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Acute GABA-A Receptor Modulation by Diazepam Following Traumatic Brain Injury in the Rat: An Immunohistochemical Study

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List of Abbreviations

ABC	avidin-biotin-complex
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
BAL	blood alcohol level
BBB	blood-brain barrier
β2/3	GABA-A receptor subunits β2 and β3
BZ	benzodiazepine
CBF	cerebral blood flow
CCI	controlled cortical impact
ChAT	choline acetyltransferase
Cl-	chloride
CNS	central nervous system
Co^{2+}	cobalt
CSF	cerebrospinal fluid

CT	computerized tomography
DAB	diaminobenzidine
DAI	diffuse axonal injury
DG	dentate gyrus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAA	excitatory amino acids
EC	entorhinal cortex
ECF	extracellular fluid
EEG	electroencephalograph
FP	fluid percussion
FPI	fluid percussion injury
GABA	gamma-aminobutyric acid
GABAaR	GABA-A receptor
GABA-T	gamma-aminuobutyric acid transferase
GAD	glutamic acid decarboxylase
GCS	Glasgow Coma Scale
ICP	intracranial pressure
IHC	immunohistochemistry
IP3	inositol 4,5-triphosphate
IR	immunoreactive

IR	immunoreactivity
K+	potassium
LEA	lateral entorhinal area
LTP	long term potentiation
MABP	mean arterial blood pressure
MAP2	microtubule associated protein 2
MCID	micro computer imaging device
MEA	medial entorhinal area
mGluRs	metabotropic glutamate receptors
mRNA	messenger ribonucleic acid
MWM	morris water maze
NMDA	N-methyl-D-aspartate
NT	neurotransmitter
PID	post-injury day
PTZ	pentylenetetrazol
SEM	standard error of the mean
SDH	subdural hematoma
SLM	stratum lacunosum-moleculare
SO	stratum oriens
SP	stratum pyramidal
SR	stratum radiatum

TBI	traumatic brain injury
Zn+	zinc

Abstract

ACUTE GABA-A RECEPTOR MODULATION BY DIAZEPAM FOLLOWING TRAUMATIC BRAIN INJURY IN THE RAT: AN IMMUNOHISTOCHEMICAL STUDY

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Traumatic brain injury (TBI) disrupts ionic balance and produces acute widespread depolarization. Restoration of ionic balance and neuronal function after TBI may be achieved by increasing inhibitory neurotransmission (e.g., stimulating GABA-A receptors). This study used antibodies specific for β2/3 subunits to examine changes in GABA-A receptors in the rat hippocampus 24 hours following moderate fluid percussion TBI. The β2/3 antibody primarily stained dendritic processes. No injury related changes were found in the CA1 but extensive morphological dendritic alterations were found in the CA3 region of the hippocampus. Analysis revealed decreased length of immunoreactive processes in CA3 apical dendrites of injured animals. These changes may represent a sublethal cytoskeletal response to excessive neuroexcitation.

Administration of diazepam 15 minutes prior to injury augmented IR β2/3 processes compared to injured/vehicle and sham groups. This study illustrates that GABA-A receptors are altered following TBI and these alterations may be attenuated by increasing inhibitory neurotransmission.

Chapter I: Traumatic Brain Injury

Human Traumatic Brain Injury

Incidence

Traumatic brain injury (TBI) affects approximately two million people per year in the United States, resulting in 500,000 hospitalizations (Ommaya, Ommaya, Dannenburg, & Salazar, 1996). The 10% overall mortality rate (Pope & Tarlov, 1991) translates into TBI as the third leading cause of death (Sleet, 1987). This represents 2% of all deaths and 26.1% of all injury-related deaths. Overall estimates indicate that approximately 4% of the population will suffer a TBI by the age of 19. TBI may affect children's intellectual capabilities, with about 33% of children who remain unconscious greater than one week having IQs less than 70 (Poper & Tarlov, 1991). Overall, 4% (75,000) of TBI patients will be left with long-term neurological deficits (Cole & Edgerton, 1990).

Public concerns regarding the costs of TBI have become apparent in recent years. In 1987, The US Department of Education, National Institute on Disability and Rehabilitation Research funded the TBI Model Systems of Care, for the development of a comprehensive model care system for TBI patients and their families. This ongoing project is a longitudinal, multi-center study, continuously updated in a standardized national database (Harrison-Felix, Newton, Hall, & Kreutzer, 1996). In 1996, the

Traumatic Brain Injury (TBI) Act (Public Law 104-166) was adopted by Congress in order to expand studies and establish new TBI-oriented programs. By 1999, Health Resources and Services Administration, Maternal and Child Health Bureau required implementation of TBI State Demonstration grant programs for TBI patients and families to improve health and community services in the areas of planning and implementation grants to states (Traumatic brain injury state demonstration grants, 1998 & 1999).

Children and the elderly are at high risk for TBI but the peak risk lies between 15 and 24 years. Males represent up to 70% of TBIs, and nonwhite, urban populations are at the greatest risk (Pope & Tarlov, 1991). Fatality rates are three times higher for males than for females (Sosin, Sacks, & Smith, 1989). Demographics indicate that the average age for a TBI patient is 35. Patients less than 15 years tend to have the shortest hospital stays, those 15-24 have the longest stays, and those over 45 have relatively long hospital stays. More than 50% of TBI patients have a positive blood alcohol level (BAL) at admission, with 39% greater than 0.1 BAL. More than half of all TBIs occur on weekends between 8 p.m. and 4 a.m. (Harrison-Felix et al., 1996).

In 1996, automobile crashes, the leading cause of TBI, accounted for 56% of all cases, with falls representing 10% and violence/ intentional injuries also a major contributor (30%) (Harrison-Felix et al., 1996). Compared to falls, more severe injuries are likely to be associated with auto crashes, resulting in higher incidence of concussion and lower incidence of hematoma (Kalsbeek, McLaurin, Harris, & Miller, 1980). Auto crashes account for 57% of TBI-related mortality (Sosin, Sacks, & Smith 1989) and

hospital stays twice as long as other causes (Kalsbeek et al., 1980). TBI due to motor vehicle crashes is 39% higher for Caucasians than for African Americans (Sosin, Sacks, & Smith 1989), is more likely to be associated with diffuse injuries (Pope & Tarlov, 1991), and accounts for approximately 49% of the total costs of TBI. This is likely due to the more severe nature of the injuries (Grabow, Offord, & Reider, 1984).

Epidemiology - Mild, Moderate and Severe Injury

Many discrepancies have been noted in the classification of injury severity. 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. Various hospital emergency rooms and research studies may define the cut-off points differently. Many other factors are considered that may influence the severity judgement. 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. The GCS score factors 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 TBI

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. Links between posttraumatic headaches and cognitive deficits were examined in one retrospective study, designed to evaluate symptoms such as concentration, memory and thinking deficits. In a sample of patients with post traumatic headaches, 65% reported cognitive problems (62% of these were confirmed by a doctor). The cognitive areas least affected by a mild injury included overall intelligence, language, perceptual and motor functions. The most prevailing cognitive deficits included problems with memory, attention, and information processing. Attention (concentration) was the most affected, followed by memory. Information processing problems typically diminished within three months of injury, whereas memory problems were not always noticeable during the first three months following injury. Age and gender did not seem to be significant factors in mild injury, although females tended to have more symptoms. Most cognitive impairments diminished gradually over the course of the first year post injury (Packard, Weaver, & Ham, 1993). Memory, attention and information processing deficits were likely to have an impact on the patient's lifestyle and job performance ability.

Moderate TBI

Moderate TBI tends to result 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 does, 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 effect 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

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). Several pilot programs are in place across the country to provide supported employment to victims of severe brain injury. One such study, conducted in Richmond, VA, accepted 115 clients who had suffered a severe injury (GCS score less than eight for more than six hours), resulting in severe impairments. Clients were placed in payed, real-work positions and all vocational intervention, job training and behavioral modification were provided at the job site. Most of the clients had deficits of attention, motor speed, verbal learning, verbal memory and visual memory. Average scores on the Wide Range Achievement Test-Revised showed deficiencies in arithmetic, spelling and problem solving (scores less than the 20th percentile). After five years of this program, 70% had been placed in competitive employment for an average of 45 weeks. The mean annual income of these 80 clients was $7079 per year (Wehman et al., 1993).

The Costs of TBI

The monetary costs of TBI are staggering. In 1985, direct costs from mortality and morbidity totaled $37.8 billion (Ommaya et al., 1996). According to a 1985 report to Congress on the incidence of injuries in the U.S., although only 13% of injuries are due

to TBI, they account for 25% of injury-related mortality and 29% of the total injury costs (Rice, Mackenzie, & Associates, 1989). 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 motorcycles ($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). In one year in the US, more than 40,000 patients are estimated to have spent three weeks or more in the hospital due to TBI. 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 include short term intellectual, motor, and learning impairments and long term rehabilitation, revenue loss, and intellectual limitations such as memory and concentration deficits.

Injury Pathology

Types of Injuries

The mechanism of injury determines the type of injury and 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 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 is most likely due to non-penetrating acceleration-deceleration or rotational type injuries but 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 due to the mechanical forces involved at the moment of impact. Primary damage includes 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 (with or without extradural hematoma), 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 following 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. Please see Table 1 for a summary of primary and secondary consequences of TBI.

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. Damaged neurons release excessive excitatory amino acids (EAA), resulting in further

Table 1. Common types of primary and secondary damage following TBI.

Common Forms 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)

depolarization and calcium entry into nearby neurons. Secondary changes are more complicated and prolonged.

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. The initial core of immediate damage, therefore, often spreads to a secondary "penumbra" of neuronal damage and then to a potentially reversible "outer zone" of neuronal dysfunction (Selzer, 1995).

Excitotoxicity and Excitatory Amino Acids

Glutamate is the most widely distributed excitatory neurotransmitter in the brain. High concentrations of glutamate are toxic. 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 is probably due to excessive neurotransmitter release following TBI-induced depolarization of neurons. 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 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).

Chapter II: Experimental Traumatic Brain Injury

Animal Models of TBI

Biomechanics of Experimental Injury

It is important that models of TBI 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 is 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 and their resulting Neuropathologic Sequella

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 places within the skull, causing additive pressure and potential skull fracture in areas other than 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, laceration 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)Eeffects and their resulting Neuropathological Sequella

Acceleration due to impact or impulsive loading produces a different 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 tránslational 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 not considered as important as the resulting sequella. Knowing the injury mechanism in humans is, however, important for safety measure development and prevention.

There are several important aspects of injury that should be represented in a good animal model. These include: the mechanism of injury, the location of brain damage, the type of damage produced, the severity and time course of injury, and long term and short term changes. The model should also be able to effectively assess morphological changes, cerebrovascular changes, metabolic receptor changes, and behavioral changes (Gennarelli, 1994).

Overview of the models by Injury Classification

Acceleration Concussion and Percussion Concussion

Denny-Brown and Russell (1941) distinguished two categories of injury: (a) acceleration concussion and (b) percussion concussion. Acceleration concussion is modeled 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 on 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 (FP) injury 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 FP, 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 such as cognition, memory and long-term outcome; physiology as in cerebral metabolism; and biochemistry, such as gene expression and ionic changes (Gennarelli, 1994).

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, N-methyl-D-aspartate (NMDA), or α-amin-3-hydroxy-5 methyl-4 isoxazale proponic acid (AMPA) do not have this 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 inhibitor 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 FP injury, likely due to the increased vulnerability of neurons to glutamate following injury (Bullock & Di, 1997). Although glutamate is the best characterized excitotoxin, ACh and kainic acid 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 which binds to the NMDA receptor subtype (Regan, 1996).

Experimental Models of Focal and Diffuse Brain Injury

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 have their own sequence of changes and cascades of events, some of which are similar and some of which are distinctively different.

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 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 (Dixon et al., 1991; Soares, Thomas, Cloherty, & McIntosh, 1992; Smith et al., 1995). A common feature of each of these models is that the 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 less brain stem damage, compared to the cortical weight drop model (Povlishock et al., 1994). Central rigid percussion produces coma, contusion to the parasagital cortex under the impact site and non-diffuse axonal

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

Current Uses of 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

damage. Lateral CCI injuries produce a brief coma, a small amount of diffuse axonal damage and contusion at impact site (Gennarelli, 1994).

In the FP 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 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 central FP model producing a more diffuse type of injury Gennarelli, 1994; Povlishock et al., 1994).

Lateral FP injuries (FPI) are more likely to result in focal contusion and 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 FP injuries are 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 FP model (Gennarelli, 1994; McIntosh et al.,

1996, Povlishock et al., 1994). FP injury, however, provides only limited biomechanical control and does not represent the full human TBI spectrum of injury (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 to diffuse forebrain ischemia, resulting in selective neuronal deficits without overt cell loss (Hayes, Jenkins & Lyeth, 1992b).

Species Studied

The choice of species in TBI modeling is important. There may be different patterns and distribution 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 fluid percussion injuries in rats have 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).

Links Between Fluid Percussion Injury and Human TBI

The pressure forces exerted on the brain during experimental FP injuries are similar to those recorded from human cadaver skulls upon impact (Lindgren & Rinder, 1966). Acute neurological symptoms and suppression of behavioral reflexes mimic human unconsciousness/coma (Teasdale, 1976). Dixon et al. (1987) characterized the FP model in rats, 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., 1994).

The Hippocampus

Subsectors of the Hippocampus

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).

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 pathway also has some feed-forward projections to the CA1 and

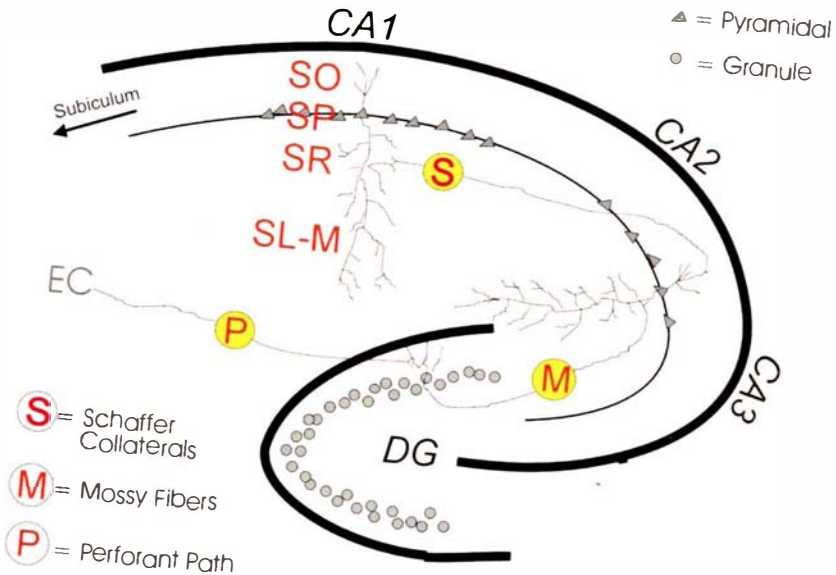
Figure 1. A diagram of a coronal section of the mid-dorsal hippocampal formation of the rat. The primary connective pathways are shown: Schaffer collaterals (S), mossy fibers (M), and the perforant pathway (P). The hippocampus proper is subdivided into CA1, CA2, and CA3 regions and the dentate gyrus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

Key: SO = stratum oriens

SP = stratum pyramidal (pyramidal cell layer)

SR = stratum radiatum

SLM = stratum lacunosum moleculare


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

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 the 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, which is found only in the CA3 and consists of mossy fiber projections from the DG.

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 is 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 cortical 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 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).

Histopathology of TBI

The hippocampus is known to be vulnerable to ischemia, seizures and brain trauma. Cognitive deficits 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 contusions, necrosis, cavitation at injury site, short unconsciousness, BBB disruption (primarily in the contused region), cerebral edema, decreased 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 (PID 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 loss 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 FP model of injury, 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 and Ischemia: Similarities and Differences

Models of TBI and cerebral ischemia both produce injuries that involve neurotransmitters (NTs). The same excitotoxic hypothesis is applied to both types of injury. Ischemia, unlike TBI, however, is associated with ATP energy stores depletion, as well as changes in brain temperature and pH (Hayes, Jenkins, & Lyeth, 1992).

TBI models with focal contusions are analogous to ischemia models with focal cerebral infarction, both producing overt structural damage. Diffuse models of TBI without contusion produce injuries similar to diffuse forebrain ischemia models. Some differences in histopathology exist between the two types of CNS injuries, and TBI does not produce the brain temperature reductions that are associated with ischemia. Although ischemia may be present in many severely injured TBI patients, TBI models must distinguish between damage caused by TBI and damage caused by ischemia (Hayes, Jenkins, & Lyeth, 1992).

Experimental TBI: Time points and the Biphasic Model

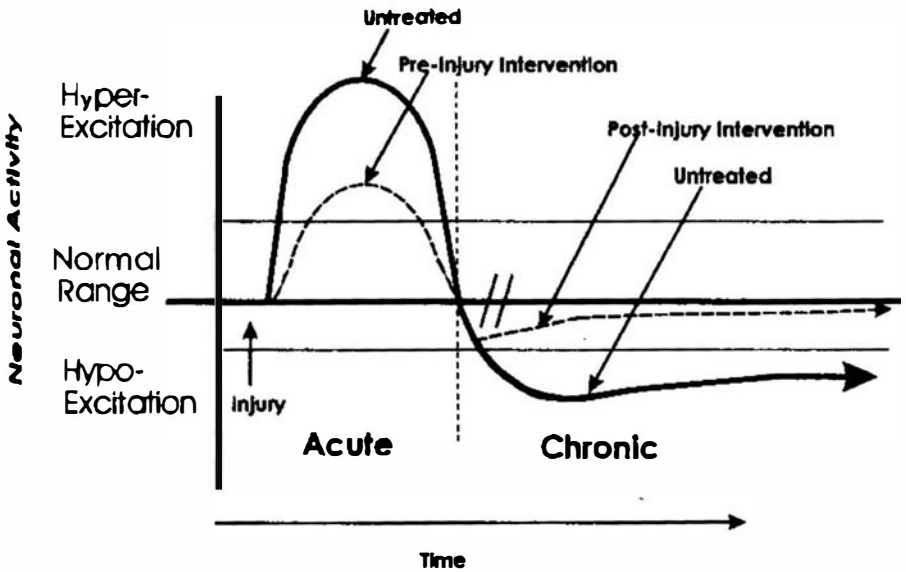
Experimental TBI initiates a cascade of events that alters normal cell signaling and neuronal over-excitation (Hayes, Jenkins, & Lyeth, 1992). Excessive neuronal excitation produces large measurable increases in extracellular potassium, which result in further neurotransmitter (NT) release, leading to further depolarization (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 cerebral metabolism have been demonstrated. During the acute phase, methods of intervention that may be useful include excitatory NT 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 NT 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).

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



Biphasic Model

Impact depolarization and BBB alterations

Ionic Flux

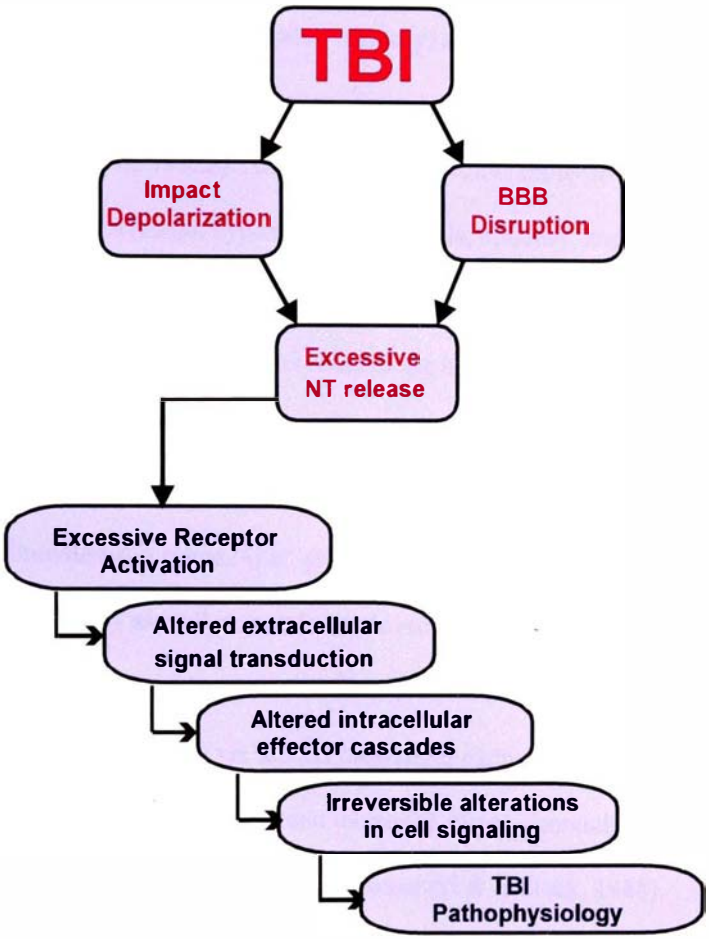
As demonstrated in Figure 3, impact is followed by injury-induced ionic flux across the cellular membrane, leading to widespread neuronal depolarization and indiscriminate NT release (Selzer, 1995). Ionic homeostasis changes following TBI are related to delayed neuronal death and degeneration. Calcium levels increase after injury, especially in damaged regions, and may persist for 48 hours following FP TBI in rats. Increased calcium activates proteases such as calpain, which may ultimately lead to cytoskeletal degradation and neuronal death.

Excitotoxicity: 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 neurotransmitters and amino acids. Pathologically high levels of excitatory neurotransmitters (ACh) and excitatory amino acids (EAA) (glutamate and aspartate) are released, leading to excitotoxicity within the cells (McIntosh et al., 1996).

Glutamate can produce powerful neurotoxic effects following CNS injuries such as ischemia, hypoxia, and 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.

Figure 3. A flow-chart of the neuronal cascade of events following TBI.


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

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). Selective vulnerability to sublethal cellular injury following non-contusional TBI include the CA1, CA3 and dentate gyrus areas of the hippocampus and specific layers of the neocortex (Hayes, Jenkins, & Lyeth, 1992b).

Neurotransmitter Changes Following TBI

Indiscriminate EAA release (i.e., glutamate) activates NMDA and AMPA/KA type ionotropic receptors as well as metabotropic receptors. Receptor activation leads to opening of ion channels and subsequent calcium and sodium influx. 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).

Increases in glutamate following FPI in rats peak within minutes and may last up to one hour (Faden et al., 1989). 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). 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) and produces a large potassium influx (Kawamata et al., 1992).

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 leads to BBB opening in the cortex and hippocampus (Povlishock & Lyeth, 1989; Jiang et al., 1991a). Injury allows greater permeability to exogenous (blood-borne) NTs and neuromodulators (Koide, Wieloch, & Siesjo, 1986). Acute BBB permeability in the cortex and hippocampus have been documented for moderate FP TBI in rats (Povlishock & Lyeth, 1989; Jiang et al., 1992). BBB permeability may last up to15 hours (Ellis, Chao, & Heizer, 1989). Blood plasma ACh levels are 7-fold greater than CSF ACh levels (Robinson et al., 1990) and may contribute up to 39% of 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.

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 3 hours post-injury (Oleniak et al., 1988). The NMDA glutamate receptor subtype has a voltage dependent magnesium (Mg) blockade. The Mg blockade determines the extent of NMDA receptor involvement in TBI pathology following injury and excessive glutamate release. NMDA receptor binding has been shown to decrease 12-15% within three hours in the CA1 of the hippocampus following moderate TBI in rats. Most changes in glutamatergic binding affinity occur in the NMDA-type receptors (Hayes, Jenkins, & Lyeth, 1992b). NMDA and nonNMDA (AMPA/KA) receptors are directly correlated to selective patterns of vulnerability and damage in specific brain areas following TBI. Acute decreases in receptor binding for NMDA-type receptors in the inner and outer layers of the neocortex and the CA1 striatum radiatum and molecular layer of the DG in the hippocampus three hours post-injury, are not seen in AMPA/KA or quisqualate-type 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 LTP suppression (Hayes, Jenkins, & Lyeth, 1992b).

Receptor antagonists have been shown to have some limited usefulness in attenuating the detrimental effects of TBI pathophysiology. NMDA antagonists such as MK-801, phencyclidine (PCP) and dextrorphan have been shown to have some effectiveness 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 and the very short therapeutic window (maximum 15-30 minutes in rats) associated with attempts to reduce excitotoxicity due to NT release (Hayes, Jenkins, & Lyeth, 1992b; McIntosh et al., 1996; Faden et al., 1989).

Treatment of the inhibitory system has been studied using chronic endpoints, although the effects are less well characterized than the excitatory NT and receptor antagonists. Chronic injections of pentylenetetrazol (PTZ), a GABAaR antagonist, following injury induced seizure activity, which enhanced injured and harmed sham cognitive performance in the 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 on MWM improvement (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.

Chapter III: The GABA-A Receptor

The Balance Between Excitation and Inhibition

Understanding Inhibitory Neuroprotection

The overexcitation induced by TBI is also characteristic of other types of CNS insults. Simply identifying excessive excitation is unlikely to present the entire cascade of events that occurs following injury. It is more likely that the normal 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 is found following hypoxia combined with lowered brain temperature (Fujisaki, Wakatsuki, Kodoh, & Shibuki, 1999).

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 (GABAaR) 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 GABAaR binding detriments (Arika, Kanai, Murakami, Kato, & Kogure, 1993; Inglefield, Wilson, & Schwartz-Bloom, 1997) and injury-induced decreases in chloride ion (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 GABAaR 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 lateral TBI selectively destroys CA3 and hilar neurons (Hayes, Jenkins, & Lyeth, 1992).

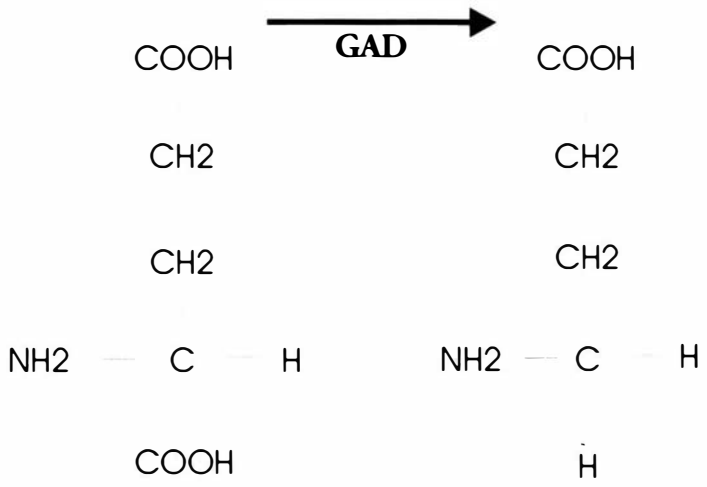
The excitatory input in the hippocampus is balanced by inhibitory processes, often on the same cell. Pyramidal neurons receive synaptic input from both glutamate and GABA receptors (Li, Siegel, & Schwartz, 1993). Glutamate is a precursor to GABA.

GABA is formed when glutamic acid decarboxylase (GAD), which is only found in neurons, removes a carboxyl group from glutamate (see Figure 4) (Luddens, Korpi, & Seeburg, 1995). GABA is the most prevalent inhibitory neurotransmitter in the brain, exerting its effects primarily through the GABAaR complex. The GABAaR acts to increase membrane hyperpolarization via modulation of a Cl- channel. The GABA-B receptor differs from the GABAaR 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).

The GABAaR is potentiated by ligands which bind to sites specific for benzodiazepines (BZs), barbiturates, alcohol, steroids, zinc ions (Zn+), or various anesthetics (Marrow, 1995; 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 GABAaRs 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 GABAaRs in hippocampal interneurons indicates that inhibition is an important mechanism for maintaining the excitatory balance in neurons (Gao & Fritschy, 1994).

Figure 4. A diagram of the transformation of glutamate into GABA by the enzymatic action of GAD.



Glutamate **GABA**

Relationship Between EAA and GABA-A

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 bicuculline (a GABA-A antagonist), indicating that the GABA-A receptor complex plays a modulatory role in ACh release (Supavilai & Karobath, 1985).

Group I metabotropic glutamate receptors (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 GABAaRs. Group II mGluRs are located on inhibitory terminals and act to reduce GABA release. In the CA1 of the hippocampus, Group I mGluRs modulate pyramidal cell input from GABAergic interneurons. Activation of Group I 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 GABAaRs are co-localized in hippocampal neurons (Craig, Blackstone, Huganir, & Banker, 1994). Modulation of NMDA glutamate receptors by MK-801 antagonism decreases GABAaR-mediated Cl- uptake by 44% in the hippocampus (Matthews, Dralic, Devaud, Fritschy, & Marrow, 2000). Stimulation of GABAaRs 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).

The GABA-A Receptor

The GABAaR 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). GABAaRs 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). GABAaRs 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). GABAaR activation also reduces glucose metabolism and mediates cerebral blood vessel dilation, improving blood flow (Lyden, 1997).

GABAaR Subunits

The GABAaR 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). Five subunits, each with several isoforms have been identified, including: $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$, δ, 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).

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., α1 and α2) have 70% identical amino acid sequences. Amino acid GABAaR 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 et al., 1992). Of the five identified subunits, α, β, and γ are considered the "main" subunit types, while δ and ρ are considered "minor" subunit types (Lyden, 1997; Mohler et al., 1996). The δ 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. GABAaRs 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).

GABAaRs with αβγ subunit combinations are the most widely distributed type receptor type and this combination is important for proper benzodiazepine (BZ) binding (Sieghart, 1995). The most abundant GABAaRs 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

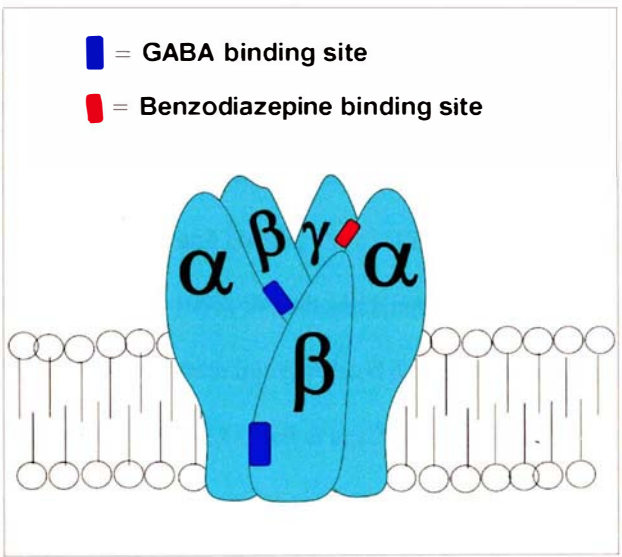
Table 3). Specific brain regions, such as the hippocampus, contain more or less abundant populations of GABAaR subtypes. One of the most expressed receptor subtypes in the hippocampus is $\alpha 2\beta 3\gamma 2$, although in other areas of the brain the $\alpha 1\beta 2\gamma 2$ GABAaRs are most abundant (Sieghart, 1995). Overall, 75% of all GABAaRs are made up of three receptor subtypes: $\alpha 1\beta 2\gamma 2$, $\alpha 2\beta 3\gamma 2$, and $\alpha 3\beta 3\gamma 2$ (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 GABAaR. The binding site for BZs lies on the γ subunit at the α junction (Sieghart, 1995) (See Figure 5). The BZ binding site location determines its function, indicating that although α contributes to binding specificity, the presence of either $\gamma 2$ or $\gamma 3$ 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 $\gamma 2$ subunits (40-50% of GABAaRs) 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 $\alpha\beta$ junction (Refer to Figure 5), it also follows that α plays an important role in GABA binding affinity, although this

Table 3. The characteristics of each of the known subunits of the GABAaR.

Subunit	Properties
α	Important to BZ ligand binding 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 GABAaRs Co-localizes with β2 or β3 and γ2 in 70-75% of GABAaRs 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 hippocampus)
α_6	Only found in cerebellar granule cells
β	Found in all in vivo GABAaRs investigated Contains binding site for GABA (on β, at α β junction)
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 a γ to form BZ Type I receptors
β_3	Tends to co-localize with α2 and a γ to form BZ Type II receptors
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

Figure 5. A diagram of a common composition of the GABAaR identifying GABA and BZ binding sites.


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

remains to be definitively determined (Huh, Delorey, Endo, & Olsen, 1995; Huh, Endo, & Olsen, 1996; Stephenson, 1995).

Modulation of the GABAaR by Ligand Binding

The numerous binding sites on the GABAaR translate into complex pharmacological potential. Modulation of the GABA-B receptor does not show neuroprotection in ischemia (Araki, Kato, & Kogure, 1991; Ito, Watanabe, Isshiki, & Uchino, 1999). GABAaR agonists, however, have consistently demonstrated their effectiveness in several types of CNS insults (Arika et al., 1993; Cross et al., 1991; 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 (Hernandez, Heninger, Wilson, & Gallager, 1989; Ito et al., 1999; O'Dell et al., 20000; Ohkuma et al., 1994).

Although GABAaR 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-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 GABAaR 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 (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 GABAaR, 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 GABAaR 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 the interneurons (Gamrani et al., 1986; Somogyi et al., 1996; Terai, Tooyama, & Kimura, 1998). Subunit-specific antibodies that stain the GABAaR demonstrate that GABA-A is most often located along the membranes of somata and on the axonal and dendritic processes (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 GABAaR 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).

One of the best-known and most often used antibodies in GABAaR immunohistochemical staining is bd17 (Boehringer Mannheim), which recognizes both GABAaR β2 and β3 (β2/3) subunits (Benke et al., 1991; Ewert et al., 1990; Pesold et al., 1997). The least expressed of the β subunits is β1 (Stephenson, 1995). Although all β

subunits are present in the hippocampus, β1 IR is rare and β3 is much less abundant than β2 (Huh et al., 1995). Since β subunits are important for GABA binding and have been found in nearly all GABAaR types in vivo (Stephenson, 1995), the bd17 antibody is likely to stain nearly all of the GABAaRs in the hippocampus. Development of β2- and β3- isoform-specific antibodies has introduced difficulties not found in other subunit types. The β2 and β3 subunits both lie within the 50-58 kDa weight range (Matthew et al., 2000). To further complicate matters, the β intracellular loops are identical and β subunits do not have the hydrophilic C-terminus which is present in other subunits (Stephenson, 1995).

Chapter IV: Acute GABA-A Receptor Modulation by Diazepam Following Traumatic Brain Injury in the rat: An Immunohistochemical Study

Introduction

As demonstrated in the previous chapter, inhibitory agonists are effective in counter-acting ischemia-induced excitotoxicity. Although ischemia and TBI produce similar injuries, there are some important differences (see Chapter II). It is unknown whether treatments that show neuroprotection in ischemia will be effective in attenuating TBI-induced deficits.

Although the primary focus in acute TBI treatment has been to reduce excessive neuroexcitation, restoring the normal excitatory/ inhibitory balance by increasing inhibition may also be beneficial. In a model of mild weight drop-induced closed head injury followed by hypoxia, Katoh et al. (1998) measured glutamatergic and GABAergic changes in the hippocampus using microdialysis and autoradiography methods. Elevations in glutamate and GABA were observed in the CA1 and CA3 regions of the hippocampus. Autoradiography revealed increased binding to NMDA-type glutamate receptors and decreased binding of muscimol to GABAaRs in the CA1 at 1 and 24 hours following ischemia (Katoh, Shima, Nawashiro, Wada, & Chigasaki, 1998). Increased glutamatergic and decreased GABAergic binding in the hippocampus during the first 24

hours following injury indicates a disruption in excitatory/ inhibitory homeostasis may be occurring.

The hippocampus, which is selectively vulnerable to TBI, may be better able to maintain an appropriate excitatory/ inhibitory balance post-injury if the overexcitation of neurons is attenuated by increasing GABAergic neurotransmission. O'Dell (1995) investigated three GABAaR agonists following moderate FPI in rats. Although little benefit was found following muscimol or midazolam treatment, pre-injury injections of 5 mg/kg of diazepam effectively attenuated TBI-induced morris water maze (MWM) deficits. Using anti-GABA antibodies, O'Dell (1995) also found that DZ significantly increased GABA-positive IR in the hippocampus 24 hours following TBI. Cognitive deficits and cell loss following ischemia, which produces a similar excitotoxic pathology, have also been shown to be attenuated by DZ and other inhibitory agonists.

Although the specific mechanism mediating various behavioral effects of GABAergic drugs is unknown, stimulation of GABA-induced Cl- channel opening by DZ during the acute phase of TBI may help to reduce excitotoxic damage. Acute DZ treatment has been shown to attenuate TBI-induced MWM deficits in a study of central FPI in rats. The central FPI produces a diffuse injury with sublethal morphological alterations. Both pre- and post- injury DZ (5mg/kg) treatment effectively attenuated TBI-induced MWM deficits 15 days following TBI in rats. The benefits of GABAaR agonist activity by DZ were in direct contrast to the exacerbation of deficits by administration of the GABAaR antagonist bicuculline (O'Dell et al., 2000). Although 10 mg/kg is the

standard DZ dose for ischemia (Inglefield, Wilson, & Schwartz-Bloom, 1997; Li, Siegel, & Schwartz, 1993; Schwartz et al., 1994), this dose has not been found effective in attenuating TBI-induced deficits (O'Dell, 1995). However, 5 mg/kg has been shown to be effective in TBI, without significantly lowering brain temperature (O'Dell et al., 2000). DZ has been shown to be neuroprotective when administered within 72 hours following ischemia in the rat (Johansen & Diemer, 1991) and up to four hours following ischemia in the gerbil (Schwartz et al., 1998). In TBI, 5 mg/kg DZ was equally effective in cognitive attenuation when administered either 15 minutes prior to or 15 minutes following FPI (O'Dell et al., 2000).

Cognitive deficits following injury may be preceded by morphological changes in selectively vulnerable areas such as the hippocampus. Ischemia literature has shown that α1 and β2 mRNA decreases in the CA1, CA3 and DG of the hippocampus by four hours following reperfusion. mRNA returns to normal in the CA3 and DG by twelve hours, although the CA1 continues to decrease steadily over the next three days, ultimately decreasing by 85% of normal values. The CA3 and DG do not characteristically show changes following ischemia, whereas, the CA1 region shows delayed degeneration and cell death by four days post-injury (Li, Siegel, & Schwartz, 1993). GABAaR α1 subunit immunoreactive changes do not become apparent until three to four days following ischemic insult, coinciding with cell loss in the CA1 (Inglefield, Wilson, & Schwartz-Bloom, 1997).

The pathological morphology of ischemia is likely to differ from other types of CNS insults. In a study of TBI in the rat, Reeves et al. (1997) found that two days post-injury population spike inhibition was reduced in the CA3 commissural pathway to the CA1 and GABAergic immunobinding increased in the SLM and SP layers of the CA1. Perforant pathway lesions produce changes in interneurons positively labeled for β2/3 subunits in the molecular layer of the DG by 24 hours post-lesion (Mizukami et al., 1997). In TBI, changes in neurons stained with anti-GABA antibodies also become apparent by 24 hours following injury (O'Dell, 1995). The 24 hour time point allows initial impact depolarization and excessive neurotransmitter changes to occur, but still falls within the acute time period for TBI in rats. The excitatory/ inhibitory ratio is likely to be disrupted and changes in receptor binding (Katoh et al., 1988) and GABAergic immunoreactivity (O'Dell, 1995; Reeves et al., 1997) are evident.

GABAaRs are present on nearly all CNS neurons and mediate the majority of inhibition. Changes in GABAergic neurotransmission following injury would indicate that receptor changes may also occur. Changes in GABAaR α1 subunits in the CA1 have been demonstrated following ischemia (Inglefield, Wilson, & Schwartz-Bloom, 1997) and sub-laminar-specific changes in β2/3 subunits in the molecular layer of the DG have been found following perforant pathway lesion (Mizukami et al., 1997). It would follow that TBI-induced GABAaR changes may also occur.

Since the bd17 antibody has been shown to specifically stain the β2/3 subunits of the GABAaR (Matthew et al., 2000) and since β subunits are constituent parts of all

known GABAaRs, it would follow that the bd17 antibody would stain virtually all of the GABAaRs in the hippocampus (β1 is rare in the hippocampus - See Chapter III).

The current study was designed to further investigate the role of inhibition following TBI. GABAaR IR may be altered following FPI and acute treatment with DZ should attenuate these alterations. Injury-induced changes in GABAaR β2/3 subunit IR were expected 24 hours following TBI, since GABAergic changes have previously been documented for this time point (O'Dell, 1995).

Methods

Subjects

Adult male Sprague-Dawley rats were divided into four groups of 5 animals (4 groups x 5 animals = 20 animals total): sham/vehicle, sham/DZ-treated, injured/vehicle, and injured/DZ-treated. The number of subjects per group was chosen because previous immunohistochemical studies have found 4-5 animals to be sufficient for statistical significance (Inglefield, Wilson, & Schwartz-Bloom, 1997; Li, Siegel, & Schwartz, 1993; Neumann-Haefelin et al., 1998). Animals were individually housed in a vivarium on a 12:12 hour light/dark cycle and received food and water ad libitum.

Pharmacological Manipulation

Diazepam (5 mg/kg) was obtained from the local hospital pharmacy and administered to animals in one bolus injection (intra-peritoneal) 15 minutes prior to injury or sham-injury. This dose has been shown to be effective in attenuating cognitive deficits in the MWM without significantly altering MABP or brain temperature after injury

(O'Dell et al., 2000). The pre-injury injection was chosen as appropriate for analyzing morphological changes to the GABAaR following injury. Also, no significant differences were found in cognitive endpoints between pre- and post-injury injections. The pre-injury injection did, however, attenuate immediate post-injury mortality (O'Dell et al., 2000).

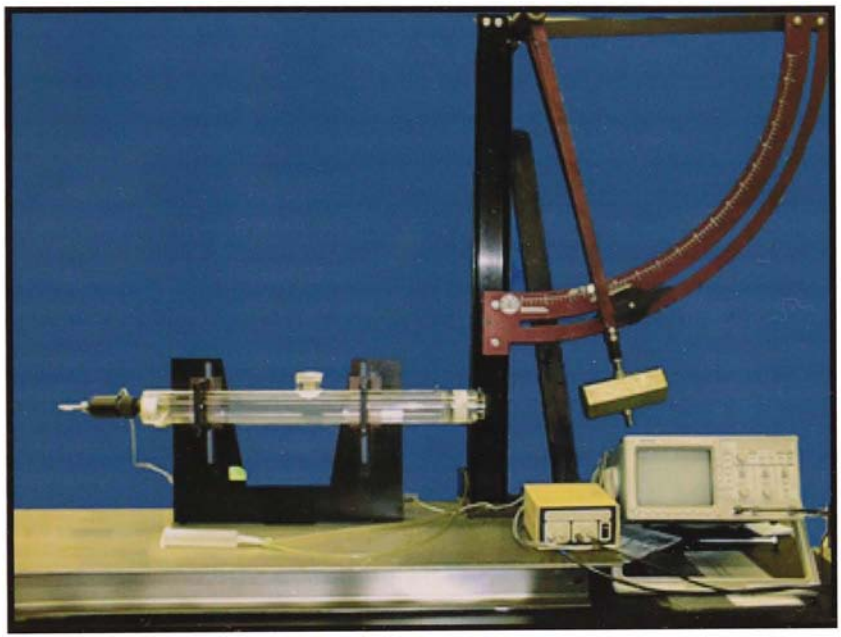
Surgical Preparation

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.

Fluid Percussion Injury

The fluid percussion injury device has been described in detail elsewhere (Dixon et al., 1987; McIntosh, Noble, Andrews, & Faden, 1987) and is shown in Figure 6.

Figure 6. A picture of the fluid percussion injury device.



Briefly, a Plexiglass cylinder 60 cm long and 4.5 cm in diameter 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 vehicle 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).

Animals were anesthetized under 4% isofluorane in a carrier gas consisting of 70% N_2O and 30% O_2, twenty-four hours following surgical preparation. The previous 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 was applied. Neurological assessments including cornea, pinnae, toe, tail and righting reflexes were monitored. The animals were closely monitored until they had sufficiently recovered and were then transferred back to the vivarium where food and water was available.

GABAaR Immunohistochemistry

Twenty-four hours following injury, brain tissue was fixed via cardiac perfusion. A solution of 4% paraformaldehyde and 15% of a solution of picric acid in 0.1 M phosphate buffer followed a 50 mL rinse with PBS. Immediately following perfusion, the brain was removed and placed in the paraformaldehyde solution for three hours. Blocked sections, 10 to 15 mm thick, were incubated overnight in a 0.1 M citric phosphate buffer, pH 4.5. The blocked sections were then boiled in the same solution in a 650 Watt microwave oven for two to three minutes. After the tissue cooled, it was incubated in PBS containing 10% Dimethyl Sulfoxide (DMSO) (Sigma) for three hours.

Consecutive coronal vibratome sections (40 μm thick) were taken from the mid-dorsal hippocampus. Four mid-dorsal sections per animal, 200 μm apart, were selected and incubated overnight at room temperature in primary antibody solution. The GABAaR β2/3 specific antibody bd17 (Boehringer Mannheim) was diluted 1:500 in Tris-saline (pH 7.4), as determined by preliminary concentration tests. Normal serum (2%) made in horse (anti-mouse) was added to the primary antibody incubation solution. Sections were then rinsed 3 x 10 minutes in 50 mM Tris-saline buffer with 0.05% Triton X-100, pH 7.4. Rinsed sections were incubated in biotinylated antibody (anti-mouse) diluted in a 50 mM solution of Tris-saline, pH 7.4 for 30 minutes at room temperature. Following the incubation period, tissue was rinsed 3 x 10 minutes in Tris-saline with Triton and incubated for 15-20 minutes in ABC Reagent (Vector ABC Kit). Then, sections were washed 3 x 10 minutes in Tris-saline with Triton and incubated in 0.1% 3,3'-

diaminobenzidine dihydrochloride (DAB) diluted in 50 mM Tris-saline, pH 7.7. The reaction took place in a Tris-saline solution (pH 7.7) containing 0.05% DAB, 0.2% nickel (II) sulfoxide, 2% β-D-glucose, 0.04% ammonium chloride and 0.0005% glucose oxidase (Sigma, type VII). The reaction was stopped by a quick rinse in Tris-saline, pH 7.4 at 4°C. Sections were washed 3 x 10 minutes in Tris-saline with Triton and mounted on gel coated slides. This procedure was selected for use based on reports that it can decrease background staining and enhance details of immunostained GABAaRs (Fritschy et al., 1996). One of the unique features of the immunohistochemistry (IHC) method described above is the microwave antigen retrieval. This method was shown in preliminary studies to be effective in reducing background and increasing the signal-to-noise ratio. Tissue shrinkage which may occur due to boiling should be uniform across groups, since all sham and injured tissue was subjected to the same protocol. Detailed immunohistochemical protocols are available at www.unizh.ch/phar/neuromorpho/Protocols.htm. The final visualization process used was modified from the original protocol, utilizing glucose oxidase rather than peroxide to drive the reaction. Preliminary studies indicated that the glucose oxidase reaction was longer-lasting and produced more consistent results than peroxide.

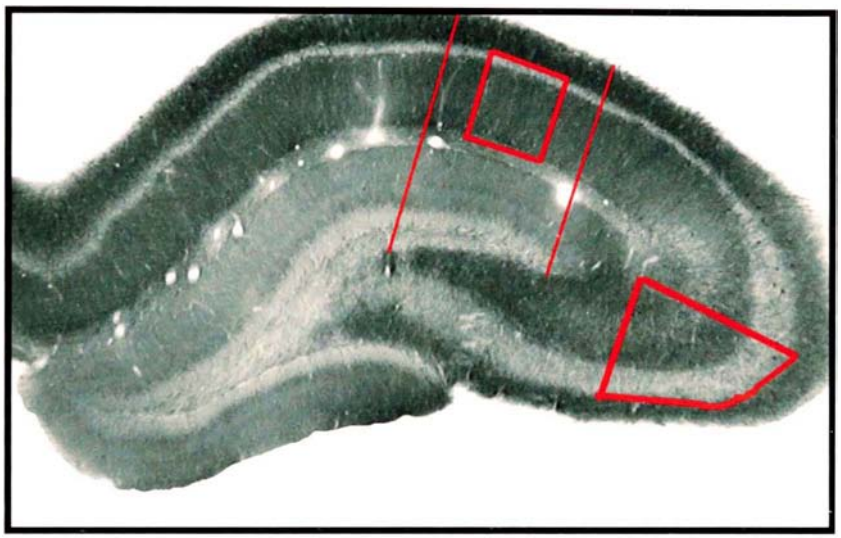
Image Analysis

The hippocampal CA1 and CA3 sectors were examined for the presence of GABAaR-IR cells. The CA1 region was chosen based upon data presented in the ischemia literature, which shows selective vulnerability in this area. Also, GABAergic

changes have been found in specific laminae of the CA1 following TBI (O'Dell, 1995). The CA3 region was chosen for analysis because of its potential for change following TBI and since qualitative observations in preliminary studies indicated injury-induced changes in this area. GABAaRs primarily stained pyramidal and interneuron dendritic processes, ruling out the possibility of counting cell bodies (profile count). Due to the non-uniform orientation of the dendrites and the variation in staining intensity, standard optical density measures (relative optical density x area) were not a viable option for analysis. For these reasons, number and length of IR dendritic processes in three laminae of the CA1 and CA3 fields, including the pyramidal cell layer, SR and SLM were quantified. The SO layer was not included because it was too densely stained to effectively assess individual processes and no qualitative differences were observed in that layer. The remaining three layers (pyramidal cell, SR and SLM) were analyzed by an MCID image analysis system, which was calibrated specifically for the 20x objective used to capture the images.

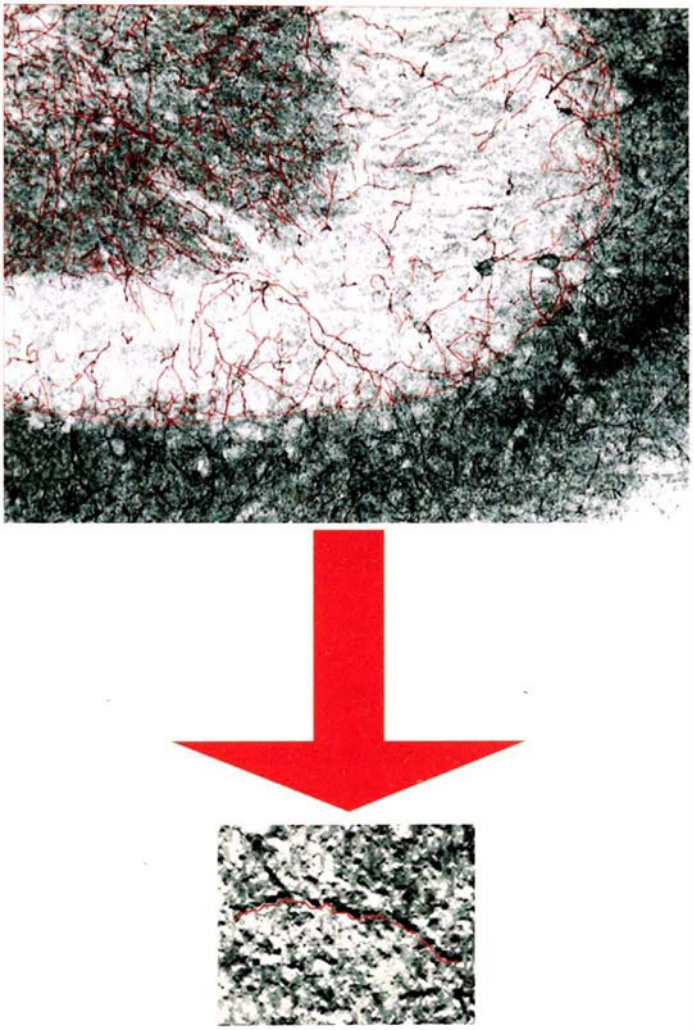
Sections included in the analysis were coronal slices (2-3 per animal), separated by a minimum of 200 μm. These sections were from the mid-dorsal hippocampus and were analyzed in both the CA1 and CA3 regions (see Figure 7). Since the central FPI does not show significant morphological differences in the hippocampal formation

Figure 7. A picture of the mid-dorsal hippocampus of a sham animal immunolabeled with the bd17 antibody. Areas in red boxes represent CA1 and CA3 regions selected for analysis.



between hemispheres, only one hippocampus (randomly chosen) was analyzed per tissue section.. Images were captured at 20x magnification, and were then enlarged by 50% in the CA3 in order to allow for a representative cross-selection of the SP, SR, and SLM fields. Due to the increased length of the CA1 apical dendritic trees in the above-mentioned layers, images were enlarged 33%. The image magnification and sample area were held constant for all sham and injured groups. Each IR process within the pre-designated area was traced, allowing for the analysis of both number and length of processes. In order to insure random sampling of stained tissue, all visible processes had an equal chance of inclusion (Coggeshall, 1999). Since the width of individual dendrites varied due to proteins embedded in the membrane, processes were traced along the external edge of one side, following the contours of the process (see Figure 8). The area of analysis was standardized per 10,000 μm^2 to account for group differences and potential variation in area demarcation. Since injury may result in shrinkage of tissue, in order to determine that changes were due to injury-induced alterations rather than a change in the reference space itself (Coggeshall & Lekan, 1996), CA1 sham and injured lamina were measured and compared. Injured tissue was slightly shrunken in the SR and SLM layers (0.4%) and in the SP layer (7.5%). The majority of IR processes were analyzed in the SR and SLM layers, therefore, injury-induced alterations between sham and injured tissue in excess of 0.4% were considered valid. The mean length and number of processes for each animal were obtained for both the CA1 and CA3 regions by first averaging the tissue section results for each subject and then analyzing group differences.

Figure 8. An enlarged example (100%) of an analyzed CA3 dendrite (captured at 20x magnification). The red line is an example of a measurement of the distribution of GABAaRs along the length of the dendrite, following the outer contours of the process.



Statistical Analysis

The study was arranged as a 2 x 2 completely randomized design. The two variables were injury level (injured or sham) and drug treatment (saline or 5 mg/kg DZ). A Chi-Square test of homogeneity was used to analyze mortality rates between groups. Two factor ANOVAs were used to analyze potential injury and treatment differences in number and length of IR processes in both the CA1 and CA3 regions. The number of processes were analyzed by number per 10,000 square μm to account for potential differences in areas selected for analysis. A simple effect analysis was used to compare groups when interaction effects were found and a significance level of $p<.05$ was used for all tests.

Results

Post-Injury Reflexes

A two-way ANOVA (injury x treatment) indicated that injury significantly suppressed reflexes. Toe pinch (measured in minutes), was suppressed in injured groups ($\underline{M} = 4.974$) compared to sham groups ($\underline{M} = 1.885$), $F_{(1, 15)} = 5.506$, $p < .05$. Corneal reflex (measured in minutes) was also suppressed in injured groups ($\underline{M} = 6.431$) compared to sham groups ($\underline{M} = 1.780$), $F_{(1, 15)} = 8.084$, $p < .05$. Neither the toe pinch nor the corneal response, both of which are simple reflexes, demonstrated significant treatment effects. The more complex righting reflex (measured in minutes) was delayed following injury ($\underline{M} = 24.682$), compared to sham groups ($\underline{M} = 6.444$), $F_{(1, 15)} = 31.785$, $p < .0001$. The pre-injury injections also produced significant suppression of reflexes for

DZ-treated (M = 23.602) compared to saline-treated (M = 5.618) animals, $F_{(1, 15)} = 33.852$, $p < .0001$. A significant interaction was found between injury and treatment groups for righting latency, $F_{(1, 15)} = 5.276$, $p < .05$. A simple effects analysis revealed that saline-treated groups had significantly longer righting latencies following injury (M = 11.178) than following sham treatment (M = 1.170), $F_{(1, 7)} = 6.189$, $p < .05$. DZ-treated animals also had delayed righting latencies following injury (M = 35.486), compared to sham treatment (M = 11.718), $F_{(1, 8)} = 29.605$, $p < .01$. Although both saline-treated and DZ-treated animals demonstrated significant injury effects on righting reflex, DZ extended the righting latency in both injured and sham animals. Therefore, although pre-injury DZ treatment did not significantly effect simple reflexes, the more complex righting response was altered due to treatment.

Mortality Rates

Mortality rates for animals in each group are shown in Table 4. A Chi-Square test of homogeneity revealed a marginally significant difference in mortality between groups, $\chi^2_{(3, N = 22)} = 7.444$, $p = .059$. The only group that experienced mortality over the course of this experiment was the injured-saline group (43% mortality). DZ effectively prevented injury-related death (0% mortality). The primary cause of mortality in injured/saline-treated animals was pulmonary edema.

Qualitative Tissue Evaluation

As expected, GABAaR β2/3 subunit IR was located primarily in the dendritic processes of pyramidal cells and interneurons. In the hippocampus proper, the SP layer

was mostly devoid of staining, although the membranes of some pyramidal cell somata were IR. Positively labeled receptors were extremely dense in the SO layer, and the SR and SLM layers were also heavily stained. In the DG, only the molecular layer showed discernable IR, although it was not as profusely labeled as the dendritic layers of the hippocampus proper (See Figure 9).

Evaluation of processes in the CA3 region revealed pervasive dendritic segmentation and an overall reduction in stain quality in injured/saline-treated animals compared to sham/saline-, sham/DZ-, and injured/DZ-treated groups (See Figure 10). The appearance of segmental beading (varicosities) along processes was specific to the injured/saline group and was only found in the CA3 region.

GABAaR Distribution in CA1 Dendritic Processes

Although injury-induced alterations were expected in the CA1 of the hippocampus, two factor ANOVAs (injury x treatment) did not reveal a significant injury effect on number ($F_{(1,9)} = 0.154$, $p > .05$) or length ($F_{(1,9)} = 0.027$, $p > .05$) of GABAaR β2/3 IR dendrites (see Figure 11 for photomicrographs of the CA1). Since no significant injury-induced changes were found, alterations could not be attenuated by DZ, and treatment effects were not revealed for either number ($F_{(1,9)} = 0.004$, $p > .05$) or length of IR dendritic processes ($F_{(1,9)} = 0.015$, $p > .05$). Also, no significant interaction effect for number ($F_{(1,9)} < 0.001$, $p > .05$) or length ($F_{(1,9)} = 0.242$, $p > .05$) of processes were found in the CA1. Collectively, these analyses indicate that no significant GABAaR β2/3 subunit alterations are evident in the CA1 region of the hippocampus 24 hours following

TBI. Figure 12 shows the mean number and Figure 13 shows the mean length of IR processes per group in the CA1 region of the hippocampus.

GABAaR Distribution in CA3 Dendritic Processes

A two factor ANOVA (injury x treatment) revealed that the overall number of dendritic processes in the CA3 was not altered by injury ($F_{(1, 10)} = 0.820$, $p > .05$). Drug treatment also did not significantly effect the number of processes in this region ($F_{(1, 10)} = 1.727$, $p > .05$). An interaction effect between injury and treatment was not found ($F_{(1, 10)} = 0.068$, $p > .05$). Qualitative evaluation of the CA3, however, indicated changes in this region (see Figures 10 and 14). Figure 15 shows the mean number of CA3 processes per group.

Although the mean number of dendrites in the CA3 was not significantly altered, a two factor ANOVA (injury x treatment) revealed that TBI did significantly reduce the length of β2/3 IR processes, $F_{(1, 10)} = 37.418$, $p < .0001$. The apical dendrites of injured animals ($\underline{M} = 19.639$) were significantly shorter than those of sham animals ($\underline{M} = 22.772$). Analysis also revealed that DZ attenuated these alterations in IR dendritic length, $F_{(1, 10)} = 31.032$, $p < .0001$. Dendritic processes in the CA3 were significantly shorter for saline-treated animals ($\underline{M} = 19.822$) than for DZ-treated animals ($\underline{M} = 22.588$). An interaction between injury level and treatment effect was found, $F_{(1, 10)} = 17.594$, $p < .01$, and a simple effect analysis indicated that injured animals treated with saline ($\underline{M} = 16.470$) had significantly shorter CA3 dendritic processes than the injured animals treated with DZ ($\underline{M} = 22.015$), $F_{(1, 5)} = 57.164$, $p < .01$. Sham animals did not significantly differ

in CA3 dendritic length ($F_{(1,5)} = 1.213$, $p > .05$) whether they were treated with saline ($\underline{M}$ = 22.337) or DZ ($\underline{M}$ = 23.352). Positively labeled GABAaRs along the dendrites of the CA3 (IR length), therefore, was similar for all groups, except the injured/saline-treated group, in which mean length was reduced (see Figure 16).

Discussion

The current study was designed to investigate potential alterations to GABAaR β2/3 subunit IR in the hippocampus of rats 24 hours following TBI. The only injury-related alteration found was decreased length of IR dendritic processes in the CA3 region of the hippocampus. The 'length' of the dendrite in this study refers to the IR distribution of β2/3 proteins along the process. Positively labeled dendrites of the CA3 formed varicose beading along their length. This response to injury was probably a sublethal cytoskeletal rearrangement driven by a calcium-based mechanism which was induced by excessive neuroexcitation. Restoration of an appropriate balance between excitation and inhibition may be achieved by either decreasing NMDA-mediated excitation with an antagonist (e.g., MK-801) or by increasing GABAergic inhibition with an agonist such as DZ. Restoration of balance between excitation and inhibition in the hippocampus attenuated injury-induced changes, including dendritic cytoskeletal alterations.

Surprisingly, there were no changes in the CA1 region in number, length, or appearance of IR processes between groups. There may be several reasons why no qualitative or quantitative changes were found in this area. First, previously documented changes in anti-GABA IHC 24 hours following TBI were found in the SLM layer but not

Table 4. The mortality percentages per group. A chi-square test of homogeneity was not significant, χ^2 (3) = 7.44, p > .05.

Group	Subjects	Died	% Mortality
Sham/saline	5	0	0
Sham/DZ	5	0	0
Injured/saline	7	3	43
Injured/DZ	5	0	0

Figure 9. A representative photomicrograph of a hippocampus stained with the bd17 antibody.

Figure 10. Representative photomicrographs of hippocampal CA1 sections 24 hours following TBI (magnification = 20x).

A = Sham/Saline

B = Sham/DZ-treated

C = Injured/Saline

D = Injured/DZ-treated

No significant injury or treatment effects were found between groups on either number or length of IR processes in the CA1 at 24 hours following injury.

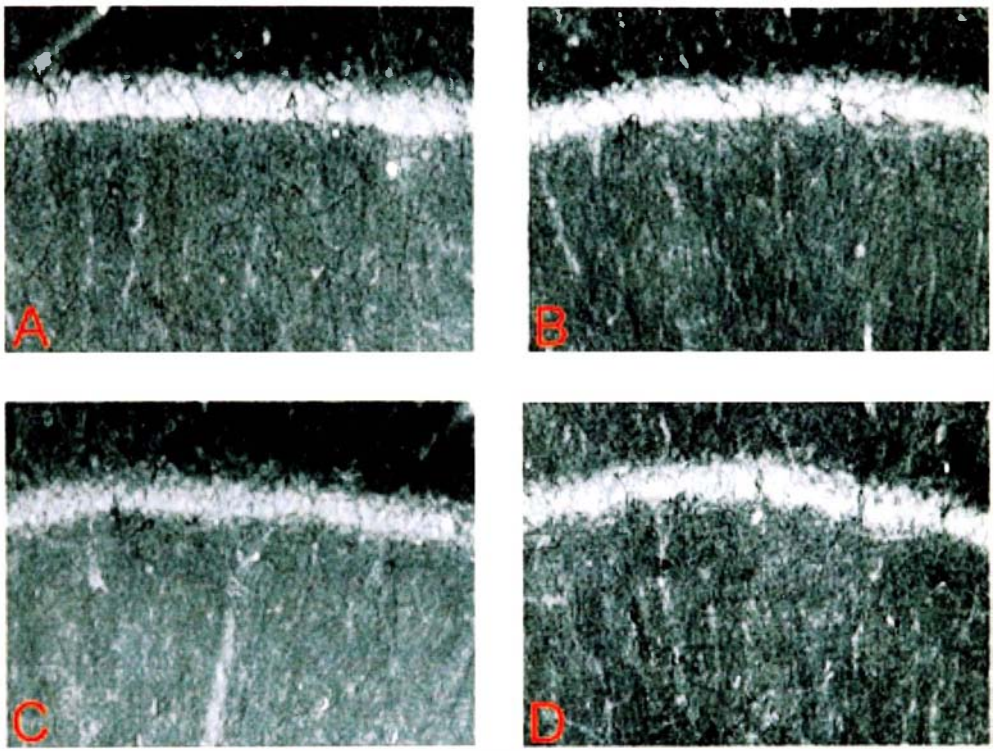

A
B
C
D

Figure 11. Mean IR number of processes (and S.E.M.) per group in the hippocampal CA1 region 24 hours following TBI. No significant treatment or injury effects were found for number of CA1 processes.

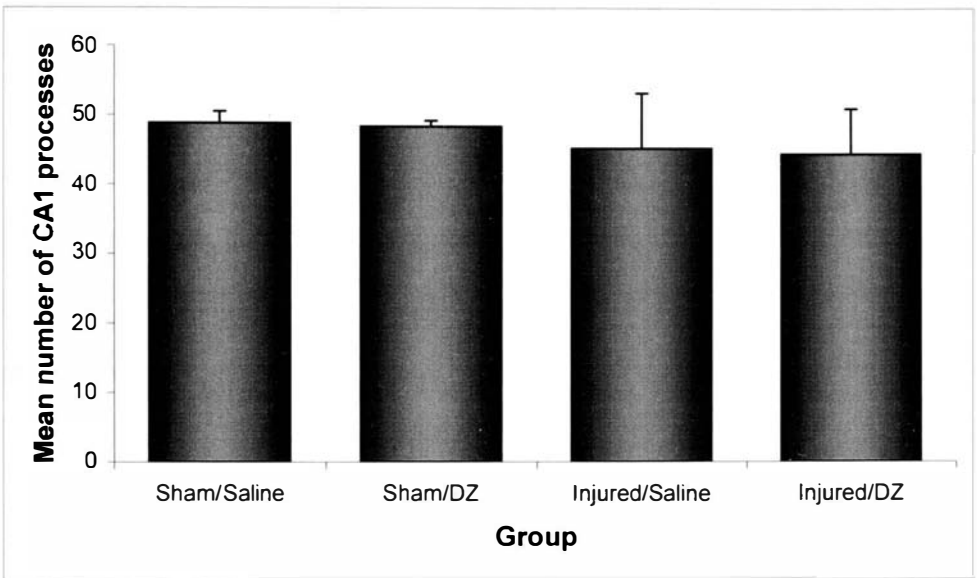

Mean number of CA1 processes
60
50
40
30
20
10
0
Sham/Saline
Sham/DZ
Injured/Saline
Injured/DZ
Group

Figure 12. Mean IR length of processes (and S.E.M.) per group in the hippocampal CA1 region 24 hours following TBI. No significant treatment or injury effects were found for IR length of CA1 processes.

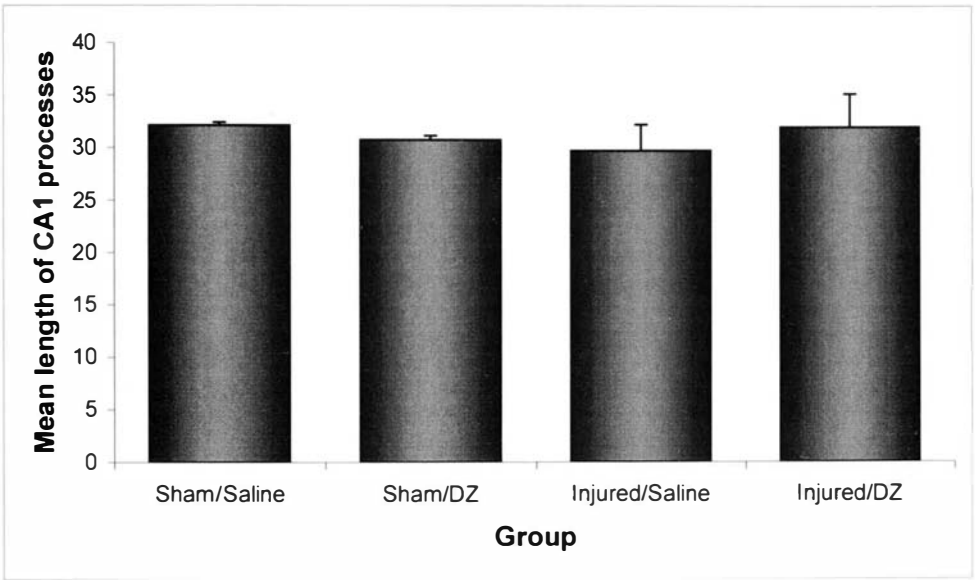

Mean length of CA1 processes
40
35
30
25
20
15
10
5
0
Sham/Saline
Sham/DZ
Injured/Saline
Injured/DZ
Group

Figure 13. Representative photomicrographs of hippocampal CA3 sections 24 hours following TBI (magnification = 20x).

A = Sham/Saline

B = Sham/DZ-treated

C = Injured/Saline

D = Injured/DZ-treated

A: The sham/saline group had significantly longer CA3 processes than the injured/saline group at 24 h. B: Sham/DZ-treated animals were not significantly different from sham/saline animals in number or length of IR processes. C: The injured/ saline group had significantly shorter processes than all other groups at 24 hours following injury. Overall number of processes did not differ between groups. D: Injured/DZ-treated animals had significantly longer processes than the injured/saline group. There were no significant differences between the injured/DZ-treated group and the sham/saline or sham/DZ-treated groups in number or length of processes at 24 hours following injury.

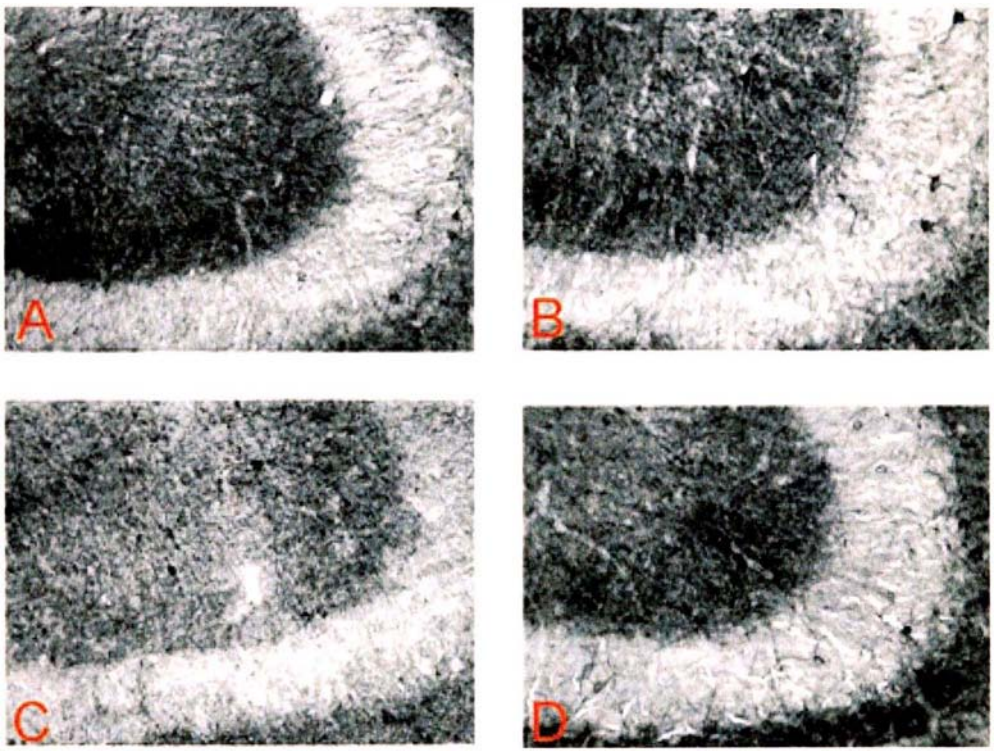

A
B
C
D

Figure 14. Mean IR number of processes (and S.E.M.) per group in the hippocampal CA3 region 24 hours following TBI. No significant treatment or injury effects were found for number of CA3 processes.

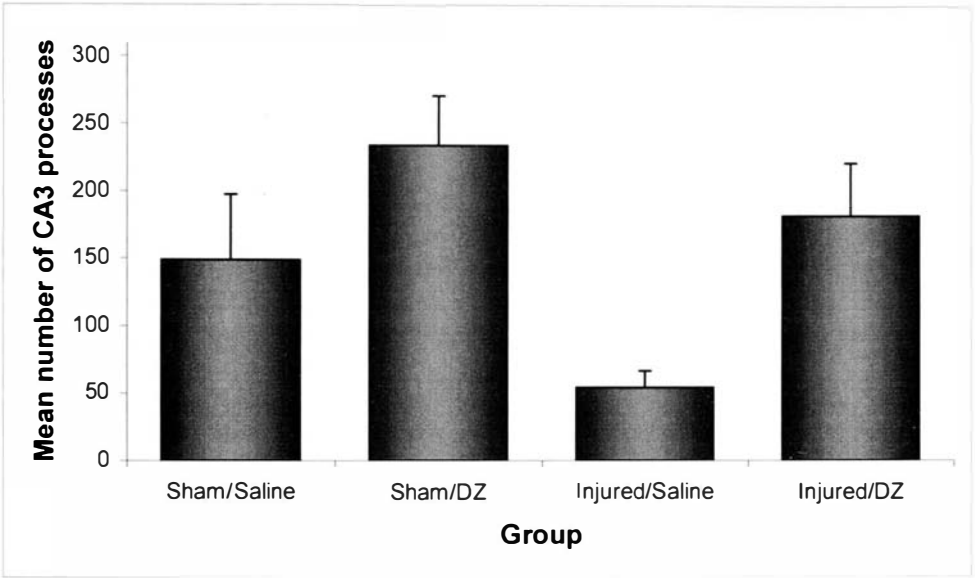

Mean number of CA3 processes
300
250
200
150
100
50
0
Sham/Saline
Sham/DZ
Injured/Saline
Injured/DZ
Group

Figure 15. Mean IR length of IR processes (and S.E.M.) per group in the hippocampal CA3 region 24 hours following TBI. The injured/saline group had significantly shorter processes than the injured/DZ-treated, sham/saline, or sham/DZ-treated groups. No other group differences were revealed.

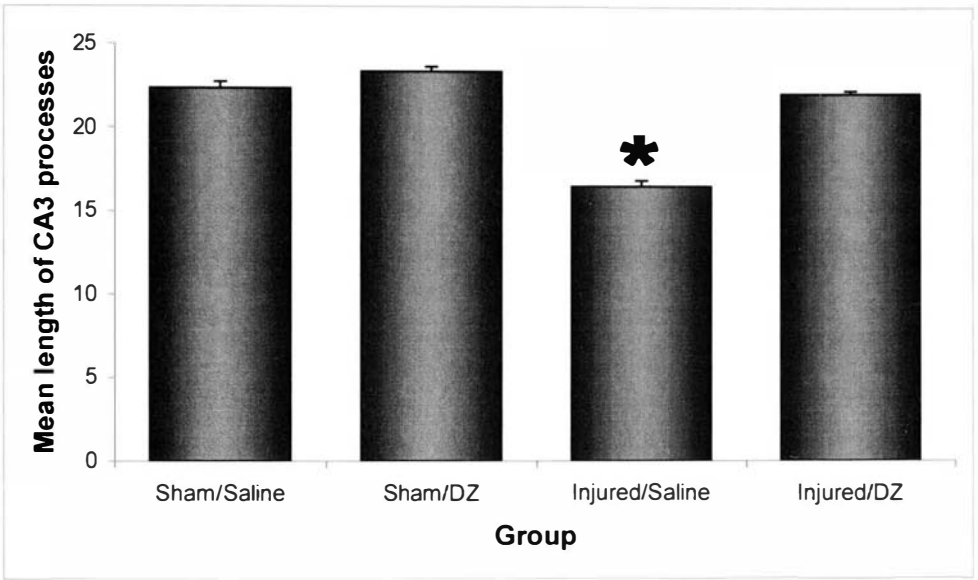
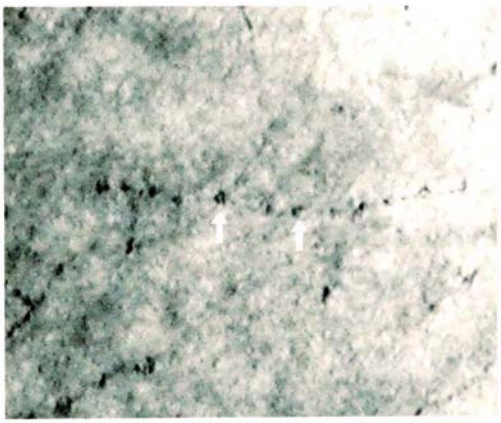

Mean length of CA3 processes
25
20
15
10
5
0
*
Sham/Saline
Sham/DZ
Injured/Saline
Injured/DZ
Group

Figure 16. An enlarged (33%) example of beaded dendrites found in the hippocampal CA3 region 24 hours following injury (40x magnification). Only the injured/saline group demonstrated dendritic beading.

A = Injured/saline

B = Sham/saline

Sham



Injured

the SR layer of the hippocampal CA1 region (O'Dell, 1995). GABAaR staining of processes did not allow for recognizable laminar demarcation between the SR and SLM regions, therefore, collapsing across lamina may have reduced the sensitivity of analysis.

This is unlikely, however, since no qualitative changes in number or appearance of CA1 processes were evident, and all quantitative counts were extremely close between all groups (refer to Figures 11, 12 and 13). Another reason for lack of alterations in the CA1 may be that the 24 hour time point was not sensitive to changes in GABAaR IR. Receptor changes may be delayed, as in ischemia, or may already have normalized by 24 hours. This is also unlikely, however, since GABAergic changes have been documented with anti-GABA IR in the CA1 at 24 hours (O'Dell, 1995). A third possibility, which is the most likely explanation, is that β2/3 protein expression was not altered in the CA1 24 hours following injury.

Although the length of IR processes in the CA3 region was altered following injury, the number of processes were not significantly different between groups. The number of processes in this area may have been artificially inflated due to extensively varicose and segmented dendritic appearance. This would, however, only have affected the injured/ saline-treated animals since varicosities were not found in any other group. Similar findings have been demonstrated in CA1 dendrites stained with GABAaR α1 antibody following ischemia. Although processes were found to be beaded ("string of beads"), the overall number of processes did not differ between ischemia and control groups (Inglefield, Wilson, and Schwartz-Bloom, 1997).

Significant decreases in IR dendritic length were found in the CA3 of the hippocampus for the injured-saline group. There may be several explanations for this finding. TBI-induced impact depolarization is followed by a surge of synaptic release of neurotransmitters, including GABA (Selzer, 1995). Post-injury GABAaR down-regulation may occur due to excessive activation, altering expression preferentially in the distal portions of dendrites. Alternatively, the number of receptors may remain constant but the conformation of the receptor may be altered. Conformational alterations may result in decreased expression of some subunits and increased expression of others. The differential expression of these subunits may be due to altered subunit composition of the GABAaR or a positional alteration that unmasks certain subunits more than other subunits. There was, however, no change in the number of β2/3 IR processes and differential subunit expression does not sufficiently account for the reduction in length of CA3 apical dendrites.

Another explanation may be dendrotomy, which refers to the complete separation of the proximal and distal portions of the dendrite. Axotomy has been well documented following TBI (Maxwell, Wyatt, Graham, & Gennarelli, 1993; Povlishock, 1992; Povlishock, Becker, Cheng, & Vaughan, 1983; Yaghmai & Povlishock, 1992). In DAI, compaction of cytoplasm and neurofilaments occurs, producing pathologically altered axonal transport (Povlishock, 1992; Yaghmai & Povlishock, 1992). Eventually, the buildup of molecular material in the axon forms a retraction ball that separates from the remaining distal portion of the axon (Povlishock et al., 1983; Yaghmai & Povlishock,

1992). Ultrastructural changes have been documented for dendrites as well as axons (Posmantur et al., 1996; Posmantur et al., 1997; Saatman, Graham & McIntosh, 1998). Immunolabeling of neurofilaments (NF68 and NF200) and microtubule associated protein 2 (MAP2), which is only found in somata and dendrites, revealed cytoskeletal protein alterations in the apical dendrites of cortical pyramidal cells 3 and 24 hours following lateral CCI (Posmantur et al., 1996; Posmantur et al., 1997). Fragmented swellings of microtubule and neurofilament IR were also found in the CA3 region of the hippocampus and in the DG 24 hours following lateral FPI in the rat (Saatman, Graham & McIntosh, 1998). In both models of TBI, microtubule and neurofilament fragmentation within dendrites occurred in areas remote from the injury site and did not always precede cell death, indicating that these changes may represent a sublethal cytoskeletal disruption (Posmantur et al., 1996; Saatman, Graham, & McIntosh, 1998). Unlike injury-induced axotomy, dendritic varicosities and alterations are more likely to be associated with sublethal cytoskeletal rearrangements than with complete separation of proximal and distal dendrites. Therefore, alterations in TBI-induced CA3 apical dendritic length are probably not due to dendrotomy. Retraction of dendrites from degenerating pre-synaptic neurons has been well documented in the DG following deafferentation of the perforant pathway (Mizukami et al., 1997; Nitsch & Frotscher, 1992; Phillips et al., 1997; Phillips, Lyeth, Hamm, Reeves, & Povlishock, 1998). The most likely explanation for the shortening of injured dendrites is that ultrastructural fragmentation of microtubules and

neurofilaments occurs within the varicosities, producing compaction of cytostructural components along the process and thereby reducing the overall length.

Inglefield, Wilson, and Schwartz-Bloom (1997) found similar beading of interneuron dendritic processes IR for the GABAaR subunit α1 following ischemia. The formation of varicosities in ischemia, however, was delayed until three to four days post-injury and corresponded with delayed degeneration of CA1 pyramidal cells. GABAaR subunit IR appears to be sensitive to morphological dendritic pathology, and similar dendritic alterations have also been found in microtubule and neurofilament immunohistochemistry (Matesic & Lin, 1994; Posmantur et al., 1996; Posmantur et al., 1997; Saatman, Graham & McIntosh, 1998), electron microscopy (Fekuda, Nakano, Yoshiya, & Hashimoto, 1993; Petito & Pulsinelli, 1984; Yamamoto, Hayakawa, Mogami, Akai, & Yanagihara, 1990), autoradiography (Johansen, Jorgensen, Ekstrom von Lubitz, & Diemer, 1984) and tissue cultures (Adamec, Beermann, & Nixon, 1998; Baar, 2000; Emery & Lucas, 1995; Ochs & Jersild, 1987; Park, Bateman & Goldberg, 1996). The connection between all of these markers of ultrastructural change is that they were all induced by some sort of excitotoxic neuronal insult (e.g., TBI, ischemia, hypoxia).

Emery and Lucas (1995) produced dendritic varicosities in cultured neurons exposed to hypothermia, NMDA or A23187 (a calcium ionophore). All three injuries produced identical dendritic pathology. Fractured microtubules and swollen mitochondria and vacuoles were found densely packed within the varicosities. The diameter of the varicosities, however, was within normal dendritic range. The interconnecting portions of

the dendrite (between the varicosities) were found to contain densely packed (mostly unfragmented) microtubules with no large organelles present. This region was constricted and the diameter was less than normal dendritic range. The somata of neurons with beaded dendrites also showed nuclear changes, chromatin clumping, and cytoplasm containing dilated vacuoles and dark mitochondria. In the hypothermic cultures, rewarming eliminated the varicosities, indicating that this response to injury can be reversed, at least in some cases (Emery & Lucas, 1995).

Although the exact mechanism driving dendritic cytoskeletal alterations is unknown, Mattson, Wang, and Michaelis (1991) found that proteins associated with hippocampal NMDA receptors are located in clusters along dendritic membranes. The varicosities, therefore, may be associated with discontinuous receptor distribution. Although NMDA involvement is likely, this does not explain the dose-dependent expression of varicose dendrites following A23187 calcium ionophore exposure. Exposure to low doses of A23187 showed no significant ultrastructural alterations in 40% of cells, although higher doses resulted in necrosis, swollen or collapsed mitochondria and nuclear changes similar to neurons exposed to NMDA. Rate-dependent beading of dendrites was found in 47% of neurons. Therefore, a likely explanation for dendritic beading would be a calcium-mediated mechanism induced by excessive neuroexcitation (Emery & Lucas, 1995).

It is well known that TBI-induced neuroexcitation is mediated by NMDA receptor activation. NMDA receptor activation leads to the opening of ion channels and

subsequent calcium influx (Alessandri & Bullock, 1988; McIntosh et al., 1996). NMDA receptor- mediated calcium elevation is associated with protease activation (e.g., calpain), which may be involved in the cascade that leads to dendritic cytoskeletal fragmentation (Alessandri & Bullock, 1988; Kampfl et al., 1997; Posmantur et al., 1997; Seubert, Lee, & Lynch, 1989; Siman, Noszek, & Kegerise, 1989). Protease activation by calcium has been shown to facilitate varicosities and interconnecting organelle loss and membrane shrinkage (Ochs & Jersild, 1987).

Lankiewicz et al. (2000) demonstrated that calcium activation of calpain I was detectable in cultured rat hippocampal neurons that were briefly exposed to NMDA. Mediated by the NMDA receptor, glutamate activates calpain, which breaks down spectrin, a protein that links membrane proteins to the actin cytoskeleton (Adamec, Beermann, & Nixon, 1998). Spectrin has also been shown to selectively interact with the C-terminal of NMDA NR2 subunits (Wechsler & Teichberg, 1998). Furthermore, glutamate mediation of calcium, rather than just an excess of calcium, is necessary for calpain activation. Calpain I inhibitors, however, interfere with the normalization of varicosities in hippocampal rat neuronal cultures exposed to sublethal levels of glutamate, indicating that activation of appropriate levels of calpain I may play a role in restoring normal cytoskeletal organization (Adamec, Beermenn, & Nixon, 1998). Calpain may provide feedback regulation of NMDA receptors by limiting NMDA receptor activation via truncation of the C-terminal domain of N2 subunits (Bi et al., 1998). Collectively, these data would indicate that NMDA-mediated glutamate activity acts to increase

intracellular calcium, which in turn activates calpain. Calpain alters cytoskeletal structure by breaking down spectrin, although this may be a sublethal response that ultimately restores cytoskeletal organization to normal by decreasing further NMDA receptor-mediated neurotoxicity

In TBI and other CNS injuries, NMDA receptor-mediated glutamate activity disturbs calcium homeostasis, which then activates a cascade of events that contributes to cytoskeletal alterations and neurodegeneration. The role of calcium and calpain are important contributors to this cascade. Calpain inhibitors administered within 24 hours of injury have been shown to attenuate structural and functional derangements of neurons (Kempfl et al., 1997). NMDA receptor antagonists such as MK-801 reduce glutamatergic receptor activation, ultimately altering the neurotoxic cascade.

The role of NMDA in dendritic pathology was investigated in mouse neuronal cultures exposed to hypoxia. Segmental dendritic beading occurred but was blocked by the NMDA antagonist MK-801. Within five minutes of NMDA or glutamate exposure, the varicosities returned and were again normalized with MK-801 treatment. Dendrites which received longer exposure (15 or more minutes) demonstrated more extensive beading of distal and proximal dendrites and a loss of dendritic spines. Cells exposed to 15 minutes of hypoxia were normal by 24 hours following injury. Although cell death due to hypoxia or NMDA exposure was always preceded by dendritic beading, reduced length of exposure resulted in sublethal dendritic cytoskeletal alterations (Park, Bateman & Goldberg, 1996).

Similar to NMDA receptor antagonism, GABAaR agonists reduce overall excitation, essentially preventing a detrimental cascade of subcellular events. Cultured hippocampal pyramidal cells exposed to glutamate showed shortened dendrites and inhibited outgrowth at sublethal levels and cell death at higher levels. A combination of GABA and DZ significantly reduced these dendritic alterations and prevented cell death. The calcium channel blocker Co^{2+} demonstrated similar neuroprotection, indicating that GABAaR-mediated neuroprotection may have calcium-related effects (Mattson & Kater, 1989). Indeed, whole-cell patch-clamp recordings from both tracheal smooth muscles (Yamakage et al., 1999) and neuronal cultures (Akaike, Oyama, & Yakuskiji, 1989; Ishizawa, Furwya, Yamagashi, & Dohi, 1997) indicate that DZ and other benzodiazepines decrease influx of voltage-dependent calcium currents. DZ was more effective than midazolam in reducing calcium currents and the response was dose-dependent (Ishizawa et al., 1997; Yamakage et al., 1999). Conversely, DZ-binding inhibitors produce an increase in intracellular calcium (Cosentino et al., 2000). Therefore, DZ treatment may provide neuroprotection by increasing inhibitory tone and decreasing intracellular calcium and subsequent calpain activation.

Elimination of dendritic pathology by MK-801 in tissue culture and by DZ in the current study are likely both due to restoration of the balance between excitation and inhibition, which ultimately reduces glutamatergic neurotoxic cascades. Since the FPI used in the present experiment is not associated with overt cell death (Delahunty et al., 1995) it is likely that injury-induced morphological alterations are a sublethal response to

the injury. Sublethal responses in the hippocampus may be associated with long term deficits in spatial cognition. MK-801 (Hamm, O'Dell, Pike, & Lyeth, 1993) and DZ (O'Dell et al., 2000) treatment have both been shown to attenuate MWM deficits 15 days following central FPI. Alterations in the hippocampus have been implicated in producing these cognitive deficits (Hayes, Jenkins & Lyeth, 1992b). Sublethal NMDA receptor-mediated changes in long term potentiation (LTP) have been linked to cytoskeletal alterations. Calpain, which is associated with LTP (Lynch & Baudry, 1987) induces the breakdown of spectrin. Interactions between spectrin and NMDA receptors are believed to play an important role in LTP, as are glutamate-induced morphological alterations to dendritic spines (Wechsler & Teichberg, 1998).

Alterations in the CA3 may have been produced by glutamatergic mossy fiber input from the DG and may in turn affect LTP associated with CA3 to CA1 Schaffer collaterals. Thus, the normalization by DZ of TBI-induced sublethal alterations in hippocampal CA3 apical dendrites may have been due to a reduction in mossy fiber glutamatergic neuroexcitation of the this region. Attenuation by DZ of changes in the CA3 found at 24 hours may, therefore, be an important precursor to attenuation by DZ of MWM deficits at 15 days (O'Dell et al., 2000).

The current study provided evidence that immunolabeling of β2/3 subunits of the GABAaR was altered following TBI. The pattern of changes in GABAaR IR was different from changes found with anti-GABA antibodies. Dendritic pathology visualized by GABAaR staining was attenuated by DZ treatment, demonstrating the importance of

acute normalization of the balance between excitation and inhibition in the hippocampus. The beaded and segmented appearance of injured apical dendrites in the CA3 was a surprising result, which is likely due to cytoskeletal dendritic pathology that needs to be further characterized.

Since this was the initial investigation into GABAaR changes following TBI, further studies are needed for a greater understanding of GABAaR alteration and manipulation following injury. Immunohistochemical characterization of other GABAaR subunits such as $\alpha 1$ and $\gamma 2$ may provide greater understanding of injury-induced alterations of the GABAaR. Correlation of GABAaR IR with microtubule and neurofilament changes may show that varicose and segmented portions of CA3 apical dendrites coincide with ultrastructural changes. Electron microscopic analysis of dendritic cytoskeletal rearrangements could be used to verify IR alterations. Also, protein expression is likely to be preceded by molecular alterations. Subunit specific modifications in the DNA encoding for GABAaR proteins may provide further understanding of GABAaR changes due to TBI. Another important aspect is the time course following injury. A greater understanding of acute ultrastructural dendritic and receptor changes may be found in the DG, CA1 and/or CA3 regions at earlier time points and the persistence of sublethal hippocampal pathology at chronic time points may help explain long term cognitive deficits.

In conclusion, attenuation of TBI-induced GABAaR alterations with DZ may provide a viable approach to the normalization of excitatory/ inhibitory balance in the

hippocampus. Excessive neuroexcitation produces sublethal pathology that may include ultrastructural alterations to hippocampal apical dendrites and these alterations may be associated with subsequent cognitive deficits. This study provides further support for the hypothesis that the inhibitory system is altered following TBI.

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