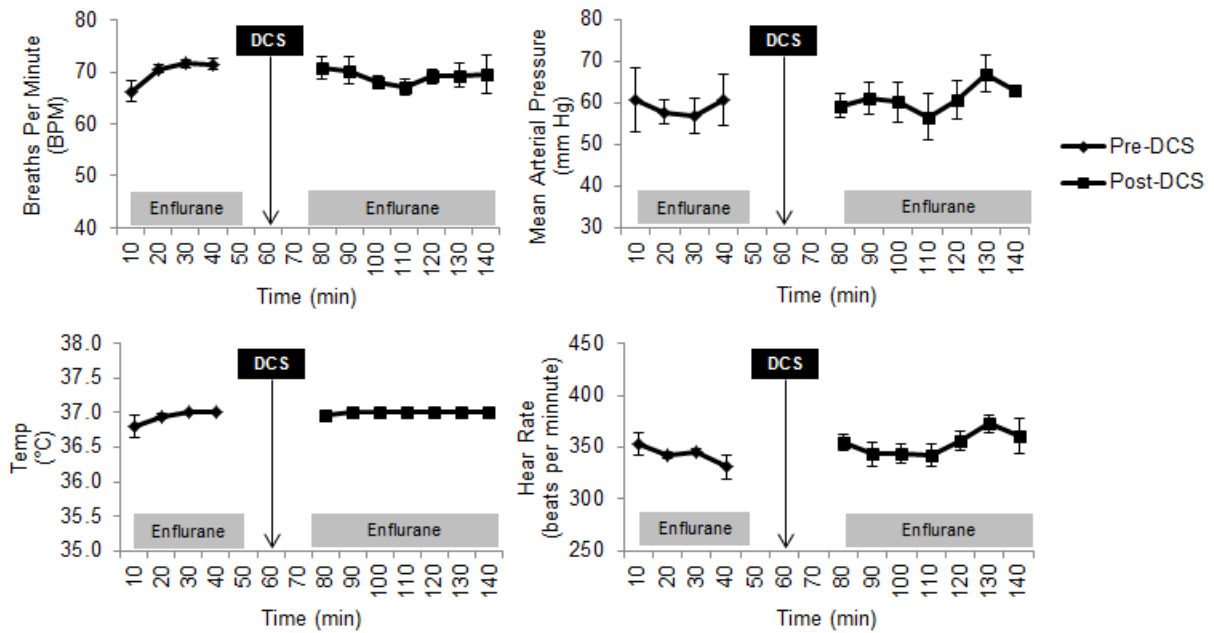
Figure 35. Bench-Side Physiological Monitoring (Chronic)
(Mean + SEM) Mean arterial pressure, breathing rate, heart rate and temperature were measured in a separate group of postnatal day 34 naïve control rats (N=3) No DCS-induced physiological perturbations were observed in the chronic time point.

A4. phMRI: DCS as a Drug Challenge – rCBV Concern

All animals underwent the DSCE-MRI protocol described in Chapter 3. In one day, animals were scanned 2 times. Scans were 90 minutes apart. rCBV values were generated from each scan from tracking the bolus injection (i.v.) of gadolinium (Magnevist). Although gadolinium has a half-life of 20 minutes, a concern was raised that there were still lingering levels of gadolinium in the body by the second post scan. A brain with contrast agent present would be dark (i.e. have reduced signal) in a T2-weighted image. Therefore, this brain would have a darker starting signal, and injection of more contrast agent during the second scan would result in artificially reduced rCBV values because maximal signal change could not be achieved.

This same concept of contrast agent pooling is quite relevant in TBI, in that TBI disrupts the blood brain barrier (BBB). Opening of the BBB would cause pooling of the contrast agent in post-injury tissues, which would artificially elevate levels of computed rCBV. If this occurs in our region of interest, DCS would fail to induce enhanced ΔrCBV (i.e. ceiling effect). This is great concern because the failure to evoked ΔrCBV is not due to the dysfunction of NMDAR-mediated neurotransmission, but instead due to pooling of contrast agent in tissue. This concern in turn raised two questions: 1) was the BBB intact during the post-injury imaging session in FPI animals? and 2) if the brain were already experiencing signal changes with contrast pooling, how can the DCS-evoked change in rCBV be parsed out from the overall brain rCBV change?

In addressing these issues, the experimental design for the phMRI sessions included timing of scans to coincide with reduced NMDAR expression and function as well as when the BBB integrity has been previously reported to be intact after a diffuse traumatic brain injury (Tanno et al., 1992; Osteen et al., 2001; Beaumont et al., 2006b). We also monitored the signal

intensity time curves during contrast agent bolus injections and observed that control and FPI subjects showed similar contrast agent bolus profiles. This indicated that the vasculature and BBB were intact during imaging. Additionally, rCBV values per session were normalized to the global rCBV mean, so that any region-specific changes would not be explained by a global change in cerebral blood flow or volume ((Panizzutti et al., 2005; Rausch et al., 2005)).

To investigate in more detail the effects of contrast agent pooling in our cohort, regions of interest (ROI) analysis was performed on the DSCoMAN generated raw rCBV maps from pre- and post-DCS injection. These values were then compared to the rCBV values normalized to the global rCBV mean. Data from Sham (N = 4) and FPI (N=6) data from Chapter 3 were used. Additional control (shams, N = 3) were also imaged before and after an i.p. saline injection. We observed that raw rCBV values appeared less in post-DCS than in pre-DCS scans (Figure 36 A and C). This meant that the trapezoidal integral under the $\Delta R2$ curve was indeed reduced in the post scans compared to the pre scans. However, these raw values did not account for the global effect of prolonged anesthesia exposure and DCS challenge. Therefore, normalizing to the global mean was indeed essential to control for signal amplitudes between imaging sessions. Normalized values revealed DCS-induced increase in hippocampal rCBV (Figure 36B). Control animals that received saline also showed reduced raw rCBV, but no change in normalized hippocampal rCBV (Figure 36D). Final percent change in rCBV (ΔrCBV) outcome measures presented in Chapter 3 were computed from the rCBV values normalized to the global mean (ΔrCBV = (norm_rCBVpost - norm_rCBVpre) / norm_rCBVpre) × 100).
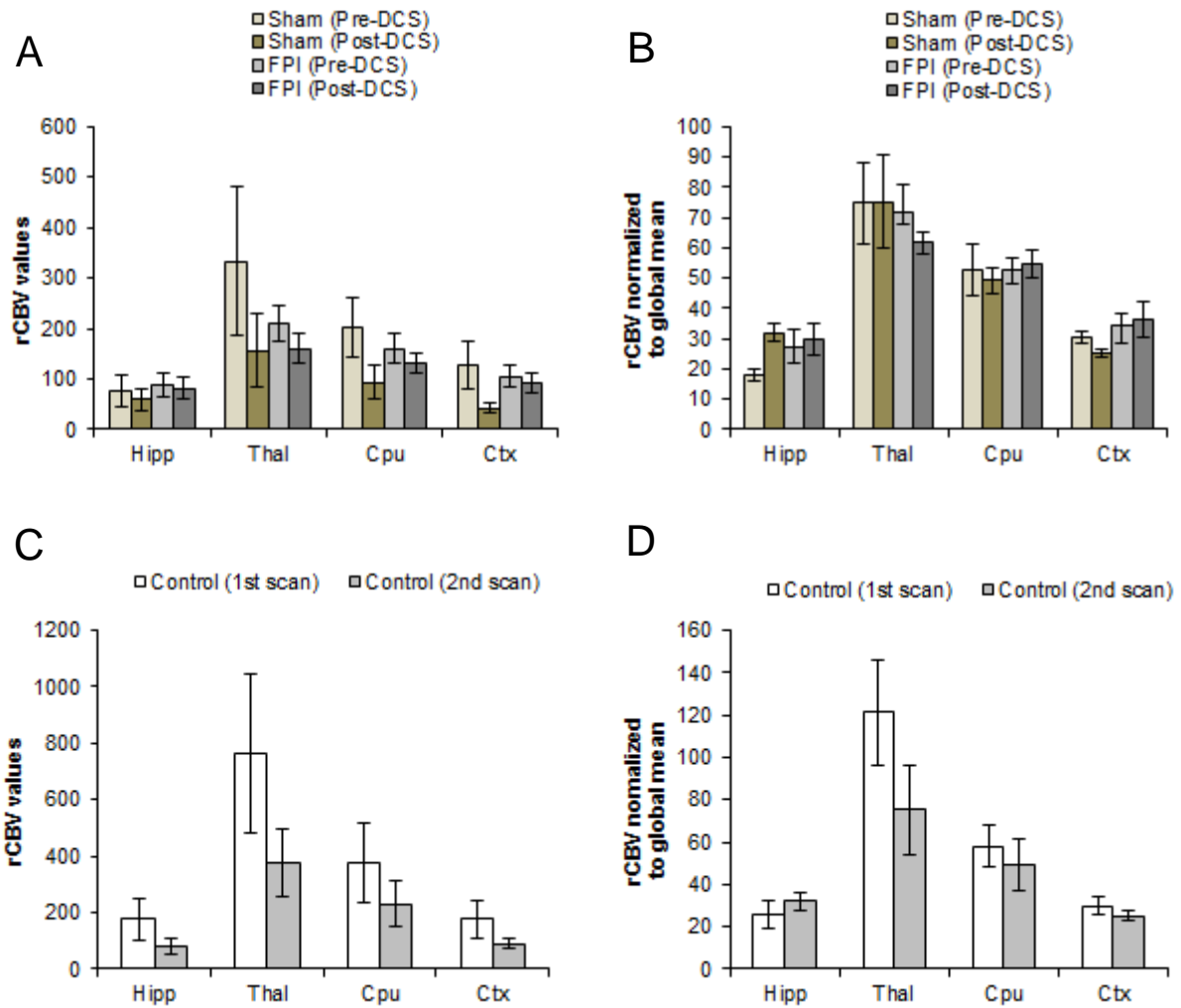
Figure 36. Raw and Normalized rCBV
(Mean ± SEM) rCBV (A and C) and normalized to global mean values (B and D) from DSCE-MRI scanned P22-24 animals (Sham, N = 4; FPI, N = 6) from Chapter 3 and Control (Shams) that received i.p. saline injection between the 1st and 2nd scans.

A5. phMRI: DCS as a Drug Challenge – rCBF

An alternative neurovascular coupled perfusion parameter, relative cerebral blood flow (rCBF), could also be obtained from tracking the gadolinium bolus during DSCE-MRI. rCBF was obtained from the ratio of the rCBF and the relative mean transit time (rMTT) The rMTT was estimated in DSCoMAN as the first moment of the ΔR2 curve. rCBF in control (Sham, N = 1; naïve, N = 2) and FPI (N = 3) animals that underwent DSCE-MRI in Chapter 3 were analyzed. DCS induced increases in normalized rCBF and ΔrCBF values (Figure 38). Taking both hemispheres together, FPI animals had significantly reduced rCBF (normalized to global mean) in hippocampus ($p < 0.05$) (Figure 38B). This result corresponds to reported reduced blood flow following TBI. The percent change in rCBF (ΔrCBF) values between the pre and post DCS administration did not significantly differ between control and FPI groups ($p = 0.1$). However, with the small sample size for each group (N = 3/group), analysis of the ΔrCBF parameter may be underpowered.
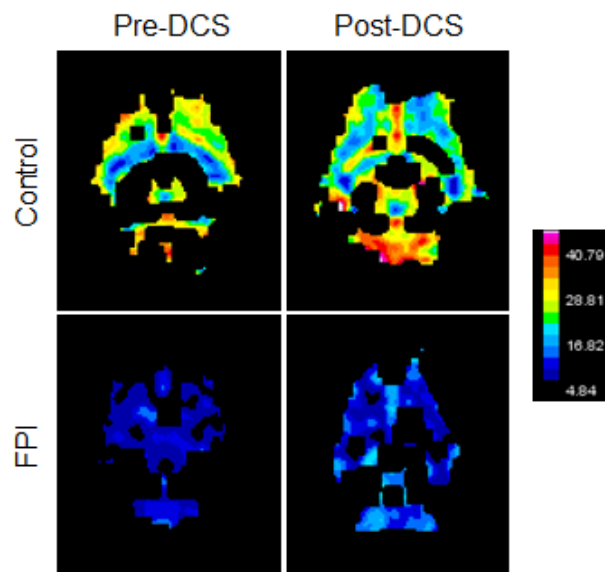

Figure 37. Representative rCBF Maps
Sample P23 Control and FPI rCBF maps on PID4.
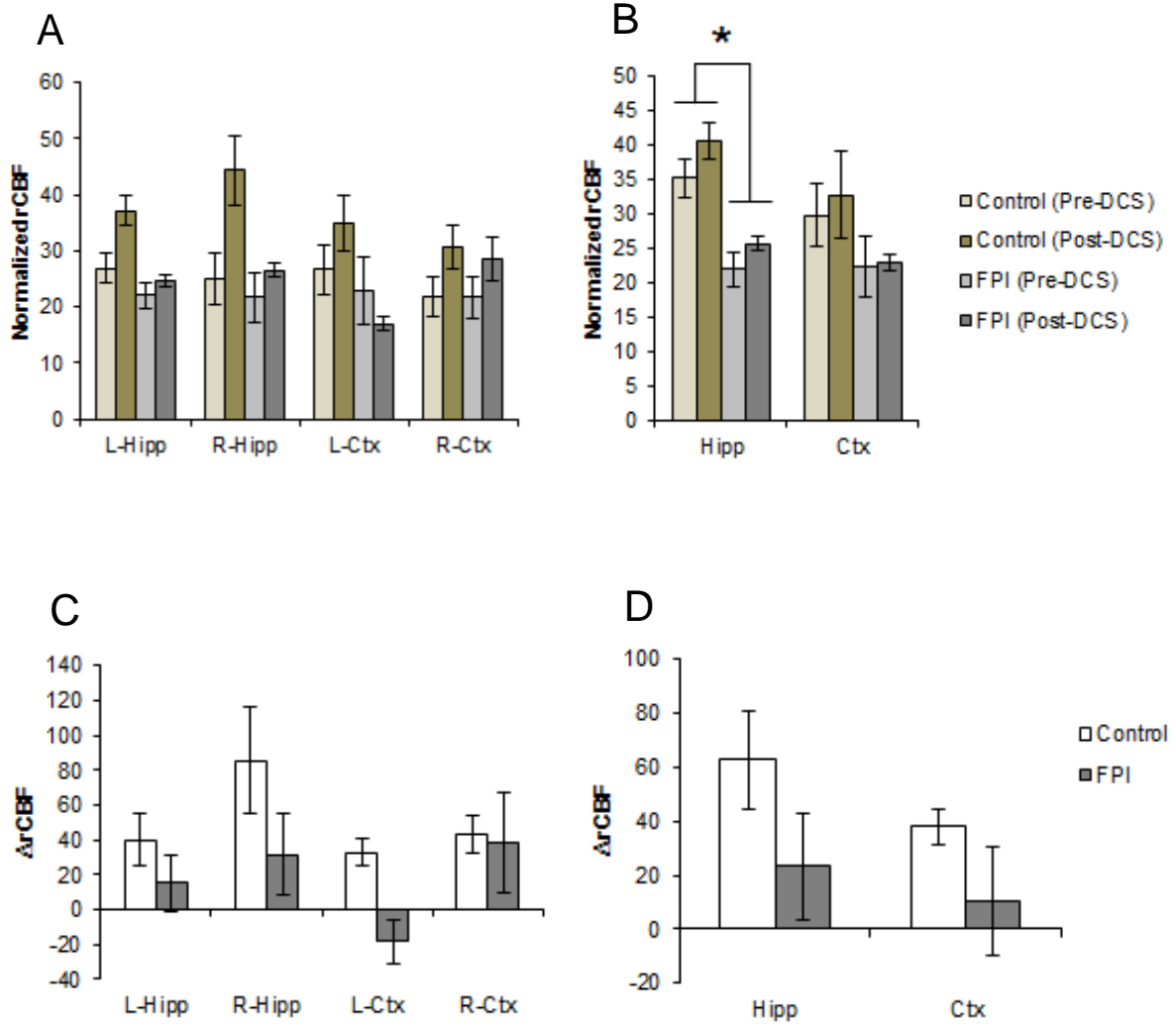
Figure 38. Normalized rCBF and ΔrCBF

(Mean ± SEM) normalized rCBF (A and B) and ΔrCBF (C and D) generated from Chapter 3 imaged P22-24 Controls (Sham, N = 1; naïve = 2) and FPI (N = 3) animals. A and C shows rCBF values for each left and right hippocampus and cortex.

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