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GABA-A Receptor Subunit Alterations Following Experimental Traumatic Brain Injury and the Effects of an NMDA Antagonist: A Western Blot Analysis

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A dissertation proposal submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AMPA	α-amin-3-hydroxy-5 methyl-4 isoxazale proponic acid
AMPA/KA	AMPA/kainate
ANOVA	analysis of variance
ATM	atmospheres
BBB	blood-brain barrier
β2/3	GABA-A receptor subunits β2 and β3
BZ	benzodiazepine
$[Ca^{2+}]_i$	intracellular calcium concentration
CaM kinase II	calcium/ calmodulin protein kinase type II
CBF	cerebral blood flow
CCI	controlled cortical impact
ChAT	choline acetyltransferase
Cl-	chloride
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computerized tomography
DAI	diffuse axonal injury
DG	dentate gyrus
DNA	deoxyribonucleic acid

DZ	diazepam
EAA	excitatory amino acids
EC	entorhinal cortex
ECF	extracellular fluid
EEG	electroencephalograph
eEF2	eukaryotic translation elongation factor 2
ER	endoplasmic reticulum
FP	fluid percussion
FPI	fluid percussion injury
GABA	gamma-aminobutyric acid
GABA-A	gamma-aminobutyric acid type A receptor
GABA-B	gamma-aminobutyric acid type B receptor
GABA-C	gamma-aminobutyric acid type C receptor
GABARAP	GABA-A-receptor-associated protein
GABA-T	GABA transferase
GAD	glutamic acid decarboxylase
GCS	Glasgow coma scale
ICP	intracranial pressure
IPSC	inhibitory post-synaptic current
IgG	immunoglobulin
IR	immunoreactive/ immunoreactivity
KA	kainic acid
LEA	lateral entorhinal area
LTP	long term potentiation
MABP	mean arterial blood pressure
MEA	medial entorhinal area

mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
MWM	Morris water maze
NMDA	N-methyl-D-aspartate
NT	neurotransmitter
PCP	phencyclidine
RNA	ribonucleic acid
SDH	subdural hematoma
SEM	standard error of the mean
SLM	stratum lacunosum-moleculare
SO	stratum oriens
SP	stratum pyramidal
SR	stratum radiatum
TBI	traumatic brain injury
tRNA	transfer ribonucleic acid
Zn+	zinc ion

Abstract

GABA-A RECEPTOR SUBUNIT ALTERATIONS FOLLOWING EXPERIMENTAL TRAUMATIC BRAIN INJURY AND THE EFFECTS OF AN NMDA ANTAGONIST: A WESTERN BLOT ANALYSIS

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Traumatic brain injury (TBI) produces an acute phase of neuronal excitation followed by a chronic phase of depressed neuronal function. Alterations in excitatory and inhibitory receptor interactions may be dynamically involved in subsequent long-term detriments in neuronal and cognitive functioning. TBI- induced elevations in intracellular calcium concentrations ($[Ca^{2+}]_i$) are mediated primarily by the NMDA receptor. Elevated $[Ca^{2+}]_i$ may trigger intracellular mechanisms which drive changes in GABA-A receptor protein synthesis and expression, ultimately resulting in receptor dysfunction.

Western blot analysis was used to investigate alterations to GABA-A receptor subunits $\alpha 1$ and $\beta 3$ in the hippocampus of rats 3 hours, 24 hours, or 7

days following TBI. No injury- induced alterations in protein expression were found for the β3 subunit, which in the hippocampus is primarily located on principal neurons (i.e., pyramidal and granule cells). No significant alterations to the α1 receptor were found 3 hours following TBI, but a significant increase in α1 protein was found 24 hours post- injury, and this increase persisted for at least 7 days. GABA-A receptors containing the α1 subunit are primarily located on interneurons, implying a potential strengthening of interneuron-mediated inhibitory tone during the chronic phase of TBI.

Study 2 used pre-injury injections of MK-801 (0.3 mg/kg) to block calcium influx through the NMDA receptor. This treatment normalized α1 protein expression 24 hours following injury (the time point of greatest change in study 1). NMDA-mediated calcium influx may, therefore, be responsible for triggering the cascade that results in increased GABA-A receptor α1 protein expression chronically following TBI.

These studies demonstrate that TBI produces an increase in GABA-A receptor α1, but not β3, subunits 24 hours and 7 days post-injury. The differential directions of the subunit changes may indicate a strengthening of inhibitory tone during the chronic phase of TBI, a period characterized by a depression of neuronal function. Although the exact mechanism of change to the

α1 subunit is unknown, study 2 indicates that it is driven by NMDA-mediated elevations in $[Ca^{2+}]_i$. The functional consequences of increased inhibitory tone may contribute to long-term detriments in cognitive and behavioral outcome following injury.

Introduction

Traumatic Brain Injury

Incidence and Causes

Traumatic brain injury (TBI) is the leading cause of death in people less than 40 years of age (Zauner & Bullock, 1998). Each year in the U.S., approximately 1.5 million people sustain a TBI, resulting in 50,000 deaths, 230,000 hospitalizations, and nearly 90,000 new cases of long-term disability. TBI is the leading cause of death and permanent disability among children and young adults. Currently, 5.3 million Americans (2% of the population) are living with TBI-related deficits, although this may be a low estimate (Center for Disease Control, 1999).

The incidence of TBI clearly demonstrates its importance as a public health problem. Because of its low visibility and high incidence, TBI has been referred to as the "invisible epidemic" (CDC, 1999), costing Americans approximately 37.8 billion dollars per year (Max, MacKenzie, & Rice, 1991). The risk of TBI is substantial across all age groups, with peak incidences among adolescents, young adults, and the elderly (75 years or older).

Although the incidence of TBI due to motor vehicle crashes has declined by 38% over the last two decades, transportation-related accidents still account for 49% of all TBI hospitalizations. Firearm violence accounts for only 10% of all TBIs, an 11% increase since1980, yet firearms have been the leading cause of

TBI-related death since 1990 (CDC, 1999). Falls are the leading cause of TBI among the elderly (CDC, 1999; Harrison-Felix et al., 1996).

Injury Severity

Although there are some discrepancies in TBI severity classifications, on average 60% of injuries are mild, 20% are moderate and 20% are severe (Frankowski, 1986). The Glasgow Coma Scale (GCS) is the most accepted rating scale for measuring injury severity. Mild injury is generally classified as a GCS score of 13-15, moderate injury is represented by a score of 9-12 and severe injury receives a score of 3-8. These are general categories and do not represent a strict definition. Because so many variables are involved, and the GCS score is subject to each doctor's interpretation, there is much controversy in the literature regarding the reliability of the scale. Factors contributing to the GCS score include eye, verbal and motor reactions. Also taken into account are length of unconsciousness, duration of amnesia or confusion, surgical requirements (such as removing blood clots), secondary insults such as seizures or ischemia, skull fractures and computerized tomography (CT) scan results (Colohan & Oyesiku, 1992). Mild, moderate, and severe injuries have varied pathologies and neuropsychological consequences.

Mild injuries generally do not include a period of unconsciousness, yet the average cost of treatment is $44,014 (Lehmkuhl, Hall, Mann, & Gordon, 1993). Cognitive impairments following mild head injury have not received much

attention because the deficits may be subtle. Many patients complain of post-concussive symptoms such as dizziness, headache, nausea, and confusion. The cognitive areas least affected by a mild injury include overall intelligence, language, perceptual and motor functions. The most prevailing cognitive deficits include problems with memory, attention, and information processing. Information processing problems typically diminish within three months of injury, whereas memory problems are not always noticeable during the first three months following injury. Most cognitive impairments diminish gradually over the course of the first year post-injury (Packard, Weaver, & Ham, 1993). Memory, attention and information processing deficits are likely to have an impact on the patient's lifestyle and job performance ability.

Moderate TBI results in an average of five days of unconsciousness and a mean treatment cost of $85,682 (Lehmkuhl et al., 1993). The outcome from moderate TBI varies widely. GCS scores of nine or ten have a 7-9% mortality rate and a much slower recovery compared to GCS scores of 11 or 12, which rarely result in death and are often moved to the mild category within 24 hours. Failure to return to work is estimated at 69%, a substantial increase over the estimated 34% for mild injuries (Rimel, Giordani, Barth, & Jane, 1982). Moderate injury often results in more impairment than mild injury, with 49% retaining a moderate disability, 10% a severe disability, and a 3% mortality rate from injury-related complications. This leaves only 38% who are classified as 'good

recovery.' Memory problems, which affect 90% of moderate injury patients, are the primary reason for unemployment. Predominating impairments tend to be in areas related to higher-level cognitive skills such as problem-solving, attention, visual reaction times, and memory for auditory and visual tasks. Overall intelligence does not seem to be greatly affected. The location and size of brain lesion resulting from the injury are related to the type and severity of the impairments (Colohan & Oyesiku, 1992).

Severe TBI is represented by 12-34 days of unconsciousness, with an average cost between $111,000 and $154,000 (Lehmkuhl et al., 1993). Severe head injury is more complicated and life-threatening than either mild or moderate injury. In one study of severely injured patients, 68% died during their hospital stay, 13.3% died shortly after discharge, and 6.1% remained in a vegetative state six months later. Only 12.5% were classified as functional survivors. Older patients (100% of those over 50) were the most likely to die. Functional recovery was only seen in patients less than 30 years of age. Low GCS scores and abnormal pupil responses were strong indicators of mortality (Quigley et al., 1997). Functional survivors of severe head injury are likely to face a lifetime of neuropsychological and physical disabilities. Only 3% return to comparable employment levels (Wehman et al., 1993).

The Costs of TBI

The monetary costs of TBI are staggering. The U.S. spends more than $37.8 billion per year in direct and indirect TBI-related expenses (Max, MacKenzie, & Rice, 1991). According to a 1999 report to Congress, TBI accounts for one third of all injury-related mortality (CDC, 1999). Fatal cases (6.3%) account for 84% of the total, including funeral costs and loss of revenue (Grabow, Offord, & Reider, 1984). Gunshots ($164,250) and motorcycle crashes ($165,294) account for the highest mean charges, while assaults ($89,940) account for the lowest charges (Lehmkuhl et al., 1993).

In 1995, the average acute care cost was approximately $105,800 and the average rehabilitative care was $58,400 (Harrison-Felix et al., 1998). A large portion of these costs are paid for by public funds. In 1992, military hospitals paid almost 42 million dollars for TBI-related injuries, the median cost per patient being $35,400 (Ommaya et al., 1996). Estimates indicate that 29-31% of acute care and 31-39% of rehabilitative care are paid for by Medicare or Medicaid (Harrison-Felix et al, 1996; Lehmkuhl et al., 1993). Further costs to the public include loss of employment. Approximately 39% of those employed at the time of injury are unable to return to work one year later (Harrison-Felix et al., 1996).

The indirect costs of TBI are likely to have the greatest impact on patients' lives. They include short term intellectual, motor, and learning impairments and long term rehabilitation, revenue loss, and intellectual limitations in the areas of memory and concentration.

Injury Pathology

Types of Injuries

The mechanism of injury determines the resulting pathology. There are two main types of brain injuries. First, injuries are classified as either penetrating or non-penetrating. A penetrating injury, from a bullet or other object, may cause tissue destruction, brain lacerations and hemorrhage. Non-penetrating injuries usually result from blunt impact of the brain with the inside of the skull. These closed head injuries are known as acceleration-deceleration and may occur either linearly or rotationally within the skull. The second major injury type refers to focal or diffuse injuries. A focal injury is limited to a specific region of the brain, resulting in contusion, laceration, hemorrhage, and/or infarction. Focal injuries may result from penetrating or non-penetrating blows to the head. The frontal and temporal lobes are especially vulnerable to focal injuries. Diffuse injury may also include axonal injury and secondary loss due to hypoxia or ischemia. Secondary insults often result from increased intracranial pressure (ICP) or cardiovascular collapse following a closed head injury. Diffuse injuries typically lead to specific patterns of necrosis in the cortex and infarctions of the hippocampus and basal ganglia (Selzer, 1995).

Primary and Secondary Injuries

Primary brain injury occurs at the moment of impact. The initial mechanical insult produces primary damage, including surface contusion and

laceration, diffuse axonal injury (DAI) and intracranial hemorrhage. Primary (mechanical) damage affects blood vessels, axons, neurons and glia and may represent a focal, multi-focal or diffuse pattern of injury. DAI, focal contusions and intracranial hematomas due to hemorrhage provide the potential for secondary damage such as ischemia, hypoxia, cerebral swelling/edema, increased ICP, hypotension, and seizures (Alessandri & Bullock, 1998).

Primary methods of injury include contact of the skull with a foreign object or unrestricted head movement due to acceleration-deceleration forces of the brain within the skull. Contact injuries primarily result from falls and result in focal damage such as local or regional lesions, skull fracture, surface contusions and intracranial hemorrhage. Acceleration-deceleration injuries primarily result from motor vehicle crashes and produce diffuse types of injuries. Shear, tensile and compressive strains often occur due to diffuse injuries and may result in subdural hematomas (SDH) and widespread axonal damage (McIntosh et al., 1996).

Secondary damage involves changes that are initialized at the time of impact. The biochemical cascade of events that follows involves changes leading to neuronal damage. Neuronal cell body injury in the gray matter occurs in stages. Neurons in direct contact with an object or the skull die immediately and form a core of primary damage. Membrane ionic balance is disrupted in damaged neurons, which release excessive excitatory amino acids (EAA), resulting in further depolarization and calcium entry into nearby neurons.

Further complicating the injury potential, delayed secondary damage may be more severe than the initial loss. Secondary damage may include ischemia, swelling (edema), and alterations of normal neurochemical functions and mechanisms. Secondary damage may not present clinically for hours or even days, providing a potential window for intervention. Recovery begins with neurons that may have been damaged but not killed (Selzer, 1995). Primary and secondary consequences of TBI are summarized in Table 1.

The Excitotoxicity Hypothesis

Glutamate, the most widely distributed excitatory neurotransmitter (NT) in the brain, is toxic at high concentrations. High levels (10 to 200 μM) are released into the extra-cellular fluid (ECF) following injury (Alessandri & Bullock, 1998). The excitotoxicity hypothesis, as it relates to TBI, postulates that activation of muscarinic cholinergic and/or N-methyl-D-aspartate (NMDA) type glutamate receptors occurs due to excessive TBI-induced neuronal depolarization and subsequent neurotransmitter (NT) release. This over-excitation then contributes to resulting TBI pathophysiology (Hayes, Jenkins, & Lyeth, 1992b). Glutamatergic receptor antagonists administered before injury have been shown to reduce histological, functional and behavioral

Table 1. TBI-induced primary and secondary damage

Types of Primary and Secondary Damage	
Type of Injury	**Type of Damage**
Laceration (cuts)	Primary
Contusion (bruising)	Primary
Disturbed ion gradients	Primary
Edema (swelling)	Primary / Secondary
Hematoma (due to hemorrhage)	Secondary
Ischemia (decreased blood flow)	Secondary
Hypoxia (decreased oxygen)	Secondary
Diffuse Axonal Injury (DAI)	Secondary (Primary at membrane)

consequences in animal models of TBI (Alessandri & Bullock, 1998). Neurochemical alterations following TBI are related to excitotoxic processes and abnormal agonist-receptor interactions. Treatment potential lies in the possibility that neurochemical processes which mediate brain pathophysiology associated with TBI may respond to pharmacological therapies (Hayes, Jenkins, & Lyeth, 1992b).

Experimental Models of TBI

The Biomechanics of Experimental Brain Injury

Valid TBI models mimic the mechanical forces, causes, and consequences which occur in human patients. The mechanical loading forces include impact forces and inertial (acceleration) forces. Brief (usually < 50 msec) mechanical deformation results in brain injury. The type and extent of brain injury are dependent on the location, magnitude and direction of the loading forces.

The most common mechanical causes of TBI are due to impact loading forces. Impact loading refers to direct contact between the head and a solid object. These contact effects result in local deformation of the skull, overall brain movement within the skull, and potentially devastating tissue strain to the underlying neuronal tissue. Inertial loading refers to indirect head movements resulting from an impact to other areas of the body. Inertial acceleration effects produce an overall pressure distortion and neuronal tissue strain which results in primary tissue damage (McIntosh et al., 1996).

Contact Effects due to Impact

Impact loading forces may result in skull fracture due to focally distributed forces. Local skull depression produces distinctive stress zones caused by waves of distortion radiating from the point of impact. These waves disperse and meet at various places within the skull, causing additive pressure and potential skull fracture in areas remote from the impact site. Local skull distortion may also occur without overt fracture. The amount of local skull displacement is an indication of the pressure and distortion experienced by underlying tissue and the tissue affected by stress waves radiating from the point of contact.

Impact forces produce contact effects such as skull bending, intracranial pressure (ICP), and focal lesions due to contusions, lacerations and hematomas. An intracranial hematoma is often considered a more extensive form of contusion and a subdural hematoma is due to vascular damage underlying the impact site. Epidural hematoma is more likely caused by torn vessels due to fracture (85%) than to ICP (15%) (McIntosh et al., 1996).

Inertial (Acceleration) Effects

Inertial acceleration of the brain within the skull produces a separate type of injury with different mechanical effects. There are two primary types of inertial effects: translational acceleration (acceleration-deceleration movement along a straight path) and rotational acceleration (brain rotation within the skull). Translational acceleration produces movement of the brain within the skull and

increased ICP. The magnitude of peak ICP is directly due to the level of translational acceleration. Rotational acceleration produces widespread tissue strain within the brain and the shear strain is directly related to the level of rotational acceleration, direction of motion, and potential intracranial impact with dural compartments such as the tentorium cerebri (McIntosh et al., 1996).

Assumptions and Hypotheses in Animal Modeling

Animal models rely on the hypothesis that human injury can be duplicated in nonhumans. Some assumptions must be made in order to allow generalization from a model to human conditions. Species differences are assumed to be of minimal consequence, and the injury production mechanism is a means by which to model the resulting sequella. The injury mechanism in humans takes on greater importance due to safety measure development and prevention.

There are several important aspects of injury that should be represented in a good animal model, including the location of brain damage, the type of damage produced, the severity and time course of injury, and long term and short term alterations. The model should also be able to effectively assess changes in morphology, cerebrovascular processes, metabolic receptors, and behavior (Gennarelli, 1994).

Acceleration Concussion and Percussion Concussion

Two categories of injury were distinguished by Denny-Brown and Russell in 1941: acceleration concussion and percussion concussion. Acceleration

concussion is modeled primarily by inertial injury models. These models produce acceleration without impact or with diffuse loading and have been used in primates, cats, and swine. Another acceleration concussion model is impact acceleration, which is also referred to as the weight drop model. Impact acceleration involves an impactor such as a piston or weight, which is dropped directly onto the skull or onto a steel plate covering the skull. The latter minimizes localized skull loading and fracture. This model has been demonstrated effectively in primates, cats, and rats (Gennarelli, 1994).

Fluid percussion injury (FPI) is a widely used model of percussion concussion. This model was used in cats until 1987, when it was modified for use in rats due to their compatibility with behavioral tasks. The central, lateral, or lateral with contralateral dura opening variations of the model have also been used in dog, rabbit, and swine. In this model, a small fluid volume is injected into subdural or supradural space. Impact is provided via a fluid column or rapid pump infusion.

Another model of percussion concussion is rigid indentation, also known as controlled cortical impact (CCI). In this model, a piston strikes the brain directly through a large craniotomy hole. Impact is controlled at about 2 to 3 m/sec and penetrates 2 to 3 mm deep. Variations of this model include central, lateral, or lateral with contralateral open dura injuries.

Other models of injury that have very specific usefulness include injection models, where a small amount of fluid or blood is injected into epidural, subdural, or intracerebral brain locations. Local tensile models produce injury under pressure or suction of the open dura (Gennarelli, 1994).

The most frequently used animal models of TBI include central or lateral FPI, central or lateral CCI, weight drop, and injection models (for subdural hematoma injuries). Areas of particular interest to the excitotoxicity hypothesis of injury include: DAI, contusion, fracture, acute subdural, epidural, or intracerebral hematoma, and brain swelling (Alessandri & Bullock, 1998). Animal models have done well in their attempts to model morphological changes following TBI but areas that are less well characterized include: neurological changes in the areas of cognition, memory and long-term outcome; physiology, as in cerebral metabolism; and biochemistry, such as gene expression and ionic changes (Gennarelli, 1994).

Models of Excitotoxicity

Models that involve glutamate infusion have been shown to activate all glutamate receptor subtypes. Glutamate is then removed from the synapse by astrocytes. Other excitotoxic infusions such as kainic acid (KA), N-methyl-D-aspartate (NMDA), or α-amin-3-hydroxy-5 methyl-4 isoxazale proponic acid (AMPA) do not have this synaptic removal property (Alessandri & Bullock, 1998). Excitotoxicity of glutamate has been demonstrated to produce hypermetabolism,

which is neuro-protected with NMDA and AMPA/Kainate (AMPA/KA) receptor antagonists such as D-CPPene, MK-801, and NBQX, as well as with the free radical and lipid peroxidase inhibitors I74006F and D-amphetamine (Fujisawa, Landolt, & Bullock, 1996; Hovda et al., 1995; Sutton, Hovda, Chen, & Feeney, 2000). These in-vivo models of excitotoxicity provide support for the potential damage of excessive glutamatergic activity. Glutamate infusion damages cortical tissue synergistically following FPI, likely due to the increased vulnerability of neurons to glutamate following injury (Bullock & Di, 1997). Although glutamate is the best characterized excitotoxin, acetylcholine (ACh) and kainic acid (KA) also have excitotoxic properties that have been demonstrated both in vitro and in vivo (Regan & Choi, 1991). The concentrations needed to kill cortical neurons are lowest for NMDA, then AMPA, and kainic acid, respectively. The strongest excitotoxic effects are produced by glutamate (Regan, 1996).

Models of Focal and Diffuse TBI

Physical, computer and cell culture models have all contributed to the understanding of specific aspects of head injury. However, animate models are the only true representations of the complex changes that occur within a living organism in response to brain trauma. Two distinctions have been made in model type: focal and diffuse (McIntosh et al., 1996). Each of these types of injuries has their own sequence of changes and cascades of events, some of

which are similar and some of which are distinctively different (see Table 2 for a complete summary).

Focal injury models describe the pathology concerning contact effects from impact forces. Cortical contusion is common to focal injuries and has been well-characterized in rats (Dixon et al., 1987; Dixon, Glifton, Lighthall, Yaghamai, & Hayes, 1991; Feeney, Boyeson, Linn, Murray, & Dail, 1981; McIntosh et al., 1989; Nilsson, Ponten, & Voight, 1977; Shapira et al., 1988; Toulmond, Duval, Serrano, Scatton, & Benavides, 1993), mice, cats, ferrets, pigs, and other primates (Lighthall, 1988; Lindgren & Rinder, 1965; Ommaya, Hirsch, & Flamm, 1966; Smith et al., 1995; Sullivan et al., 1976). Primary skull displacement lasting approximately 10-30 msec occurs due to contact (focal) loading forces (McIntosh et al., 1996). TBI with contusion may be analogous to ischemic focal forebrain infarction (Hayes, Jenkins & Lyeth, 1992b). TBI models that produce cortical contusion may also cause damage to areas remote from the injury site (McIntosh et al., 1996).

Several models are available that can produce a focal injury (see Table 2), including impact acceleration (weight drop) (Feeney et al, 1981; McIntosh et al., 1989; Nilsson, Ponten, & Voight, 1977), fluid percussion (Dixon et al., 1987; Lindgren & Rinder, 1965; McIntosh et al., 1989; Toulmond et al.,1993), and rigid indentation (CCI) (Dixon et al., 1991; Soares, Thomas, Cloherty, & McIntosh, 1992; Smith et al., 1995). A common feature of each of these models is that the

Table 2. The injury characteristics of commonly used animal models of TBI

Injury Characteristics of TBI Animal Models			
Animal Model	**Focal/ Diffuse Injury**	**Uses- Injury Type**	**Species Studied**
Cortical weight drop/ Impact Acceleration	Focal	Contusion Neuronal loss Potential seizures Impaired motor ability	rat
Cortical impact (CCI)	Focal	Contusion Axonal damage	rat, ferret
Fluid Percussion (FP)	Both Lateral and Central effects: Focal (Lateral) Diffuse (Central)	Behavioral dysfunction Brief coma Impaired motor ability Axonal injury Vascular abnormalities Neurochemical changes Possible contusion Neuronal cell loss Unilateral damage Brain stem involvement Bilateral damage	rat, cat, micropig, non-human primates
Inertial injury	Diffuse	Full spectrum of human head injury, morbidity, and coma	micropigs and non-human primates

head is held steady in one position as the injury occurs (McIntosh et al., 1996). The weight drop model often produces contusion at the injury site, neuronal loss of the hippocampi, thalamus and brain stem nuclei, and may produce skull fracture, prolonged coma, DAI and seizures (Beaumont et al., 1999; Foda & Marmarou, 1994; Gennarelli, 1994; Marmarou et al., 1994; Povlishock, Hayes, Michel & McIntosh, 1994). The rigid indentation model (CCI) normally produces a focal contusion at the injury site, axonal damage, and lower mortality due to reduced brain stem damage, compared to the cortical weight drop model (Povlishock et al., 1994). Central CCI produces unconsciousness, contusion to the parasagital cortex under the impact site and non-diffuse axonal damage. Lateral CCI injuries produce a brief coma, a small amount of diffuse axonal damage and contusion at impact site (Gennarelli, 1994).

In the FPI model, fluid is injected through a sealed cannula-type hub into the closed cranium. This model has been well characterized. It has been associated with behavioral detriments, transient coma, impaired motor function, and altered learning and memory (Povlishock et al., 1994). Structural abnormalities have been noted, including subarachnoid hemorrhage and axonal injury. Cell loss in the cortices and hippocampi is common in moderate severity injuries, with contusion likely at higher severities, especially in lateral injuries (Gennarelli, 1994). Cerebral vascular abnormalities in blood flow and blood-brain barrier (BBB) permeability, neurochemical changes controlling ionic

homeostasis, and metabolic alterations have also been documented (Povlishock et al., 1994). For focal injuries, the lateral FP model is preferable, with the central FP model producing a more diffuse type of injury (Gennarelli, 1994; Povlishock et al., 1994).

Lateral FPI is more likely to result in focal contusion. Cortical changes are often unilateral to the side of injury, sparing the contralateral cortex and brain stem. Ipsilateral changes in white matter axons and occasional deep tissue tears at gray-white matter junctions may occur. Unilateral hippocampal damage has been well characterized (Gennarelli, 1994).

Central FPI is less likely to produce contusion and overt cell loss at the impact site. Bilateral cortical responses and brain stem involvement (including axonal injury) are common features of the central FPI model (Gennarelli, 1994; McIntosh et al., 1996, Povlishock et al., 1994). FPI, however, provides only limited biomechanical control and does not represent the full spectrum of human TBI (e.g., prolonged unconsciousness). Higher severity levels of injury are often complicated by brain stem involvement and rats are more susceptible to the confound of pulmonary edema (Povlishock et al., 1994). Diffuse TBI-induced depolarization without contusion has similar neuropathological consequences as diffuse forebrain ischemia, resulting in selective neuronal deficits without overt cell loss (Hayes, Jenkins & Lyeth, 1992b).

Species Selection in Animal Modeling

The choice of species in TBI modeling is important. There may be different patterns and distributions of receptors between humans and various other species. This must be weighed against the societal and financial expenses involved in using phylogenetically closer species. Rodent animal models, particularly rats, have been well-characterized and have the benefit of providing extensive normative data. Their small size and availability permits exhaustive structural and functional studies to be performed (Povlishock et al., 1994). Their age, genetic background, and environment can all be controlled to reduce experimental variability, and their high infection resistence and compatibility with neurochemical and neuropharmacological techniques have made them a popular choice in animal models. Central FPI in rats has been shown to be reliable at mild to moderate levels, without the complications of focal tissue damage (Dixon et al., 1987).

Important similarities between humans and rodents include impact depolarization and a high correlation between rodents' receptor pharmacology and neurochemical changes and humans' TBI-induced behavioral deficits (Hayes, Jenkins & Lyeth, 1992b). Rodents' physiologic responses may differ from humans, however, and they do not provide complete modeling of complex human changes due to their smaller neocortex and their lack of complex gyri and sulci (Povlishock et al., 1994).

Parallels Between Experimental Fluid Percussion Injury and Human TBI

The pressure forces recorded from human cadaver skulls upon impact are similar to the forces modeled by experimental FPI (Lindgren & Rinder, 1966). Acute neurological symptoms and suppression of behavioral reflexes mimic human unconsciousness/coma (Teasdale, 1976). Dixon et al. characterized the FPI model in rats in 1987, using neurological and histopathological endpoints following various injury severity levels. Acute neurological evaluations indicated that mortality was positively correlated with injury severity, with an average mortality for moderate injury around 30-35%. A primary cause of mortality was pulmonary edema, which was not significantly correlated with injury severity. Convulsions were only seen at higher injury levels (>2.1 ATM) and a positive correlation was found between injury magnitude and apneic episodes (respiration cessation and resumption >10 sec). Acute somatomotor responses were developed to be similar to human reflexes on which GCS scores are based. Somatomotor nonpostural responses correlated to injury magnitude included corneal and pinna reflexes. Somatomotor postural responses, also correlated to injury magnitude, included paw flexion, tail flexion, startle reflex, and righting reflex. Sham animals were normal within one minute of removal from the injury device. Systemic cardiovascular variables included: increased mean arterial blood pressure (MABP), which peaked within 10 sec and was not graded by injury severity level; brief bradycardia (5-10 sec), with the heart rates of all injury levels lowered to 50% of baseline, and decreases in pO_2 and pCO_2 at high

severity levels only. Plasma glucose increased at 5 min for all injury groups and pulmonary edema ("pink fulminating exudate") increased carbon dioxide, lowered oxygen and produced 100% mortality. Most of the measures documented in this study (and others) show similarities to moderate human head injury, as measured by the GCS (Dixon et al., 1991).

The Hippocampus

The hippocampus resides within the temporal lobe and is one of several brain structures that are selectively vulnerable to TBI. The hippocampal formation is an elongated, C-shaped structure that wraps around the diencephalon from the septal nuclei of the forebrain to the temporal lobe. The hippocampal formation consists of four primary structures: the dentate gyrus (DG), the entorhinal cortex (EC), the hippocampus proper, and the subicular complex. The hippocampus proper is further divided into CA1, CA2 and CA3 regions. The structures of the hippocampus have a distinctive laminar orientation. Although most of the hippocampal connections are feed forward, GABAergic interneurons also provide feedback information to the original structure. The EC provides the major hippocampal input via the perforant pathway, terminating primarily in the DG. The DG projects mossy fibers to the CA3 fold of the hippocampus proper, which in turn projects to the CA1 region. The subiculum receives the primary output from the hippocampus proper. Hippocampal structures communicate ipsilaterally via associational projections

and by commissural projections to the contralateral structures (Amaral & Witter, 1995) (See Figure 1).

Subsectors of the Hippocampal Formation

Entorhinal Cortex

Information from cortical structures such as the perirhinal cortex, the tetrosplenial cortex and the medial frontal cortex are relayed to the hippocampus via the EC. Feed-forward projections from the EC innervate the DG and the CA1 and CA3 regions of the hippocampus. The EC receives feedback information from the CA1 and from the subiculum. Projections from the EC to the hippocampal regions are collectively referred to as the perforant pathway. The perforant pathway consists of projections from many EC cell types, including stellate, pyramidal, GABAergic, and others (Amaral & Witter, 1995).

The EC has two main subdivisions: the lateral entorhinal area (LEA) and the medial entorhinal area (MEA) (Amaral & Witter, 1995). The primary perforant pathway projections are to the outer two-thirds of the molecular layer of the DG, where terminals synapse on dendritic spines of granule cells. There are also some synapses on GABAergic interneurons. The LEA projects to the outer one-third of the molecular layer of the DG and the MEA projects to the middle one-third of the DG (Hjorth-Simonsen,1972; Nafstad, 1967; Steward, 1976; Witter, 1993; Wyss, 1981). The inner one-third of the DG receives mossy cell projections from the polymorphic layer (Amaral & Witter, 1995). The perforant

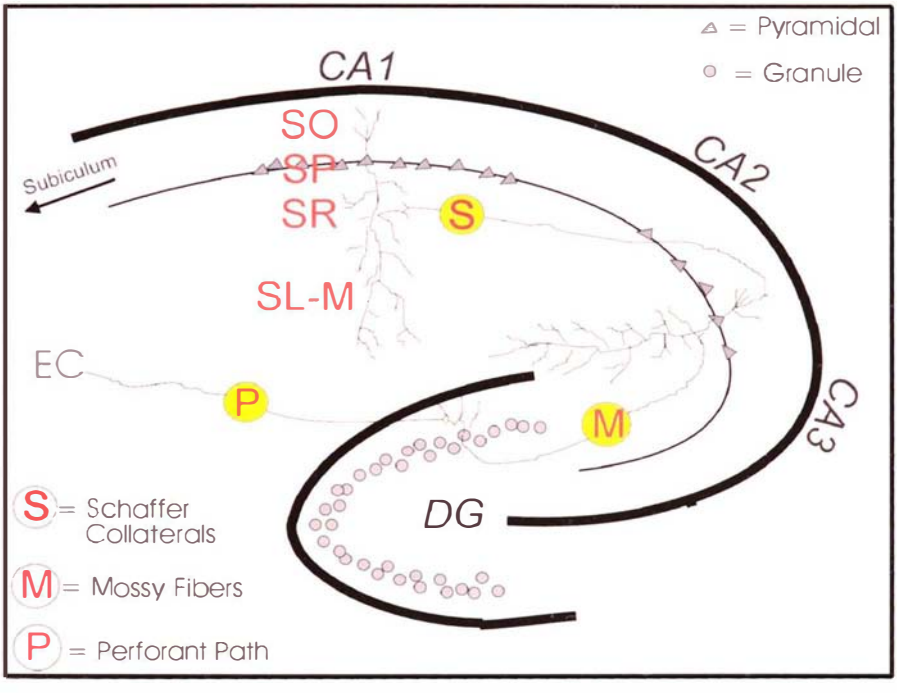
Figure 1. A diagram of a coronal section of the mid-dorsal hippocampal formation of the rat. The primary connective pathways are shown: Schaffer collaterals (S), mossy fibers (M), and the perforant pathway (P). The hippocampus proper is subdivided into CA1, CA2, and CA3 regions and the dentate gyrus (DG) is also shown.

Key: SO = stratum oriens

SP = stratum pyramidal (pyramidal cell layer)

SR = stratum radiatum

SLM = stratum lacunosum moleculare


= Pyramidal
= Granule
CA1
CA2
CA3
DG
SO
SP
SR
SL-M
Subiculum
EC
S
P
M
S = Schaffer Collaterals
M = Mossy Fibers
P = Perforant Path

pathway also has some feed-forward projections to the CA1 and CA3 regions of the hippocampus proper, although only the CA1 reciprocates these projections (Amaral & Witter, 1995; Nafstad, 1967; Steward & Scoville, 1976).

Dentate Gyrus

The laminar structure of the DG consists of the molecular layer, the granule cell layer, and the polymorphic layer. The molecular layer primarily contains apical dendrites of granule cells as well as some smaller stellate cell bodies. The granule cell layer, consists primarily of granule cell bodies, although GABAergic basket cells are found nestled between the granule and polymorphic layers (Amaral & Witter, 1995). There is approximately one basket cell to every 180 granule cells (Amaral, Ishizuka, & Claiborne, 1990). The polymorphic layer, commonly referred to as the hilus, contains mossy cells as well as basal dendrites and axonal projections of granule cells.

Although relatively cell-free, the molecular layer does contain some basket cells and axo-axonic interneurons known as chandelier cells (Amaral & Witter, 1995). These interneurons are primarily GABAergic, providing pre-synaptic input to the perforant pathway and synaptic input to dendrites of the granule cells in the granule cell layer. GABAergic chandelier cells may contribute to regulation of granule cell excitatory input from the EC (Somogyi et al., 1985; Soriano & Fotscher, 1989).

The granule cell layer consists primarily of granule cell somata which extend their axons to the CA3 region. The stratum lucidum layer, found only in the CA3 region of the hippocampus, consists of mossy fiber projections from the granule cells of the DG. Mossy fibers bend temporally, forming an "end bulb," which demarcates the CA3 and CA2 regions of the hippocampus. Besides axo-axonic feedback from interneurons, granule cells also receive synaptic input from basket cells, which are also primarily GABAergic. Because of the wrapped orientation of the hippocampus, the molecular and granule cell layer meet and form a 'V' shape. The suprapyramidal blade refers to the layered portion closest to the CA1 and the infrapyramidal blade is furthest from the CA1. Between the blades of this region, basal to the granule cell layer, is the polymorphic layer (Amaral & Witter, 1995).

Mossy cells are the most common cell type in the polymorphic cell layer. Mossy cells have large triangular or multipolar shaped bodies with proximal dendrites covered in spines or "thorny excrescences", which are termination sites of mossy fiber axons (Ribak, Seress, & Amaral, 1985; Frotscher, Seress, Schwerdtfeger, & Buhl, 1991). These spines are also seen in proximal dendrites of CA3 pyramidal cells. Primarily glutamatergic, mossy cell projections may form as many as 37 synapses with a single CA3 pyramidal cell dendrite (Amaral & Witter, 1995; Chicurel & Harris, 1992).

Hippocampus Proper

The hippocampus proper consists of CA1, CA2, and CA3 regions. CA2 and CA3 areas contain primarily large pyramidal cells, whereas pyramidal cells of the CA1 are noticeably smaller. The narrow CA2 differs in poorly characterized connectional and functional ways from CA1 and CA3 regions. The border of CA2 and CA3 is marked by the termination of the stratum lucidum.

Hippocampal laminar organization is similar in all areas of the hippocampus proper. The pyramidal cell layer is the main cellular layer. Basal dendrites extend into the stratum oriens (SO) layer. Apical dendritic trees extend through the stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) layers. The SR layer receives input from CA3 to CA1 Schaffer collaterals. CA3 to CA3 associational (ipsilateral) and commissural (contralateral) terminals are also located in the SR layer. Some EC perforant pathway fibers travel and terminate in the SLM layer of the CA1, although the majority of perforant pathway input is to the DG (Amaral & Witter, 1995).

Although not well characterized, there are a variety of primarily GABAergic local interneurons located throughout the layers of the hippocampus proper. The pyramidal cell layer consists primarily of pyramidal cells but GABAergic basket cells are also present, located along the SP/ SO border (Seress & Ribak, 1984). These basket cells are the most common type of interneuron in the CA1. Basket cells of the SP/ SO border directly inhibit pyramidal cells in this region (Thompson, 1994). The dendrites of the basket cells appear beaded and have

few dendritic spines. In contrast to the basket cells at the SP/ SO border, the interneurons at the SR/ SLM border do not receive CA1 input. These interneurons terminate on the distal dendrites of the SO layer in the CA1 (Amaral & Witter, 1995). Interneurons of the DG and CA3 receive direct input from septal projections (Amaral & Witter, 1995; Freund & Antal, 1988). CA3 interconnections to both CA3 and CA1 are divergent and extensive. As many as 6000 CA3 neurons (1.9% of the CA3 cell population) may innervate a single other CA3 neuron. Also, a single CA1 neuron may be innervated by as many as 5500 CA3 neurons (1.8% of the CA3 cell population). CA3 projections may, therefore, play important roles in hippocampal inter-communication (Amaral, Ishizuka, & Claiborne, 1990).

Subiculum

The primary output from the hippocampus proper originates in the CA1 and is relayed to the subiculum. The subiculum projects to the EC and also has minor projections to other cortical areas, including the limbic cortex, the nucleus accumbens and the lateral septal region. The aforementioned cortical connections to the subiculum in turn project to the hypothalamus and the amygdala. The SR layer is not as prominent in the subiculum and the SO and molecular layers widen to accommodate the enlarged pyramidal cells (Amaral & Witter, 1995).

Histopathological Changes to the Hippocampus Following FPI

The hippocampus is known to be vulnerable to ischemia, seizures and brain trauma. Cognitive deficits, some of which are mediated by the hippocampus, are the most common long-term consequence of TBI in human patients (McIntosh et al., 1996). Unlike ischemia, which produces damage in the CA1 region, lateral TBI results in damage primarily in the CA3 and the hilus of the DG. Focal models often result in cell loss in these areas (Cortez, McIntosh, & Noble, 1989; Hicks, Smith, Lowenstein, SaintMarie, & McIntosh, 1993; Soares, Hicks, Smith, & McIntosh, 1995; Smith, Okiyama, Thomas, Claussen, & McIntosh, 1991). Loss of CA3 dendritic processes is common in focal models of TBI (Hicks, Smith, & McIntosh, 1995; Taft, Yank, Dixon, & Hayes, 1992). Axonal damage throughout the hippocampus and thalamus is often found, even in the absence of DAI (McIntosh et al., 1996). Also, receptor binding properties are disrupted and the seizure threshold within the hippocampus is lowered (Dixon et al., 1991; Feeney et al., 1981).

Focal models of TBI have distinctive patterns of histopathological changes, including: focal contusion, hemorrhagic contusion, necrosis, cavitation at injury site, short unconsciousness, BBB disruption (primarily in the contused region), cerebral edema, decreased cerebral blood flow (CBF), and increased metabolism, mircoglial and macrophage proliferation and recruitment, and the potential for delayed secondary insults (McIntosh et al., 1996). Histopathological studies have shown that the presence of subarachnoid blood may be the only

observable pathology at low injury levels. Moderate and severe injuries are likely to produce acute and chronic changes such as bilateral intraparenchymal hemorrhage in the hippocampi. At post-acute survival times (post-injury days 4-7), necrosis and cavitation become evident at the site of injury (Dixon et al., 1987). Lateral injuries produce radial contusion, structural damage, and cortical and hippocampal cell losses which correlate with specific behavioral deficits (Delahunty, Jiang, Gong, Black, & Lyeth, 1995).

Diffuse models of TBI are less likely to result in overt cell loss. Using the FPI model, Delahunty et al. (1995) showed that central injuries produced muscarinic and metabotropic dysfunction without any overt cell loss. Small or no contusions were observed.

TBI-Induced Changes

Temporal Changes: The Biphasic Model

Experimental TBI initiates a cascade of events that alters normal cell signaling and produces neuronal over-excitation (Hayes, Jenkins, & Lyeth, 1992). Impact interferes with normal ionic homeostasis. TBI produces an ionic flux (e.g., calcium influx) across the membrane, leading to neuronal excitation and depolarization. Excessive neuronal excitation produces large measurable increases in extracellular potassium, which result in further depolarization and NT release (Faden, Demediuk, Panter, & Vink 1989; Gorman, O'Beirne, Regan, & Williams 1989; Katayama, Becker, Tamura, & Hovda, 1990). Excessive

depolarization and NT release may produce changes to the intracellular signaling mechanisms, resulting in irreversible or long-lasting alterations in cell functioning (Hamm, Temple, Buck, Floyd, & DeFord, 1999). Following the acute excessive excitation of neurons, a chronic phase of functional neuronal depression begins (Hubschmann, 1985).

The biphasic hypothesis (see Figure 2) posits that there is an acute phase (< 24h in rats) of excessive neuronal depolarization. Increased levels of excitatory NTs and enhanced cerebral metabolism have been demonstrated. During the acute phase, methods of intervention that may be useful include excitatory receptor antagonists, reductions in glutamate and ACh levels, and decreased elevations in metabolism during the first six hours following injury. Inhibitory agonists may also help reduce neuronal excitation (Hamm et al., 1999). Massive ionic fluxes and EAA release following TBI require high metabolic energy, measurable by increased glucose utilization (Alessandri & Bullock, 1998). This hypermetabolism may last for minutes or hours in rat animal models and is followed by a hypometabolism lasting days or weeks (Yoshino, Hovda, Kawamata, Katayama, & Becker, 1991).

The chronic phase (> 24h in rats) of depressed neuronal activity has a duration that is dependent on the severity level of injury. Decreases have been demonstrated in cerebral metabolism, choline uptake, scopolamine-evoked release, and choline acetyltransferase (ChAT) and acetylcholinesterase (AChE)

immunoreactivity (IR). Excitatory receptor antagonists have been shown to be detrimental during the chronic phase, although drugs that attenuate the depressed activation of the cholinergic and metabolic systems may be of some usefulness (Hamm et al., 1999).

Ionic Flux and the Role of Calcium

As demonstrated in Figure 3, impact is followed by mechanically- and chemically-induced ionic flux across the cellular membrane. Ionic alterations lead to widespread neuronal depolarization, and indiscriminate NT release (Selzer, 1995). Calcium levels increase after injury, especially in damaged regions, and may persist for 48 hours following FPI in rats (Fineman et al, 1993).

Mechanically-induced alterations in calcium channel permeability contribute to increases in intracellular calcium concentration ($[Ca^{2+}]_i$), which lead to depolarization and further calcium influx through both NMDA-gated and voltage-gated ion channels. NMDA receptor-mediated calcium influx is well documented and clearly a critical contributor to excessive intracellular free calcium levels (Thomas et al., 1990; Fineman et al., 1993; Hayes, Jenkins, & Lyeth, 1992). Elevated $[Ca^{2+}]_i$ propagates neuronal depolarization and NT release, as well as modulating intracellular enzymatic action. Increased calcium activates proteases such as calpain, which may ultimately lead to cytoskeletal degradation and neuronal death. Additionally, calcium activates phosphatases such as calcineurin, and kinases such as calcium/ calmodulin kinase type II

Figure 2. Acute and chronic neuronal activity according to the Biphasic Hypothesis

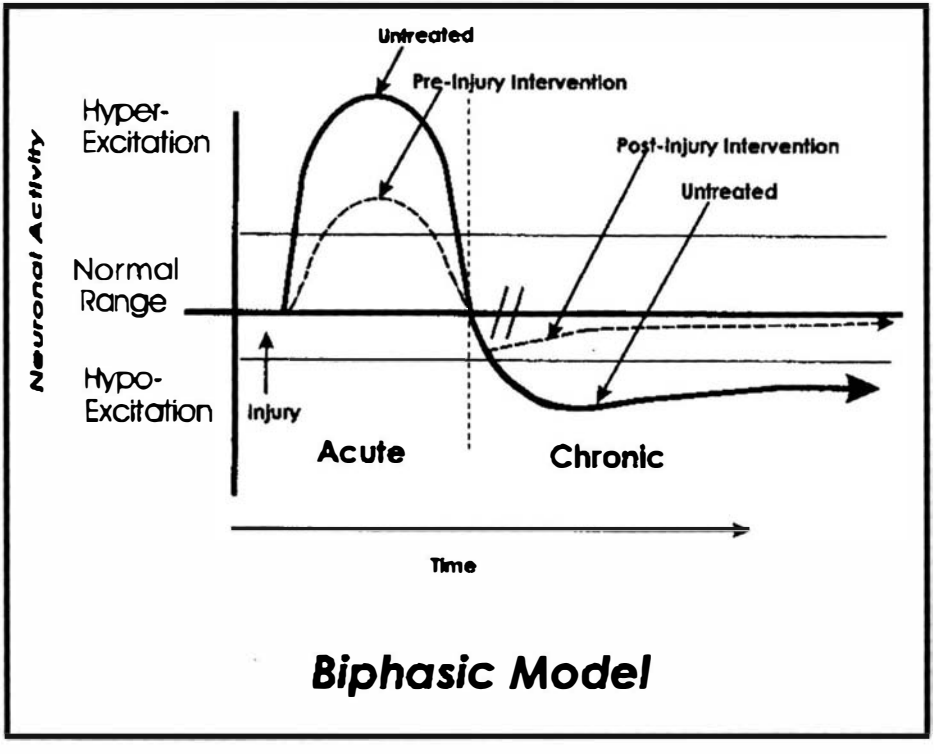
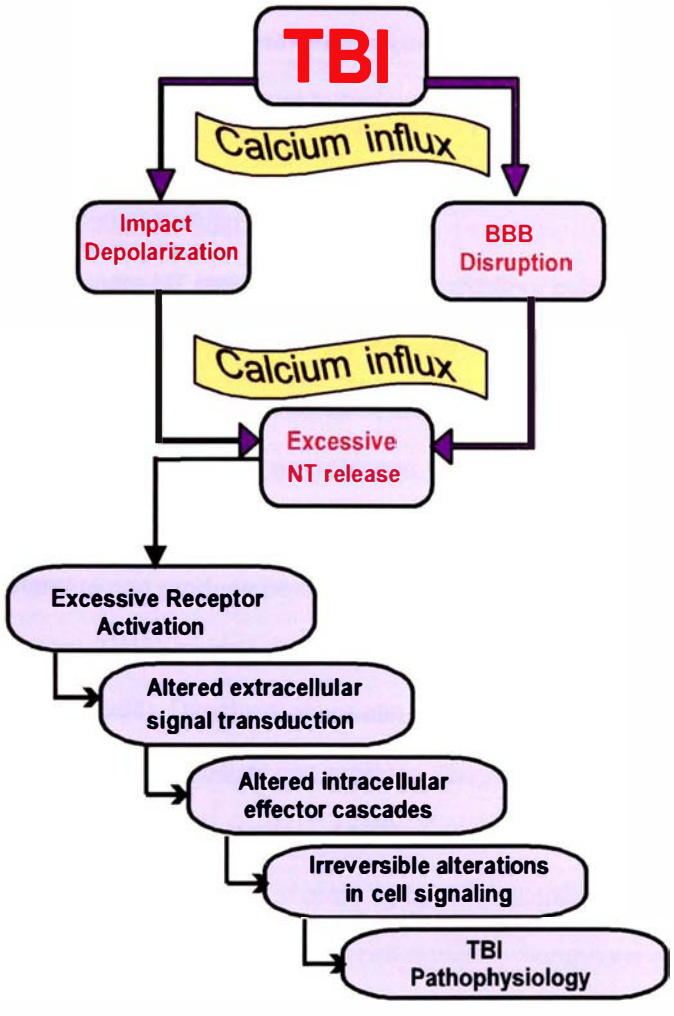

Untreated
Pre-Injury Intervention
Post-Injury Intervention
Untreated
Hyper-
Excitation
Normal
Range
Hypo-
Excitation
Neuronal Activity
Injury
Acute
Chronic
Time
Biphasic Model

Figure 3. The neuronal cascade following TBI.


TBI
Calcium influx
Impact Depolarization
BBB Disruption
Calcium influx
Excessive NT release
Excessive Receptor Activation
Altered extracellular signal transduction
Altered intracellular effector cascades
Irreversible alterations in cell signaling
TBI Pathophysiology

(CaM kinase II) (Morioka et al., 1999). The role of intracellular enzymes have not been conclusively demonstrated following TBI, although preliminary evidence indicates that both basal and maximal calcineurin activity is increased in the cortex and hippocampus 24 hours following central FPI (Kurz, Gibson, Hamm & Churn, 2001).

Lethal and Sublethal Cellular Changes

Indiscriminate NT release includes the inhibitory opioids and GABA, which may help to modulate the excitotoxicity induced by release of excitatory NTs and amino acids. Pathologically high levels of excitatory NTs (e.g., ACh) and EAA (e.g., glutamate and aspartate) are released, leading to excitotoxicity within the cells (McIntosh et al., 1996).

Glutamate can produce powerful neurotoxic effects following central nervous system (CNS) injuries such as ischemia, hypoxia, and brain trauma (Regan & Choi, 1994). Direct exposure can kill neurons (Rothman, 1985) and intermittent excessive exposure can produce delayed cell death (Choi, 1985; Choi, Maulucci-Gedde, & Kriegstein, 1987). Excitotoxicity has been documented acutely for many different types of brain injury. The resulting cascade may produce deficits due to either sublethal cell signaling disruptions or overt cell death.

Sublethal excitotoxic injury has been documented in mild to moderate TBI, due partly to processes which depend on muscarinic and NMDA receptors.

Dysfunctional cell changes due to excitotoxicity, neurological deficits, hippocampal disturbances in cholinergic circuits (which mediate spatial memory) and increased vulnerability to secondary insults may occur in the absence of overt cellular death or axonal injury (Hayes, Jenkins, & Lyeth, 1992b). Areas of selective vulnerability to CNS insult have been demonstrated in ischemia, hypoxia, hypoglycemia, epilepsy, and TBI (Brierly, 1976). Areas selectively vulnerable to sublethal cellular injury following non-contusional TBI include CA1, CA3 and DG regions of the hippocampus and specific layers of the neocortex (Hayes, Jenkins, & Lyeth, 1992b).

Glutamatergic Changes

Glutamate activates three receptor subtypes: NMDA and AMPA/KA (non-NMDA) ionotropic receptors as well as metabotropic glutamate receptors (mGluR). Ionotropic receptor activation leads to the opening of ion channels and subsequent calcium and sodium influx into the cell. Additional calcium is released from intracellular stores via second messenger pathways. Ultimately, the cascade results in alterations in gene expression and increased energy demand from high-affinity glutamate carriers in neurons and astrocytes (Alessandri & Bullock, 1988).

Extracellular increases in glutamate following FPI in rats peak within minutes and may last up to one hour (Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990). These increases may be 7-8 fold in the cortex and 3-4 fold

in the hippocampus of humans following TBI (Bullock, Maxwell, Graham, Teasdale, & Adams, 1991), and high levels may persist for up to several days (Bullock et al., 1995; Fineman et al., 1993; Persson et al., 1996; Robertson et al., 1995; Zauner & Bullock, 1995). Experimental FPI in rats produces increases in glutamate in the extracellular space that exceed 100% of normal within the first few minutes following injury. Katayama et al. (1990) found a 90% increase in glutamate in the hippocampus within two minutes following moderate FPI in rats. Glutamate levels in the aforementioned study returned to baseline within four minutes. Once released, extracellular glutamate binds to a ligand-gated receptor (i.e., NMDA or AMPA/KA), activating the ion channel and producing a large flux of cations across the cellular membrane (e.g., calcium and sodium influx, and potassium efflux) (Kawamata et al., 1992; McIntosh, Juhler, & Wieloch, 1998). Elevated intracellular cation concentration alters the membrane potential, contributing to depolarization and further NT release.

Blood Brain Barrier Alterations

Alterations in the BBB introduce exogenous sources of NTs to the brain (Hayes, Jenkins, & Lyeth, 1992a). Moderate TBI without contusion in the rat compromises the BBB in the cortex and hippocampus (Povlishock & Lyeth, 1989; Jiang et al., 1991). Injury allows greater permeability to exogenous (blood-borne) NTs and neuromodulators (Koide, Wieloch, & Siesjo, 1986), and this BBB disruption may persist up to15 hours (Ellis, Chao, & Heizer, 1989). Blood plasma

ACh levels are 7-fold greater than CSF levels (Robinson et al., 1990) and may contribute up to 39% to elevated ACh levels found in the CSF following TBI (Robinson et al., 1990). BBB changes allow blood plasma constituents (e.g., ACh) to access and influence the brain after injury, exacerbating excitotoxic effects and receptor dysfunction.

Excitatory Receptor Changes

Several receptor mechanisms exist which may mediate and propagate acute post-injury excitotoxicity. Muscarinic cholinergic and ionotropic NMDA glutamate receptors show decreased binding following injury (Hayes, Jenkins, & Lyeth, 1992b). Muscarinic cholinergic binding decreases 30-40% in the CA1 and DG of the hippocampus by three hours post-injury (Oleniak et al., 1988). Most changes in glutamatergic binding affinity occur in the NMDA-type receptors. NMDA receptor binding has been shown to decrease 12-15% within three hours of moderate TBI in the CA1 of the hippocampus (Hayes, Jenkins, & Lyeth, 1992b). NMDA and non-NMDA (AMPA/KA) receptors are directly correlated to selective patterns of vulnerability and damage in specific brain areas following TBI. Three hours post-injury, acute decreases in receptor binding for NMDA-type receptors have been found in the neocortex (inner and outer layers) and the hippocampus (CA1 stratum radiatum and DG molecular layer). Glutamate binding decreases are specific to NMDA receptors and are not seen in AMPA/KA glutamate receptors (McIntosh et al., 1996; Miller et al., 1990).

Muscarinic and NMDA receptor interactions (via G proteins and the IP3 pathway) have been implicated in aberrant intracellular effector cascades following TBI (e.g., changes in intracellular effectors, coupling efficiency, and early effector genes). The above interactions may play an important role in sublethal TBI pathology. Sublethal injury has been associated with decreased CA1 muscarinic and NMDA receptor binding, increased CA1 sensitivity to forebrain ischemia (first 24 hours), EEG spike frequency from the affected CA1 region, spatial memory deficits in the intact hippocampus and Schaeffer-collateral CA1 long-term potentiation (LTP) suppression (Hayes, Jenkins, & Lyeth, 1992b).

Excitatory receptor antagonists have been shown to have some usefulness acutely in attenuating the detrimental effects of TBI pathophysiology. NMDA antagonists such as MK-801, phencyclidine (PCP) and dextrorphan have been shown to be beneficial in rat TBI models. Another potentially beneficial treatment is the muscarinic ACh receptor antagonist scopolomine. These drugs are unlikely to be highly effective in a clinical setting, however, due to their potential toxicity, detrimental side effects, and the very short therapeutic window (maximum 15-30 minutes in rats) (Hayes, Jenkins, & Lyeth, 1992b; McIntosh et al., 1996; Faden et al., 1989). In a pre-clinical setting, however, they may provide valuable insight into mechanisms that drive neuronal excitotoxicity.

The Role of Intracellular Calcium

Glutamate Receptors

Glutamate is the most abundant excitatory neurotransmitter in the brain, with receptors on nearly all neurons (Kandel, Schwartz, & Jessell, 1991). AMPA/KA glutamate receptors gate a low conductance channel permeable to sodium and potassium (but not calcium). The NMDA glutamate receptor subtype gates a high conductance cation channel permeable to sodium, potassium, and calcium. Glutamate binds to the NMDA receptor, opening the channel. The NMDA ion channel, however, contains a voltage dependent Mg blockade that must be displaced before efficient ion conductance can occur (Kandel, Schwartz, & Jessell, 1991; McIntosh, Juhler, & Wieloch, 1998). AMPA/KA receptors are primarily responsible for depolarization of the cell. NMDA receptors have delayed activation, depending on cellular depolarization to remove the Mg block (Bading et al., 1995; Hestrin, Nicoll, Perkel, & Sah, 1990). Non-competitive NMDA antagonists such as phencyclidine (PCP) and dizocilipine (MK-801) bind to sites within the ion channel, effectively blocking calcium and sodium influx (Foster & Wong, 1987; Kandel, Schwartz, & Jessell, 1991; Olney, Price, Salles, Labruyere, & Frierdich, 1987; Ransom & Stec, 1988). The NMDA receptor is the only ionotropic glutamate receptor that allows calcium influx. Calcium influx through the NMDA channel is the primary source of glutamate-related excitotoxicity (Bading et al., 1995; Hayes, Jenkins, & Lyeth, 1992b; Kandel, Schwartz, & Jessell, 1991; Regan, 1996). High $[Ca^{2+}]_i$ may produce free radicals (e.g.,

hydroxyl radicals) (Laplanche, Kamenka, & Barbanel, 2000); activate proteases, phosphatases, and lipases (Kandel, Schwartz, & Jessell, 1991; Morioka et al., 1999); alter gene expression at the transcriptional and translational levels (Bading et al., 1995; Barish, 1998; Marin et al., 1997; Rosen, Ginty, & Greenberg, 1995; Scheetz, Nairn, & Constantine-Paton, 1997); and interfere with transportation and insertion of proteins into the cellular membrane (Barish et al., 1998).

Genetic Factors

The Process of Transcription and Translation

At the molecular level, proteins are a product of a sequence of calcium-regulated events. Transcription refers to the formation of RNA from DNA. During this process, nucleotides transcribe a portion of a DNA strand into mRNA. There are four primary steps involved in transcription: 1) RNA polymerase binds to DNA, 2) transcription is initiated on the DNA strand; 3) the RNA chain is elongated, and 4) transcription is terminated and the RNA is released (Kolb & Wishaw, 2001; Parker, 1994; Pinel, 2000). Many transcriptional regulators, which mediate each phase of this process, are regulated by cytosolic [Ca^{2+}] (Barish, 1998).

Translation refers to the formation of a protein (protein synthesis) from transcribed mRNA. Ribosomes in the endoplasmic reticulum (ER) translate mRNA codones (a group of three consecutive nucleotide bases) into amino

acids, which are added to a peptide chain by tRNA to form a polypeptide chain. The steps in translation include: initiation, elongation of the polypeptide chain, and termination. Intracellular stores of calcium in organelles such as the ER play important roles in the regulation of each step of protein synthesis (Barish, 1998; Palfrey and Nairn, 1995).

Calcium, gene expression, and the NMDA Receptor

The regional pattern of $[Ca^{2+}]_i$ and the route of Ca^{2+} entry are critical factors in gene regulation. NMDA-mediated elevations in $[Ca^{2+}]_i$ in cultured hippocampal neurons have been shown to produce an up-regulation of immediate early gene (IEG) expression at the level of transcription. Calcium influx through voltage-gated receptors was not sufficient to produce these transcriptional changes (Bading et al., 1995). At the translational level, NMDA receptor activation induces the phosphorylation of eukaryotic translation elongation factor 2 (eEF2), which results in a depression of protein synthesis. Phosphorylation of eEF2 is dependent on NMDA receptor activation and may represent a sublethal protective mechanism against glutamatergic excitotoxicity (Marin et al., 1997; Scheetz, Nairn, & Constantine-Paton, 1997).

Protein Activation, Transport, and Membrane Insertion

Following translation, the completed polypeptide chain folds into a specific conformation. The finished protein may be held in the ER until it is transported and inserted into the plasma membrane. Calcium regulates the enzymes (e.g.,

kinases and phosphatases) responsible for activation and deactivation of proteins via phosphorylation factors (Brandon et al., 2000; Churn & DeLorenzo, 1998; Krishek et al., 1994; McDonald et al., 1998; Morioka et al., 1999; Robello, Amico, & Cupello, 1997; Stelzer & Shi, 1994).

Although little is known about the role of calcium in membrane protein transport and insertion, $[Ca^{2+}]_i$ may regulate the phosphorylation states of the motor proteins (kinesin and dynein) responsible for transporting proteins to the surface membrane (Barish, 1998; Hirokawa, 1998). Calcium may also play a role in insertion of proteins into the membrane (Barish, 1998).

Receptor interactions

Transient increases in $[Ca^{2+}]_i$ can be blocked by voltage-gated calcium channel blockers. Excessive $[Ca^{2+}]_i$ elevations, however, are primarily mediated by NMDA receptors (Bading et al., 1995; Fekuda et al., 1998). Preliminary evidence from Sun et al. (2001) indicates that basal $[Ca^{2+}]_i$ is elevated in acutely isolated hippocampal neurons 24 hours and 6 days following TBI. Additionally, injured neurons take significantly longer to return to basal $[Ca^{2+}]_i$ following glutamate exposure (50μM, 2 min).

Elevated $[Ca^{2+}]_i$ plays a role in desensitizing the γ-aminobutyric acid type A (GABA-A) receptor. Mozrzymas and Cherubini (1998) used the whole cell patch-clamp technique to study the effects of high (2.2×10^{-6} M) or low (1.2×10^{-8} M) levels of $[Ca^{2+}]_i$ on GABA-A receptors in acutely dissociated rat hippocampal

slices. High levels of $[Ca^{2+}]_i$ significantly reduced GABA-evoked chloride (Cl-) currents and increased desensitization kinetics of GABA-A receptors. Similar results were found in mouse cortical neurons. Increases in NMDA receptor-mediated $[Ca^{2+}]_i$ reduced Cl- current amplitude. Transient increases in low levels of $[Ca^{2+}]_i$, however, enhanced the maximal response to GABA. This enhancement was blocked by a CaM kinase II inhibitor (Aguayo, Espinoza, Kunos, & Satin, 1998). Inhibition of GABA-A receptor Cl- current gradients exacerbates $[Ca^{2+}]_i$ elevations, although this effect can be blocked by NMDA receptor antagonists. Therefore, GABA, the primary inhibitory NT in the brain, may exacerbate neuronal excitotoxicity if there is a reduction in the Cl- equilibrium potential (Fukuda et al., 1998).

Treatment of the inhibitory system following TBI has been studied using chronic endpoints, although the effects are less well characterized than excitatory NT and receptor antagonists. Following injury, chronic injections of pentylenetetrazol, a GABA-A receptor antagonist, induced seizure activity which enhanced injured and harmed sham cognitive performance in the Morris water maze (MWM) (Hamm, Pike, Temple, O'Dell, & Lyeth, 1995). Treatment with MDL 26,479/ Suritozole, a GABA-A receptor inverse agonist, was found to be effective in attenuating MWM deficits (O'Dell & Hamm, 1995). A single acute injection of Diazepam within 15 minutes of injury has also been shown to effectively improve MWM performance (O'Dell, Gibson, Wilson, DeFord, & Hamm, 2000).

Collectively, these findings would indicate that acute modulation of the GABAergic system has beneficial effects on attenuating TBI-induced chronic cognitive deficits.

Understanding Inhibitory Neuroprotection

The overexcitation induced by TBI is also characteristic of other types of CNS insults. Excessive excitation alone, however, is unlikely to represent the entire story behind the cascade of events that occurs following injury. It is likely that the normal neuronal balance between excitation and inhibition has been disrupted, resulting in neurotoxicity. Alternative to decreasing excitation, balance may also be restored by increasing inhibition. Impaired inhibition of neocortical pyramidal neurons has been found following hypoxia combined with lowered brain temperature (Fujisaki, Wakatsuki, Kodoh, & Shibuki, 1999). Additionally, lateral FPI produces regionally specific alterations in inhibition. Reeves et al. (1997) demonstrated a reduction in the strength of paired-pulse depression in the hippocampal CA3 to CA1 commissural input, which returned to baseline by 15 days post-injury. The EC input to the DG and CA1, however, showed a strengthening of inhibition that remained essentially constant from two to fifteen days following injury and was qualitatively associated with an increase in GABA IR. The SLM layer in the CA1 of the hippocampus receives input from the EC, and persistent elevations of GABA have been demonstrated in this layer at one and fifteen days following TBI (O'Dell, 1995). Increased inhibitory input from the

EC during the chronic phase of TBI pathophysiology may be a compensatory mechanism responding to the acute excitatory phase.

Inhibitory agonists have been studied extensively in ischemia and are neuroprotective when administered within the first four hours following injury (Cross, Jones, Baldwin & Green, 1991; Inglefield, Wilson & Schwartz-Bloom, 1997; Li, Siegel, & Schwartz, 1993; Schwartz, Huff, Yu, Carter, & Bishop, 1994; Schwartz et al., 1994; Shauib & Kanthan, 1997). Diazepam and other GABA-A receptor agonists, when administered before or shortly after ischemic injury, have been shown to be protective against morphological cell damage (Nishikawa, Takahashi, & Ogawa, 1994) and CA1 pyramidal cell loss (Cross et al., 1991; Fujisaki, Wakatsuki, Kudoh, & Shibuki, 1999; Inglefield, Wilson, & Schwartz-Bloom, 1997; Johansen & Diemer, 1991; Schwartz-Bloom et al., 1998; Wahlgren, 1997), as well as attenuating GABA-A receptor binding detriments (Arika, Kanai, Murakami, Kato, & Kogure, 1993; Inglefield, Wilson, & Schwartz-Bloom, 1997) and injury-induced decreases in Cl- channel currents (Sigel, Baur, Trube, Mohler, & Malherbe, 1990). Diazepam has also demonstrated its effectiveness in cognitive protection in TBI (O'Dell et al., 2000).

A majority of the research regarding GABA-A receptor agonist-mediated neuroprotection has been done in ischemia. TBI and ischemia share similar pathologies. Both injuries culminate in excitotoxic consequences affecting selectively vulnerable brain regions. Important differences exist between the two

types of CNS injuries. Global ischemia lowers MABP and brain temperature (Inglefield, Wilson, & Schwartz-Bloom, 1997) but moderate diffuse TBI does not significantly alter either of these measures (O'Dell et al., 2000). The most studied brain region in ischemia research is the hippocampus (Shauib & Kanthan, 1997), where an important morphological difference between injury types is evident. The CA1 region of the hippocampus shows delayed degeneration at three to four days post-ischemia (Li, Siegel, & Schwartz, 1993), whereas diffuse TBI does not tend to exhibit overt cell loss and focal TBI selectively destroys CA3 and DG hilar neurons (Hayes, Jenkins, & Lyeth, 1992).

The excitatory input in the hippocampus is balanced by inhibitory processes, often on the same cell. Pyramidal neurons receive synaptic input from both glutamate and GABA receptors (Li, Siegel, & Schwartz, 1993). Glutamate is a precursor to GABA, and is formed when glutamic acid decarboxylase (GAD), which is only found in neurons, removes a carboxyl group from glutamate (Luddens, Korpi, & Seeburg, 1995). GABA is the most prevalent inhibitory neurotransmitter in the brain, exerting its effects primarily through the GABA-A receptor complex.

The GABA-A receptor acts to increase membrane hyperpolarization via modulation of a Cl- channel. The GABA-B receptor differs from the GABA-A receptor in both mechanism of action and neuroprotective potential. The GABA-B receptor acts primarily through a G-protein-coupled second messenger system

to reduce the presynaptic release of various neurotransmitters (Karlsson & Olpe, 1989). Modulation of the GABA-B receptor does not show neuroprotection in ischemia (Araki, Kato, & Kogure, 1991; Ito, Watanabe, Isshiki, & Uchino, 1999). The third type of GABA receptor, GABA-C, is an ionotropic receptor found only in retinal bipolar and horizontal cells (Feigenspan, Wassle and Bormann, 1993; Quian and Dowling, 1993; Lukasiewicz, 1996) and is insensitive to benzodiazepines and other GABA-A agonists (Sivilotti and Nistri, 1991; Bormann and Feigenspan, 1995; Johnston, 1996).

The GABA-A receptor is potentiated by ligands which bind to sites specific for benzodiazepines (BZs), barbiturates, alcohol, steroids, zinc ions (Zn+), or various anesthetics (Marrow, 1990; Roberts, 1974). As many as 20-50% of all synapses in the CNS use GABA as a neurotransmitter (Bloom & Iversen, 1971). Other estimates contend that GABA-A receptors may be present on all neurons in the brain (Wahlgren, 1997).

Specific areas of the hippocampus, such as the SO and SR layers of the hippocampus proper, have been estimated to use GABA in 80-95% of the synapses. Although the pyramidal cell layer represents a much lower percentage of GABA-positive cells (5-8%), approximately 11% of the general hippocampal neuronal population is GABAergic (Woodson, Nitecka, & Ben-Ari, 1989). The prominent expression of GABA-A receptors in hippocampal interneurons

indicates that inhibition is an important mechanism for maintaining excitatory/ inhibitory balance in neurons (Gao & Fritschy, 1994).

The Relationship Between Excitatory and Inhibitory Processes

The relationship between excitatory and inhibitory processes is complex. ACh is closely associated with GABA, and GABAergic inhibition decreases ACh levels. The mechanism involved in the ACh-GABA relationship is poorly understood, but it is believed to be mediated via GABA receptors on cholinergic neurons (DeBoer & Westerink, 1994). GABA and muscimol (a GABA-A agonist) enhance cholinergic release and this effect is blocked by biciculline (a GABA-A antagonist), indicating that the GABA-A receptor complex plays a modulatory role in ACh release (Supavilai & Karobath, 1985).

Group I mGluRs have been implicated in the excitation of neurons in the CA1 of the hippocampus. Classified by their pharmacological profiles, Group I mGluRs include mGluR1 and mGluR5. The Group I mGluRs not only excite neurons via depolarization by glutamate release, but they also play a role in regulating GABA release. GABAergic interneurons in the hippocampus are activated by mGluRs either pre- or post-synaptically. Group I mGluRs increase neuronal excitation by co-localizing on neurons which also contain GABA-A receptors. Group II mGluRs are located on inhibitory terminals and act to reduce pre-synaptic GABA release. In the CA1 of the hippocampus, Group I mGluRs modulate pyramidal cell input from GABAergic interneurons. Activation of Group

l mGluRs on inhibitory interneurons in the hippocampus contributes to the overexcitation which is associated with epilepsy and may play other important roles in disrupting the excitatory- inhibitory balance (Bordi & Ugolini, 1999).

As would be expected by the importance of mGluRs in GABAergic balance, NMDA-type glutamate receptors and GABA-A receptors are co-localized in hippocampal neurons (Craig, Blackstone, Huganir, & Banker, 1994). Modulation of NMDA glutamate receptors by MK-801 antagonism decreases GABA-A receptor-mediated Cl- uptake by 44% in the hippocampus (Matthews, Dralic, Devaud, Fritschy, & Marrow, 2000). Stimulation of GABA-A receptors has been shown to be protective against neuronal injury induced via NMDA receptor activity. GABA-A agonists block NMDA-induced damage and this protection can be reversed by GABA-A antagonists such as biciculline (Ohkuma, Chen, Katsura, Chen, & Kuriyama, 1994).

Interactions between NMDA receptors and GABAergic inhibition are also important to LTP. Central FPI produces impaired LTP capacity in the CA1 of the hippocampus at two and fifteen days post-injury. Qualitatively associated with these changes in LTP are decreased NMDA receptor (NMDAR1 subunit) IR and increased GABAergic IR in the CA1. Decreased NMDA receptor activity and increased GABAergic activity correspond well with decreased LTP chronically following TBI, providing some insight into the physiological and neurochemical

mechanisms associated with TBI-induced deficits in spatial learning (Reeves, Zhu, Povlishock, & Phillips, 1997).

Whole cell voltage clamp studies have demonstrated that NMDA and glutamate application diminish GABA-A receptor activation in acutely isolated hippocampal cells (Chen & Wong, 1995; Stelzer & Shi, 1994) and in cerebellar granule cells (Robello, Amico, & Cupello, 1997). NMDA reduced GABA-A outward current, although this effect was reversed when extracellular calcium was replaced with barium. Intracellular perfusion of calcineurin, a calcium-dependent phosphatase, also suppressed GABA-A responses (Chen & Wong, 1995; Stelzer and Shi, 1994), and this suppression was reversed by the calcineurin inhibitor deltamethrin (Robello, Amico, & Cupello, 1997). Calcium currents activated by voltage dependent channels did not alter GABA-A receptor responses. These data indicate that NMDA-mediated calcium influx alters GABA-A receptor activity. Activation of the phosphatase calcineurin has been implicated in the dephosphorylation of the GABA-A receptor complex (Chen & Wong, 1995; Robello, Amico, & Cupello, 1997; Stelzer & Shi, 1994).

The GABA-A Receptor

The GABA-A receptor is part of a superfamily of neurotransmitter-gated ion channels which includes nicotinic ACh receptors, glycine receptors and glutamate receptors (Sigel & Buhr, 1997; Schwartz, 1988). GABA-A receptors mediate the majority of CNS inhibitory neurotransmission (Mohler et al., 1996).

The fast-acting GABA-A ion channel reacts within milliseconds to receptor activation by ligand-binding, initiating the opening or gating of a Cl- channel (Stephenson, 1995). GABA-A receptors help regulate anxiety, vigilance, memory, convulsive activity and muscle tension (Mohler et al., 1996). Although the binding sites are distinct, through ligand-mitigated action they initiate complex interactions with each other (Kandel, Schwartz, & Jessell, 1991; Sieghart, 1995). The binding of one ligand increases the affinity for other ligands (e.g., benzodiazepine binding increases receptor binding affinity for GABA). Activation of the GABA binding site produces a conformational change in the receptor, increasing the binding capabilities of other ligands and ultimately increasing Cl- flux and neuronal hyperpolarization (Li, Siegel, & Schwartz, 1993; Lyden, 1997; Sieghart, 1995). GABA-A receptor activation also reduces glucose metabolism and mediates cerebral blood vessel dilation, improving blood flow (Lyden, 1997).

GABA-A Receptor Subunits

The GABA-A receptor has a pentameric structure (Lyden, 1997). The heterogeneity of the formation of the receptor by its constituent subunits has prompted it to be referred to as a "heterooligomeric complex" (Matthews et al., 2000; Backus et al., 1993), which is a common subunit composition for ligand-gated ion channels (Backus et al., 1993). Six subunits, most with several isoforms, have been identified, including: $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$, δ, π, and $\rho_{1\text{-}3}$ (Luddens &

Wisden, 1991; Pritchett, Luddens, & Seeburg, 1989a; Pritchett et al., 1989b; Schofield, 1989; Sieghart, 1995; Smith & Olsen, 1995). Each subunit is encoded by a different gene (Sieghart, 1995), and contains both a hydrophilic NH_2 (N) terminal and a cystine (C) terminal domain. Subunits consist of four transmembrane helices (M1-M4) and a large intracellular loop located between M3 and M4 (Burt & Kamatchi, 1991; MacDonald, Saxena, & Angelotti, 1996; Olsen & Tobin, 1990). The N-terminal lies between the M1 and M3 domains and the M2 domain lines the inside of the Cl- channel (Kandel, Schwartz, & Jessell, 1991; Stephenson, 1995). The M2 domain is positively charged and believed to be responsible for anion selectivity of the Cl- pore (Kandel, Schwartz, & Jessell, 1991). The large intracellular domain of the GABA-A receptor subunit contains multiple potential sites for protein phosphorylation by protein kinases (Moss, Doherty, & Huganir, 1992).

Each type of subunit (e.g., α and β) shares 30-40% of their amino acid sequence. Within a subunit type, the different isoforms (e.g., $\alpha 1$ and $\alpha 2$) have 70% identical amino acid sequences. Amino acid GABA-A receptor sequences are conserved approximately 90% across mammalian species (Stephenson, 1995). Each type of subunit is distinctively different in its encoded sequence and its function. However, due to the similarity between the subunit constituent sequences, changes to a single amino acid residue can drastically change receptor stoichiometry and binding properties (Buhr & Sigel, 1997). Although

recombinant receptors containing one, two, three, four, or five subunits have been identified, the most common form in vivo contains three subunits (Persohn, Malherbe, & Richards, 1992; Wisden & Seeburg, 1992). Of the five identified subunits, α, β, and γ are considered the "main" subunit types, while δ, π, and ρ are considered "minor" subunit types (Lyden, 1997; Mohler et al., 1996). The δ, π, and ρ subunits are considered minor because they are rare and not widely distributed. The remaining three subunits (α, β, and γ) have several isoforms, each of which combines to form a wide array of receptor types (see Table 3 for a summary). GABA-A receptors with specific subunit combinations confer specific functions and distributions within neuronal tissue. The most abundant subunits are α1, α2, α3, β2, β3, and γ2 (Stephenson, 1995).

GABA-A receptors with αβγ subunit combinations are the most widely distributed receptor type and this combination is important for proper benzodiazepine (BZ) binding (Sieghart, 1995). The most abundant GABA-A receptors in the rat brain consist of 2α, 1β and 2γ subunits, although 2α, 2β, and 1γ is also a common subtype (Backus et al., 1993) (See Table 3). Specific brain regions, such as the hippocampus, contain more or less abundant populations of GABA-A receptor subtypes. One of the most expressed receptor subtypes in the hippocampus is α2β3γ2, although in other areas of the brain α1β2γ2 GABA-A receptors are most abundant (Sieghart, 1995). In the hippocampus, GABA-A receptors on interneurons tend to be the α1β2γ2 subytpe, whereas receptors on

pyramidal and granule cells tend to be the α2β3γ2 subtype (Barnard et al., 1998; Mohler et al., 1996). Overall, 75% of all GABA-A receptors are made up of three receptor subtypes: α1β2γ2, α2β3γ2, and α3β3γ2 (Mohler et al., 1996).

The relative abundance of subtypes and heterogenous clustering of certain types of receptors in different brain areas has increased the understanding of the role that each subunit plays in the overall function of the GABA-A receptor. The binding site for BZs lies on the γ subunit at the α junction (Sieghart, 1995) (See Figure 4). The BZ binding site location determines its function, indicating that although α contributes to binding specificity, the presence of either γ2 or γ3 is required for proper BZ action (Luddens, Korpi, & Seeburg, 1995; Persohn, Malherbe, & Richards, 1992; Somogyi, Fritschy, Benke, Roberts, & Sieghart, 1996; Stephenson, 1995; Wisden et al, 1992). The widespread action of BZs may be explained by the relative abundance of γ2 subunits (40-50% of GABA-A receptors) in rat brains (Benke, Mertens, Trzeciak, Gillessen, and Mohler, 1991). Similarly, the GABA binding site's location on the β subunit explains the presence of β in nearly every known receptor subtype. Since the GABA binding site is near the αβ junction (Refer to Figure 4), it also follows that α plays an important role in GABA binding affinity, although this remains to be definitively determined (Huh, Delorey, Endo, & Olsen, 1995; 1996; Stephenson, 1995).

Unassembled GABA-A receptor subunits are retained in the endoplasmic reticulum (Gorrie et al., 1997). GABA-A receptor surface stability is regulated by specific subunit isoforms and protein kinases (Connolly et al., 1999). Protein kinases, including protein kinase C, protein kinase A, and CaM kinase II, have been shown to be involved in the phosphorylation of β and γ subunits at specific intracellular serine residues (Ser-409, Ser-410 and in β3 only, Ser-408) (Brandon et al., 2000; Churn & DeLorenzo, 1998; Krishek et al., 1994; McDonald et al., 1998; McDonald & Moss, 1997; Moss, Doherty, & Huganir, 1992). Phosphorylation of GABA-A receptor subunits by CaM kinase II increases Cl- conductance by increasing the availability of receptors. Increased neuronal excitability due to seizure activity and glutamate excitotoxicity decreases CaM kinase II phosphorylation of GABA-A receptor subunits (Churn & DeLorenzo, 1998; Churn, Limbrick, Sombati, & DeLorenzo, 1995; Wang, Cheng, Kolaj, & Randic, 1995; Yamagata & Obata, 1998).

The β subunits play a unique role in subcellular distribution and may be involved in the relocation of GABA-A receptors between subcellular locations. The β3 subunit shows a unique ability to transcytose, providing a mechanism for receptor isoform relocation (Connolly, Wooltorton, Smart, & Moss, 1996). The γ2 subunit interacts with the cellular protein GABA-A-receptor-associated protein (GABARAP). GABARAP has similarities with microtubule-associated proteins and contains a tubulin binding motif on its N terminus. Interactions between γ2,

Figure 4. A diagram of an abundant subtype of the GABA-A receptor, identifying GABA and BZ binding sites. The subunits are arranged around a central chloride pore.

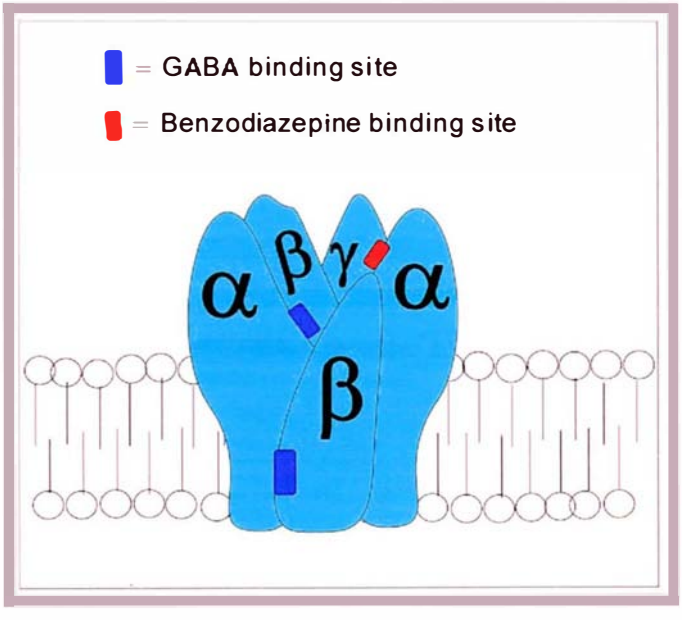

= GABA binding site
= Benzodiazepine binding site
α
β
γ
α
β

Table 3. The characteristics of GABA-A receptor subunits and their isoforms.

Properties of the GABA-A Receptor Subunits	
Subunit	**Properties**
α	Important to BZ and other ligand binding properties Tends to co-localizes with β2 or β3 and γ2 or γ3
α_1	Most abundant α subunit in rat brain Found in 90% of cortical and 40% of hippocampal GABA-A receptors Co-localizes with β2 or β3 and γ2 in 70-75% of GABA-A receptors with α1
α_2	Second most abundant α subunit in rat brain Tends to co-localize with β3 and γ2
α_3	Tends to co-localize with β2 or β3 and γ2
α_4	Rarest form of α
α_5	Low abundance (but is found in CA1 and CA3 of the hippocampus)
α_6	Only found in cerebellar granule cells and cochlear nucleus granule cells
β	Found in all in vivo GABA-A receptors investigated Contains binding site for GABA (on β, at α β junction) Role in subcellular receptor distribution
β_1	Low distribution, not much in the hippocampus
β_2	Most abundant β subunit in rat brain Tends to co-localize with α1 and γ_2
β_3	Role in receptor expression (can transcytose) Tends to co-localize with α2 and γ_2
β_4	Only found in chick brains
γ	Contains binding site for BZ (on γ, at αγ junction) - γ_2 and γ_3 only
γ_1	BZ-insensitive receptors
γ_2	Most abundant γ subunit in rat brain BZ-sensitive receptors
γ_3	Rare BZ-sensitive receptors

GABARAP, and tubulin, therefore, may be important in the anchoring of GABA-A receptors to the cytoskeleton (Wang, Bedford, Brandon, Moss, & Olsen, 1999).

Modulation of the GABA-A Receptor by Ligand Binding

The numerous binding sites on the GABA-A receptor translate into complex pharmacological potential. Agonists acting at the GABA-A receptor, however, have consistently demonstrated their acute effectiveness in several types of CNS insults (Arika et al., 1993; Cross et al., 1991; Gibson, 2000; Inglefield, Wilson, & Schwartz-Bloom; Ito et al., 1999; Johansen and Diemer, 1991; Matthews et al., 2000; O'Dell and Hamm, 1995; O'Dell et al., 2000; Schwartz et al., 1994; Schwartz-Bloom et al., 1998; Shauib and Kanthan, 1997; Wahlgren, 1997) while antagonists are likely to exacerbate injury-related deficits acutely (Hernandez, Heninger, Wilson, & Gallager, 1989; Ito et al., 1999; O'Dell et al., 20000; Ohkuma et al., 1994).

Although GABA-A receptor binding sites interact, they have unique pharmacological qualities, and some provide greater neuroprotection than others against CNS injury. Barbiturates such as pentobarbital are ineffective in preventing neuronal degradation following ischemia (Araki, Kato, & Kogure, 1991; Ito et al., 1999). Partial GABA-A agonists such as Ro 16-6028 and imidazenil show limited protection in ischemia (Hernandez et al., 1989; Schwartz-Bloom et al., 1998). Drugs that act to increase GABA (muscimol) or prevent GABA transferase (GABA-T) from removing GABA from the synapse (gamma-

vinyl GABA, No-328) also provide effective neuroprotection after injury (Ito et al., 1999; Johansen & Diemer, 1991; Katoh, Shima, Nawashiro, Wada, & Chigasaki, 1998; Shauib & Kanthan, 1997; Wahlgren, 1997). Other GABA-A receptor modulators such as chlormethiazole and MDL 26,479 (Suritozole) are at least partially neuroprotective (Cross et al., 1991; Fujisaki et al., 1999; Johansen & Diemer, 1991; Shauib & Kanthan, 1997; Wahlgren, 1997). The most widely used and most demonstrably effective drugs in neuroprotection are the benzodiazepines (BZs), diazepam (DZ) in particular. DZ has consistently demonstrated its effectiveness in providing neuroprotection following CNS insult (Gibson, 2000; Hernandez et al., 1989; Inglefield et al., 1997; Johansen & Diemer, 1991; O'Dell et al., 2000; Schallert, Hernandez, & Barth, 1986; Schwartz et al.,1994; Schwartz-Bloom et al., 1998; Sigel et al., 1990). Working memory enhancement (Moran, Kane, & Moser, 1992) and cognitive deficit attenuation following TBI (O'Dell et al., 2000) have also been attributed to DZ treatment.

Benzodiazepines such as DZ bind to the GABA-A receptor, instigating a conformational change that allows GABA to bind more readily and more tightly. Increased GABA stimulation of the receptor increases the potency and frequency of Cl- channel opening. Each receptor may have a BZ binding site located on each of the constituent γ subunits, and an additive effect may occur when combined with barbiturates or GABA agonists. The presence of GABA is

required for proper BZ action (Kandel, Schwartz, & Jessell, 1991; Sieghart, 1995).

Immunoreactivity in the Hippocampus

The hippocampus has been well-characterized with regards to GABAergic immunoreactivity (IR). Anti-GAD and anti-GABA antibodies have demonstrated that dendritic fields and interneurons in the CA1, CA3 and hilar regions of the hippocampus are GABAergic (Gamrani, Onteniente, Seguela, Gefferd, & Calas,1986; Nishikawa, Takahashi, & Ogawa, 1994; Terai, Tooyama, & Kimura, 1998; Woodson, Nitecka, & Ben-Ari, 1989). GABA and GABA-A receptor staining are not always co-localized, however. The neurotransmitter GABA is likely to be found in the nucleus, cytoplasm and in the synapses, especially of interneurons (Gao & Fritschy, 1994; Inglefield, Wilson, & Schwartz-Bloom; Li, Siegel, & Schwartz, 1993; Mizuhami et al., 1997; Somogyi et al., 1996; Terai, Tooyama, & Kimura, 1998). In an *in situ* hybridization characterization of α1 and β2 in the hippocampus, Li, Sigel and Schwartz demonstrated in 1993 that GABA-A receptor subunits reside predominantly on non-pyramidal cells. γ2 subunits, which tend to co-localize with α1 and β2 or β3, are highly expressed in dendritic layers and on interneurons (Somoygi et al., 1996). A majority of the interneurons in the hippocampus are the α1β2γ2 subtype. In pyramidal cells and DG granule cells, β3 tends to co-localize with α2 or α5 and γ2 to form GABA-A receptor complexes (Barnard et al., 1998; Mohler et al., 1996).

Using an antibody specific for β2 and β3 subunits (β2/3) of the GABA-A receptor, Gibson (2000) found changes in the CA3 region of the hippocampus 24 hours following central FPI. Although the overall number of stained processes was not altered, the IR length of CA3 apical dendrites was decreased following injury. Additionally, qualitative alterations were found in the appearance of IR processes. Specifically, the dendrites had a varicose beaded appearance. Similar beading has been found following ischemia (Inglefield, Wilson, & Schwartz-Bloom, 1997) and in cultured neurons exposed to NMDA or A23187 (a calcium ionophore) (Emery & Lucas, 1995). Administration of diazepam 15 minutes prior to injury normalized qualitative and quantitative alterations in β2/3 IR (Gibson, 2000).

TBI and the GABA-A Receptor

It is essential to proper functioning that the hippocampus maintain an appropriate excitatory/ inhibitory balance. TBI may disrupt this balance. GABA-A receptors mediate the majority of CNS inhibitory neurotransmission (Mohler et al., 1996) and may be present on all neurons in the brain (Wahlgren, 1997). The prominent expression of GABA-A receptors in hippocampal interneurons indicates that inhibition is an important mechanism for maintaining neuronal balance (Craig et al., 1994).

Modulation of NMDA glutamate receptors by MK-801 antagonism decreases Cl- uptake mediated by GABA-A receptors in the hippocampus

(Matthews et al., 2000). Additionally, stimulation of GABA-A receptors is protective against neuronal injury induced via NMDA receptor activity. GABA-A agonists block NMDA-induced damage and this protection can be reversed by GABA-A antagonists (Ohkuma et al., 1994). Therefore, the evidence indicates that there is an important relationship between NMDA receptor-mediated excitation and GABA-A receptor-mediated inhibition. Interactions between NMDA and GABA-A receptors following injury may be time dependent. During the more acute stage of neuroexcitation, GABA-A receptor expression may be reduced. Chronic hypo-excitation during the chronic phase, however, would indicate that the inhibitory system is strengthened over time. This strengthening of cortical input into the DG of the hippocampus was demonstrated by Reeves et al. in 1997. GABA-A receptor alterations may, therefore, be an essential, yet uncharacterized, component to the maintenance of the neuronal balance between excitation and inhibition both acutely and chronically following TBI.

The most abundant subunits in the brain include the α1, α2, β2, β3, and γ2 subunits (Stephenson, 1995). Of the α subunits, α1 tends to co-localize with β2 and γ2 on interneurons in the hippocampus, while α2 tends to co-localize with β3 and γ2 on principal neurons such as pyramidal cells and DG granule cells. Whereas GABA-A receptor subunits on principal hippocampal neurons are regulated by Ca^{2+}-related phosphorylation factors (e.g., CaM kinase II phosphorylation and calcineurin dephosphorylation), there is some evidence to

indicate that interneurons in the hippocampus may be governed by non-Ca^{2+}-mediated cascades (Sik, Hajos, Gulacsi, Mody, & Freund, 1998). These differences may be due to receptor composition. Principal cells (pyramidal and granule) tend to express GABA-A receptors containing β3 subunits, which contain an additional intracellular serine residue for phosphorylation (Brandon et al., 2000; McDonald et al., 1998) and have the unique ability to transcytose, providing a mechanism for receptor isoform relocation (Connollly et al., 1996). Therefore, it is important to distinguish between interneurons and principal neurons when exploring GABA-A receptor alterations following TBI.

The α1 subunits, which are found primarily on interneurons in the hippocampus, have been shown to be altered following ischemic injury (Inglefield, Wilson, & Schwartz-Bloom, 1997; Li, Siegel, & Schwartz, 1993) and BZ tolerance studies (Pesold et al., 1997). Additionally, β2/3 subunits have been shown to have regional alterations in the hippocampus 24 hours following central FPI (Gibson, 2000), although this study did not distinguish between β2 and β3 subunits.

Summary

TBI-induced depolarization results in Ca^{2+} influx through both NMDA-gated and voltage-gated ion channels. NMDA receptor-mediated Ca^{2+} influx is well documented and clearly a critical contributor to excessive intracellular free Ca^{2+} levels (Thomas et al., 1990; Fineman et al., 1993). Elevated $[Ca^{2+}]_i$ may

alter gene expression at both the transcriptional and translational levels (Bading et al., 1995; Barish, 1998), and/or may trigger an enzymatically-driven intracellular cascade that results in the dephosphorylation of GABA-A receptor subunits (Morioka et al., 1990). Alterations to the GABA-A receptor have yet to be characterized following TBI, although they are well documented following ischemia (Inglefield, Wilson, & Schwartz-Bloom, 1997; Johansen & Diemer, 1991; Schwartz-Bloom et al., 1998).

The α subunits play important modulatory roles in GABA-A receptor binding properties and α1 mRNA (Li, Siegel, & Schwartz, 1993) and protein expression (Inglefield, Wilson, & Schwartz-Bloom, 1997) are decreased following ischemic injury. Additionally, α1 is most abundant in the hippocampus on interneurons (Mohler et al., 1996), providing an indication of how GABA-A receptor expression in interneurons is altered following TBI.

The β subunits contain the GABA binding site and are found in nearly all GABA-A receptors (Huh et al., 1995, 1996; Stephenson, 1995). Their importance is further highlighted by their implication in phosphorylation factors, a quality they share with γ2 subunits. The β3 subunit is abundant in the hippocampus and it tends to localize in GABA-A receptors on principal neurons such as pyramidal cells and DG granule cells (Barnard et al.,1998; Mohler et al., 1996). Alterations to β3 subunits, therefore, may be an indication of changes occurring primarily to receptors located on principal neurons. In TBI, preliminary evidence (Gibson,

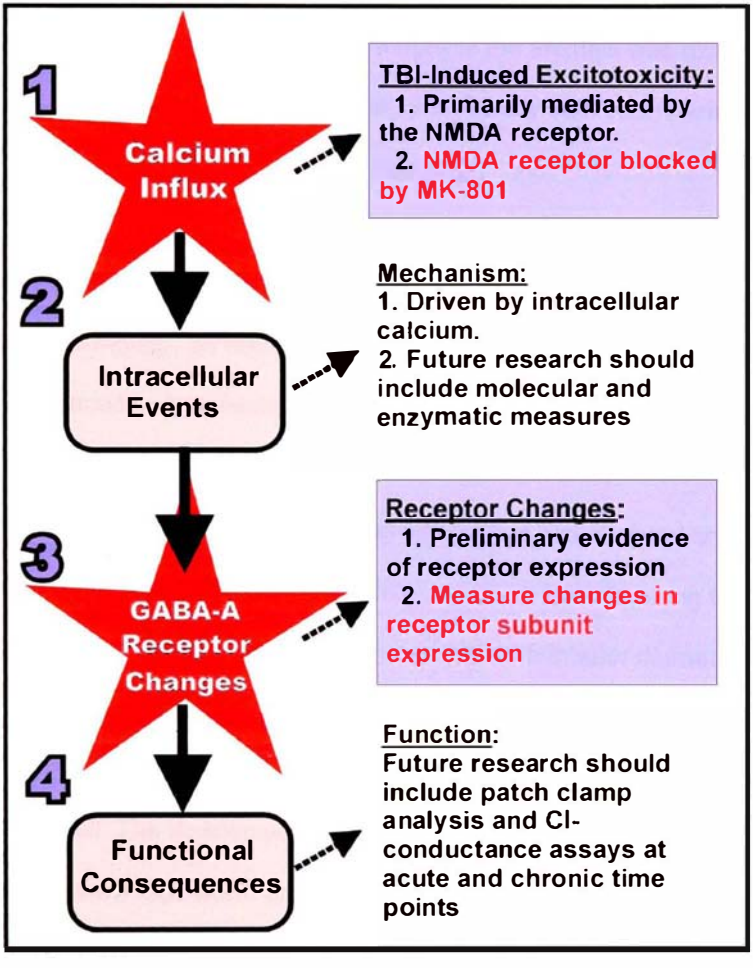
2000) indicates that the expression of β2/3 subunits of the GABA-A receptor is decreased 24 hours following injury. Alterations to the structure of the GABA-A receptor may be associated with functional consequences such as decreased Cl- conductance and consequent reductions in cellular hyperpolarization. Disrupted homeostasis mediated by dysfunctional GABA-A receptors may, therefore, contribute to excessive neuroexcitation and subsequent dysfunction following TBI.

The current overall hypothesis is that TBI induces: 1) a well-documented massive Ca^{2+} influx, which 2) triggers intracellular calcium-mediated mechanisms that 3) alter the structural expression of the GABA-A receptor and 4) result in receptor dysfunction. The primary focus of the current research project involves the first (Ca^{2+}) and third (GABA-A receptor) steps in the above cascade (see Figure 5 for a summary). First, Study 1 characterized changes to the α1 and β3 GABA-A receptor subunits in the hippocampus following central FPI in rats and examined the time course of these changes. Then, Study 2 used pharmacological intervention (MK-801) to normalize GABA-A receptors by preventing the NMDA-mediated Ca^{2+} cascade that contributes to excessive neuroexcitation and neuronal dysfunction.

Methods

Subjects

Figure 5. The potential cascade of events involving GABA-A receptors following TBI. The steps addressed are highlighted in red.


1
Calcium Influx
TBI-Induced Excitotoxicity:
1. Primarily mediated by the NMDA receptor.
2. NMDA receptor blocked by MK-801
2
Intracellular Events
Mechanism:
1. Driven by intracellular calcium.
2. Future research should include molecular and enzymatic measures
3
GABA-A Receptor Changes
Receptor Changes:
1. Preliminary evidence of receptor expression
2. Measure changes in receptor subunit expression
4
Functional Consequences
Function:
Future research should include patch clamp analysis and Cl- conductance assays at acute and chronic time points

Adult male Sprague-Dawley rats weighing approximately 320-340g were used for all experiments. Animals were housed individually in a vivarium in shoebox-type cages on a 12:12 hour light/dark cycle and received food and water ad libitum. The general health and weight of the animals was monitored daily and the general care was maintained by university staff veterinarians.

Surgical Preparation

All surgical preparations were performed with sterile instruments under antiseptic conditions. Subjects were surgically prepared under sodium pentobarbital (54 mg/kg), 24 hours prior to injury. While under anesthesia, animals were placed in a stereotaxic frame and a sagittal incision was applied to the scalp. A craniotomy hole was made over the central suture, midway between bregma and lambda. Burr holes were drilled to hold two nickel plated screws (2-56 x 6 mm) 1 mm rostral to bregma and 1 mm caudal to lambda along the central suture. A modified Leur-Loc syringe hub (2.6 mm interior diameter) was placed over the exposed dura and sealed with cyanoacrylate adhesive. Dental acrylic was applied over the entire device (leaving the hub accessible) to secure the hub to the skull. The incision was sutured and bacitracin applied to the wound. Animals were kept warm and continuously monitored until they had fully recovered from the anesthesia. Upon recovery, animals were returned to the vivarium where food and water were available ad libitum.

Central Fluid Percussion Injury

Although there are no rat models of TBI that effectively replicate all features of human head injury, the central fluid percussion model has been shown to produce a diffuse injury that reliably depicts many important aspects of human TBI.

Injury Device

The fluid percussion injury device has been described in detail elsewhere (Dixon et al., 1987; McIntosh et al., 1987). Briefly, the injury device consists of a Plexiglass cylinder 60 cm long and 4.5 cm in diameter, which is filled with saline. A rubber-covered piston at one end of the device is mounted on O-rings. On the other end, metal housing contains a pressure transducer (Entran Devices, Inc., model EPN-0300*-100A). A 5-mm syringe with an interior diameter of 2.6 mm terminates in a male Leur-Loc fitting, which is located on the end of the pressure transducer. The male fitting is connected to the modified female Leur-Loc hub implanted over the open dura of the rat. A metal pendulum (4.54 kg) is released from a pre-determined elevation, impacting the piston of the injury device. The impact delivers a pressure pulse through the continuous water-filled cylinder into the closed cranium of the rat. Brief displacement and deformation of brain tissue results and the pressure pulse is measured by the pressure transducer in atmospheres (atm) and displayed on a storage oscilloscope (Tektronix 5111: Beaerton, OR).

Injury Procedure

Animals were anesthetized under 4% isoflurane in a carrier gas consisting of 70% N_2O and 30% O_2, twenty-four hours following surgical preparation. The surgical incision was re-opened and the animals were connected to the fluid percussion device via the female-to-male connection described above. Animals in the injury groups received a moderate fluid pulse (2.1 ± .1 atm). Sham animals were attached to the injury device but no fluid pulse was delivered. The incision was sutured and bacitracin applied. Neurological assessments including tail, cornea, and righting reflexes were evaluated. The animals were closely monitored until they had sufficiently recovered and were then transferred back to the vivarium where food and water was available ad libitum.

Brain Tissue Preparation

Animals were anesthetized under 4% isoflurane in a carrier gas consisting of 70% N_2O and 30% O_2 at the time point indicated by the study design. The rats were quickly decapitated and bilateral hippocampi were dissected away on ice. The hippocampi were weighed and homogenized with a motorized homogenizer in a buffer consisting of 3ml RIPA lysis buffer (US Biological) and 30 µl Complete cocktail protease inhibitor (Roche Molecular Biochemicals) per gram of tissue.

Study 1: Western Blot Analysis of TBI-Induced Alterations to GABA-A Receptor Subunits α1 and β3 at 3 hours, 24 hours, and 7 days Post-Injury

Western Blot analyses of TBI-induced alterations to the GABA-A receptor subunits α1 and β3 in the hippocampus at several post-injury time points (3 hr, 24 hr, and 7 days) were conducted.

Design and Analysis

The experimental design was a 2 (TBI or sham) x 3 (time) factorial. Tissue obtained from each animal was used to analyze both α1 and β3 subunit expression. Due to gel size constraints, density measurements had to be pooled across several blots. In order to control between-gel variance (e.g., differences in staining intensity), data were normalized. The mean sham density for each gel was adjusted to represent 100%. The percent difference of each individual protein density was then calculated against the sham mean. Once normalized, data were pooled across gels. Results from each subunit were analyzed separately by an ANOVA (Group x Time). In order to determine which time point produced the greatest change, a Fisher's LSD post-hoc was used to analyze pairwise comparisons for each time point.

Western Blot Procedure

The Western Blot procedure was adapted from Santa Cruz Biotechnology, Inc. All materials (e.g., gels, buffers) were Invitrogen's NuPage products, unless otherwise specified. Following homogenization, bilateral hippocampi were centrifuged at 10,000 xg for 10 minutes. The supernatant was

removed and spun a second time at 10,000 xg for 10 minutes. Aliquots of 10 ul of lysate (the supernatant) were stored at -20°C until they were used.

A Bio-rad micro assay was used to assess the amount of protein in each sample by using a standard regression equation to calculate the amount of protein from the spectrophotometer readings of optical density. Each lane of the gel contained a standard amount of sample buffer (6.3 µl) and reducing agent (2.5 µl). The amount of water added was adjusted to ensure that equal amounts of protein were loaded in each lane (10 µg protein/ lane, determined empirically). Samples were heated at 70°C for 10 minutes.

All treatment groups were run concurrently. Proteins were separated on a 4-12% Bis-Tris mini-gel using a MOPS running buffer in the Novex Mini-Cell electrophoresis system. Separated proteins were then transferred to a nitrocellulose membrane (Invitrogen; 90 min at 30V). Standard weights were run alongside each condition, including the negative controls. Negative controls included a lane that received all treatments, except no primary antibody was applied to the membrane. Additionally, control immunoglobulin (IgG) (Santa Cruz) was run from the same species in which the primary antibody was raised. Following transfer, the gel was stained with Coomassie Blue to verify complete transfer to the membrane. The membrane was rinsed twice in TBS (25mM Tris-base, 140 mM NaCl, 3mM KCl, pH 7.6), enclosed in plastic and stored at -20°C until immunoblotted.

Non-specific binding was blocked by Blotto A (5% milk in Tris with 0.05% Tween 20; Santa Cruz) for 1 hr at room temperature (RT). The blot was then probed with GABA-A receptor anti-peptide α1 (1:100) or anti-peptide β3 (1:75) (Santa Cruz) diluted in Blotto A for 1 hr at RT. Primary antibody concentrations were empirically determined. The membranes were washed 4 x 15 minutes in TBS containing 0.05% Tween 20 (TBST). Blots were then incubated for 1 hour at RT in horseradish peroxidase-conjugated donkey anti-goat secondary antibodies (1:2000). Secondary antibody concentrations were empirically determined. Blots were then washed 4 x 15 minutes in TBST and 1 x 15 minutes in TBS. Specific peptide labeling was detected by chemiluminescent luminol reagent (Santa Cruz). The luminol reagent was applied to the blot for 1 minute. Following development, the blot was drained and exposed to BioMax x-ray film for visualization. Several exposure times, ranging from 15 sec to 5 minutes were tested to determine the clearest visualization. Densitometry was used to measure the optical density of the bands corresponding to the appropriate molecular weight for each subunit (Scion Image for Windows, Scion Corporation). Following immunoblotting, membranes were stained with BLOT-FastStain (Geno Technology, Inc.) to ensure there was even loading of proteins across lanes.

Study 2: Pharmacological Blockade of TBI-Induced NMDA-Mediated Calcium Influx and its Effect on the GABA-A Receptor α1 Subunit at 24 hours Post-Injury.

Design and analysis

Study 2 was designed to manipulate the injury cascade at the time point of greatest change. A one factor ANOVA was used to compare 4 groups (injured, sham, injured-treated, and sham-treated) 24h following TBI (time point determined by Study 1). Drug injections (MK-801, 0.3 mg/kg) were given 15 minutes prior to FPI in order to block NMDA-mediated Ca^{2+} influx. All drug treatment groups were then run concurrently with sham and injured groups during Western Blot procedures to control for variation in group effects.

Drug Administration

NMDA-mediated Ca^{2+} influx was blocked by 0.3 mg/kg/mL MK-801 (Research Biochemicals International), in saline solution. This dose was previously shown to be protective against motor deficits (Hayes, Jenkins & Lyeth, 1992b) and cognitive deficits following TBI alone (Hamm, O'Dell, Pike, & Lyeth, 1993) or in combination with secondary bilateral entorhinal cortex lesions (Phillips, Lyeth, Hamm, Reeves, & Povlishock, 1998).

Results

Experimental Controls

No protein bands were visible on any blots run under minus primary conditions (see Figure 6). Several blots included a negative control IgG from the

same species as the primary antibody. Immunoblotting did not produce any noticeable staining of the control IgG condition (see Figure 6).

Following transfer of the proteins to the membrane, all gels were stained with Coomassie blue to ensure uniform transfer. Protein transfer was equivalent across lanes, if not always complete (See Figure 7). Following immunoblotting and analysis, the membrane was stained to confirm an even distribution of protein across lanes. Protein transfers were found to be evenly distributed across all lanes (see Figure 8). Any blots revealing uneven distribution of protein were excluded from the studies.

Study 1: Western Blot Analysis of TBI-Induced Alterations to GABA-A Receptor Subunits α1 and β3 at 3 hours, 24 hours, and 7 days Post-Injury

GABA-A Receptor β3 Subunit

Post-Injury Reflexes

A 2 x 2 ANOVA (group: sham and injured x time: 3h and 24h) indicated that injury significantly suppressed reflexes. All reflexes were measured in minutes. Tail pinch, was suppressed in the injured group ($\underline{M}$ = 3.57) compared to the sham group ($\underline{M}$ = 1.0), $F_{(1, 13)} = 7.581$, $p < .05$. Corneal reflex was also suppressed in the injured group ($\underline{M}$ = 6.20) compared to the sham group ($\underline{M}$ = 1.0), $F_{(1, 13)} = 8.688$, $p < .05$. Tail pinch and corneal response are simple reflexes. The more complex righting reflex was delayed following injury ($\underline{M}$ = 7.88), compared to the sham animals ($\underline{M}$ = 2.00), $F_{(1, 13)} = 15.707$, $p < .05$. There

were no differences on any measure for the variable time, indicating that equivalent injuries were included in both time points (3 and 24 hours).

Western Blot Protein Density Analysis

A band recognized by the β3 antibody co-migrated with the 51-kDa molecular weight standard. A 2 x 2 ANOVA (group x time) did not reveal a significant injury effect ($F_{(1, 13)} = 0.003$, $p > .05$) on β3 protein density. Additionally, there was not a significant effect of time ($F_{(1, 13)} = 0.125$, $p > .05$). Although only the 3 hour and 24 hour time points were included in the analysis, analysis of the β3 subunit was terminated due to clear evidence that no injury-induced changes were occurring by 24 hours (see Figures 9 and 10).

GABA-A Receptor α1 Subunit

Post-Injury Reflexes

A 2 x 3 ANOVA (group x time) indicated that injury significantly suppressed reflexes (all reflex recovery was measured in minutes). Tail pinch was suppressed in the injured group ($\underline{M} = 3.44$) compared to the sham group ($\underline{M} = 1.0$), $F_{(1, 27)} = 20.183$, $p < .05$. Corneal reflex was also suppressed in the injured group ($\underline{M} = 5.70$) compared to the sham group ($\underline{M} = 1.0$), $F_{(1, 27)} = 57.291$, $p < .05$. The righting reflex was delayed following injury ($\underline{M} = 7.54$) compared to the sham animals ($\underline{M} = 2.00$), $F_{(1, 27)} = 113.187$, $p < .05$. There were no differences on any measure for the variable time, indicating that equivalent injuries were included in all time points (3 hours, 24 hours, and 7 days).

Mortality

Mortality rates for animals in each group are shown in Table 4. A Chi-Square test of independence revealed a significant difference in mortality between groups, $\chi^2_{(1, N = 54)} = 15.789$, $p < .05$. There also was a significant effect of time, $\chi^2_{(2, N = 54)} = 5.959$, $p = .05$, with the 24 hour time point having higher mortality (56%) than all other groups (20% at 3 hours and 29% at 7 days). The primary cause of mortality for all groups was pulmonary edema, which occurred within the first 5 minutes following injury. Although mortality was higher in the 24 hour injured group, the surviving animals had equivalent injury characteristics (severity and reflex recovery) with the other time points.

Western Blot Protein Density Analysis

A clear band at approximately 48-kDa was recognized by the α1 antibody. A 2 x 3 ANOVA (group x time) revealed a significant effect of injury on α1 protein density, $F_{(1, 27)} = 21.503$, $p < .001$. Injured animals ($M = 126.36$) had significantly denser GABA-A receptor α1 subunit protein than sham animals ($M = 100.54$). The main effect of time approached significance ($F_{(2, 27)} = 3.043$, $p = .06$), and no interaction was found ($F_{(2, 27)} = 2.613$, $p > .05$). In order to determine at which time point injury had the greatest effect on protein density, a Fisher LSD post-hoc was used to analyze differences between groups. Due to unequal group sizes, a harmonic sample mean was used for all analyses. The Fisher LSD revealed that injured animals had significantly higher protein densities at 24

hours (M = 141.15) and 7 days (M = 132.17) compared to 3 hours (M = 109.18) post-injury (see Figures 11 and 12). No other significant differences were found.

Study 2: Pharmacological Blockade of TBI-Induced NMDA-Mediated Calcium Influx and its Effect on the GABA-A Receptor α1 Subunit at 24 hours Post-Injury.

Post-Injury Reflexes

A one factor ANOVA (group: sham, injured, sham-treated, and injured-treated) revealed that the tail pinch reflex was significantly suppressed in injured animals (M = 3.65), as compared to sham (M = 1.00), sham-treated (M = 1.00), and injured-treated (M = 1.55) groups, $F_{(3, 17)} = 6.931$, $p < .05$. The corneal reflex was also suppressed in injured animals (M = 6.01), compared to sham (M = 1.00), sham-treated (M = 1.00), and injured-treated (M = 3.49) groups, $F_{(3, 17)} = 13.952$, $p < .05$. The pre-injury injections of MK-801 may have normalized the suppression of simple reflexes (tail pinch and cornea) in the injured-treated group, but this effect was not present for the more complex righting reflex. Analysis of the righting reflex revealed that it was significantly suppressed in both injured (M = 8.48) and injured-treated (M = 6.73) groups, as compared to sham (M = 2.00) and sham-treated (M = 2.00) groups, $F_{(3, 17)} = 31.960$, $p < .05$.

Mortality

Mortality rates for animals in each group are shown in Table 4. A Chi-Square test of homogeneity revealed a significant difference in mortality between groups, $\chi^2_{(3, N = 39)} = 15.234$, $p < .05$. The sham and sham-treated groups had

0% mortality, whereas the injured (74%) and injured-treated (44%) groups had relatively high death rates (see Table 5). The primary cause of mortality in injured animals was pulmonary edema.

Western Blot Protein Density Analysis

As determined by study 1, the 24 hour post-injury time point revealed the greatest increase in α1 protein. Therefore, the effects of MK-801 treatment in study 2 were analyzed 24 hours following injury. A one factor ANOVA revealed significant group differences in protein density for the α1 subunit of the GABA-A receptor, $F_{(3, 17)} = 16.455$, $p < .001$. A Fisher LSD post-hoc revealed that the injured group ($M = 141.15$) had significantly denser protein than sham ($M = 101.52$), sham-treated ($M = 80.77$) and injured-treated ($M = 79.13$) groups (see Figures 13 and 14). No other significant differences were found.

Figure 6. Representative photographs of Western blot negative controls. Respectively from top to bottom, the straight black lines represent standard weights 191, 64, 51, 39, 28, 19, and 14 kDa.

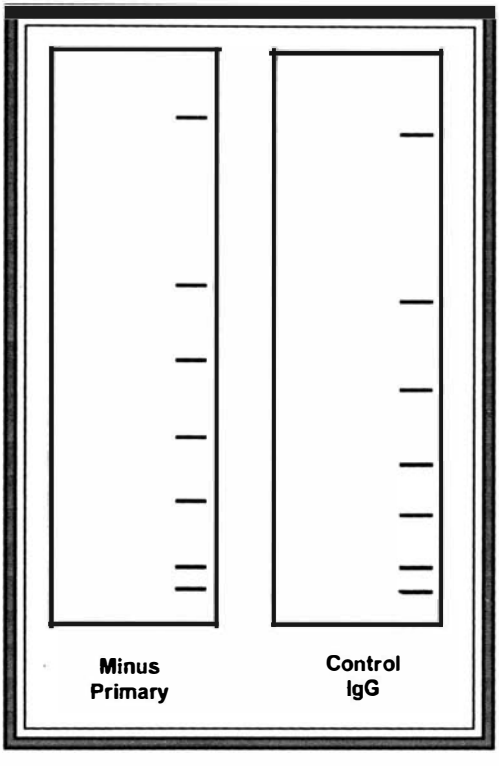

Minus
Primary
Control
IgG

Figure 7. A representative gel stained with Coomassie Blue following transfer of proteins to the membrane.

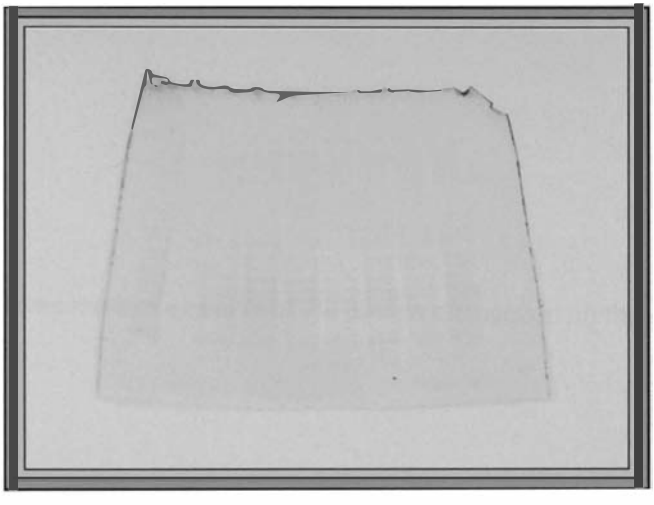


Figure 8. A representative example of the protein distribution on the membrane.

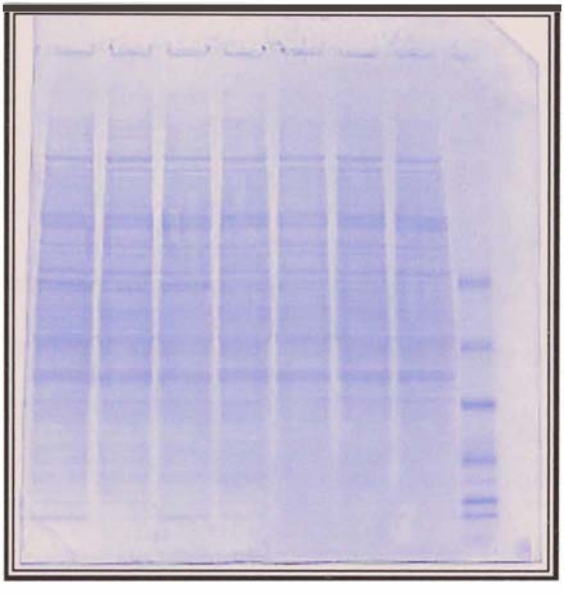


Figure 9. The mean percent density (and S.E.M.) of GABA-A receptor β3 subunit protein in the hippocampus for Study 1. No significant injury or time effects were found.

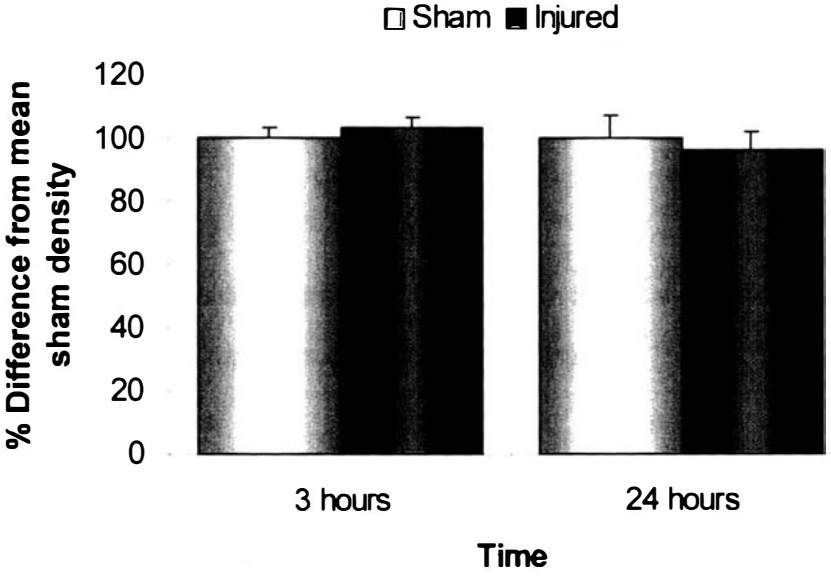

Sham
Injured
120
100
80
60
40
20
0
% Difference from mean sham density
3 hours
24 hours
Time

Figure 10. A representative photograph of Western blot analysis for GABA-A receptor β3 subunits at 24 hours post-injury in Study 1.


Sham
Sham
Injured
Injured
Injured
Injured

Table 4. The mortality percentages per group for Study 1. A chi-square test of independence was significant, $X^2_{(1)} = 15.789$, $p < .05$.

% Mortality			
	3 Hours	24 Hours	7 Days
Sham	0	0	0
Injured	20	56	29

Figure 11. The mean percent density (and S.E.M.) of GABA-A receptor α1 subunit protein in the hippocampus for Study 1. TBI significantly increased protein density compared to sham, $F_{(1, 27)} = 21.503$, $p < .001$.

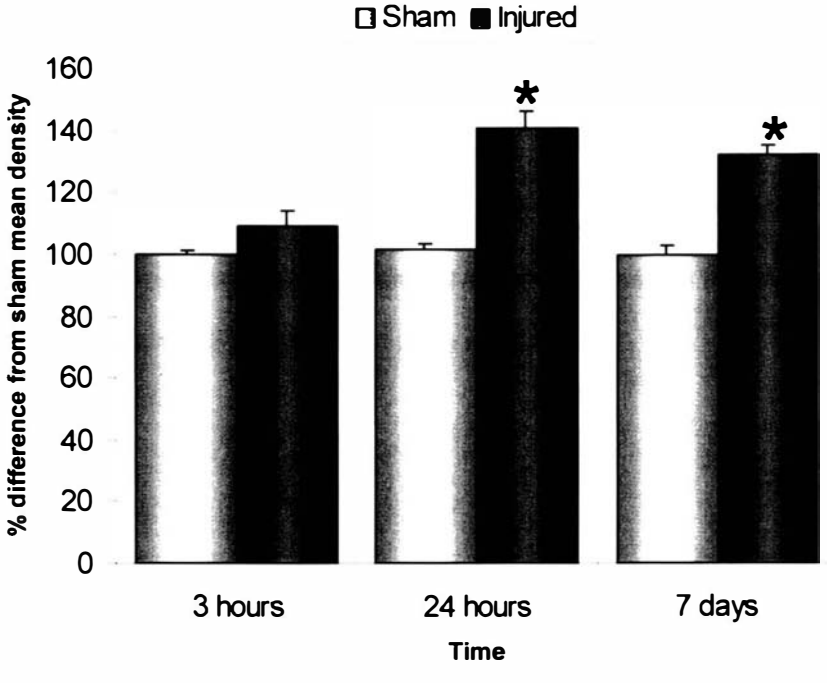

Sham
Injured
% difference from sham mean density
160
140
120
100
80
60
40
20
0
*
*
3 hours
24 hours
7 days
Time

Figure 12. A representative photograph of Western blot analysis for GABA-A receptor α1 subunits at 24 hours post-injury in Study 1.

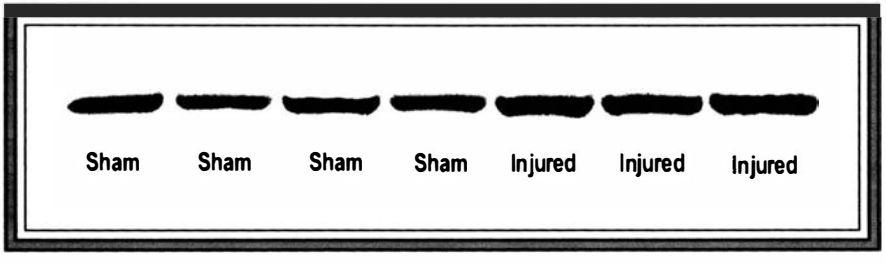

Sham
Sham
Sham
Sham
Injured
Injured
Injured

Table 5. The mortality percentages per group for Study 2. A chi-square test of independence was significant, $\chi^2_{(3)} = 15.234$, $p < .05$.

% Mortality			
Sham	Injured	Sham-Tx	Injured-Tx
0	49	0	44

Figure 13. The mean percent density (and S.E.M.) of GABA-A receptor α1 subunit protein in the hippocampus for Study 2. The NMDA anatagonist MK-801 normalized TBI-induced increases in protein density, $F_{(3, 17)} = 16.455$, $p < .001$.

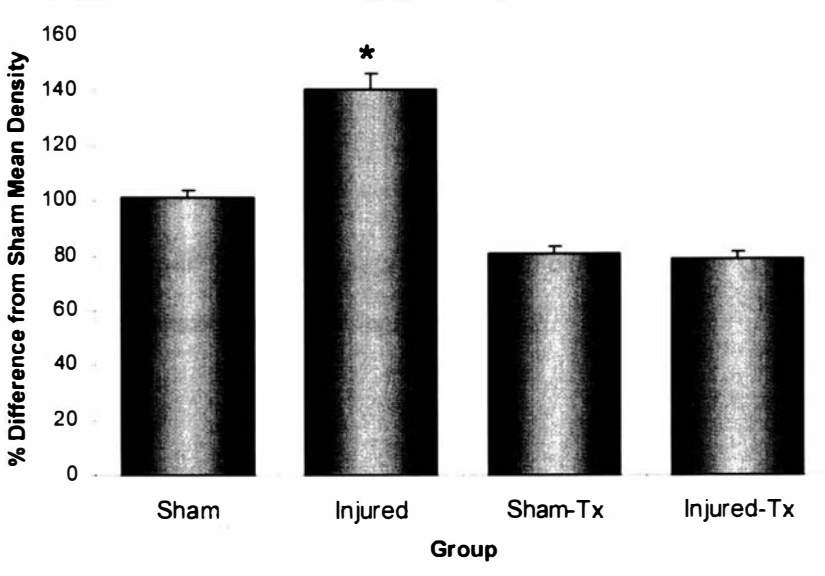
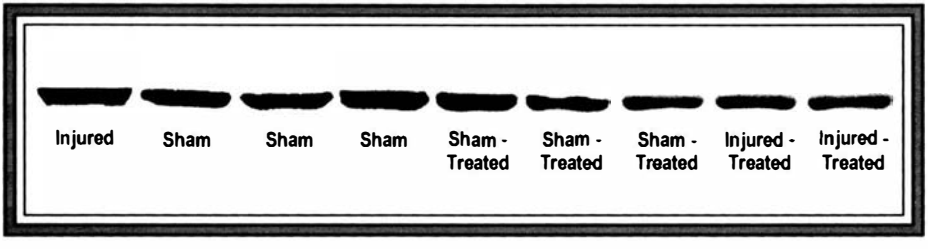

% Difference from Sham Mean Density
160
140
120
100
80
60
40
20
0
*
Sham
Injured
Sham-Tx
Injured-Tx
Group

Figure 14. A representative photograph of Western blot analysis for GABA-A receptor α1 subunits24 hours post-injury in Study 2.


Injured
Sham
Sham
Sham
Sham - Treated
Sham - Treated
Sham - Treated
Injured - Treated
Injured - Treated

Discussion

Study 1 showed that the GABA-A receptor subunits α1 and β3 change differentially due to TBI. The β3 subunit was not altered significantly by injury at either 3 or 24 hours. The α1 protein, however, was enhanced by 24 hours and this increase persisted for at least 7 days. The differential effects of injury on various subunits may be related to the differential distribution and function of specific receptor subtypes. In the hippocampus, the β3 subunit tends to co-localize with α2 and γ2 on principal neurons (i.e., pyramidal and granule cells), which are primarily excitatory neurons. The α1 subunit, however, tends to co-localize with β2 and γ2 on inhibitory interneurons (Barnard et al., 1998; Mohler et al., 1996; Sperk et al., 1998). A single interneuron in the hippocampus may ultimately influence thousands of post-synaptic principal cells. Interneurons in the DG maintain membrane depolarization in response to perforant pathway stimulation. This long-term depolarization allows interneurons to produce action potentials to previously subthreshold excitatory potentials, providing an inhibitory regulatory mechanism that is unique to the hippocampus (Ross & Soltesz, 2001).

Increases in α1 and β2 in the cerebellum and cortex early in development (9 weeks) translate into increased inhibitory tone and delay the onset of isoniazid- induced seizures (Follesa et al., 1999). GABA-A mRNA changes precede receptor expression and functional alterations that drive epileptogenesis (Brooks-Kayal et al., 1998). In the hippocampus, reduced inhibitory tone in

seizure-prone animals is associated with reduced α1 mRNA and protein expression (Poulter et al., 1999). Increased inhibitory tone due to increases in interneuronal subunits (α1& β2) is consistent with the chronic phase of TBI, which is characterized by a suppression in neuronal activity. It should be noted, however, that an increase in inhibitory tone on interneurons would reduce depolarization. Since interneurons in the hippocampus provide inhibitory regulation of excitability in principal cells, a reduction in GABA release from interneurons may produce a net increase in excitation.

In the rat, the chronic phase of neuronal hypofunctioning begins around 24 hours (Hubschmann, 1985). In the current study, increases in α1 protein were found at 24 hours and 7 days, but were not present during the acute phase (3 hours post-injury). Therefore, the acute phase of neuronal over-excitation may trigger a strengthening of interneuronal inhibitory tone in the hippocampus, and increased α1 protein expression may be an important contributor to the neuronal dysfunction that occurs chronically following TBI.

Increases in interneuronal inhibitory tone and corresponding increases in interneuronal subunits may involve a shift in GABA-A receptor subunit expression, similar to the shift that occurs during development. Both in vitro and in vivo studies have shown a compensatory shift in subunit expression in hippocampal neurons during development. Relative expression of α2, α3, and α5 are highly expressed in embryonic neurons (Poulter et al, 1999). Postnatally,

α1, β2, and γ2 subunit mRNAs increase two- to five- fold during the first two weeks (Brooks-Kayal, Jin, Price, & Dichter, 1998; Brooks-Kayal et al., 2001). Miniature inhibitory post-synaptic currents (IPSCs) in cerebellar neurons on post-natal day 35 (P35) demonstrate significantly slower decay rates in α1 subunit-deficient mice, compared to wild-type mice, indicating that increased α1-containing subunit assembly may be responsible for fast inhibitory synaptic currents (Vicini et al., 2001). In the hippocampus, DG interneurons exhibit calcium-dependent long-term depolarization after high-frequency perforant pathway stimulation (Ross & Soltesz, 2001). GABA-A receptor subunit mRNA and protein expression in the DG and subsequent subunit shifts and receptor subtype changes during postnatal development are correlated to functional alterations. Increases in specific subunits (including α1) translate into increases in receptor blockade by zinc ions and reduced receptor augmentation by both positive and negative BZ modulators (Brooks-Kayal et al., 2001). Therefore, developmental shifts that increase interneuronal GABA-A receptor subunit expression may have functional consequences that include changes in receptor ligand modulation and increases in inhibitory tone.

Not only can GABA-A subunit concentration alter ligand activity, but chronic ligand exposure may, in turn, alter receptor subunit expression. Chronic GABA exposure may alter binding of transcriptional factors, which may alter GABA-A receptor subunit composition and expression. Persistent exposure of in

vitro neurons to GABA decreases α1 and β1 mRNA and subsequent protein expression (Lyons, Gibbs, & Farb, 2000; Russek, Bandyopadhyay, & Farb, 2000). Changes to the β1 subunit mRNA occur at the transcriptional level, by an alteration in the binding of a sequence-specific transcription factor (Inr) to its initiator component (Russek, Bandyopadhyay, & Farb, 2000). GABA- induced decreases in both α1 and β1 may be associated with their interactive functions. Cystein residues on α1 and β1 near the middle of M2 (the pore-lining segment of the polypeptide chain) asymmetrically rotate and form a disulfide bond that locks the channel open in the presence of GABA (Horenstein et al., 2001). Alterations of a single amino acid residue (leucine) in the 9' position of the M2 region alters β1, but not α1, conformation and function, ultimately reducing Cl- currents (Dalziel et al., 2000). Modifications of amino acid residues alter the conformational folding of the receptor, and may mask or unmask binding sites such as those for GABA. The GABA binding pocket is located in a narrowing cleft, which constricts during activation (Wagner & Czajkowski, 2001). Therefore, even subunits with similar functions may be altered differently by solitary changes to a single amino acid in the polypeptide chain.

Regulatory changes may differ among GABA-A receptor subunits. Although the current studies only addressed α1 and β3 subunits, other subunits may also change due to TBI. Cultured cerebellar granule cells exposed to protein kinase A demonstrate a down-regulation of α6 and β3 mRNA and an up-

regulation of α1 and β2 mRNA. Changes occurred at both the transcriptional and translational levels. Protein kinase A inhibitors prevented these effects on α1 but not on α6, indicating differential regulatory mechanisms for different subunits (Thompson et al., 2000).

Epilepsy research also demonstrates disparate alterations in subunits. Although β3 mRNA decreases in the DG (6 hours), CA1 (progressive), and CA3 (24 hours) following kainic acid- induced seizures, α1 mRNA increases in the interneurons of the DG and in the CA3 of the hippocampus. Protein expression, however, does not always reflect mRNA changes, although interneuronal (e.g., α1) mRNA and protein may be selectively spared following seizures due to a compensatory strengthening of inhibitory tone (Sperk et al., 1998).

There may also be diverse regulatory mechanisms involved in the interaction between NMDA receptors and GABA-A receptors. Chronic blockade of calcium influx through the NMDA receptor with MK-801treatment reduces β3 and increases β2 mRNA in the hippocampus. Protein expression of neither β2 nor β3 is altered (Kim et al., 2000). Chronic MK-801 treatment does not alter GABA-A α1 or NMDA receptor subunit mRNA or protein expression in the hippocampus but GABA-A receptor-mediated Cl- uptake is significantly decreased (Matthew et al., 2000). Therefore, NMDA receptor ligand binding may result in disparate alterations to one or more GABA-A receptor subunits, and these changes may have specific functional consequences.

Disparate alterations to GABA-A α1 and β3 subunits indicate that interneuronal alterations may occur following TBI, and these changes may have important consequences. Interneurons receive GABAergic input from both local and distant circuitry. DG interneurons receive input from the EC, the septum, and the hilar region of the DG. Considering that each of these areas has been shown to be susceptible to TBI (Hicks et al., 1993; Lewen et al., 1999; Smith et al., 1997), dysfunctional signaling from damaged regions may contribute to interneuronal GABA-A subunit alterations in the hippocampal formation. Since the hilus is especially vulnerable to FPI, local circuitry may be essentially involved in GABA-A receptor changes.

Cell loss in the DG hilar region following lateral TBI has been correlated with memory deficits (Hicks et al., 1993). Cholinergic systems in the hippocampus and septum, areas related to memory formation, are also altered by TBI (Dixon et al., 1997; Gorman et al., 1996; Pike & Hamm, 1997). Considering the disruptive effects TBI has on excitatory systems (e.g., cholinergic and glutamatergic), the alterations to GABA-A receptor subunits may be a symptom of a pathology that affects hippocampal GABAergic circuitry. Therefore, GABA-A receptor α1 protein changes may be a reflection of aberrant GABAergic function due to injury.

The α1 subunit not only contributes to GABA-A receptor interneuron composition and function, but also has distinct functions of its own. Mice with α1

subunit point-mutations of a single amino acid (i.e., His to Arg at the BZ binding site) are rendered BZ-insensitive. Studies with these "knock-in" mice demonstrate that the α1 subunit is responsible for the sedative effects of BZs such as DZ, whereas the α2 subunit is responsible for the anxiolytic effects of BZs. Additionally, α1 plays a critical role in the production of anxious reactions in novel environments, an important element in behavioral testing of animals (Rudolph, Crestani, & Mohler, 2001; Tobler, Kopp, Deboer, & Rudolph, 2001).

The mechanism(s) driving the changes in GABA-A receptor subunit protein expression may occur anywhere along the pathway from mRNA transcription to insertion of the finished protein into the cellular membrane. Transcriptional regulators (Barish, 1998), each translational step (Barish, 1998; Palfrey & Nairn, 1995), protein folding, protein expression, and insertion of the finished protein into the cellular membrane are all regulated by $[Ca^{2+}]_i$ (Barish, 1998). Elevated $[Ca^{2+}]_i$ suppresses GABA-mediated Cl- conductance by decreasing the affinity of the receptor for GABA (Inoue, 1986). Additionally, localized increases in $[Ca^{2+}]_i$ fluctuations may suppress GABA-A mediated IPSCs (Pitler & Alger, 1992). Calcium influxes through NMDA receptor channels, but not voltage-gated channels, produce a persistent dephosphorylation-dependent suppression of GABA-A receptor currents in CA1 pyramidal cell bodies and apical dendrites (Stelzer & Shi, 1994). The largest increases in $[Ca^{2+}]_i$ occur in

the apical dendritic shaft in CA1 pyramidal cells, which primarily receive synaptic input from GABAergic interneurons (Papp et al., 2000).

Electrophysiology studies in TBI research demonstrate that injury alters GABA-A receptor function. Lateral FPI reduces paired-pulse depression strength in CA3 to CA1 commissural input but inhibition is strengthened in DG and CA1 EC input (Reeves et al., 1997). Concordantly, basal $[Ca^{2+}]_i$ is elevated in isolated hippocampal neurons one and six days after central FPI. Following glutamate exposure, TBI neurons take significantly longer to return to basal $[Ca^{2+}]_i$, compared to shams (Sun et al., 2001).

Study 2 used a single pre-injury injection of MK-801 to normalize GABA-A receptor subunit expression. The blockade of calcium influx through the NMDA receptor effectively attenuated GABA-A receptor $\alpha 1$ subunit increases 24 hours post-injury, indicating that alterations to the $\alpha 1$ subunit were induced by injury-related elevations in $[Ca^{2+}]_i$. Although this study was not designed to determine which calcium- related mechanism drove the alterations to the $\alpha 1$ receptor, we do know that elevated $[Ca^{2+}]_i$ suppresses GABA-mediated Cl- conductance, thereby reducing inhibition. Increases in $\alpha 1$ subunits and potential increases in interneuronal inhibitory tone may play a role in dysfunctional excitatory/ inhibitory homeostasis following injury. Blockade of NMDA-mediated elevations in $[Ca^{2+}]_i$ may prevent interneuronal strengthening and subsequent changes in inhibitory tone.

Future studies to determine the mechanism responsible should include molecular assays such as RT-PCR to determine whether GABA-A receptor subunit mRNA is altered following TBI. Immunohistochemical analysis combined with RT-PCR could indicate whether changes are occurring at the transcriptional and/or translational level(s). Immunohistochemistry and autoradiography studies could be used to determine whether persistent alterations to subunits are, as suspected, located on interneurons in the hippocampus and if these changes are regionally specific. The use of several antibodies which bind to different regions of the α1 receptor are recommended in order to determine if changes are occurring due to the masking or unmasking of particular binding sites. Additionally, specific enzyme assays (e.g., CaM Kinase II and calciuneurin) and Western blot analysis of only membrane-bound subunits may help clarify whether changes are occurring during transport and membrane insertion of finished proteins. The functional consequences of TBI-induced alterations to GABA-A receptor subunits could also be explored by use of electrophysiology studies, Cl- conductance assays, and genetically altered mouse studies.

The current studies demonstrate that TBI induces an increase in GABA-A receptor α1, but not β3, subunits at 24 hours and 7 days post-injury. The differential directions of the alterations may indicate a strengthening of interneurons in the hippocampus chronically following injury, although this remains to be definitively determined. Although an increase in inhibitory tone

during the acute phase of neuronal over-excitation would likely be beneficial, increased inhibition during the chronic phase of neuronal hypofunctioning is likely to be detrimental. Speculatively, the increases in GABA-A receptor α1 protein expression found in these studies may represent a mechanism by which the inhibitory system in the hippocampus is strengthened. Although the strengthening of the inhibitory system may originate as an adaptive response to acute neurotoxicity, it may ultimately contribute to maladaptive hypofunctioning chronically following TBI. The functional consequences of increased inhibitory tone may contribute to long-term changes in LTP and behavioral outcome mediated by the hippocampus.

Following TBI, acutely administered NMDA receptor antagonists are beneficial to cognitive recovery (Phillips et al., 1997). Likewise, GABA-A receptor agonists have been shown to be beneficial to MWM performance (O'Dell et al., 2000), indicating that alterations to the GABA-A receptor may contribute to cognitive deficits and these behavioral changes may involve an important interaction between excitatory (NMDA) and inhibitory (GABA-A) receptors.

The changes to α1 were likely driven by an elevated $[Ca^{2+}]_i$- induced mechanism, since the subunit was normalized when NMDA-mediated calcium influx was blocked by MK-801 prior to injury. Acute neuronal activity and the subsequent influx of intracellular calcium mediated by the NMDA receptor have been well- documented following TBI. Study 2 indicates that pharmacological

blockade of the NMDA receptor is not only beneficial in reducing excitation acutely, but may also play a role in normalizing inhibitory function chronically. These studies, therefore, support the hypotheses that TBI alters the GABA-A receptor and that this alteration is driven by a calcium-mediated mechanism.

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Vita

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