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THE EFFECTS OF ANIRACETAM TREATMENT ON COGNITIVE
PERFORMANCE AND AMPA RECEPTOR GLUR2 SUBUNIT EXPRESSION
AFTER MODERATE FLUID PERCUSSION INJURY IN RATS

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

By

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To Denis Kashin

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List of Abbreviations

ABC   Avidin-biotin-peroxidase complex

Ach   Acetylcholine

AchR   Acetylcholine receptor

AMPA   α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA   Analysis of Variance

ATM   Atmospheres

Ca++   Calcium

CCI   Controlled cortical impact

ChaT   Choline acetyltransferase

CHI   Closed head injury

CNS   Central nervous system

CT   Computed tomography

DAI   Diffuse axonal injury

EAA   Excitatory amino acid

FPI   Fluid percussion injury

GCS   Glasgow Coma scale

Glu   Glutamate

GluR1   AMPA receptor subunit 1

GluR2   AMPA receptor subunit 2

GluR3   AMPA receptor subunit 3

GluR4   AMPA receptor subunit 4

HC	Hippocampus
ICP	Intracranial pressure
i.p.	Intraperitoneal
M	Mean
ML	Molecular layer
mRNA	Messenger ribonucleic acid
MWM	Morris water maze
N_2O	Nitrous oxide
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NE	Norepinephrine
PID	Post-injury-day
PML	Polymorphic layer
Q	Glutamine
R	Arganine
RNA	Ribonucleic acid
SLM	Stratum lacunosum-moleculare
SO	Stratum oriens
SR	Stratum radiatum
SEM	Standard error of the mean
TBI	Traumatic brain injury

ABSTRACT

THE EFFECTS OF ANIRACETAM TREATMENT ON COGNITIVE
PERFORMANCE AND AMPA RECEPTOR GLUR2 SUBUNIT EXPRESSION
AFTER EXPERIMENTAL TBI IN RATS

By Anna Baranova

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

Virginia Commonwealth University, 2004

Major Director: Robert J. Hamm, Ph. D., Department of Psychology

In addition to the acute pathology produced by traumatic brain injury, there are

chronic alterations that occur after the trauma, including a depressed state of neuronal

activity (Feeney, 1991). This study included a preclinical testing of a novel treatment

strategy focusing on increasing neuronal activity during the chronic hypofunctional

posttraumatic stage. The present investigation tested the effects of repeated post-injury

aniracetam administration on cognitive performance in the Morris water maze (MWM)

and on the GluR2 - immunoreactivity and protein expression by Western blot analysis in

the hippocampus. The first study examined the optimal dose of aniracetam in the MWM

task. Animals received aniracetam (25 mg/kg, 50 mg/kg) or vehicle once daily for fifteen

days and on days 11-15 were tested in the MWM. The results indicated that injured

aniracetam-treated rats had a significant improvement in MWM performance compared

to injured saline-treated animals. When the drug was delayed for 11 days post-injury in

the second experiment, its beneficial effects were still present, as injured aniracetam-treated rats performed significantly better that injured saline treated rats on the MWM task. In the third experiment, chronic daily aniracetam administration was terminated after 15 days immediately before MWM testing on days 16-20. The results indicated that termination of aniracetam did not enhance MWM performance as injured terminated aniracetam-treated rats did not have significant improvement over injured saline-treated rats. In the fourth study we investigated the mechanism of aniracetam's effects by examining the expression of the AMPA receptor GluR2 subunit, the only AMPA receptor subunit that is Ca++ impermeable. Using a monoclonal antibody selective for the GluR2 subunit, immunohistochemical results indicated that injured rats treated with aniracetam (50mg/kg for 15 days post-injury) had a slight reduction in the GluR2- IR. The fifth study investigated a change in the GluR2 protein expression in the hippocampus with a Western blot analysis. The results were consistent with the immunohistochemical study outcome as the injured vehicle and injured aniracetam treated animals showed a reduced protein expression in the hippocampus. The changes were not significantly different from the controls.

The results of these experiments suggested that chronic aniracetam treatment significantly attenuated injury induced spatial memory deficits when administered continually during the hypofunctional posttraumatic stage and when the treatment was delayed for 11 days, but not when the treatment was terminated before the MWM testing. These effects suggest that the compound does not induce chronic receptor changes and has to be biologically active in an organism for it to exert its beneficial properties. Results

from the present studies suggest that aniracetam may become a potential treatment option for brain injury induced cognitive deficits.

INTRODUCTION

Overview of TBI

In October 1998, National Institute of Health (NIH) produced a Consensus Statement on Rehabilitation of Persons with Traumatic Brain Injury (TBI). The NIH Consensus Development Program was developed in order to evaluate available scientific information and resolve safety and efficacy issues related to a biomedical technology. Traumatic brain injury is a disorder of major national health significance. It deserves further investigation in many areas such as finding treatment and identifying factors that affect vulnerability to TBI. Approximately 1.5 to 2 million people was affected by TBI annually in the US, with over 500,000 hospitalizations, and over 50,000 deaths (Pope and Tarlov, 1991). TBI results mostly from motor vehicle accidents, falls, acts of violence, and sports-related injuries. The number of people that survive the TBI has increased in the recent years. This has been attributed to faster and more effective emergency care, quicker and safer transportation to treatment facilities specializing in TBI care, and better medical management. However, victims of TBI that survive the injury do encounter impairments that may last throughout their lives.

Traumatic brain injury affects people of all ages. However, the highest incidence rates were amongst young persons 15 to 24 years of age, with males being twice more likely to experience TBI than females (Sosin et al., 1989). Adults 75 years of age and

older were also at a high risk for incurring a TBI because of a high incidence of falls in that population. TBI is also the leading cause of long-term disability in children and young adults.

Approximately 70,000 to 90,000 people a year incur a TBI that leaves individuals with long-term, extensive loss of functioning. In addition, there were about 300,000 hospital admissions annually for individuals incurring mild or moderate head injury. Also, a number of traumatic brain injuries, especially sports related ones, remain unrecorded but may lead to a long-term disability in the future.

EPIDEMIOLOGY OF TBI

Incidence. Data from Centers for Disease Control and Prevention (CDC) indicates annual incidence rates of 100 per 100,000. Approximately 2.5 to 6.5 million people live with consequences of TBI. Such vast range is due to discrepancies in records, since most of the data is obtained from hospitalized patients or those who die before hospitalization.

Car accidents, including motor, bicycle, or pedestrian, contribute to 50 % of TBI's (Harrison-Felix et al., 1996). Changes in speed limits, road designs, and increased traffic control have diminished motor vehicle related deaths and TBI incidents. Also, in recent years, revolutionary technology advances in safety belts, air bags, infant and child car seats have resulted in reduced rates of TBI and in reduced number of deaths from head injury, leaving TBI victims living but sustaining physical and psychological injuries, for which they may require medical attention for a prolonged period of time. That is why it is necessary to research all possible treatment options in order to be able to treat and/or prevent traumatic brain injury.

The second leading cause of TBI is falls among the elderly and the very young. Some of the risk factors for falls among the elderly population include alcohol, medication and osteoporosis. Few preventative measures are available for this group of people. However, some changes have occurred in the design of walkers, strollers, and shopping carts to decrease the number of falls and injures. Assault is another cause of TBI in the very young. Unintentional injures account for three quarters of TBI in this group, and the remainder is unfortunately due to child abuse. Shaken baby syndrome causes TBI and spinal cord injury. Domestic violence also affects both males and females and deserves greater attention. Programs focusing on domestic violence should be addressed in schools for both parents and children.

Approximately 20 % of the TBI incidents were due to violence. Such assaults are divided almost equally into firearm related incidents and non-firearm related incidents. Young people are the ones at the highest risk for violence related assaults. Programs to prevent street violence should be strengthened through legislation and education.

Sports and recreation-related injuries account for about 3% of hospitalized persons with TBI, however it should be noted that the majority (approximately 90 %) of sports-related injuries are mild and may remain unreported, which leads to the underestimated number of the actual incidence rate of sports-related TBI. The group at risk here is the young people, ages 5 to 24. Risk factors are inadequately defined. However, there is great promise in prevention of sports-related injuries. Safer sport equipment such as helmets, pads, wrist and knee guards are technical improvements that promise to reduce the rate of sports-related injuries.

All of the above risk factors indicate that TBI is very heterogeneous. Multiple strategies are needed to accommodate these complexities.

The Cost of TBI

The economic consequences of TBI are overwhelming. Direct and indirect TBI-related expenses add up to approximately $37.8 billion dollars per year (Max, MacKenzie, & Rice, 1991). According to a 1999 report to Congress, TBI accounts for one third of all injury-related mortality (CDC, 1999). The annual cost of acute care and rehabilitation for TBI amounts to $9 to $10 billion in the United States. Average lifetime cost of care for a person with severe TBI ranges from $600,000 to $1,875,000. Older patients, ages 55 and up, tend to stay longer at the hospital, and therefore have higher total rehabilitation cost, and have a lower rate of functional changes, compared to younger patients, ages 18 to 54 (Cifu et al., 1996). A substantial portion of the cost is paid by the public funds. Approximately 30% of acute care and 35% of rehabilitative care are paid by Medicare or Medicaid (Harrison-Felix et al., 1996; Lehmkuhl et al., 1993). Other sources (Ommaya et al., 1996; Rivara et al, 1988) indicate that the public pays up 64% of acute care cost, and almost 100% of rehabilitation cost. Access to initial medical care and rehabilitation facilities for patients with TBI depends on many factors. Some of these factors include insurance coverage, emergency personnel, health care staff, family and community, geographic location, and knowledge of available resources. Cost of care for TBI patients creates a huge economic burden to their families. However, the true price of TBI cannot be estimated in the monetary value. It is the neuropsychological deficits that leave patients having to readjust their lives (Cole and Edgerton, 1990).

Types of Injury

Injuries can be penetrating, for example, from a bullet, and non-penetrating, for example, from a blunt object, that does not penetrate the skull. Penetrating injuries cause blood loss, tissue rapture and brain lacerations. Non-penetrating injuries (or impact-loading) injuries cause acceleration-deceleration to the brain, which can occur with a linear or a rotational force. Brain lesions and lacerations develop from the brain shifting within the skull.

The initial trauma to the head is referred to as the primary injury. According to Rilley and Bullock (1991), all the biochemical events following the primary injury, such as chemical alterations in neurons, axonal damage, tissue destruction that continues for days, weeks, and maybe months after the initial trauma is referred to as secondary injury. Primary injuries are visible from the very beginning. They initiate tissue laceration, hemorrhage, edema, and other changes in the functioning of the brain, and at the same time initiate the secondary injuries and the underlying pathophysiological processes. The secondary injuries on the other hand may not be detectable for hours, days, or even weeks (Selzer, 1995).

Further injuries are subdivided into focal and diffuse injuries. Focal injuries are limited to a point of contact between the head and object striking it. Focal injuries often result from falls, and more often occur at frontal and temporal lobes. Severe blood loss, swelling, and contusion are common consequences of the focal head injury. Diffuse injuries often result from motor vehicle accidents. Such injuries lead to a skull deformation at the point of trauma. The trauma itself causes proliferation of stress waves

that originate from the point of contact with the skull and follow through the brain tissue, causing damage to nerve cells and axons. Subdural hematoma is a commom pathology of a diffuse head trauma. Diffuse head injuries lead to increased intracranial pressure (ICP), an important indicator of a patient's recovery (Clifton and Robertson, 1986). Diffuse trauma often causes nerve cell death in the cortex, as well as hippocampus, and basal ganglia (Selzer, 1995).

Severity of Injury

Approximately 60% of all head injuries are classifies as mild injuries, 20 % are moderate, and 20% are severe (Frankowski, 1986). The Glasgow Coma Scale (GCS) is the one used most often to assess injury severity. It is the scale that measure patients' response to pain, their verbal ability, and motor ability. Also duration of unconsciousness is taken into account as well as the length of amnesia, surgical requirements (such as removal of blood clots), secondary insults like ischemia and seizures, skull fractures and computerized tomography (CT) scan reports (Colohan & Oyesiku, 1992). Lower Glasgow Comas Scores represent more severe injuries. Mild injuries then correspond to a score range of 13 to 15, moderate injuries are represented by a score of 9-12, and severe injuries receive a score of 3-8. The score is subject to each doctor's interpretation, and therefore receives much debate over the objectivity of the score. The type and severity of the impairments depend on the location and size of the lesion resulting from TBI (Colohan & Oyesiku, 1992). Mild, moderate and severe TBIs have different pathologies and motor and cognitive consequences.

Mild TBI

Mild injuries usually do not include a state of unconsciousness. However the average cost of treatment is about $44,000 (Lehmkuhl, Hall, Mann & Gordon, 1993). Unfortunately, very little research is conducted in the area of mild TBI, and therefore, cognitive impairments following mild TBI have not received much attention. Many patients report post-concussive symptoms, such as headache, nausea, dizziness, and confusion. Intelligence, language, motor function and perceptual function are least affected by mild injury. However, other cognitive deficits prevail in the area of memory, attention, and information processing. Information processing difficulties typically disappear within three months after the injury. Memory problems, on the other hand, are not always noticeable within the first three months post injury, but may leave long lasting cognitive impairments. Deficits with memory, attention and information processing may negatively affect persons' social as well as private life. However, for mild TBI, most cognitive deficits diminish gradually during the first year post-injury (Packard, Weaver, & Ham, 1993).

Moderate TBI

Moderate injuries usually result in about 5 days of unconsciousness on the average. The average cost of treatment is approximately $86,000 (Lehmkuhl et al., 1993). There is a great variation in the outcome of moderate TBI. Mortality rate is estimated around 7 - 9% for those with a GSC score of nine or ten. The recovery period is significantly longer for patients in the moderate TBI category compared to the patients in the mild TBI category. Out of the individuals who sustained moderate TBI 69% did not

return to work, which is a dramatic increase from 34% for the mild TBI group (Rimel, Giordani, Barth, & Jane, 1982). Memory problems affect approximately 90% of moderate TBI patients. This remains the primary reason for unemployment in this group of people.

Severe TBI

Severe TBI results in approximately 12-34 days of unconsciousness. The average cost of care ranges from $111,000 to $154,000 (Lehmkuhl et al., 1993). Research indicates that only 3% of patients that sustain severe TBI returned to work (Wehman et al., 1993). Patients who acquired severe brain injury reported deficits in attention, motor speed, verbal memory, visual memory, and verbal learning. They scored in the 20th percentile of the Wide Range Achievement Test-Revised, and showed problems in mathematics, spelling, and problem solving (Wehman et al., 1993). Survivors of severe TBI are at the highest risk, compared to survivors of mild and moderate TBI, for suffering permanent physical and psychological deficits that can impair their performance at work and their personal everyday life.

Cognitive and Behavioral Deficits

Rarely do the consequences of TBI leave patients with impairments in only one part of a person's life. Instead, the consequences of TBI often affect the person's life in a broad range from altered physiological functions of cells to psychological problems and disabilities that influence the individual with TBI, their friends and family, their work community, and the whole society in general. It is very likely that more problems will

occur, as TBI patients get older, as more challenges arise with the aging process. (Ferrell and Tanev, 2002).

The neurological consequences of TBI are broad and complex (Cole and Edgerton, 1990). Any motor, sensory or autonomic function may be compromised after TBI. Most of such complications are apparent within the first few days depending on the severity of the injury. Long-term deficits may include movement disorders, headaches, seizures, visual impairments, and sleep disorders. Non-neurological medical complications may include and are not limited to pulmonary, gastrointestinal, metabolic, musculoskeletal, and dermatologic problems. (Holland et al., 2003; Lai et al., 1997)

The cognitive deficits of TBI are also very broad and complex. Cognitive consequences can occur immediately or after some time post injury. They vary from an individual to an individual, and can occur singly or in combination. They also change in their severity and presentation over time. Some of the most persistent problems include memory impairment, concentration and attention difficulties (Arcieniegas et al, 1999). Visual perception may be affected, as well as language skills. Also, frontal lobe functions executive skills such as problem-solving, abstract reasoning, insight, judgment, planning, information processing, and organization are vulnerable to TBI. (Watts and Perlesz, 1999).

Some behavioral deficits may include a number of motor problems. Patients may experience difficulty initiating responses, some agitation, and impulsivity. Verbal and physical aggression is sometimes common after the TBI. Learning difficulties, altered sexual functioning, social disinhibition are also characteristic to TBI recovery. Other

behavioral alterations that may be prevalent after TBI include: mood disorders, personality changes, altered emotional control, anxiety, and depression. (Kaitaro et al., 1995).

Social consequences of all types of TBI have very serious side effects. Patients are at very high risk of divorce, suicide, economic strain, unemployment, and substance abuse. Such consequences place a dramatic burden on the patients as well as on their families (Bushnik et al., 2003).

<div align="center">THERAPEUTIC INTERVENTIONS</div>

Successful treatment strategies for patients with traumatic brain injury are still to be found. Despite improved understanding of mechanisms of cellular response to trauma, standardized clinical treatment guidelines, enhanced auto safety technologies, and years of clinical trials, traumatic brain injury still prevails and the search aimed at identifying therapeutic agents for TBI patients continues today.

Pharmacological strategies under investigation include agents that target sites involved in the secondary injury cascade. Some of the examples of treatments include ion channel antagonists, calcium channel antagonists, growth factors, antioxidants, stem cells, apoptosis inhibitors and inhibitors of other signal modulators. The complexity of TBI pathology may require an optimal treatment to be a combination therapy taking into consideration such factors as the most advantageous time of drug delivery, an appropriate sequence of inhibitory and excitatory neurotransmitter agonists or antagonists that will ultimately lead to the recovery from devastating neurological deficits of TBI.

Pharmacological Strategies

Inhibiting apoptosis. Drugs targeting programmed cell death include caspase inhibitors, neuronal apoptosis inhibitory protein (NAIP) modulators and poly (ADP-ribose) polymerase (PARP) inhibitors. Activation of the caspase-3 appears to be a major event in the process of apoptosis in the CNS (Robertson et al. 2000). Caspase-3 belongs to a group II caspases (2, 3 and 7) and is found in structural, metabolic and repair proteins that are essential for cellular homeostasis. Caspase-3 activation has been observed in spinal cord injury, stroke, Alzheimer's disease and head injury. Robertson and colleagues indicated that NAIP modulation prevented brain cell death in an animal stroke model and reduced cell loss of CA1 hippocampal neurons following transient forebrain ischemia. As of today, NPS 1506 (NAIP modulator) is in phase IIb clinical trials. CV 1013 and GPI 6150 are PARP inhibitors that have demonstrated a reduction in brain damage in animal models and are expected to enter phase I clinical trials in the near future (Cosi and Marien., 1999).

a-Adenoreceptor agonists. α2-Adenoreceptor agonists induce cerebral vasoconstriction and reduce ICP in experimental models of head injury (Jolkkonen et al., 2000). Atipamezol (1mg/kg, s.c.) or desipramine (5 mg/kg, i.pi.) noreadrenaline reuptake blockers were administered to animals after ischemia, and facilitated the sensorimotor recovery following focal cerebral ischemia in rats. Dexmedetomidine decreased ischemic volume by 40% in rats; however hypotension and hyperglacemia were observed in some of the animals. Cherian and colleagues (1999) observed an increase in ICP, and a decrease in mean arterial pressure, cerebral perfusion pressure (CPP), and laser Dopler flow (LDF)

immediately after the impact injury (velocity, 5m/sec; deformation, 3mm) over the right

parietal cortex. In search of the therapeutic intervention, phenylephrine (0.3 mg/kg/min

for 10 min.) and L-arginine (300 mg/kg in 1ml saline for 10 min.) were administered

intravenously to the animals after the impact injury. The results indicated that

phenylephrine increased cerebral blood flow (CBF) by increasing CPP. However, L-

arginine increased CBF without altering CPP. Improved CBF accompanied a decrease in

the neurological injury. The researchers concluded that there are possible ways to control

cerebral blood flow without having compromising effects of inducing hypertension.

Cholinergic agents. It is a well established fact that the level of acetylcholine

increases in the brain and in CSF immediately after the traumatic brain injury (Teasdale

and Graham, 1998). Research indicates that a chronic reduction in acetylcholine activity

during the hypofunctional posttraumatic phase may be related to long-term cognitive

deficits seen in patients with TBI (Dixon et al., 1994). Chen and colleagues (1998)

administered Rivastigmine (2mg/kg), a selective acetylcholinesterase inhibitor to mice 5

minutes after the closed head injury. The results indicated that Rivastigmine improved

motor function recovery as well as the performance in the Morris water maze. Such

benefits were abolished when muscarinic and nicotinic receptor antagonists (2.5 mg/kg

mecamylamine and 0.5 mg/kg scopolamine) were given simultaneously with

Rivastigmine. Pike and Hamm (1995) examined the effects of selective blockade of the

presynaptic muscarinic M2 autoreceptor with BIBN 99 on cognitive recovery following

moderate fluid percussion injury in rats. Results showed that BIBN 99 (both doses of 0.5

mg/kg and 1.0 mg/kg) attenuated cognitive deficits produced by the injury, when it was

administered chronically once daily for 15 days beginning 24 hours after the injury. Such observations indicate that reduced cholinergic activity after TBI contributes to the secondary neurological deficits following the injury.

Kinin antagonists. Bradykinin is an endogenous neuropeptide known for its dilating properties. It increases vascular permeability and dilates cerebral vasculature by activating bradykinin receptors located on vascular endothelium. One of the bradykinin2 receptor antagonists LF-160687 (100 microg/kg/min) given 1 hour after weight-drop trauma for 23 hours reduced brain edema as indicated by a 68% increase in specific gravity and a 64% decrease of water content at the site of the trauma. The same dose of LF-106687 significantly improved neurological function as measured by the neurological severity score (NSS). Such findings indicate that the blockade of bradykinin B2 receptors may be an effective approach to reduce the cerebral edema and to improve the neurological result after the focal head injury. In another study (Stover et al., 2000) researchers found that a single injection of the same bradykinin B2 receptor antagonist LF-160687 (3mg/kg and 30 mg/kg) given 5 minutes after the injury reduced brain swelling by 25% and 27% respectively, however the water content was increased. The results also revealed increased glutamate levels in the CSF which could have partially contributed to the swelling effect.

Clinical trials of one of the bradykinin antagonist deltibant (CP 0127) have showed to be beneficial in patients with mild to moderate TBI (GCS scores between 3 and 7). Detibant helped control ICP and demonstrated a smaller decline in the GCS scores when it was infused for 7 days at 3μg/kg/min within 24 to 96 hours of injury when

it was given to twenty patients with mild and moderate closed head injury (GCS score of 9 to 14).The drug was tolerated well, and patients did not report severe abnormalities. Later, it was tested with a group of 139 patients with severe head injury (GCS score of 3 to 8) and was administered to patients within 12 hours of the injury and continuously infused for 5 days (Marmarou et al., 1999). Consistent positive trends were seen for ICP and neuropsychological tests in the treated groups, however, such differences were not significantly different from the placebo group.

Cyclo-oxygenase-2 inhibitors. Cyclo-oxygenase-2 (COX-2) is a primary inflammatory mediator that converts arachadonic acid into precursors of vasoactive prostaglandins, which produce reactive oxygen species in the process. Under normal circumstances the levels of COX-2 in the brain are almost undetectable, however after traumatic brain injury COX-2 levels significantly increase in neurons and astrocytes in cortex and hippocampus (Strauss et al., 2000). COX-2 stimulation after TBI may result in selective beneficial response, however chronic increase in COX-2 production may lead to free radical production, cell damage, vascular dysfunction, and alterations in cellular metabolism. Such outcome may contribute to secondary injuries to the brain tissue and promote further neuropathy and decreased behavioral functioning. Research indicates that inhibitors of COX-2 show mixed results. Colecoxib (marketed as Celebrex) worsened motor performance but not cognitive test performance, suggesting that COX-2 induction following TBI may be protective from the cognitive deficits associated with brain trauma (Dash et al., 2000). Another COX-2 inhibitor nimesulide (Koyfman et al., 2000) decreased cortical and hypothalamic prostaglandin-E2 formation when it was

administered to rats (30 mg/kg i.p.) after closed head trauma (CHT). However, nimesulide did not improve edema formation or functional activity measured by the Neurological Severity Score (NSS). The anti-inflammatory process triggered by TBI also activates platelet-activating factor (PAF), which under pathological conditions such as trauma or ischemia behaves as a neuronal injury messenger by increasing glutamate release and or activating COX-2 induction (Bazan 1998). When over produced, PAF acts as an endogenous neurotoxin. Antagonists of PAF (BN 50730) have been used to block seizure-induced COX-2 induction. Bentzer and colleagues (2001) investigated the effects of prostacyclin infusions (1ng/kg), which is an important regulator of micro vascular function after fluid percussion injury in rats. The main functions of prostacyclin include inhibition of platelet/leukocyte aggregation and adhesion, and vasodilation. The results of the study indicated that a low dose prostacyclin reduced the cortical lesion volume by 43% following TBI when it was compared to a vehicle at 7 days post injury. However no differences were found in neuromotor function between the treated and the vehicle groups.

Intracellular adhesion molecule antagonists. Research indicates that cytidine 5'-diphosphocholine (CDPC), or citicoline, which is a naturally occurring endogenous compound, displayed neuroprotective effects after experimental cerebral ischemia. However, no such effects have been reported after traumatic brain injury (Baskaya et al., 2000). The researchers investigated the effects of citicoline on brain edema and blood-brain barrier (BBB) breakdown after the control cortical impact (CCI) injury (velocity 3m/second; deformation 2mm). Results indicated that 100 mg/kg of citicoline reduced

brain edema in the injured cortex but not the ipsilateral hippocampus. A higher dose of citicoline of 400 mg/kg did reduce brain edema and BBB breakdown in both ipsilateral cortex and hippocampus. The researchers concluded that citicoline (CDPD) is an effective neuroprotective treatment for secondary injuries that appear in the cortex and hippocampus brain regions vulnerable to trauma after experimental injury. Citicoline has gone into clinical trials (phase II/III) in stroke patients however it did not demonstrate significant treatment effects and its future in TBI research remains to be investigated deeper.

   N-Methyl-D-Aspartate (NMDA) receptor antagonists. A great part of neuroprotective research has been devoted to the NMDA receptor. Agents acting as antagonists at NMDA receptors are broadly categorized into competitive and noncompetitive antagonists and glycine blockers. Competitive NMDA antagonists have a high affinity for the glutamate receptor. If given in adequate concentrations, such drugs will block glutamate from binding to its receptor and therefore prevent the NMDA channel from opening. Research indicates that glutamate levels in the extracellular fluid increase 10 to 50 fold above the normal levels after the injury (Rothman and Olney, 1986). Rapid access of glutamate antagonists to the brain is a critical issue in treatment of TBI. Clinical trials of competitive NMDA antagonists have been filled with problems in doses and concentration of the drugs because of the adverse psychological effects on patients. NMDA antagonists have been reported to induce hallucinations, psychosis and other CNS adverse effects in conscious patients (Olney et al, 1989). Another study indicated that NMDA noncompetitive antagonists MK (+) 801 induced vacuole formation

and necrosis in neurons of the rat cortex at 4 hrs after the administration of the drug. Such findings raise concerns in the development of the therapeutic intervention for human TBI. Dose and the time of the drug administration play a significant role in the outcome of the brain injured patient. Despite the negative effects of the drugs, NMDA antagonists also offer anticonvulsant effects and potential enhancement of cerebral blood flow. Pre-treatment with D-CPPP-ene, a competitive NMDA antagonist, resulted in amelioration of hypermetabolism caused by acute subdural haematoma in a response to elevated extracellular glutamate concentrations after ischemic brain damage (Lodge et al., 1988).

Noncompetetive NMDA antagonists bind within the channel itself. When glutamate levels are increased, as is the case after the traumatic brain injury, such binding is enhanced. This relationship is commonly referred to as a "use-dependent" effect, meaning that efficacy depends on the channel access that is limited unless the receptor has been activated. Noncompetitive NMDA antagonists have very rapid access to the brain (depending on the cerebral blood flow) compared to the lower penetrating competitive NMDA antagonists. Duration in the CNS is another distinctive characteristic between the two classes with noncompetitive antagonists staying in the CNS for a much shorter period of time. Competitive agonists must be available in sufficient concentrations to overcome the glutamate surge within the injured brain tissue. However, other problems may occur that could prevent the drugs from displacing glutamate from the injury site once activation of the glutamate receptor has occurred. Also, concentration of the drug at the traumatic tissue may be limited due to the compromised blood flow after the injury. Such problems could have contributed to the failure of the experimental

competitive NMDA antagonist drug selfotel (CGS 19755) in the treatment of severe head injury (Morris et al., 1999). A total of 693 severe head injury patients with a Glasgow Coma Scale (GCS) of 4 to 8 were enrolled into a double blind study. The results indicated that there were no significant differences between the treatment groups, and the study had to be stopped prematurely because of the increasing concern for other adverse brain effects and the likelihood that the drug's treatment effect was nil.

Noncompetitive NMDA antagonists, also known as ion channel blockers have stronger lipohilic characteristics. These NMDA antagonists also demonstrate a use-dependent receptor binding that may enhance the uptake of the drugs by ischemic tissue. Researchers investigated eliprodil, a noncompetitive NMDA antagonist that acts on the polyamine modulatory site and also acts as a calcium channel blocker (Hogg et al., 1998). Results indicated that eliprodil produced a 50% reduction in a deficit in a conditioned freezing response caused by a lateral fluid percussion-induced lesion when it was administered three times (1 mg/kg, i.v.) at 15 minutes, 6 hours, and 24 hours following the injury. When eliprodil was administered two times (1 mg/kg) at 6 and 24 hours and equally at 12 and 24 hours post lesion it produced a similar result of protection (56% and 59% respectively). However no protective effect was found when a single treatment of eliprodil (3mg/kg) was administered 24 hours after the injury. Researchers concluded that eliprodil has an ability to reduce the lesion volume following traumatic brain injury in addition to its neuroprotective effects on functional outcome. Even though eliprodil has been shown promising effects in animal trials, it has not been successful in human trials. In another study, researchers (Okiyama et al., 1997) examined the effects of other NMDA

noncompetitive blockers, ifenprodil derivatives, CP-101,606 and CP-101,581 and its

racemic mixture CP-98,113 on spatial memory and cerebral edema after the experimental

fluid percussion injury in the rats. Animals received the compounds (CP-98,113 – 5

mg/kg, i.p.; CP-101,581 – 5 mg/kg, i.p.; CP-101,606 – 6.5 mg/kg, i.p.) or vehicle fifteen

minutes after the injury, followed by the continuous infusion of the drugs at a rate of 1.5

mg/kg/hour via Alzet osmotic mini pumps. The results indicated that all the compounds

significantly attenuated fluid percussion injury induced cognitive deficits at 2 days post

trauma when tested in the Morris water maze visuospatial memory task. Also,

administration of CP-89,113 significantly reduced cerebral edema in the ipsilateral

cortex, hippocampus and thalamus. Such findings indicate that NMDA receptor blockade

may significantly attenuate cognitive deficits associated with TBI. Out of all the isomers,

CP-101,606 seems to hold the most potential for the therapeutic treatment. It

demonstrates selective binding at the N2RB receptor subtype in neurons in cortex and

hippocampus (Bullock et al., 1999a). The selectivity of the compound is very important

as the location of the neurons is vulnerable to traumatic brain injury and ischemic injury.

In clinical trials, CP-101,606 produced no hematological or electrocardiogram

abnormalities in patients with severe traumatic head injury or spontaneous intracerebral

hemorrhage. Researchers (Bullock et al., 1999a) found that CP-101,606 infused with for

up to 72 hours was well tolerated, penetrated CSF and brain and suggested a positive

trend in brain injury outcome as indicated by the enhanced Glasgow Coma Scale. Similar

results were found in another study (Merchant et al., 1999) where CP-101,606 was well-

tolerated and produced no psychotropic effects in patients who have sustained a mild or moderate traumatic brain injury or hemorrhagic stroke.

Aptiganel (CNS 1102) is another noncompetitive NMDA receptor antagonist evaluated in clinical trials for TBI patients. CNS 1102 has a high affinity and selectively binds with the transmembrane ion channel coupled with the NMDA receptor. This drug's mechanism encompasses the blockade of the open channel and by that limiting the calcium transport. Such agents have an ability to change cerebrovascular tone within arterioles either as a result of the sympathetic stimulation or because of the increased metabolic demand localized within brain regions. Pharmacokinetic studies (Muir et al., 1995) showed that the adverse effects of CNS 1102 were related to the total dose administration than the duration of the administration. Some of the adverse effects of this drug (Aptiganel) included increased blood pressure, light-headedness, disorientation, and hallucinations in voluntary subjects. However all symptoms resolved within 24 hours. In another study (Wagstaff et al., 1999) aptiganel was administered to patients with severe head injury (GCS scores of 4 to 10) as a bolus of 250mg/kg, followed by a 4 hour infusion, all within 72 hours of injury. All the patients in the study were in the intensive care unit and ventilated. The results of the drug included a drop in the ICP and temperature, however both returned to baselines within 12 hours. The drug has been discarded from a phase III trial after the premature analysis showed no significant difference from a placebo (Cawley et al., 1998).

Other agents investigated for improvement post-TBI include compounds targeting the glycine site on the NMDA receptor (Lees 1997). Studies show that glycine site

antagonists produced only minimal vacuolation and neuronal necrosis. Some of the obstacles of the glycine site receptor therapeutic agents include poor affinity to the receptor site, limited brain tissue penetration and relative insolubility. Clinical phase II trials demonstrated glycine site antagonist gavestinel (GV 150526) to improve functional outcome in patients four weeks after stroke (Lees et al., 2000). However, increase levels of bilirubin were reported in the treated group when compared to the placebo group, and statistical analysis did not support significance in the functional outcome between the treated and the placebo groups even though a positive trend was noted. Another glycine site NMDA antagonist licostinel (ACEA 1021) demonstrated a reduction in cellular swelling after subdural haematoma in a rat model of TBI (Di and Bullock, 1996). Licostinel administered at 30 minutes post ischemic stroke was found to successfully reduce cellular swelling, however a number of animals died unexpectedly. Pulmonary edema possibly contributed to animal loss. Unlike other NMDA antagonists at neuroprotective doses, agents targeting the glycine site of the NMDA receptor have a reduced incidence rate of psychomotor side effects in conscious patients. Further studies of this compound should be completed in head injury patients as such agents have a high level of neuroprotection and a lower level of major behavioral side effects.

Remacemide, another low affinity NMDA receptor antagonist works by blocking the sodium channel within the membrane. It has been studied extensively in the field of epilepsy (Palmer et al., 1996). Remacemide metabolite remacemide desglycine maybe the mediator of the neuroprotective effects. Remacemide desglycine reduced NMDA-triggered intracellular free calcium by 70% in cultured hippocampal neurons and

prevented the loss of membrane-associated protein kinase C (PKC) activity that developed 4 hours after the exposure to 100 microM NMDA (Black et al., 1996). Researchers concluded that remacemide desglycine is a potent channel blocking NMDA receptor antagonist, while remacemide is weaker. Future studies need to be performed to further investigate clinical application of this agent.

Sipatrigine, BW619C89 is a use dependent sodium channel antagonist. During ischemia it decreases the release of glutamate in rats (Kawaguchi and Graham, 1997). The agent (50 mg/kg., i.v.) significantly reduced the infarction volume in the cortex and striatum at 72 hours after the middle cerebral artery (MCA) was occluded for two hours. The same dose of the drug also reduced the infarction volume after it was administered 30 and 60 minutes after the onset of ischemia. However, no significant results were seen after the agent was delivered 5 minutes after reperfusion. In a different study, researcher investigated the effects of another sodium channel blocker and glutamate release inhibitor BW1003C87 (10 mg/kg, i.v.) on experimental cerebral edema following moderate fluid percussion injury in rats (Okiyama et al., 1995). Fifteen minutes post injury the animals received a constant infusion of the agent for 15 minutes. Regional tissue water content was assessed at 48 hours after the injury. The results indicated that glutamate release inhibitor BW1003C87 significantly reduced focal brain edema in the cortical area adjacent to the injury site and the ipsilateral hippocampus. These animal studies indicate that glutamate release inhibitors and sodium channel blockers may have neuroprotective effects during ischemia and traumatic brain injury; however clinical studies need to be carried out to further investigate its potential therapeutic values.

Apart from studying NMDA receptor antagonists, a novel strategy of therapeutic intervention includes a blockade of N-acetylated alpha-linked acidic dipeptidase (NAALADade), an enzyme that hydrolyzes N-acetyl-aspartyl-glutamate (NAAG), which acts like a modulatory neurotransmitter of glutamate or its storage form. In other words, researchers (Vornov et al., 1999) here attempted to reduce glutamate accumulation during ischemia by inhibiting the enzyme which acts to liberate glutamate. Using the transient middle cerebral artery occlusion model in rats, the investigator concluded that the decrease in glutamate was significant both during a two hour occlusion and during reperfusion. Authors concluded that such approach has a potential benefit of targeting the sites where the excessive accumulation of glutamate occurs rather than the whole brain. Such findings suggest possible improvements in the safety profile of glutamate modulators and offer significantly less adverse behavioral side effects.

AMPA receptor antagonist. Talampanel (LY300164) is a selective noncompetitive AMPA receptor antagonist with strong anticonvulsant properties. Preclinical investigations showed that this agent significantly diminished seizure severity score, seizure and afterdischarge durations. Researchers concluded that blockade of glutamate mediated events at AMPA receptors may offer a potential therapeutic value in traumatic brain injury where its significance is yet to be evaluated. Other AMPA receptor antagonists under investigation for potential neuroprotective effect include A205804, E5531, and ORG24292 (Hatton, 2001).

Magnesium sulfate. Magnesium aids in regulating calcium access to the cell through the NMDA receptor. After traumatic brain injury intracellular levels of

magnesium are decreased, and correlate with the injury severity after the diffuse axonal injury (Heath and Vink, 1996; 1998b; 1999b). According to the researchers, severe impact-acceleration induced injury resulted in a significant decrease in intracellular free magnesium concentration that lasted for up to four days post injury with noted recovery back to normal levels by day six.

In another study (Bareyre et al., 1999), investigators found that blood ionized magnesium concentration significantly declined by 30 minutes post injury and stayed depressed for 24 hours. However, in the same study total magnesium concentration remained at normal levels. Magnesium chloride treatment (125 micromol/rat) administered 1 h post injury restored magnesium levels by 2 hours and maintained at normal levels for up to 24 hours. Magnesium treatment also significantly decreased posttraumatic neuromotor impairments measured at 1 and 2 weeks, but failed to improve spatial learning performance. Investigators concluded that acute ionized magnesium measurement may be a predictor of long-term neurobehavioral outcome after head injury, and that delayed magnesium chloride treatment can restore magnesium concentration and enhance neurologic motor deficits in injured rats. Researchers concluded that both intravenous and intramuscular injections of magnesium sulphate significantly improved the outcome in rotarod and angleboard tests (Heath and Vink, 1997).

It is also important to note that both the sulphate and the chloride magnesium salts (100 micromoles/kg) administered at 30 minutes after severe closed-head injury improved intracellular free calcium concentration and neurologic outcome, suggesting that both salts can penetrate the blood-brain barrier after traumatic brain injury (Heath

and Vink, 1998a). Time point at which magnesium is administered is of great importance. When magnesium was given between 8 and 12 hours following injury, the rats' neurological motor outcome improved (Heath and Vink, 1999a). However, when the administration of magnesium was delayed for 24 hours after the injury, motor outcome did not improve and further doses did not facilitate test performance.

In a clinical study investigators (Muir and Lees, 1998) administered loading doses of magnesium sulphate (8, 16 and 24 mmol) followed by 65 mmol administered over 24 hours to patients 20 hours (mean time) after the onset of stroke. Results indicated that each dose restored serum concentrations to normal levels, and no tolerability problems or adverse affects were evident. The Intravenous Magnesium in Acute Stroke (IMAGES) study enrolled 2589 patients who were randomized within 12 hours of acute stroke to receive 16 mmol of magnesium sulphate intravenously over 15 minutes continued with 65 mmol over the following 24 hours, or a placebo. Unfortunately no significant differences were found between the placebo and the magnesium treated groups. A number of preclinical studies have led to the clinical trials examining the effect of magnesium treatment for stroke and traumatic brain injury.

The department of rehabilitation medicine at the University of Washington is conducting a magnesium sulphate study, where half of the patients enrolled in this study are randomly selected to receive magnesium sulfate and the other half to receive a placebo. The participants are examined at 1, 3, and 6 months on a comprehensive battery of measures to determine the significance of the treatment. Researchers hope that this treatment improves quality of life of TBI patients. National Institute of Neurological

Disorders and Stroke (NINDS) has sponsored a phase III clinical double-blind, placebo controlled study of magnesium sulfate for neuroprotection after brain trauma. The purpose of the research is to determine if magnesium sulfate will improve medical, mental, and psychological recovery. The study will also evaluate magnesium sulfate's ability to reduce the risk of developing seizures and its ability to increase survival rates after a traumatic brain injury when the participants will be given neuropsychological and psychosocial evaluations.

Dexanabinol. Dexanabinol (HU-211) is a nonpsychotropic synthetic cannabinoid, which exhibits pharmacological properties similar to noncompetitive NMDA receptor antagonist and acts as a cerebroprotectant (Shohami et al., 1997). Researchers found that dexanabinol (HU-211) improved the outcome of closed head injury. This putative neuroprotective agent was evaluated in a number of animal models including closed head injury, optic nerve crush, focal ischemia and global ischemia. In these investigations, a single injection of HU-211 administered after the insult showed significant long-term functional improvement and a significant increase in neuronal survival. Also, HU-211 is a potent scavenger of peroxy and hydroxy radicals in vitro and it protects cultured neurons from toxicity of radical generators. Such attributes make it a unique neuroprotective agent since it combines NMDA blocking activity along with free radical scavenging properties in one compound (Biegon and Joseph, 1995).

A clinical phase II study was completed with 101 patients treated with Dexanabinol or placebo (Pop, 2000). The researchers concluded that the drug was well tolerated and appeared toxicologically safe. Dexanabinol was found to be effective in

limiting intracranial hypertensive episodes during the first four days following injury

when it was administered within six hours of injury. Patients received three doses of the

drug 48, 150 or 200 mg, and at 6 months post injury the neurological outcome for

patients treated with lower dose was significantly better than patients treated with

placebo. Dexanabinol is currently in phase III clinical trials for patients with severe head

injury. It is a promising agent that seems to be able to provide the therapeutic benefits of

noncompetitive NMDA antagonist without displaying the adverse psychotrophic affects

(Darlington, 2003).

　　　　Estratrienes. Research indicates that steroid hormones and sex hormones decrease

in the accumulation of astrocytes in the proximity of the wound after brain injury

(Garcia-Estrada et al., 1999). Under normal conditions, astrocytes do not express

aromatase, an enzyme that catalyzes the conversion of androgens to estrogens. However,

investigators (Garcia-Segura et al., 1999) found that aromatase expression was increased

significantly in the injured hippocampus. In another study, estrogen displayed

neuroprotection against glutamate-induced toxicity (Green et al., 1998). The study also

revealed that the presence of an antioxidant in the extracellular space is required for the

neuroprotection to take place. Investigators also demonstrated that estrogen was

neuroprotective against anoxia-reoxygenation and AMPA-induced toxicity when the

studies were carried out using primary rat cortical neuronal cultures (Zaulyanov et al.,

1999). The exact mechanist is not well understood; however down regulation of gliotic

tissue has been noted which leads to the decreased accumulation of astrocytes near the

injury site. Emerson et al, (1993) administered estrogen or vehicle to male and female

rats prior to the fluid percussion injury. Researchers found that estrogen significantly increased magnesium concentration in males but not in females, and enhanced post traumatic motor function when it was measured at 1 week after trauma. It was concluded that estrogen protected male rats after traumatic brain injury but exacerbated the effects of trauma and increased mortality in female rats. Estratrienes are a new class of neurosteroids that can potentially offer therapeutic value in the treatment of traumatic brain injury.

Calcium antagonists. Calcium is known for triggering multitude of chemical sequences leading to secondary injury. Antagonizing calcium is one of the many approaches scientists attempt to investigate in their search of neuroprotection after traumatic brain injury (McBurney et al., 1992). Clinical trial examined the cerebral specific calcium channel antagonist, nimodipine (Teasdale et al., 1992). The data indicated that patients who received nimodipine (2 mg/h i.v. for 2 days) did not differ significantly from patients who received placebo. Another study suggested that nimodipine potentially may be neuroprotective to patients with subarachnoid hemorrhage (Murray et al., 1996). One of the side effects of nimodipine and other calcium antagonists included a decrease in the blood pressure which led to the decrease in cerebral perfusion blood pressure. Researchers concluded that the decrease in pressure contributed to the lack of benefit of a similar compound, nicardipine when it was administered at 2.5 mg/h for 24 hours and then every 12 hours for 2 days (Compton et al., 1990).

Phase I trial has been completed for a calcium channel antagonist DP b99. Ziconotide (SNX 111/CI 1009) blocked presynaptic N-type calcium channels and

prevented release of excitatory neurotransmitters following traumatic brain injury

(Samuii et al., 1999) when it was administered to rats 1 hour after the injury. The

accumulation of calcium was significantly reduced in the ipsilateral cortex, hippocampus

and thalamus. Unfortunately phase III clinical trials were suspended in the US

prematurely in 1999. Other calcium antagonists are in development. Researchers continue

in their attempts to demonstrate that reducing the release of excitatory neurotransmitters

by blocking the calcium channels may aid in aborting the process that leads to nerve cell

death.

LOE 908. Loe-908 is a novel compound that controls the levels of intracellular

calcium by inhibiting cation channels. When this compound was administered to rats 15

minutes post fluid percussion injury, researchers (Cheney et al., 2000) found that the

animals significantly improved on a neuromotor function test 48 hours after the injury.

However the rats who received LOE-908 did not differ significantly on a visual spatial

cognitive test in the Morris water maze from the animals who received placebo.

Histological findings revealed that LOE-908 did not affect cortical lesion volume at 48

hours post the injury. The authors concluded that the novel compound LOE-908 could be

a beneficial therapeutic treatment in alleviating motor deficits after traumatic brain injury

when administered in the acute posttraumatic phase.

MS 153. MS-153 works by inhibiting glutamate accumulations which occur after

the ischemic event. When cultured rat cells from the cortex and dorsal root ganglia were

used for recording the channel currents, MS-153 inhibited high voltage-gated calcium

channels through interaction with protein kinase C (PKC) and therefore prevented

massive release of glutamate from the nerve terminals after ischemia (Uenishi et al., 1999). The results however were quite variable partly due to the changeable activity of intracellular components and especially that of PKC. Further research is required in order to investigate the possible therapeutic properties of this compound.

  Cyclosporin. Cyclosporin is a widely used immunosuppressive compound that inhibits activation of T-lymphocytes and plays a vital role in neuronal regulation. Evidence suggests that cyclosporin may be able to protect against secondary neuronal injury (Sullivan et al., 2000). Cyclosporin inhibits mitochondrial pore opening and preventing the efflux of calcium which maintains calcium homeostasis after the animals received cyclosporin A (CsA) 15 minutes post unilateral controlled cortical impact injury (Sullivan et al., 2000). In another study (Scheff and Sullivan, 1999), cyclosporin A significantly decreased cortical damage 7 days post injury when it was administered immediately before or immediately after the cortical injury to young adult rats and mice. Systemic cyclosporin A administration after traumatic brain injury significantly enhanced neurobehavioral recovery as was measured by neuroscore, rotarod and sticky paper tests (Reiss et al., 1999). Cyclosporin A treatment also ameliorated cognitive deficits induced by traumatic brain injury in rats as was assessed in the Morris water maze test (Alessandri et al., 1999).

  Under normal conditions cyclosporin A can not penetrate into the CNS; however, traumatic brain injury alters the blood brain barrier (BBB) and regions normally not accessible to compounds such as cyclosporin A, may become accessible after the trauma (Baldwin et al., 1996). Such change may present a window of opportunity for the

therapeutic interventions to take place. Research indicates (Alessandri et al., 1999) that a clinically relevant cyclosporin dose can achieve therapeutic concentrations in the animal injury model and may lead to an improvement of neuropsychological functions. A number of studies indicate that penetration of cyclosporin through the BBB is critical for neuroprotection to take place (Liu et al., 1991; O'Keefe et al., 1992; Shiga et al., 1992; Folbergrova et al., 1997; Uchino et al., 1998). Research indicates that the BBB becomes accessible after the trauma in a biphasic manner. Such biphasic opening offers a window of opportunity for cyclosporin A to enter the CNS and access the injured brain. The results of some studies indicate that the window for therapeutic intervention may be at least 1 hour and as long as 24 hours (Baldwin et al., 1996; Scheff and Sullivan, 1999). Cyclosporin therapy has been shown to be successful in experimental animals when it was administered before or after cerebral contusion (Shiga et al., 1992; Scheff and Sullivan, 1999; Sullivan et al., 1999). Although, lesion volume was reduced in all animals receiving cyclosporin post injury, a dose effect was observed, with the highest dose of cyclosporin (20 mg/kg i.p. bolus with 10 mg/kg/day via osmotic mini pumps) offering the most neuroprotection with 74% reduction in lesion volume. The therapeutic profile of cyclosporin is complex and population dependent. Variable dynamics of cyclosporin activity combined with the complex metabolic changes that follow TBI require detailed studies investigating the issues of dose, concentration and time for drug administration in order to advance the treatment for traumatic brain injury population.

Antioxidants. There are a number of new strategies for antioxidant protection following TBI under investigation, despite the unfavorable results from the phase III

clinical trials of pegorgotein and tirilazad (Young et al., 1996; Marshall et al., 1998; Marshall and Marshall, 1995). In a clinical trial of 463 patients with severe head injury, researchers (Young et al., 1996) found no statistical significance between groups of patients receiving a single intravenous dose of pegorgotein (10000 U/kg, or 20000 U/kg) or those receiving placebo within 8 hours of the injury. In another clinical trial of 1120 patients, 85% with severe head injury and 15% with moderate injury, researchers (Marshall et al., 1998) found that the administration of tirilazad did not significantly improve patient outcome. Six months assessment of Glasgow Coma scale categories of good recovery and death did not differ significantly between tirilazad treated patients and the placebo treated group. Authors concluded that a number of inconsistent variables occurred despite a large population studied. Imbalances aroused in such areas as pretreatment hypotension, pretreatment hypoxia, and incidence of epidural hematomas.

Other therapeutic agents acting as antioxidants include OPC-14117, a superoxide free radical scavenger. Researchers (Kawamata et al., 1997) administered OPC-14117 (300 mg/kg, p.o.) to rats immediately after controlled cortical impact injury. The results revealed that OPC-14117 significantly attenuated the formation of edema, as well as the size of contusion-induced necrosis. Researchers also concluded that OPC-14117 improved performance on behavioral tests measured in the Morris water maze and the habituation of exploratory activity paradigm. Another free radical scavenger edaravone (MCI-186) significantly decreased the levels of free radical and water content in rats when it was administered (3mg/kg, i.v.) twice for 30 minutes after the controlled cortical

impact injury (Nakamura et al., 2003). Clinical trials of endaravone (MCI-186) are soon to be completed.

Nitric oxide inhibitors. Nitric oxide inhibitors and modulators have demonstrated neuroprotective properties in their ability to trap free radicals. Researchers (Beit-Yannai et al., 1996) tested the neuroprotective effects of the nitroxide stable radicals in a rat model of closed head weigh drop injury. Results indicated that nitroxide radicals significantly reduced brain edema, ameliorated BBB disruption and improved the recovery according to the Neurological Severity Score (NSS). Researchers concluded that the therapeutic window for the above improvements lasted up to 4 hours post trauma. Another novel nitric oxide inhibitor, NXY 059 has been shown to penetrate the BBB, and reduce the infarct volume and necrosis when it was administered before and after the transient focal ischemia in a rat model. The therapeutic window was noted to be from 3 to 6 hours post injury. However, another nitric oxide synthase pathway modulator, Lubeluzole, did not significantly attenuate contusion volume, water content or hemispheric swelling in rats when it was administered (0.8 mg/kg, i.v.) 15 and 75 minutes after cortical contusion impact injury (Kroppenstedt et al., 1999). The outcome measurements were carried out at 6 and 24 hours post injury, suggesting that lubeluzole did not offer beneficial effects in the treatment post injury.

Corticotrophin-releasing factor. Corticotrophin releasing factor is a known hypothalamic neuropeptide with protective properties inhibiting leakage of plasma-derived fluids and tissue edema in response to injury. Researchers (Beaumont and Marmarou, 1998) found that CRF reduced cerebral edema 24 hours following contusion

when it was administered to rats at 50 mg/kg and 100 mg/kg immediately after the injury.

Corticorelin which functions as a releasing factor that may increase the release of

corticotrophin similar to CRF, is entering the phase II clinical trials in patients with

peritumoral brain edema (Hatton, 2001).

Growth factors. Some of the examples of the glial–secreted trophic factors with

neuroprotective properties include BDNF, CTNF, IGF-1, fibrin growth factor (FGF),

bone morphogenetic protein (BMP-4), BMP-7, amphiregulin, cerebellum-derived growth

factor (neuregulin-2), and GDNF. One of the continuing problems includes the delivery

of the adequate concentration to the CNS. Another barrier to the advancement of these

agents to the clinical trials has been the lack of recorded deficiencies of specific

neurotrophic factors following traumatic brain injury. More studies need to be carried out

to show that supplementing with such exogenous trophic factors will benefit TBI patient.

Concentration, dosage, time of initial drug administration, and the duration of therapy

still remain under investigation.

Researchers (Dietrich et al., 1996) investigated the effects of BFGF after fluid

percussion injury in rats. The neurotrophic factors were administered continuously for 3

hours beginning 30 minutes after the injury. The results indicated that BFGF treated rats

(45 micrograms/kg/h) had a significantly decreased number of necrotic cortical neurons

and a reduced size of the contusion site compared to the vehicle-treated rats. In another

study, investigators (Saatman et al., 1997) administered insulin-like growth factor-1

(IGF-1) by subcutaneous injection 15 minutes post injury and similarly every 12 hours

for 14 days. Results showed that IGF-treated animals improved neuromotor function,

enhanced learning ability and memory retention compared to vehicle treated animals. Researchers concluded that chronic posttraumatic treatment with IGF-1 may be efficacious in attenuating neurobehavioral deficits induced by traumatic brain injury.

In a clinical trial Hatton et al., (1997) investigated the effects of IGF-1 on the neurological outcome after TBI in patients with GCS scores between 4 and 10. Patients were divided into two groups who were not receiving corticosteroid treatment. One received nutrition support alone, and another group received nutrition support in combination with IGF-1 continuous infusion (0.01 mg/kg/h) initiated within 72 hours post trauma and continued for 14 days. The drug was tolerated well and metabolic improvement was documented within three days of the injury in patients who received the neurotrophic factors. Another study (Young et al., 1998) investigated the effects of IGF-1 in severe TBI patients. Patients again received either nutrition support alone therapy or nutrition support in combination with IGF-1 therapy. Again, metabolic parameters were significantly better in the combination therapy group compared to the nutrition support therapy group. However, neurological outcome was not significantly different between the combination therapy group and the nutrition support only group.

Stem cells. The future of the stem cell research is promising. The potential of the neural stem cells in repopulating injured CNS sites following a traumatic injury needs to be further investigated in greater details. Today, researchers have been able to show signs of DNA synthesis of a neural progenitor in donor cells within 4 days post injury. Research indicated that the engraftment of donor cells and proliferation of host cells appears to be best within 3 to 7 days post trauma. Studies showed that neurotrophin 3

expression in stem cells leads to differentiation into glutamatergic, GABA-ergic and cholinergic neurons in 80% of cells. Stem cell transplantation research offers a tremendous hope into the potential therapeutic treatment of a number of CNS diseases including traumatic brain injury.

The complexity of TBI makes the search for an appropriate treatment very difficult. A number of therapeutic strategies are under investigation, each targeting various sites of intervention. The optimal treatment plan is still to be found as many questions remain unanswered. The time of drug delivery, the concentration of the therapeutic agent, the dose, the length of therapy, are all important questions under investigation. It is possible that a single agent will not attenuate all the TBI induced deficits, but a combination approach may be the answer. The type of injury, the severity, the initial trauma may serve as a possible baseline from which the clinician will generate a treatment plan that may differ from patient to patient, but that will hopefully improve each patient's neurological outcome.

EXPERIMENTAL BRAIN INJURY

A great number of hospitals and university laboratories in the US and across the world conduct scientific experiments in many areas of TBI, ranging from cell functioning to mental status of patients that acquire head injury. Clinical studies with human subjects are carried out by most teaching hospitals while experimental studies with animals are performed by university labs and some private organizations. All of the researchers provide essential information in the field of TBI pathology and recovery, and both clinical and animal studies have its advantages and disadvantages. Human studies offer

tremendous help in understanding the pathophysiology and recovery from TBI, however

they do not offer the control of a number of variables that animal studies do. Such

variables may include the severity of head injury, the location, the magnitude, the

direction of the forces leading to TBI, and other important variables. A number of TBI

models (such as fluid percussion, cortical weight drop, impact acceleration) have been

developed to mimic the physiology associated with human head trauma (Povlishock,

Hayes, Michel & McIntosh, 1994). Even though no one animal model can completely

replicate the human TBI and the processes associated with the injury (especially the

emotional factors surrounding the TBI patient), each of the developed nonhuman models

offers valuable and essential information about the underlying course of TBI. A number

of variables are taken into consideration to characterize animal TBI models. Such

variables include the location of injury within the brain, the mechanism of injury

production, the severity of trauma, and the time course (Gennarelli, 1994).

Injury Pathology

Traumatic brain injury causes severe deformation of the neuronal tissue, which

leads to membrane depolarization and an increased release of the excitatory

neurotransmitters (Hayes; Jenkins & Lyeth, 1992). The increased release of glutamate

and acetylcholine leads to the activation of N-methyl-D-aspartate (NMDA) and

muscarinic cholinergic receptors, which in term set the brain into the state of

neuroexcitation. Human studies have indicated increased glutamate levels after TBI using

the microdialysis technique (Perssons and Hillered, 1992). Animal studies have supported

this idea with similar findings. Katayama et al., 1990 and Faden et al., 1989 also found

an increase in glutamate release in animals after the experimental traumatic brain injury. Excessive neuronal excitation produces increase in extracellular potassium levels, which results in a further neurotransmitter release (Faden et al., 1989; Gorman et al., 1989; Katayama et al., 1990). Excessive neurotransmitter release may produce alterations in the intracellular signaling mechanisms, resulting in the long-lasting changes (Hamm et al., 1999). Following the acute excitation, a chronic functional neuronal depression follows (Hubschmann, 1985).

Much research has been devoted to the acute effects of TBI, conversely the mechanisms that mediate chronic deficits after TBI have been less intensively investigated and are not fully understood. However, as early as 1905, Von Monakow hypothesized that the CNS enters a state of "functional depression" following insult, characterized by immediate decreased neuronal activity. Fenney (1991) reviewed the theory of reduced neural activity, and referred to it as RFD "remote functional depression". The theory of RFD states that the chronic functional depression of normal neuronal activity leads to the behavioral deficits observed after neurological insult.

The biphasic hypothesis describes the acute excitation phase lasting approximately 24 hours, followed by a chronic depressed phase, lasting for hours, days or weeks. Acute phase can be characterized by excitotoxicity, neuronal damage, cell death with increased levels of excitatory amino acids (Faden et al., 1989; Katayama et al., 1990). Interventions that could be beneficial in this phase would include antagonists of excitatory neurotransmitters to achieve a reduction in neuronal activity and a decrease in cerebral metabolism during the first six hours following injury (Hamm et al., 1999).

Increased ionic fluxes and excitatory neurotransmitter release following TBI require high metabolic energy, as was assessed by increased glucose utilization (Alessandri and Bullock, 1998). Such increase in metabolism may last for minutes to hours, followed by a decrease in metabolism that may last for days or weeks as was seen in an animal model (Yoshino et al., 1991).

Figure1. A diagram of the hypothesized biphasic model following traumatic brain injury

(Von Monakow, 1969; Feeney, 1991). Neuronal activity is increased during the acute

post injury phase and depressed during the chronic post trauma phase. According to the

remote functional depression (RFD) theory, intervention in the acute post injury phase

should decrease neuronal activity as antagonists of excitatory neurotransmitters do.

However, treatment during the chronic post injury phase should increase neuronal

activity and may consist of agonist of excitatory neurotransmitters. Note the hypothetical

treatment line represents aniracetam bringing the neuronal activity back to the

baseline/normal range.

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Biphasic Model of TBI
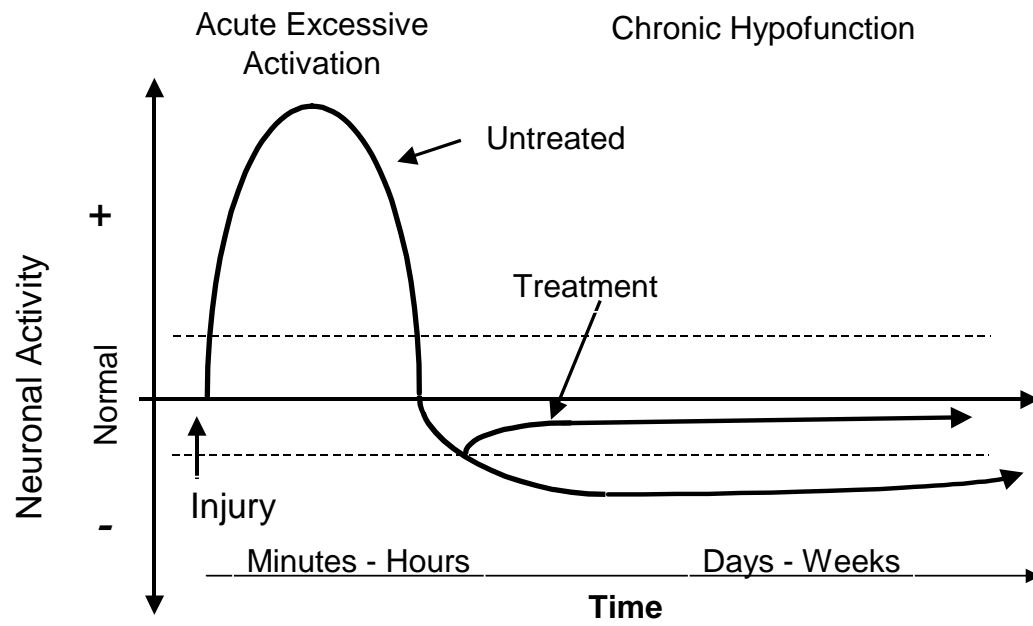
A chronic phase is described by a decreased level of activity. A number of research studies have supported the phenomena by showing a reduction in neuronal function chronically after TBI (Dixon et al., 1994; 1996; 1997; Gorman et al., 1989; Leonard et al., 1994). Metabolism slows down below normal levels and injured cells enter a hypofunctional state (Hubschmann, 1985). Besides reductions in cerebral metabolism, decreases are seen in choline uptake, scopolamine-evoked release and choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) immunoreactivity. Antagonists of excitatory neurotransmitter have shown adverse affects, however drugs that would increase metabolic and cholinergic systems may be advantageous at this stage (Hamm et al., 1999). In order to be able to study the pathology of traumatic brain injury with greater detail and control and measure numerous variables that affect the outcome of TBI, a trauma model that mimics the pathophysiological procedures of the insult is necessary. A number of injury models exist, however fluid percussion model is the preferred choice for the study for its characteristics described below.

Fluid Percussion Injury Model

Fluid Percussion injury model will be used in the present study. Several aspects of the physiological and psychological effects that result from human TBI have been steadily imitated by FPI model in animals (Bramlett, Green, & Dietrich, 1997; McIntosh et al., 1989). This model has also been characterized on many species, including rats, cats, and pigs. This model has also been used across a broad spectrum of injury severity, varying from mild to moderate to severe injury (Povlishock et al., 1994). FPI is characterized with a time period of unconsciousness, changes in cerebral blood flow,

axonal damage, edema, neuronal cell loss, deafferentiation, alterations in blood brain barrier functions (Bramlett, Green, & Dietrich, 1997; McIntosh et al., 1989). This model induces an experimental injury that mimics the cognitive deficits many patients acquire after TBI. Other deficits experienced after FPI include motor impairment, which is tested in rats in a number of motor coordination tasks, such as beam balance, beam walk, and rotarod test (Floyd et al., 2002).

The fluid percussion injury is induced by delivering a fluid, saline, under pressure to the exposed brain. In order to expose the brain on the specific location, a craniotomy is performed over that location. The rat is attached to the cylinder filled up with water, and the fluid then is delivered under pressure to the exposed rat brain. The severity of the trauma can be controlled and recorded with a transducer hooked up to the device. The oscilloscope connected to the injury device records the pressure of the fluid inducing the injury; fluid deforms the exposed brain, causing significant alterations in the neuronal tissue. The injury produced can vary depending on the location of the craniotomy and therefore the injury placement (Floyd et al., 2002).

The injury can be applied centrally (affecting both hemispheres equally), or laterally (over one hemisphere) between bregma and lambda. Usually central FPI causes bilateral damage to the hippocampus while lateral injury causes damage over one hemisphere only. Lateral injury usually induces cellular dysfunction, and also cell death (Delahunty et al., 1995; Hicks et al., 1996). Brainstem damage is usually common to the central FPI, while hippocampus damage is characteristic of lateral FPI (Gennarelli, 1994). Lateral injury has been recorded to induce spatial deficits such that can be tested in the

Morris Water Maze. Lateral FPI are likely to result in focal contusion and cortical changes unilateral to the injury side sparing the contralateral cortex, allowing for a control "sham" hemisphere within one animal. Ipsilateral axonal damage and occasional deep tissue injuries at gray white matter junctions may occur. Unilateral damage to the hippocampus in this model has been well characterized (Gennarelli, 1994). Central injury usually induces little focal damage, no cell death or seizures in the hippocampus, but still produces cellular dysfunction (Lyeth, et al., 1994). Central FPI involves bilateral cortical and hippocampal damage as well as brainstem, including traumatic axonal injury (Gennarelli, 1994; McIntosh et al., 1996; Povlishock et al., 1994). Central FPI delivers a more diffuse type of injury (Gennarelli, 1994; Povlishock et al., 1994, and therefore central FPI was chosen for the present study.

Present study focused on the alterations that occur chronically after traumatic brain injury. While many neurotransmitters are involved in the injury pathology, glutamatergic receptor system seems to play a significant role in the outcome of TBI. Of specific interest are AMPA receptor subtypes of the glutamatergic receptor system.

AMPA RECEPTORS

AMPA receptor properties. Research indicates that neurological excitation within the hippocampus induced by traumatic brain injury leads to long-term cognitive deficits (Hamm et al., 1993; Lyeth et al., 1990; Capruso & Levin, et al., 1992). Glutamate receptors mediate excitatory neurotransmission in the brain and play a pertinent role in neural plasticity, neural development and neurodegeneration (Choi and Rothman, 1990; Nakanishi, 1992). Glutamate also plays a significant role in the in posttraumatic injury

process. After TBI, glutamate levels increase up to 300% above normal baseline (Faden et al., 1989).

Glutamate receptors are divided into ionotropic and metabotropic G-protein linked receptors. Ionotropic glutamate receptors, ones of interest here, are categorized into NMDA receptors and kainite/AMPA receptors, and both contain glutamate-gated, cation-specific ion channels (Hollman and Heinemann, 1994). AMPA/Kainate receptors consist of seven structurally related subunits, labeled GluR1 – GluR4 (AMPA) and GluR5 through GluR7 (Kainate) (Schiffer et al., 1997), and are responsible for the fast excitatory neurotransmission by glutamate (Stern et al., 1992), where as NMDA receptors are primarily responsible for the slow neurotransmission with high calcium permeability (Bliss and Collingridge, 1993).

NMDA receptors have been a major focus in excitatory post-traumatic investigations in search of possible therapeutic interventions. For example, acute administration of NMDA antagonist MK-801 immediately after the injury (Hamm et al., 1993) reduced cognitive deficits induced by TBI in the Morris water maze. Also, chronic administration of glutamate agonist D-cycloserine during the hypofunctional post-traumatic (Temple and Hamm, 1996) phase significantly improved animals' performance on a spatial memory task. The results of these studies suggest that alterations in the glutamate receptor system influence cognitive function following TBI. Since NMDA receptors have received much attention in the posttraumatic TBI research, interest towards AMPA receptors and their role in the injury pathology also grew.

AMPA receptors are ligand-gated fast functioning channels found in both presynaptic (Ohta et al., 1994) and postsynaptic neurons (Leranth et al., 1996) as well as astrocytes. The structure of the AMPA receptor system includes an N-terminus extracellular domain, 3 transmembrane-spanning domains (TM1, TM3 TM4), a hydrophobic segment, a binding domain for agonists formed from the extracellular S1 and S2 regions, and a C-terminus intracellular domain (Pellegrini-Giampietro et al., 1997). AMPA receptors fire rapidly by allowing sodium to flow into the cell, and decreasing the time to achieve the threshold to reach the action potential. AMPA receptors quickly desensitize and slowly recover (Hestrin, 1992). Desensitization limits the frequency at which a receptor can produce full amplitude responses. Once AMPA receptors desensitize, they accumulate in desensitized state during their slow recovery and continue to accumulate after the channel has closed (Hestrin, 1992; Raman and Trussel, 1995). During the increased glutamate release state, such that occurs post injury, the slow recovery of the AMPA receptors may extend miniature excitatory post synaptic currents (Jones and Westbrook, 1996; Kiskin et al., 1986; Smith et al., 1991; Raman and Trussell, 1994). Modulation of AMPA receptor desensitization may offer therapeutic value (Jones and Westbrook, 1996).

GluR2 subunit. GluR2 is the AMPA receptor subunit that exists in both edited and unedited forms (Hollman et al., 1991; Nakanishi et al., 1992). Unedited forms, that are very few, refer to the naturally occurring state of the subunit that is permeable to sodium and calcium. The unedited form of the subunit contains glutamine residue (Q) on the Q/R site of the M2 domain, which allows sodium, potassium as well as calcium ions to pass

through the pore (Burnashev, 1992). In the adult brain, specifically in the hippocampus, the GluR2 subunit is present almost exclusively in the edited form, which inhibits calcium permeability. The RNA editing of the GluR2 edited subunit replaces glutamine (Q) residue within the M2 domain (Higuchi et al., 1993; Sommer et al., 1991) with a larger arginine (R) molecule, which due to is size does not allow calcium ions to pass through the now smaller channel. Research indicates that nearly 100% of the hippocampal GluR2 receptors are edited (Bennett et al., 1996; Gorter, 1997; Sommer et al., 1991). Hippocampal cells that display higher permeability to calcium show reduced mRNA for GluR2 receptor subunits (Bochet et al., 1994; Racca et al., 1996; Geiger et al., 1995). And subsequently, decreased calcium levels were seen with increased mRNA levels of GluR2 (Bennett et al., 1996).

AMPA and TBI. Research indicates that following ischemia, GluR2 gene expression is reduced and AMPA-mediated calcium influx increases (Bennett et al., 1996; Gorter et al., 1997). Another study indicated that hypoxic ischemic necrosis of basal ganglia resulted in decreased GluR1, GluR2, GluR3 and GluR4 expressions (Meng et al., 1997). Similar to alteration in GluR2 receptor after injury, changes in calcium also have been reported after the traumatic event. Several studies showed that following TBI, intracellular calcium levels increase (Cargill and Thibaut, 1996; Lazarewicz, 1996; Mattson, 1996). Calcium is extremely important in regulating neurotransmitter release. Calcium plays a role in mobilization of synaptic vesicles to the presynaptic pore as well as in the exocytosis of the vesicles into the synaptic cleft. Increased calcium release translates into the amplified neurotransmitter release, which includes elevated glutamate

release after a traumatic event, leading the cell into neuroexcitation and excitotoxicity (Zipfel et al., 2000). An influx of calcium can propagate glutamate neurotoxicity in a positive feedback loop by further stimulating the release of glutamate (Choi, 1988). According to previous research, following ischemia GluR2 mRNA expression is markedly reduced in the CA1 region (Gorter et al., 1997; Pellegrini-Giampeitro et al., 1992). Decreased GluR2 immunoreactivity suggests an increase in calcium influx leading to toxic levels of excitatory neurotransmitters causing cell damage and cell death. The importance of calcium in regulating synaptic transmission of glutamate indicates the significance for further investigational studies that would improve the outcome after TBI. While increased Ca++ influx immediately after the injury has been linked to neurodegeneration, elevated calcium may be beneficial in the chronic hypofunctional stage following trauma. Injury related reduction in the AMPA receptor GluR2 subunit may turn advantageous if it increased overall neuroexcitation by allowing greater calcium influx through the AMPA receptors lacking GluR2 subunits during the depressed stage post TBI. A decrease in the GluR2 expression may lead to an increased formation of calcium-permeable AMPA receptors, and thereby to an enhanced neuronal activity during the chronic phase after the trauma. The importance of AMPA receptor subtypes extends to aniracetam, as it modulates the second subunit of the AMPA receptor, GluR2 , also referred to as GluRb.

ANIRACETAM

Aniracetam properties. Aniracetam is a cognitive enhancer that has been used in the treatment of behavioral and psychological symptoms of dementia following stroke

and Alzheimer's disease (Nakamura, 2002). Aniracetam belongs to a class of drugs that include piracetam, oxiracetam, nefiracetam, also known as "nootropic" agents, suggesting their cognitive enhancing abilities. However, their mechanism of action and clinical efficacy still remain unclear.

In the past studies, the nootropic drugs induced improvement on such cognitive tasks as passive avoidance paradigm, active avoidance, eye blink classical conditioning (Sakurai et al., 1989; Nabeshima et al., 1990; 1991; Kojima et al., 1990; Tanaka et al., 1992; Hiramatsu et al., 1992; Abe et al., 1991; Ozawa et al., 1991; Woodruff-Pak, 1995). One of the studies demonstrated that aniracetam potentiated AMPA receptor-mediated excitatory post-synaptic potentials (EPSP's) (Ito et al., 1990). They suggested that the potentiation of the AMPA receptor-mediated synaptic responses was responsible for aniracetam's cognitive enhancing effects. Aniracetam appears to positively modulate metabotropic glutamate receptors and alpha-amino-3-hydroxy-methyl-4-isoxazolepropionic acid (AMPA)-sensitive glutamate receptors, and may facilitate cholinergic transmission.

Research indicates that such nootropic drugs as aniracetam facilitate long-term potentiation (LTP) in Schaffer collaterals in vitro (Staubli et al., 1992) and in vivo (Arai et al., 1996a) and improve animal's performance in experimental learning and memory tests. Long-term-potentiation is an activity-dependent and synapse-specific synaptic potentiation thought to be a prerequisite for encoding information for long-term memory storage. And the cellular mechanisms that enable this process are thought of as a possible foundation for learning and memory. In vitro aniracetam increased the peak and

prolonged the decay of AMPAergic EPSPs, which resulted in a threefold increase in the integrated response (Staubli et al., 1992) in hippocampal slices (1.5 mM). Such effect was present for about 60 minutes with its peak at 15 minutes after the infusion. In vivo aniracetam injections (120 mg/kg) exhibited potentiation that peaked at 45 minutes and returned to normal values within 2 hours post injection. The ability of AMPA modulators to facilitate the induction of LTP suggests that if LTP is a cellular basis for memory encoding, then AMPA modulators that facilitate LTP should also improve functional experimental procedures in learning and memory assessment. Aniracetam reduced glutamate receptor desensitization and slowed the decay of fast excitatory synaptic currents in the hippocampus (Isaacson and Nicoll, 1991).

Aniracetam in Animal Studies

In a behavioral study (Staubli et al., 1994) aniracetam administration enhanced the performance on three behavioral tests. Researchers reported that animals treated with aniracetam improved performance in the Morris water maze, a task thought to be dependent upon hippocampal LTP, as well as in 8-arm radial maze test and the discrimination task between odors for food reward. Such in vivo results suggest that AMPA modulators have the potential for enhancing memory and recall. And fortunately no adverse side effects have been reported. In another study, Togashi et al., (2002) found that aniracetam (100 mg/kg) enhanced glutamatergic transmission in the prefrontal cortex stroke-prone spontaneously hypertensive rats, however it failed to exert any significant effects in the amygdale. Aniracetam (100 mg/kg) reversed increased extracellular levels of aspartate and glutamate that were observed in the hippocampus following forebrain

global ischemia (Yu and Cai, 2003). Also, aniracetam administration enhanced the release of glutamate and aspartate in the normal gerbil hippocampus. Authors concluded that such action of aniracetam may be a way of protecting against delayed neuronal death in the ischemic hippocampus, therefore leading to enhanced memory deficits induced by an ischemic event.

In an animal behavioral study (Smith and Wehner, 2002) mice treated with aniracetam (100 kg/kg) 30 minutes before training, performed significantly better on a fear conditioning task when tested 30 minutes and 24 hours after the training session. No improvement in learning was noted 5 minutes after the training session. In one of the studies researchers (Sekiguchi et al., 2001) found that aniracetam did not significantly improve test performance in the Morris water maze, compared to PEPA (Phenylsulphonylamino-ethylthio-diflurophenoxyacetamide), an allosteric potentiator of AMPA receptors. Researchers reported that repeated intravenous administration of PEPA (1, 2, or 10 mg/kg/day for 10 days) improved the performance of the spatial memory task, but aniracetam did not produce significant differences.

One of the research studies (Zajaczkowski and Danysz, 1996) indicated that aniracetam produced beneficial effects in attenuating spatial and reference memory deficits induced by entorhinal cortex lesions in rats. Animals performance in the radial arm maze significantly improved when they were treated with aniracetam (50 mg/kg, for 10 days) when it was administered 30 minutes prior to the testing. However, the same dose of aniracetam did not have any effect in the naive animals and no effects on the scopolamine induced amnesia as measured in the passive avoidance test. Another study

reported that aniracetam demonstrated positive effects in the naive animals (Martin et al., 1992), where oral administration of aniracetam (100, 200, 400, or 800 mg/kg) significantly improved the animal performance in the 8 arm radial maze. The highest doses correlated with the most performance enhancement.

Researchers have demonstrated that aniracetam is able to block the formation of cytotoxic hydroxyl radicals during the ischemia in the mouse brain (Himori et al., 1995). Investigators induced an ischemic event for 40 minutes, as was evident by an increasing level of hydroxyl radicals with a peak at 20 minutes after the insult. Aniracetam (100mg/kg, i.p.) decreased the formation of the free radicals by 80%, suggesting that aniracetam can attenuate the formation of hydroxyl free radicals.

Aniracetam and Clinical Studies.

A number of clinical studies show aniracetam produced beneficial effects in patients with dementia. The results of a clinical trial in New Zealand (Lee and Benfield, 1994) revealed that aniracetam was beneficial at 4 and 6 months of treatment in elderly patients with mild to moderate cognitive impairments due to senile dementia of the Alzheimer's type. The authors suggested according to the preliminary evidence collected in the treatment of patients with cognitive impairment of cerebrovascular traumatic origin that such potential patients may also benefit with aniracetam treatment. In another study, aniracetam displayed therapeutic effects on emotional disturbances such as depressed mood and anxiety in patients with cerebral insufficiency (Nakamura K., 2002).

In a different clinical study in Europe (Senin et al., 1991), 109 elderly patients suffering from mild to moderate cognitive impairment of the Alzheimer type were treated

for 6 months with aniracetam (Ro 13-5057) or placebo in a double-blind randomized study. The two treatment groups were compared at baseline for demographic and behavioral parameters and symptomatology. Patients who received aniracetam differed significantly from the placebo and showed a statistically significant improvement versus baseline in the psychobehavioral parameters, compared to a placebo group, where a steady deterioration was observed. Researchers also reported excellent tolerability to aniracetam with no adverse side effects.

Another clinical trial (Tsolaki et al., 2001) investigated the efficacy of nootropics (piracetam, aniracetam, nimodipine and dihydroergicristine) versus acetylcholinesterase inhibitors (AChE-Is) (tacrine and donepezil) in the treatment of Alzheimer's disease in 510 patients. A series of behavioral tests and questionnaires were used to determine the baseline and clinical efficacy of treatment. At 12 month after treatment patients with moderate Alzheimer's disease who received nootropics, scored significantly better on the neuropsychological tests compared to those who received AChE inhibitors. However no differences were found in the severe Alzheimer's patients group, and a reverse effect was seen in mild AD patients, where those treated with nootropics performed worse than AChE treated group. Authors concluded that overall, no significant differences were demonstrated between AChE-Is and nootropics in the treatment of Alzheimer's disease**.**

In another study (Sourander et al., 1987) forty-four patients with senile dementia of the Alzheimer type were treated with aniracetam (Ro 13-5057) or placebo daily for 3 months. Neurological examinations were made before and after treatment and the tests revealed that improvement was seen in several cognitive tests, however this evidence was

seen in both placebo and aniracetam-treated groups. Due to the confusion between the groups, treatment had to be interrupted. In clinical evaluation no difference was seen in efficacy between the two treatment groups.

Besides the devastating physical injuries that patients with traumatic brain traumas have, a vast variety of patients also end up with tremendous psychological deficits. Such problems may last indefinitely and sometimes are not observed at the initial stage after the injury. Most of the psychological deficits are cognitive in nature.

LEARNING AND MEMORY

According to research, people with TBI have more problems with tasks involving learning and memory than any other cognitive tasks. Therefore this area of TBI patients in recovery and rehabilitation deserves great attention from researchers, health care personnel and rehabilitation staff. Hippocampus is one of the structures that are associated with the ability to construct, store and retrieve new memories (Miller, 1973; Squire & Zola-Morgan, 1991). The hippocampus is also one of the brain structures that is extremely vulnerable to TBI.

Research indicates that damage to the hippocampus results in decreased performance on spatial navigation tasks in animals (Morris, Garrud, Rawlins &O'Keefe, 1982; Sutherland, Kolb & Whishaw, 1982). Ischemia studies found that hippocampus plays a very important role in memory dysfunction in animals (Mishkin, 1978), and humans (Cummings, Tomiyasu, Read & Benson, 1984). Since hippocampus is such an imperative component in learning and memory functioning, and it is vulnerable to TBI,

this brain structure has been under thorough investigation by many researchers working in the area of traumatic brain injury and rehabilitation.

Hippocampus

Hippocampus structures. Hippocampus is located within the temporal lobe. It is a C-shaped structure, and contains four primary components. They are: the dentate gyrus (DG), the entorhinal cortex (EC), the subicular complex (SC), and the hippocampus proper, which is further divided into three regions, named CA1, CA2, and CA3. Hippocampus structures have a very distinct laminar organization. The major hippocampal pathway is through the perforant pathway, originating at EC and terminating in DG. Mossy fibers project from DG to the CA3 region of the hippocampus proper. And from CA3 projections go out to the CA1 region. Hippocampus proper structures send their output primarily to the subiculum. Associational projections allow communications within the ipsilateral hippocampal structures, and commissural projections allow communications between contralateral regions. (Amaral and Witter, 1995).

Entorhinal cortex. Cortical structures send the information to the hippocampus via the EC, which is comprised of five layers. From there, the information is passed to the DG and subsequently to the CA1 and CA3 regions. Feedback information comes back from the CA1 area and the subiculum. Projections originating from the EC and going to the hippocampal regions are referred to as perforant pathway, which consists of projections from numerous cell types, such as stellate cells, pyramidal cells, GABAergic cells (Amaral and Witter, 1995) and originates in the layers II and III of the EC. The

entorhinal cortex also projects to the CA2 and CA3 of the hippocampus and to the subiculum. Layer III projections from the pyramidal cells go out to the CA1 region (Kohler, 1985a; 1985b; 1986; 1988; Ruth et al., 1989; Witter et al., 1989). The two main division of the EC include the lateral entorhinal area (LEA) and the medial entorhinal area (MEA) (Amaral and Witter, 1985). LEA projections innervate an outer part of the molecular layer of the DG, and MEA projections innervate a middle part of the DG (Hjorth-Simonsen, 1972; Nafstad, 1967; Steward, 1976; Wyss, 1981). The inner part of the DG receives input from the polymorphic layer mossy cells (Amaral and Witter, 1985).

Dentate gyrus. Dentate gyrus is comprised of three layers: molecular layer, granule cell layer, and a polymorphic layer, or a hilus. The molecular layer mostly consists of apical dendrites of the granule cell and some stellate and basket cells. There are very few cell bodies in this layer, except for some basket cells and axo-axonic interneurons, or chandelier cells that provide input to the perforant path and to the dendrites of the granule cells (Amaral and Witter, 1985). These GABAergic chandelier interneurons may contribute to the regulation of the granule cell excitatory input from EC (Somogyi et al., 1985; Soriano and Fotsher, 1989).

The granule call layer, principal layer of the dentate gyrus mostly consists of the granule cell bodies, and some basket cells (Amaral et al., 1990). Granule cells project their axons to the CA3 region of the hippocampal proper. Granule cells also receive input from the basket cells that are primarily GABAergic, from the axo-axonic chandelier cells of molecular layer, and from the polymorphic cells. These inhibitory connections act as a

regulatory mechanism for granule cell output. The molecular and the granule cell layer from a "V" shape, with the suprapyramidal blade and the infrapyramidal blade, named after their position. The apex of the dentate gyrus, where the blades meet is called the crest (Amaral and Witter, 1985).

The polymorphic layer or hilus, is located between the suprapyramidal and the infrapyramidal blades of the gyrus. It contains mossy cells, basal dendrites and axonal projections of the pyramidal and granule cells. Mossy cells are characterized with large triangular or multipolar shape bodies and spines, or "thorny excrescences", termination sites of the mossy fibers (Frotscher et al., 1991; Ribak et al;. 1985). Mossy cell projections may form numerous synapses with one CA3 pyramidal cell (Amaral and Witer, 1985; Chicurel and Harris, 1992). Between the polymorphic and the granule cell layers some basket cells can be located, which are mostly GABAergic in nature.

Hippocampus proper. The hippocampus proper consists of three regions, CA1, CA2 and CA3. All the regions contain pyramidal cells with smaller cell bodies in the CA1 regions, and larger somas in the CA2 and CA3 areas. Another distinctive characteristic of the layers includes the stratum lucidum, containing mossy fiber projections from the DG and only found in the CA3. All hippocampal laminar organization is similar throughout the structure. The pyramidal layer is the main cell layer, containing mostly pyramidal cells and some basket cells (Seress and Ribak, 1984). Stratum oriens (SO) layer contains basal dendrites of the pyramidal cells. Stratum radiatum (SR) contains apical dendrites of the pyramidal neurons, Schaffer collaterals, and stratum lacunosum-moleculare (SLM) contains perforant pathway and other fibers.

Schaffer collaterals extend from the CA3 regions to the CA1 region, mostly to the stratum radiatum and to a lesser degree to a stratum oriens. The hippocampal CA3 region receives input from the mossy fibers of the dentate granule cells, and from the septal nuclei, but to a lesser degree. Hippocampal region CA2 also projects to CA1, however those projections lack the organized structure of the Schaffer collaterals. The perforant pathway of the hippocampus extends from the entorhinal cortex to the dentate gyrus.

Subiculum. The primary output from the hippocampus proper is through the CA1 region to the subiculum. Subiculum projects to the EC as well as to other cortical areas, including limbic cortex, the nucleus accumbens and the lateral septal region. The stratum radiatum is not very prominent in the subiculum, and stratum oriens and the molecular layers widen in order to accommodate bigger pyramidal neurons.

Hippocampus and neurotransmitter systems. Numerous neurotransmitters, including ACh, glutamate, GABA play a role in the hippocampal system. A major excitatory neurotransmitter here, as well as through out the brain is glutamate. Glutamatergic receptors have also been found to play a major role in the mechanism of long term potentiation (LTP), where NMDA receptors are required for the induction of LTP, and AMPA receptors are required for the expression of LTP. (Bekkers and Stevens, 1989). Research supports this finding, as NMDA receptor antagonists impaired acquisition, but not the retrieval of information previously acquired in rats (Morris, 1989). And AMPA receptor antagonists can block retrieval of stored information (Izquierdo et al., 1993).

Hippocampus and TBI. Hippocampus is known to be vulnerable to ischemia, seizures and traumatic brain injury. Cognitive deficits induced by traumatic brain injury are the most common long-term neurological problems in patients with TBI (McIntosh et al., 1996). Ischemia produces damage mostly in the CA1 region, however TBI mostly affects the CA3 region of the hippocampus proper as well as the dentate gyrus. Focal injury models have reported cell loss in the hippocampal regions (Cortez et al., 1989; Hicks et al., 1993; Soares et al., 1993; Smith et al., 1991). Axonal damage is common throughout hippocampus and thalamus (Povlishock et al., 1996). After the injury, receptor binding properties are disrupted and the seizure threshold is lowered (Dixon, et al., 1991; Feeney, et al., 1981). CA1 region is noted to be the most vulnerable area to the TBI, and therefore is the one studied with greater attention. Research indicates that damage to the CA1 regions only produced impairments in the working memory of rats (Zola-Morgan, Squire & Amaral, 1986). Morris water maze, radial arm maze and other similar memory tasks require spatial discrimination and are sensitive to the damage of the hippocampus. Hippocampal lesions have induced severe impairments in the performance on the MWM tasks (Eichenbaum et al., 1990; Morris et al., 1980). Different models of experimental brain injury, including fluid percussion, controlled cortical impact, lesion injuries demonstrated deficits in the Morris water maze performance suggesting hippocampal damage (Hamm et al., 1993). TBI also induces dysfunction in long-term potentiation in CA1 region (Miazaki et al., 1989). Other neurological problems associated with CA1 region impairments include seizures, hypoglycemia, ischemia, Huntington's disease, Parkinson's disease, Alzheimer's and Korsakoff's disease (Lyeth et al., 1994).

SUMMARY

Much progress has been made in understanding the pathological alterations that are induced by traumatic brain injury (TBI). Research shows that the mechanical forces produced by TBI initiate a cascade of biochemical events that result in both acute and long-term neurological dysfunction. The increased understanding of the mechanisms involved in TBI has led investigators to develop and test numerous therapeutic interventions designed to address the acute and chronic deficits of TBI to ensure better treatment of TBI patients. Acute pathology after TBI is followed by chronic pathological changes in brain activity. In contrast to the acute changes that include excitotoxic events, chronic pathology includes suppressed or hypofunctional alterations in the brain. Research indicates that hypermetabolism produced as a result of injury is followed by a depressed state of cerebral metabolism (Yoshino et al., 1991). Also, indexes of the function of several neurotransmitter systems become hypofunctional after TBI (Hamm et al., 2000). If the brain enters a hypofunctional state after TBI, then delayed treatments that would chronically enhance neuronal activity would be beneficial to the TBI patients.

Since glutamate is the primary excitatory neurotransmitter in the brain, enhancing its activity without causing neurotoxicity may improve neuropsychological and neurophysiological outcome after traumatic brain injury. In order to avoid neurotoxicity that can occur as a result of increased stimulation of the glutamate system by a direct agonist, a positive modulator, aniracetam should be considered as a possible therapeutic intervention for the treatment of post-TBI deficits. According to previous data, modulatory actions of aniracetam cause a reduction of glutamate receptor desensitization

through the allosteric potentiation of AMPA-specific glutamate receptors, which leads to a decrease in channel closing rate and subsequent increase in calcium and excitatory neuroteransmitters release. Ultimately, aniracetam increases neuronal activity in the post-traumatic otherwise hypofunctional state. If a reduced level of neuronal activity is a long-term consequence of TBI, then augmenting neuronal activity with aniracetam should improve recovery from TBI.

The purpose of this research study was to investigate the efficacy of aniracetam in increasing neuronal activity during the chronic hypofunctional state following trauma, and ultimately leading to an improvement in performance on the spatial memory task. The rationale for the study was based on the previous research where chronic treatment with such compounds like L-deprenyl (Zhu, et al., 2000) administered chronically for 7 days led to improved MWM performance, and the beneficial drug effects were associated with the changes in DBH immunoreactivity. These findings suggest that the drug treatment may have induced a beneficial neural plasticity response. These effects were also supported by the findings that the termination of drug treatment before MWM testing was still effective in improving spatial memory performance. Therefore, the present study intended to investigate aniracetam as a possible treatment option for cognitive deficits following TBI.

The goal was to examine whether aniracetam could alleviate cognitive dysfunction, and to investigate the effects of various treatment parameters, whether the mechanism of action was also driven by chronic receptor-related changes as in previous chronic treatment studies. Such preliminary studies are necessary in order to improve

care and outcome for patients with TBI. Preclinical animal studies for TBI treatment are important as they may support a future clinical trial that could demonstrate a beneficial treatment for TBI patients. First, the optimal dose of aniracetam was determined using a classic chronic treatment paradigm, where drug administration began 24 hours post injury and continued for 15 days. Second, using the optimal dose of treatment, delayed chronic aniracetam administration was tested for its efficacy in improving cognitive performance of injured animals. In the second experiment, the goal was to investigate the optimum strategy by investigating the temporal therapeutic window in which the treatment would be still beneficial following TBI. In a third study, aniracetam treatment was terminated before the animals were tested in the spatial memory task. That study examined whether repeated administration of aniracetam improved cognitive performance once daily administration was terminated. Lastly, using the optimum dose and time of treatment, determined in the behavioral experiments, changes in the AMPA-glutamate receptor (GluR2 subunit) were investigated in aniracetam-treated and vehicle-treated injured animals to examine whether aniracetam's behavioral effects were mediated by its actions on the GluR2 subunit of the AMPA receptor. Alterations in the expression of the GluR2 subunit of the AMPA-glutamate receptor were determined by immunohistochemistry as well as by Western blot analysis. Functional recovery after TBI was evaluated by performance in the Morris water maze

Therefore, it was hypothesized that aniracetam would increase the neuronal activity during the chronic hypofunctional posttraumatic state of TBI and improve cognitive performance of injured animals. Beneficial aniracetam effects were expected to

be seen through the altered neuronal function reflected in a decrease in the expression of the GluR2 subunit of the AMPA-glutamate receptor after TBI. Since the GluR2 subunit is calcium impermeable, a reduction of this subunit expression was expected in order to increase calcium, accompanied by glutamate and other excitatory neurotransmitters release for the neuronal activity to enhance. Thus, the purpose of this investigation was to test the compound aniracetam on spatial memory performance after TBI, and examine GluR2 immunoreactivity and GluR2 protein expression in the hippocampus as anatomical neurochemical correlates of TBI-induced cognitive impairment.

METHODS

Subjects

Adult (3-month old) male Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottsdale, PA) weighing 280-330 grams were used in all experiments. Animals were housed individually (at 20-22°C) with lights on a 12 hour light and dark cycle with free access to food and water. All procedures were completed following the guidelines established in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services) and were approved by our Institutional Animal Care and Use Committee.

Fluid Percussion Apparatus

The fluid percussion device used to produce experimental brain injury is identical to that used previously on rodents and is described in greater detail elsewhere (Dixon et al., 1987). Briefly, the device consisted of a Plexiglas cylinder reservoir 60 cm long and 4.5 cm in diameter. Fitted at the end of the metal housing is a 5-mm tube with a 2.6 mm inner diameter that is terminated with a male Leur-Loc fitting. This fitting connects to a female Leur-Loc fitting that has been implanted over the exposed dura of the rat (see *Surgical Preparation and Injury* for more details). The entire system was filled with distilled water. The injury was produced by a metal pendulum that strikes the piston of the injury device. This injury device injected a volume of water into the closed cranial

cavity and produced a brief displacement and deformation of brain tissue. The magnitude of injury was controlled by varying the height from which the pendulum was released. The extracranial pressure pulse is expressed in atmospheres.

Injury Model: Midline Fluid Percussion

Rationale for selection. While no single rat model of TBI incorporates all the features of human head injury, we have chosen to use the midline fluid-percussion model of TBI. Fluid percussion produces pressure transients to the brain similar to those recorded in human cadaver skulls during sudden impact (Lindgren and Rinder, 1966). Fluid percussion TBI has been demonstrated to produce suppression of behavioral responses and neurological signs in the rat resembling signs of unconsciousness in humans (Dixon et al., 1987). Comparable to mild and moderate head injury in humans, fluid percussion produces vestibular and motor deficits (Dixon et al., 1987). Importantly, fluid percussion in the rat produces cognitive deficits that may persist for weeks and even months after injury (Hamm et al., 1993a; Lyeth et al., 1990). This model exhibits minimal or no focal tissue damage at the site of impact, and no evidence of cell death in the hippocampus and other brain regions (Lyeth et al., 1990). This model of TBI produces non-ischemic reductions in blood flow (DeWitt et al., 1988). In summary, we believe that this model of TBI produces a reliable model of moderate diffuse brain injury and is well suited for the examination of our research questions.

Surgical Preparation and Injury

All animals were surgically prepared under gas anesthesia (4% isoflurane) in a mixture of 70% N_2O, and 30% O_2 24 hours before the fluid percussion injury or sham

injury. A 4.8 mm hole was trephined into the skull over the sagittal suture, midway between bregma and lambda. Two stainless steel screws were placed 1 mm rostral to bregma and 1 mm caudal to lambda. A modified Leur-Loc syringe hub with a 2.6 mm inside diameter was placed over the exposed dura and bonded in place with cyanoacrylate adhesive.

Twenty-four hours after the surgical preparation, rats were anesthetized with 4% isoflurane in a mixture of 70% N_2O, and 30% O_2 and connected to the injury device. Animals in the injured condition were injured at 2.1 - 2.2 atmospheres. This level of injury produces a moderate severity of injury that is associated with long-lasting cognitive deficits. Rats assigned to the sham-injury condition were anesthetized and connected to the injury device however no injury was delivered.

Outcome Assessment

The impairment of cognitive function is one of the most significant and enduring consequences of human TBI. The Morris water maze (MWM) procedure is a standard method for assessing cognitive function of rats (Brandeis et al., 1989) and has become a standard test procedure in TBI animal model research. It offers several advantages over other potential procedures that could be used. Previous research indicates that MWM is known to be sensitive to hippocampal damage (Morris et al., 1992; Olton et al., 1978), and the hippocampus has been shown to be especially vulnerable to TBI (Miyazaki et al., 1992). Also the MWM is a relatively simple procedure that rats learn rapidly. Another advantage included the fact that the procedure did not require food deprivation. In addition, our preliminary data demonstrated that the MWM task is sensitive to TBI-

induced cognitive dysfunction for at least 65 days (Hamm et al., 1991). We have also documented that the deficits observed on this task are not confounded by visual, motor, or other non-cognitive factors (Hamm et al., 1993a). We have chosen to examine MWM performance on Days 11-15 after injury for two reasons. First of all, the TBI-induced deficits are large at this time point which makes drug effects more apparent. And secondly, the data will be comparable to other data on pharmacological interventions we have collected at this post-injury time.

Morris Water Maze

The water maze procedure utilized a metal tank 180 cm in diameter and 60 cm in height painted white and filled with white water to a depth of 28 cm. The water temperature was maintained between 23^0 C and 26^0 C. A platform 10 cm in diameter and 26 cm high was placed 2 inches under the surface of the water and used as the hidden goal platform. The pool was located in a 2.5 x 2.5 m room with numerous extra-maze cues (shelves, pipes, curtain, furniture) that remained constant throughout the experiment.

Animals were given 4 trials per day for 5 consecutive days. On each trial, rats were placed in the pool by hand at one of the four start locations (marked south, west, north, and east). The animals were placed in the water facing the wall of the tank. Each animal started a trial once from each of the 4 possible start locations on each day. The order of starting locations was randomized each day. The goal platform was positioned 45 cm from the outside wall and in the center of the north quadrant.

Rats were given a maximum of 120 seconds to find the hidden platform. If the rat failed to find the platform after 2 minutes, it was placed on the platform by the

experimenter. The animals were allowed to remain on the platform for 30 sec before being placed in a heated incubator until the next trial approximately four minutes later. The animals' movements within the maze were recorded and analyzed with a video tracking system (San Diego Instruments, Polytrack 4). This tracking equipment allowed the analysis of latency to reach the goal platform, total distance, cumulative distance, which measured the sum of the path length from the rat to the platform every two seconds, and swim speed. Latency to find the platform was the primary dependent variable for the assessment of cognitive performance. Swim speed was also calculated to ensure that cognitive performance was not confounded by motor deficits that could impair swimming performance.

GluR2 Immunohistochemistry

Rationale for selection. The AMPA-glutamate receptor is comprised of four subunits, GluR1-GluR4. Previous research indicated a significant decrease in GluR2 immunoreactivity after neurological insult compared to sham injured animals(Pellegrini-Giampeitro et al., 1997). Thus, we examined trauma-induced changes in this major excitatory neurotransmitter system after the injury.

Procedure. Animals were fixed via cardiac perfusion under 60 mg/kg sodium pentobarbital with saline followed with a solution of 4% paraformaldyhide in 0.1 M phosphate buffer. Immediately following perfusion the brains were removed and placed in paraformaldehyde for 24 hours, and stored in phosphate buffer afterwards. After the tissue has been fixed, brains were sliced on a vibratome into sections 50 μm thick, and slices were collected through the mid-dorsal hippocampus. Tissue was washed in

phosphate buffer saline (PBS) threes times for five minutes, and blocked in endogenous peroxidase for 30 minutes to reduce background staining. After three additional five minute washes in PBS, slices were incubated in 10% normal serum with Triton X for 30 minutes. Following a quick rinse in PBS, sections then were incubated overnight at -4^0C in a 1:200 concentration of a mouse anti-GluR2 monoclonal antibody (Chemicon International, Temecula, CA USA, catalog number MAB397). Following a thorough wash in PBS, sections were incubated in diluted secondary antibody (Vector Laboratories, Burlingame, CA, USA, PK-6102) for one hour at room temperature, and then rinsed three times for 10 min in PBS. Tissue was then incubated in the Vectastain ABC kit for one hour at room temperature. DAB solution containing 2% β-D-glucose, 0.04% ammonium chloride and 0.005% glucose oxidase was applied to the tissue for ten minutes. Towards the end of the procedure 3% H_2O_2 was added to the tissue. To end the reaction sections were washed in 0.1 M PB (phosphate buffer) solution, mounted onto gelatin-coated slides, dehydrated and cover-slipped. Positive immuno-labeling was analyzed for qualitative results in the hippocampal regions using Imaging Research Inc. MCID System M4.

Drug Treatment: Aniracetam

Rationale for selection. Aniracetam has been found to be nootropic (cognitive enhancing) in a number of models of cognitive dysfunction. It has been shown to improve fear conditioning (Lu and Wehner, 1997), object recognition (Lebrun et al., 2000), and spatial learning after cortical lesions (Zajaczkowski and Danysz, 1997). With respect to moving a drug from preclinical to clinical testing, aniracetam has been used in

elderly patients with cerebrovascular disease (Endo et al., 1997) and Alzheimer's disease (Tsolaki et al., 2001) without significant adverse side effects. Thus, the beneficial effects observed in animal models and clinical trials make aniracetam a rational choice for testing it as a treatment for cognitive impairment after TBI.

Experiment 1

Optimum Dose Determination

Design. The following experiment examined what dose of aniracetam was the most effective in reducing the cognitive deficits associated with the consequences of human traumatic brain injury. We administered different doses of the drug, low (25 mg/kg), and high (50 mg/kg), as well as vehicle. Animals were randomly divided into injured and sham injured groups. There were ten animals in each of the groups: injured low dose aniracetam treated, injured high dose aniracetam treated, and injured saline treated. Administration of the drug began twenty four hours after the injury and continued for fifteen days. Rats were injected daily at the same time in the afternoon for the duration of the study. Morris water maze performance was assessed on days 11 – 15 after TBI, while the animals were still treated with aniracetam.

Analysis. Morris water maze data was analyzed by a 4 (Group) X 5 (Day) split-plot analysis of variance (ANOVA). Following the ANOVA, the Duncan Multiple Range post-ANOVA tests were performed to examine pair-wise group differences.

Experiment 2

Delayed Chronic Administration

Investigating the delay of treatment is of great importance. Delaying chronic administration is clinically relevant as the cognitive deficits after TBI may not be apparent immediately after the injury. This experiment explored the efficacy of aniracetam in its ability to attenuate spatial memory deficits when chronic administration of the compound is delayed for 11 days after TBI. This experiment will help determine an optimum therapeutic intervention strategy that may help patients overcome cognitive disabilities that may have life-long consequences.

Design. Animals were treated with vehicle or with the optimal dose of aniracetam, determined in previous experiment. Immediately after the injury animals did not receive any treatment on days 1 through 11 after the trauma. Beginning day 11 post injury, animals were treated with aniracetam daily at the same time in the afternoon until the remainder of the experiment. Rats remained living in their cages in the animal room and were weighed daily. After fifteen days of treatment with aniracetam or vehicle animals were tested in the MWM for their spatial memory performance on days 26-30 after TBI.

Analysis. MWM data was analyzed by a 3 (Group) X 5 (Day) split-plot analysis of variance (ANOVA). Following the ANOVA, the Duncan Multiple Range post-ANOVA test was performed to examine pair-wise group differences.

Experiment 3

Termination of Chronic Administration

The present experiment investigated whether the continued administration of aniracetam is necessary for the enhancement of performance on the spatial memory test in the Morris water maze after TBI. In other words, this part of the study attempted to clarify if chronic administration of aniracetam normalized receptor function in such way that continued treatment was not necessary; or is the beneficial effect of aniracetam more pharmacological in nature so that the drug must be present in order to improve cognitive performance of injured rats? Thus, this part of the experiment was set to determine whether the compound must be biologically active in the organism during the time of testing in order to provide cognitive enhancement.

Design. Animals were treated with vehicle or an optimal dose of aniracetam, determined by the second experiment. Administration of the drug or vehicle began 24 hours after the injury, and continued daily for 15 days. Aniracetam treatment was then terminated, and spatial memory performance was assessed on days 20-24 while aniracetam was not administered to the animals during the testing days in the water maze.

Analysis: MWM data was analyzed by a 3 (Group) X 5 (Day) split-plot analysis of variance (ANOVA). Following the ANOVA, the Duncan Multiple Range test was performed to examine pair-wise group differences.

Experiment 4

Effect of Aniracetam on GluR2 Subunit Expression Following TBI

Previous research indicated that numerous indexes of neuronal function are altered after TBI. For example, a time-dependent decrease in ChAT immunoreactivity (IR) has been observed following TBI. The present experiment addressed whether chronic treatment with aniracetam that is behaviorally beneficial would also normalize GluR2 subunit expression in injured animals. This experiment is important in providing essential data on the mechanisms by which aniracetam improves outcome following brain injury.

Design. The optimal dose and time of administration that improved MWM performance was examined in the previous experiments. Following the optimal treatment, immunohistochemical procedures were conducted to evaluate GluR2 immunoreactivity. For a control group ten sham injured animals were treated with vehicle the same way the injured animals were treated with aniracetam (n=10) or vehicle (n=10) receiving daily injections for fifteen days beginning 24 hours after the injury.

Analysis. Immunohistochemical data was analyzed for gross observations. Although no quantification was performed, qualitative examination was completed in all groups.

Experiment 5

Effect of Aniracetam on GluR2 Protein Expression Following TBI

Design. Animals were injured and sham injured as in previous experiment. Following the optimum treatment dose rats were administered aniracetam or vehicle once

a day for fifteen days beginning 24 hours post injury. On the fifteenth day, animals were sacrificed and brain tissue collected for the Western blot analysis. Two naive animals were used for controls.

Tissue Preparation and Western Blot Analysis. Animals were anesthetized with 4% isofluorane and rapidly decapitated. Brains were removed on ice-cooled glass plates, and bilateral hippocampi were dissected. The hippocampi were weighed and homogenized in a buffer containing 3ml RIPA lysis buffer (US Biological), 0.2% SDS and 4% complete cocktail protease inhibitor (Roche Molecular Biochemicals, catalog number 1-697-498) per gram of tissue. Following homogenization, bilateral hippocampi were centrifuged at 15 000 g for 15 minutes at - 4° C. Supernatant was removed and stored at -80°C.

A Bio-Rad micro assay was used to determine the amount of protein in each sample by using a standard regression equation to calculate the protein quantity from the spectrophotometer readings of optical density. Each sample lane of gel contained a standard amount of sample buffer (6.3 μl) and the reducing agent (2.5 μl). The amount of water added was adjusted to guarantee equal quantities of protein loaded in each lane. Samples were heated for ten minutes at 70° C before transfer to a nitrocellulose membrane (Invitrogen; 90 minutes at 30 V). Following transfer, the gel was stained with Coomassie Blue to ensure complete transfer to the membrane. The membranes were then blocked in 0.5% non-fat dry milk and 0.1 % Tween 20 in phosphate-buffer saline for 1 hour at room temperature. Samples were incubated in 1μg/ml primary antibody (Chemicon International, Temecula, CA, USA, catalog number MAB 397) at 4° C

overnight. Negative control included a lane that received the same treatments except the primary antibody was omitted. The membrane was then rinsed six times for five minutes per rinse in PBST - 0.5 % milk. Membrane was then incubated in PBST - 0.5 % milk with horseradish peroxidase-conjugated goat anti-mouse (lot 11969, Rockland catalog number 610-103-121) secondary antibody (1:5000) for 1 hour at room temperature. Samples were then put through 4 five minute washes in PBST - 0.5 % milk and 2 five minute washes in PBST. Protein bands were visualized on a Kodak (Rochester, NY, USA) XAR-5 film with enhanced chemiluminescence. After developing the blot, it was drained and exposed to BioMax x-ray film for visualization. Exposure times ranging from a 1 to 60 seconds were analyzed to receive a clean image. Densitometry Scion Image for Windows (Scion Corporation, Frederick, MD, USA) was used to calculate the optical density of the bands. All assays were carried out under settings where densitometry signal intensity was linear with protein concentration as was calculated by the previous experiments.

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Figure2. A picture of the fluid percussion injury device (FPI)
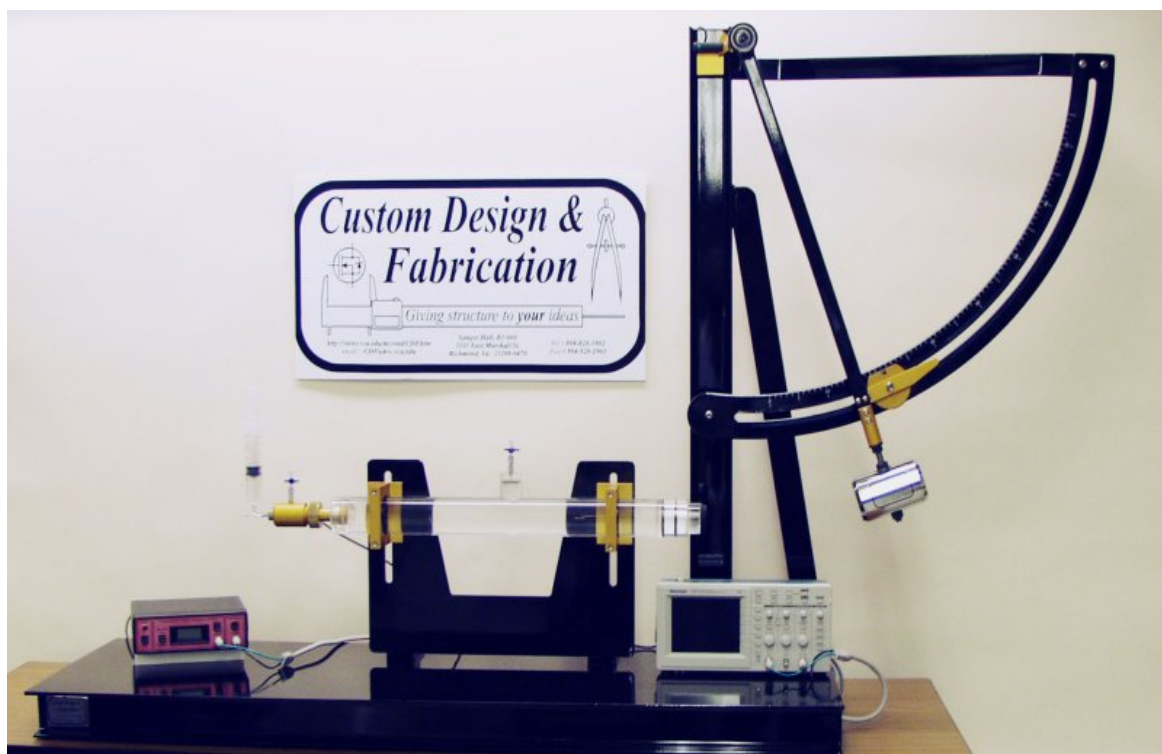
Figure3. A cartoon of the Morris Water Maze.
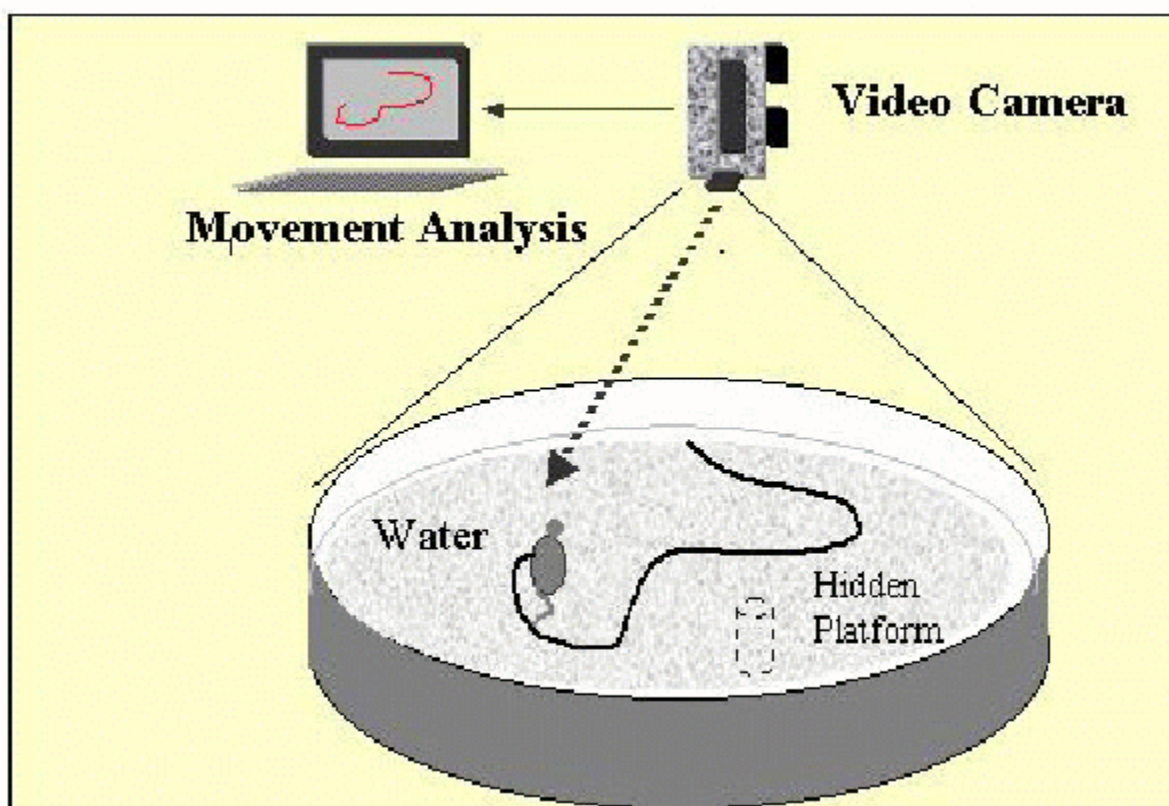
RESULTS

Experiment 1
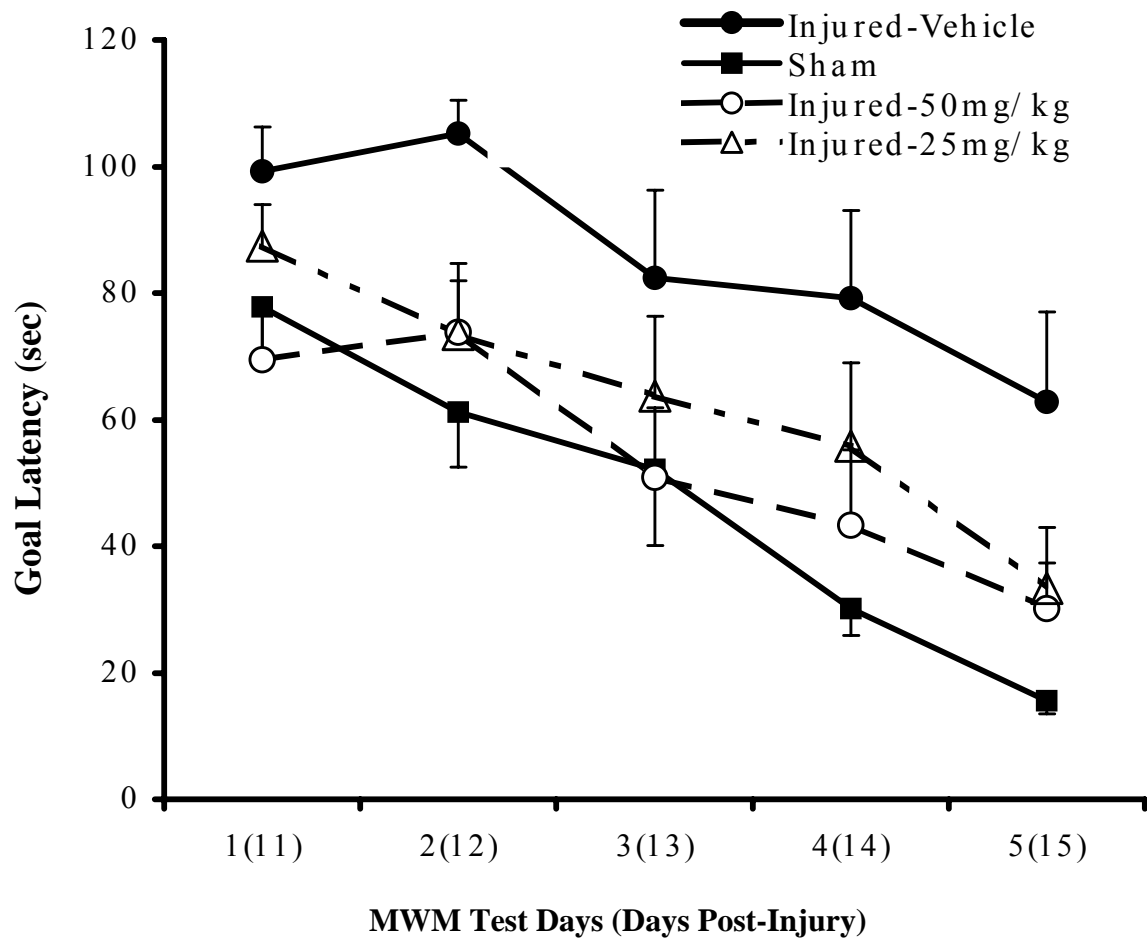
Optimum Dose Determination

The first experiment attempted to determine the optimum dose of aniracetam that would be most effective in reducing chronic cognitive deficits after traumatic brain injury. Aniracetam was orally administered to two groups of rats at a volume of 5ml/kg once a day for fifteen days. Based on previous research two doses of the drug were selected for this experiment - 25 mg/kg, 50 mg/kg as well as vehicle for the control group of animals.

Results. The results from the Morris water maze testing were analyzed by a 4 (Group) X 5 (Day) split-plot analysis of variance (ANOVA). The results indicated that there was a significant main effect of Group ($F_{3,33} = 5.29$, $p < 0.004$). In order to determine the specific group differences, the Duncan Multiple Range test was used. The results indicated that injured animals treated with 25 mg/kg and 50 mg/kg of aniracetam demonstrated significantly improved MWM performance when compared to injured animals treated with vehicle ($p < .05$). Also, MWM results in both groups of injured animals treated with aniracetam (25 mg/kg and 50 mg/kg) did not differ significantly from sham injured animals. The analysis of swim speed found no differences between the groups indicating there were no motor deficit effects from the injury. Animals treated with 50 mg/kg of aniracetam indicated a slight improvement in the Morris water test over

the animals treated with 25 mg/kg, and therefore the higher dose was chosen as an

optimum dose and was selected for the remainder of the experiments.

Figure 4. Mean latency to reach the goal platform on days 11 – 15 following TBI. A 4

(Group) X 5 (Day) split-plot analysis of variance (ANOVA) revealed that injured animals

treated with 25 mg/kg and 50 mg/kg of aniracetam demonstrated significantly shorter

latencies to reach the platform when compared to injured animals treated with vehicle (p

< .05). Both injured treated groups (25 and 50 mg/kg) did not differ significantly from

sham injured animals.

MWM Latencies

Figure shows Goal Latency (sec) on the y-axis (0 to 120) versus MWM Test Days (Days Post-Injury) on the x-axis: 1(11), 2(12), 3(13), 4(14), 5(15).

Legend:
- Injured-Vehicle (filled circles)
- Sham (filled squares)
- Injured-50mg/kg (open circles)
- Injured-25mg/kg (open triangles)

Experiment 2

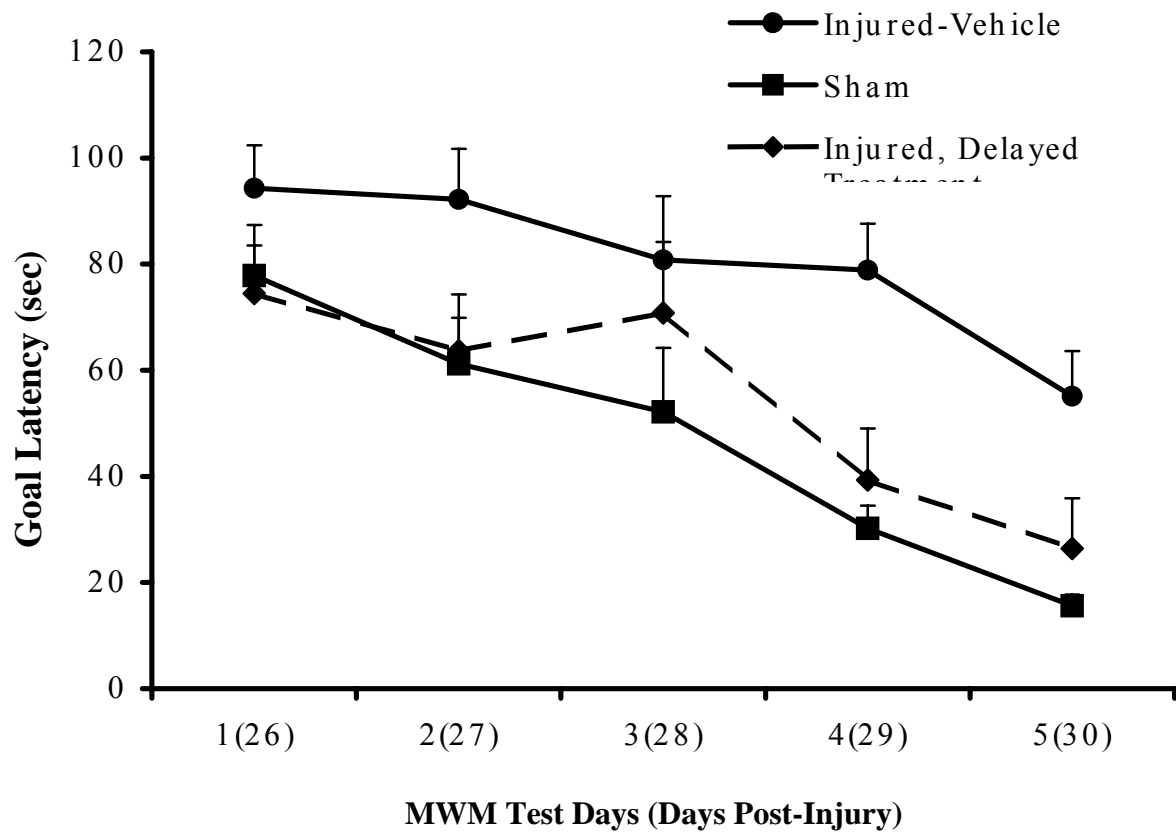*Delayed Chronic Administration*

The aim of the second experiment was to investigate the optimum therapeutic strategy by examining the temporal therapeutic window for the posttraumatic pharmacological treatment of cognitive impairment after traumatic brain injury. In the present investigation the efficacy of aniracetam in attenuating cognitive deficits was tested when chronic administration of the compound was delayed for 11 days after traumatic brain injury. Animals were treated once daily with 50 mg/kg of aniracetam or vehicle beginning 11 days after the injury. After fifteen days of treatment, animals were tested in the Morris water maze for five days, during which they continued to receive aniracetam or vehicle treatment.

Results. The results from the spatial memory task were analyzed by a 3 (Group) X 5 (Day) split-plot analysis of variance (ANOVA). The analysis for the results indicated that there was a main effect of Group variable ($F_{2,25} = 4.96$, $p < .015$). To establish the specific group differences, the Duncan Multiple Range test was used. Its results indicated that injured animals chronically treated with 50 mg/kg of aniracetam beginning 11 days post injury performed significantly better ($p < .05$) on the MWM task compared to injured animals treated with vehicle. The MWM performance of injured animals with delayed aniracetam treatment did not significantly differ from the sham injured animals.

Again, swim speed analysis indicated no differences between injured and sham groups of

animals.

Figure 5. Mean latency to reach the goal platform on days 26-30 with delayed aniracetam treatment following TBI. A 3 (Group) X 5 (Day) split-plot analysis of variance (ANOVA) revealed that injured animals treated with 50 mg/kg of aniracetam demonstrated significantly shorter latencies to reach the platform when compared to injured animals treated with vehicle ($p < .05$). The goal latencies of injured animals with delayed aniracetam treatment did not significantly differ from the latencies of the sham injured animals.

MWM Latencies

Figure showing MWM Latencies with Goal Latency (sec) on the y-axis (0 to 120) and MWM Test Days (Days Post-Injury) on the x-axis: 1(26), 2(27), 3(28), 4(29), 5(30). Legend: Injured-Vehicle (circles), Sham (squares), Injured, Delayed Treatment (diamonds).

Experiment 3: Termination of Chronic Administration

The third behavioral experiment intended to examine whether the continued administration of aniracetam is necessary for the improved cognitive performance after traumatic brain injury. The present investigation attempted to discover if the chronic administration of aniracetam alters receptor function in a way that continued treatment is not necessary during the cognitive testing phase or if aniracetam must be present in order to improve spatial memory performance after the injury. In other words, the experiment examined whether aniracetam must be biologically active in the organism during the time of testing in order to provide its beneficial effects and improve cognitive performance. Animals were treated once daily with 50 mg/kg of aniracetam or vehicle beginning 24 hours post injury for fifteen days. Animals were tested on the Morris water maze spatial memory task on days 16 through 20, during which rats did not receive aniracetam or vehicle treatment.

Results. The MWM results were analyzed by a 3 (Group) X 5 (Day) split-plot analysis of variance (ANOVA). The ANOVA indicated that there was a significant effect of Group variable ($F_{2, 24} = 3.33$, $p < .05$). To determine the specific group differences, the Duncan Multiple Range test was applied. Its results indicated that injured animals treated with vehicle demonstrated significantly impaired MWM performance ($p < .05$) compared to sham injured animals. The injured animals who were treated with 50 mg/kg of aniracetam chronically prior but not during the MWM testing, did not differ significantly from the injured animals treated with vehicle. Swim speed analysis indicated no significant differences between groups.

Figure 6. Mean latencies to reach the goal platform on days 16-20 when aniracetam

treatment was terminated before the MWM testing after TBI. A 3 (Group) X 5 (Day)

split-plot analysis of variance (ANOVA) revealed that injured animals treated with

vehicle demonstrated significantly longer goal latencies ($p < .05$) compared to sham

injured animals. Injured animals treated with 50 mg/kg of aniracetam chronically prior

but not during the MWM testing, did not differ significantly from the injured animals

treated with vehicle.

MWM Latencies
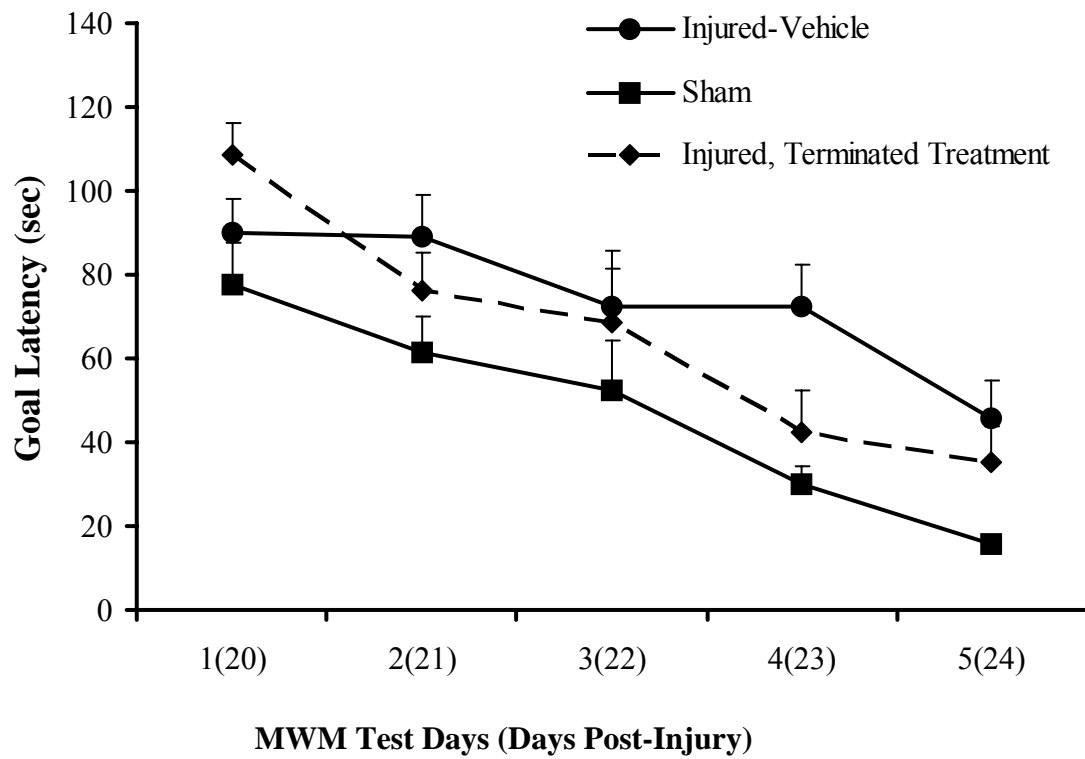
*Experiment 4: Effects of Aniracetam on GluR2 Subunit Expression Following TBI*

Present experiment examined the effects of aniracetam on the expression of GluR2 subunit after traumatic brain injury. The AMPA-glutamate receptors are ligand-gated fast-acting channels that contribute to the neuron's ability to fire rapidly by allowing Na^+ to enter the cell, and therefore decreasing the time necessary to reach threshold values for an action potential. The AMPA-glutamate receptor is comprised of four subunits (GluR1-4). Changes in the GluR2 (Ca^{++}- impermeable AMPA receptors) have been observed following other types of neurological disorders (Pellegrini-Giampieto et al., 1997). The observed alteration in the GluR2 subunit of the AMPA-glutamate receptor may be a compensation or reaction to diaschisis/RFD that follows TBI. In addition, this experiment addressed whether chronic treatment with aniracetam that is behaviorally beneficial (as observed in Experiments 1-2 above) will also alter GluR2 subunit expression in injured animals.

The same procedure used in Experiment 1 was repeated. Injured rats were treated with 50 mg/kg of aniracetam (n = 10) or vehicle (n = 10) beginning 24 hrs after injury daily for fifteen days. Sham -injured (n = 5) rats received vehicle beginning 24 hrs after injury daily for fifteen days. On Day 16, brains were removed and prepared for GluR2 immunohistochemistry as described in general methodology. The immunohistochemical analysis is essential in visualizing alterations of the GluR2 subunit following the injury and the aniracetam or vehicle treatment. Regional anatomical differences of the receptor expression were observed with this technique. This experiment provided important data on the mechanisms by which aniracetam improved outcome following brain injury

Results. A qualitative examination revealed that injured treated animals exhibited a less intense cellular and synaptic immunoreactivity especially in the CA1 region of the hipppocampus when compared to both sham animals and injured vehicle treated animals. While the injured vehicle treated tissue samples appeared to express slightly reduced amounts of protein when compared to the sham injured animals, those differences did not appear to be of significance. The highest density of immunoreactivity was observed in the molecular layer as well as the apical dendrites in the CA1 region of the hippocampus. At fifteen day post trauma, experimental brain injury appeared to have a slight reduction in the GluR2 staining, however that receptor subunit alterations did not appear to be susbstantial. To support these findings in the immunohistochemistry experiments, a western blot analysis was chosen to examine the GluR2 protein expression in the whole hippocampus of sham injured, injured aniracetam-treated and injured vehicle-treated groups.
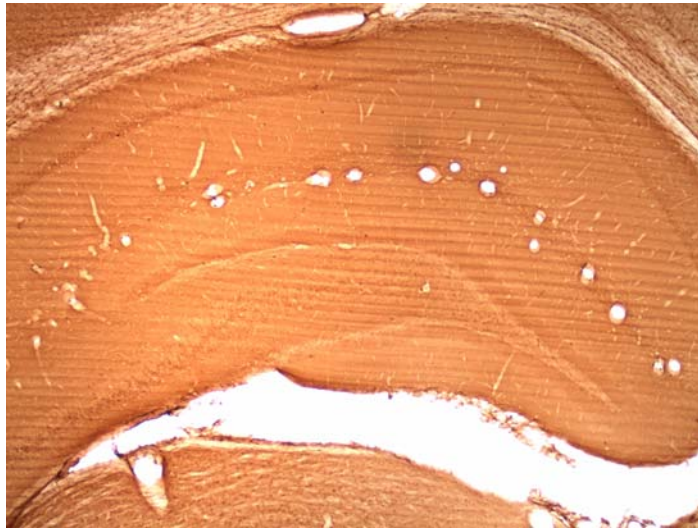
Figure7. Micrograph of immunohistochemistry for GluR2 in the hippocampus (4X).

Positive staining can be seen outlining neuron cell bodies as well as in the apical

dendrites. Sham vehicle subjects appear to have stronger positive labeling of GluR2

immunoreactivity compared to injured saline and aniracetam treated subjects.
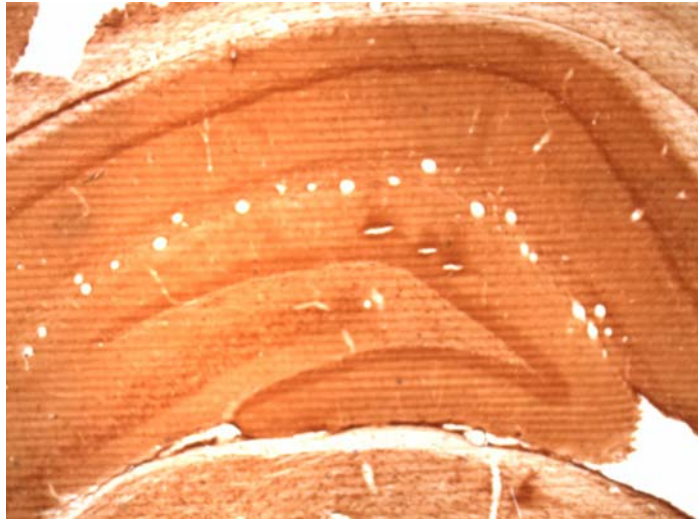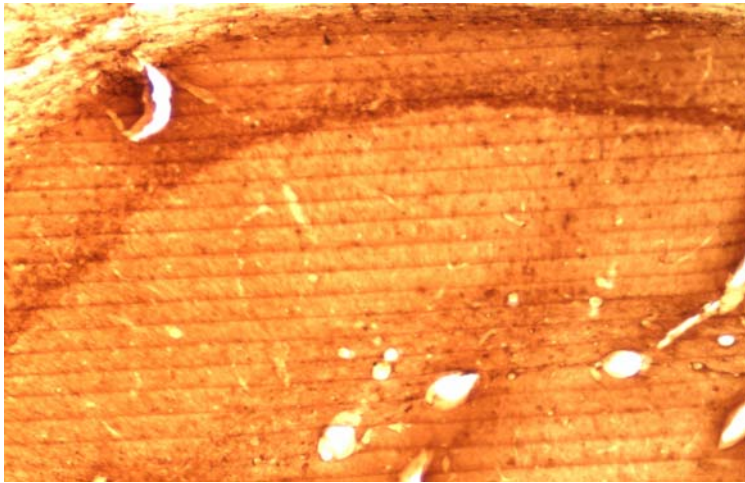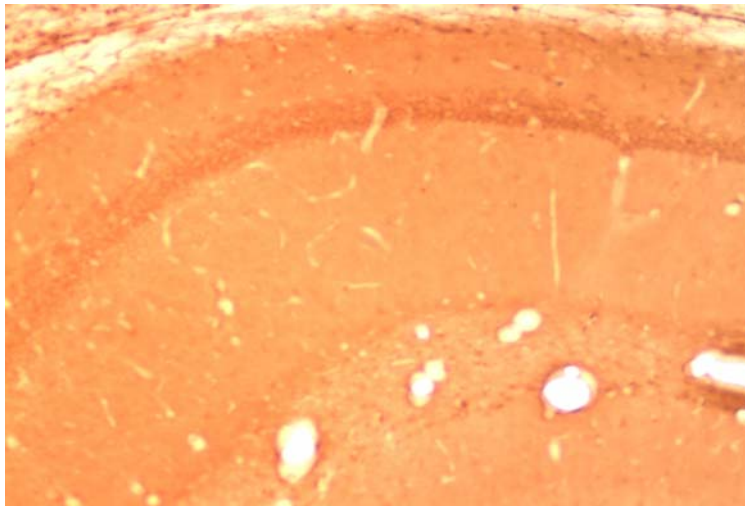
Sham saline 4X
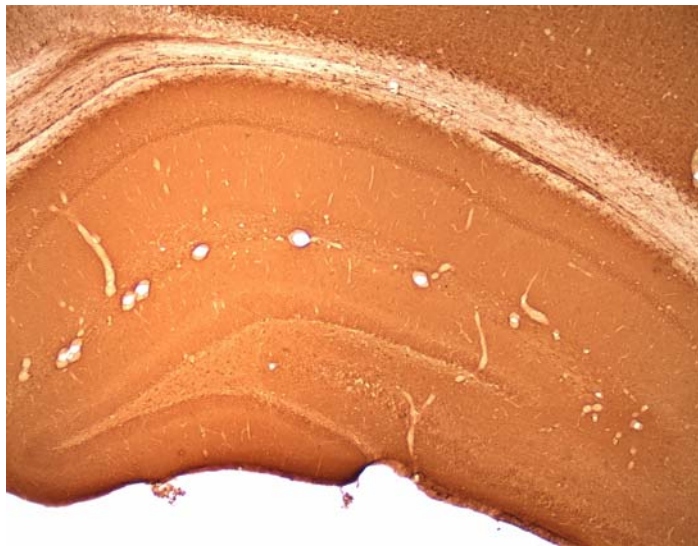
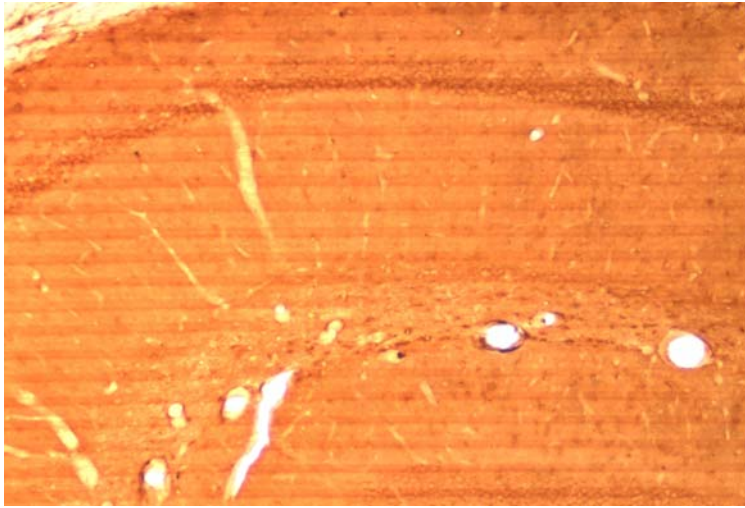Injured saline treated 4X

Injured aniracetam treated 4X

Figure 8. Micrograph of immunohistochemistry for GluR2 in the hippocampus (10X).

Injury induced reduction in the GluR2 immunoreactivity can be seen in the injured saline

and aniracetam treated cases compared to sham saline ones.

Sham saline 10X
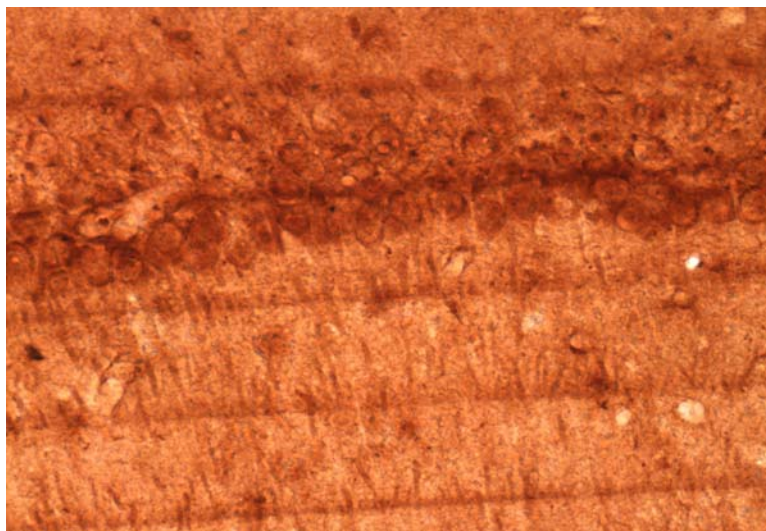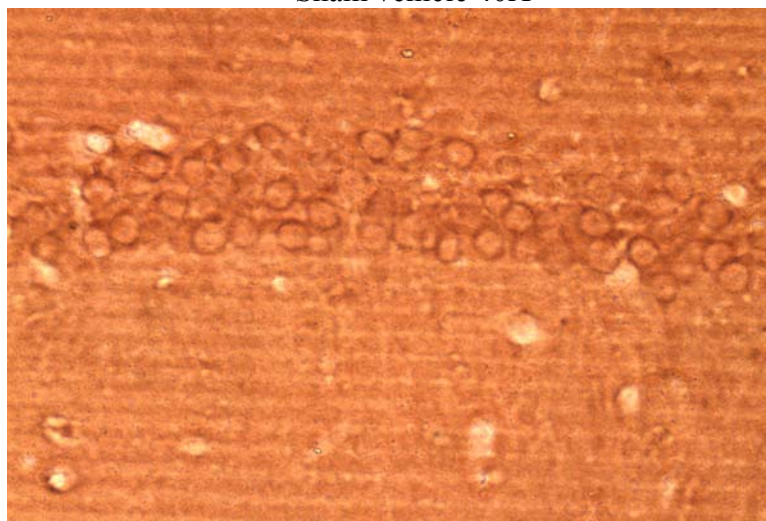

Injured saline treated 10X

Injured aniracetam treated 10X

Figure 9. A higher magnification (40X) micrograph of immunohistochemistry for GluR2 in the hippocampus. Injury induced reduction in the GluR2 immunoreactivity can be seen in the injured saline and aniracetam treated cases compared to sham saline ones.
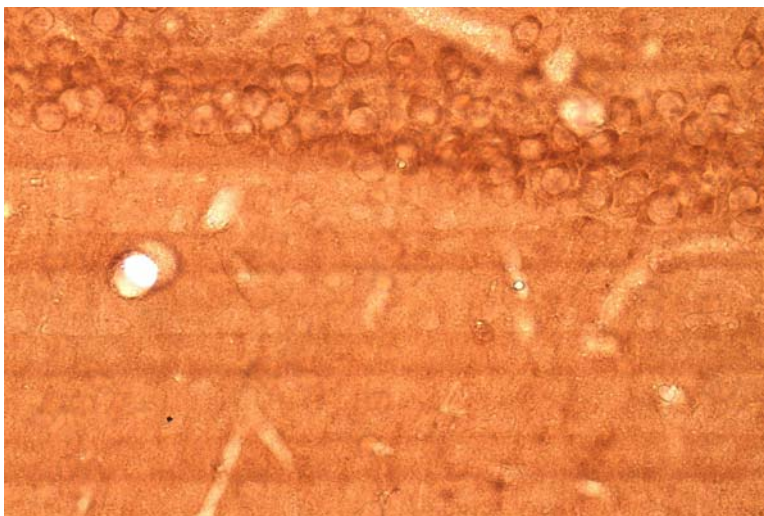
Sham vehicle 40X

Injured saline treated 40X

Injured aniracetam treated 40X

Experiment 5: Western Blot Analysis

Present investigation examined the effects of aniracetam on GluR2 expression in the hippocampus using a Western blot analysis following traumatic brain injury. Procedure similar to the one in optimal dose determination experiment was repeated. Animals were injured (n=6) and twenty four hours later were treated with 50 mg/kg aniracetam (n=3) or vehicle (n=3) once daily for fifteen days. For the control group, two naïve animals were used for Western blot analysis. On the fifteenth day of treatment, all animals were sacrificed and brains processed for Western blots analysis as described in detail in the methods section.

Results. Mouse anti-GluR2 monoclonal antibody corresponded to an approximately 100-kDa protein. The other reactive proteins with approximately 70-kDa molecular weight were assumed to be breakdown products of GluR2, and support previous data (Vissavajjhala, et al., 1996). A one-way analysis of variance (ANOVA) indicated no statistical differences between the groups (p > .05) indicating that the amount of protein expression was not significantly different between injured and naïve groups as well as between injured aniracetam-treated and injured vehicle treated. It is worth noting that injured aniracetam-treated group did have the lowest protein expression count, however that difference was not large enough to reach statistical significance.

Figure 10. Reactive bands in Western blotting for GluR2 protein in hippocampi of rats

(N – naïve, IT-injured treated, I-injured untreated).
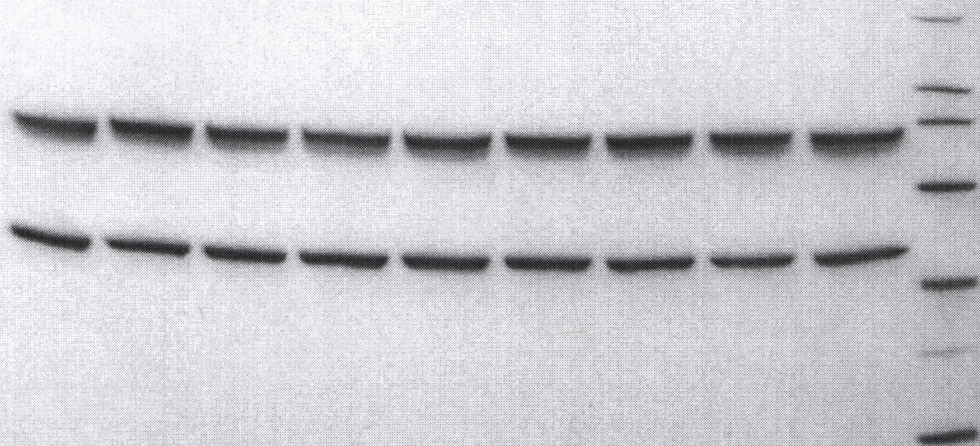
Figure 11. Quantification of GluR2 Western blot. The relative optical density of the entire top band and the area of the top band were measured with Scion computerized image analysis. The data represent the mean of injured aniracetam-treated (n=3) and injured vehicle treated (n=3) at 15 days post injury in comparison to the naïve animals. Immunoreactive optical density was reduced by 28 % in the injured aniracetam-treated group and by 11.3 % in the injured saline-treated group compared to naïve samples.

GluR2 protein expression
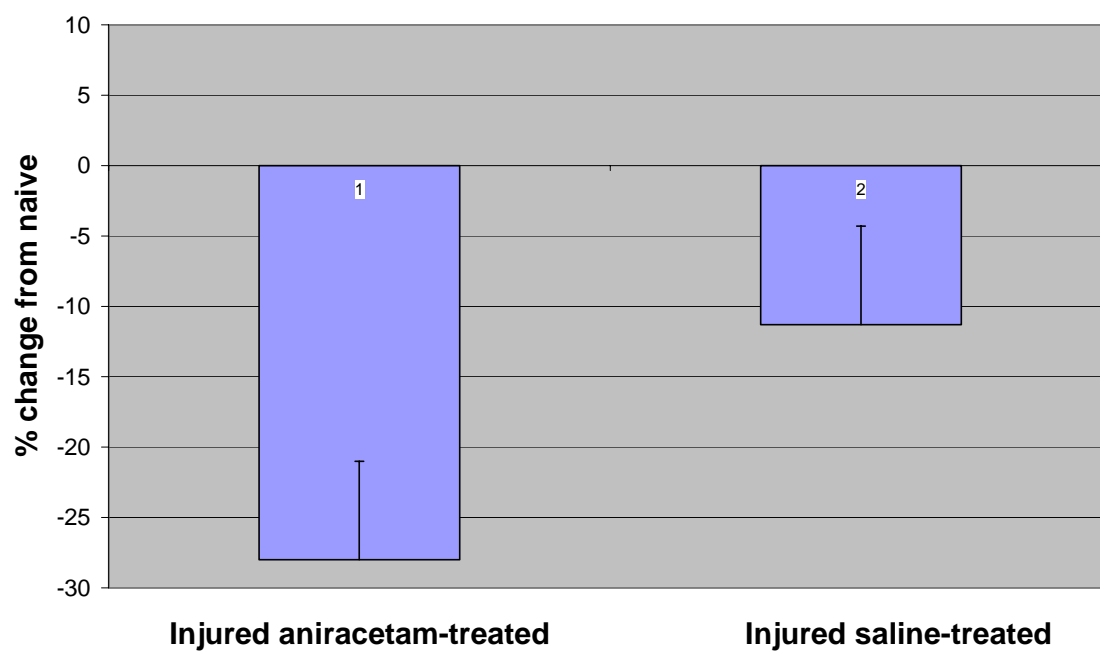
DISCUSSION

In the present study we examined the effects of the novel compound aniracetam on spatial memory deficits induced by experimental fluid percussion injury in rats. This research was driven by the idea of finding a new pharmacological treatment that would alleviate cognitive deficits associated with the aftereffects of TBI. While there are a number of experimental treatment options that focus on acute processes after TBI, fewer experiments have offered treatment alternatives during the chronic hypofunctional post injury phase. It is extremely important to understand the physiological alterations that occur immediately after the injury and all the possible acute therapy options beneficial in that stage. However, it is not always possible to deliver the acute therapeutic interventions to the TBI patient, and therefore the acute window treatment may be missed. In addition, new pathology may develop through secondary injury that was not initially present following trauma. Therefore, it is extremely important to investigate possible treatment options that can be advantageous to TBI victims when administered during the chronic hypofunctional posttraumatic phase.

This study was especially important as it looked into the therapeutic potential of this compound over a period of time following the injury. Aniracetam's beneficial effects were investigated when the administration of the drug was delayed and when it was terminated prior to cognitive assessment. Such paradigms are extremely important as they offer crucial information that could be used in cases where cognitive problems may not become apparent immediately following trauma. A longer therapeutic window, especially in the chronic post injury phase, offers an opportunity to introduce the compound when

the acute treatment window was missed. After investigating the functional outcome of aniracetam, the mechanism of action of the compound became the next issue of research. Using immunohistochemistry and Western blot analysis, we attempted to investigate how aniracetam enhanced behavioral outcome and whether its mechanism of action occurred through an alteration in AMPA receptor.

Even though, there are fewer studies investigating chronic phase of TBI compared to the acute intervention studies, it is apparent that chronic therapeutic treatments should attempt to elevate neuronal activity (Feeney, 1991). The results of the present experiments demonstrated the efficacy of using a positive modulator of AMPA receptor function in the hypofunctional stage as chronic treatment for the cognitive impairment produced by TBI. The results of Experiment 1 demonstrated that aniracetam in doses of 25 or 50 mg/kg was effective in reducing the cognitive deficits produced by TBI. Results indicated that aniracetam was beneficial when treatment was initiated 24 hrs after TBI and was continued for 15 days during the cognitive testing. Experiment 2 found that aniracetam was still effective in attenuating trauma-induced cognitive impairment even after the treatment was delayed for 11 days following injury. In fact, comparing the results for efficacy of aniracetam when it was administered 24 hours after the injury in the first experiment, or when it was delayed for 11 days after the injury in the second experiment, aniracetam appeared to be equally successful in reducing trauma-induced cognitive deficits. Such a long posttraumatic therapeutic window for aniracetam is rather important as it presents an opportunity to alleviate cognitive deficits in cases where such deficits are not apparent immediately after the trauma. A number of post-traumatic

pharmacological interventions that have shown to be beneficial in alleviating cognitive difficulties had to be administered within 24 hours of TBI (Temple and Hamm, 1996; O'Dell and Hamm, 1995; Pike and Hamm, 1995). In fact, two experiments that have explicitly tested the effectiveness of delayed treatments that were initiated 24 hours post-injury have found that compounds that were effective when administered beginning 24 hours after injury were no longer effective if treatment was initiated 11 days after the injury (O'Dell and Hamm, 1995; Pike and Hamm, 1995). In fact, aniracetam is the only compound known today that is still effective when treatment initiation is delayed for several days after the onset of traumatic brain injury. Thus, the observation of aniracetam being efficacious even when it was delayed for 11 days is quite a significant finding. Such results make aniracetam a potential candidate for a study in a clinical setting.

Previous studies in the field of chronic posttraumatic therapy influenced the idea and the design of the present study. Previous experiments investigated the effects of a chronic treatment on the behavior and correlated functional outcome to anatomical indexes. For example, Pike and Hamm (1995) observed significant improvement in rats on the MWM test after chronic administration of LU-25-109-T beginning 24 hours after the injury and continuing for fifteen days. Researchers were able to correlate the behavioral outcome to alteration in the ChAT immunoreactivity. Results indicated that Lu-26-109-T significantly attenuated injury-induced reductions in ChAT-IR. Researchers concluded that behavioral improvement was due to the increase in the cholinergic tone induced by the chronic administration of Lu-25-109-T.

Another study investigated the effects of CDP-choline treatment on MWM performance and acetylcholine release following lateral control cortical impact injury (Dixon et al., 1997). Morris water maze performance was significantly improved in rats who received CDP-choline 1 day after the injury for 18 days compared to saline treated rats. The microdialysis studies demonstrated that CDP-choline significantly increased extracellular levels of Ach. In conclusion, researchers reported that chronic treatment enhancing Ach release significantly improved spatial memory performance. In this study behavioral improvement was correlated to the increased Ach release that was induced by the chronic treatment of CDP-choline. Therefore, based on previous studies, we decided to investigate whether chronic aniracetam treatment had an effect on MWM behavior and whether that functional improvement correlated to AMPA receptor changes.

Since the results of Experiment 1 indicated that administration of aniracetam beginning 24 hours after the injury and continuing during MWM testing resulted in improved spatial memory task performance of injured rats, the next step was to examine whether terminating the treatment prior to MWM testing would yield similar results. Therefore, in the third experiment aniracetam was initiated 24 hours after TBI and continued for fifteen days, but drug treatment was terminated on days 16 through 20 prior to MWM testing. The outcome of that experiment showed that injured aniracetam-treated animals did not perform significantly better than injured vehicle-treated rats.

Although few experiments have tested the effects of terminating pharmacological treatment, one previous study provided data relevant to the consequences of the termination of an efficacious drug intervention. Zhu et al. (2000) examined the effect of

the dopamine enhancer l-deprenyl on cognitive function and neuroplasticity following TBI. The drug was administered starting 24 hours after the injury and was terminated after 7 days. When rats were tested in the MWM four days after drug termination, injured l-Deprenyl treated animals performed significantly better on a spatial memory task compared to untreated injured rats. After the beneficial behavioral component was established, researchers investigated changes in the dopamine β-hydroxylase immunoreactivity (DBH-IR) and acetylcholinesterase (AChE) histochemistry following the chronic treatment. Results indicated that l-Deprenyl treatment also attenuated the trauma-induced loss in dopamine β-hydroxylase immunoreactivity and acetylcholinesterase histochemistry staining. The authors concluded that dopaminergic enhancement induced by l-deprenyl administration facilitated cognitive recovery following brain trauma. The results also suggested that dopaminergic/noradrenergic enhancement correlated with enhanced synaptic plasticity in the injured hippocampus. Therefore, MWM behavior and immunohistochemistry results imply that l-Deprenyl produced its beneficial cognitive effects via enhanced post-injury plasticity rather than the direct receptor stimulation at the time of MWM testing, since the benefits of the drug were apparent days after its administration was discontinued.

The present experiment examined the possibility that the therapeutic effect of aniracetam was due to the aniracetam-enhanced remodeling of the neural network following trauma, similar to that observed by Zhu et al. (2000). Previous studies indicate that there is some evidence that aniracetam has an effect on neuroplasticity. For example, Fushiki et al. (1995) found that aniracetam stimulated neurite extensions in cultured

granule neurons. Therefore, one could assume that the beneficial effects of aniracetam on cognitive recovery may be mediated by a drug-induced neuroplasticity effect. However, the results of the third behavioral experiment argue against such an effect of aniracetam. In contrast to the outcome reported with chronic and terminated administration of l-deprenyl, the results of the third aniracetam behavioral experiment indicated that the termination of chronic aniracetam prior to cognitive testing resulted in aniracetam being ineffective in improving the cognitive performance of injured animals. These results suggest that aniracetam's efficacy is the consequence of its pharmacologically mediated effects at the time of cognitive testing.

Aniracetam's beneficial effects on cognitive function could be mediated via multiple mechanisms of action. In addition to aniracetam's well-documented effect on AMPA receptor desensitization, aniracetam has been shown to enhance long-term potentiation (LTP, Satoh et al., 1986). Another effect of aniracetam is through its enhancement of glucose availability and ACh synthesis in the brain. Ouchi et al. (1999) found that the administration of aniracetam following lesioning of the basal forebrain prevented the reduction in glucose metabolism typically observed after the lesion. TBI is also known to result in a period of depressed glucose metabolism following injury (Yoshino et al., 1991). Thus, aniracetam may improve behavioral function by increasing post-injury glucose metabolism.

Aniracetam has also been found to positively modulate several neurotransmitter systems, and as reviewed previously, research indicated that a number of receptor systems are hypofunctional chronically after TBI. There is increasing evidence that

aniracetam has a positive interaction with central glutamatergic systems, potentiating endogenous glutamate release (Togashi et al., 2002). Aniracetam also enhances ACh release (Nakamura and Shirane, 1999) by means of aniracetam-elicited glutamate stimulating group II metabotropic glutamate receptors (Togashi et al., 1996). In addition, administration of aniracetam to stroke-prone spontaneously hypertensive rats (SHRSP) enhanced both DA and 5-HT release, ameliorating dopaminergic hypofunction observed in SHRSP rats. These results suggest that a glutamatergic mechanism may underlie aniracetam's effect on other neurotransmitters, including ACh, DA, and 5-HT. Thus, in addition to aniracetam's effect as an inhibitor of AMPA receptor desensitization, it stimulates the release of a number of other neurotransmitters.

In conclusion, if the behavioral deficits observed chronically after TBI are the result of the depression of normal neuronal activity, as proposed by the theory of diaschisis/RFD (Feeney, 1991), efficacious post-injury interventions would enhance neuronal activity. The results of the present study did enhance neurotransmission as indicated by the improved cognitive function following TBI. The data reviewed above indicated that aniracetam positively modulated excitatory neurotransmission through multiple mechanisms, and the activation of neuronal function may underlie the beneficial effects that aniracetam has on cognitive recovery after TBI. It is difficult to pinpoint the exact mechanism of action of aniracetam. Evidence from the present study indicates that most likely aniracetam does not improve behavior through its influence on the GluR2 subunit of the AMPA receptor. Other mechanisms of actions by which aniracetam has an effect on cognitive functioning in rats post TBI should be investigated in the future.

Future Directions. Although the data presented in this study are very convincing, more experiments remain to be completed. Most importantly, aniracetam should be brought into a clinical TBI study. Aniracetam already has been tested in the clinical settings in patients with dementia and Alzheimer's-like syndrome, but not in patients with brain trauma. However, before clinical trials can begin, details about aniracetam's mechanism of action should be resolved. The effects of aniracetam on glucose metabolism should be explored with greater detail. Ouchi et al (1999) measured cerebral glucose levels using positron emission tomography (PET) and discovered that aniracetam prevented the reduction in glucose metabolism after the brain lesion. An experiment investigating the role on aniracetam on glucose metabolism in its connection to enhancement of neuronal activity and spatial memory performance should be conducted.

Once researchers uncover the mechanism of aniracetam effects, a study focusing on enhancing the effects of the drug could be designed. Another study where aniracetam treatment would be delayed longer than 11 days is crucial for cases where cognitive dysfunction is not apparent for an extended period of time. Such a temporal therapeutic window could permit many TBI victims an enhancement in cognitive problems induced by TBI where such cognitive deficits were not recognized at once. Since most of the initial treatment post TBI focuses on managing primary injuries such as blood loss, hematomas, blood pressure and intracranial pressure alterations, treatment of cognitive deficits usually falls to the rehabilitation stage. After the acute phase, chronic treatment may enhance recovery from TBI by stimulating neuronal repair and enhancing neuronal activity. Fewer studies have explored the efficacy of chronic treatment therapies

compared to acute treatments. Some of the studies that examined the effects of chronic therapies included excitatory neurotransmitter agonists (Pike and Hamm, 1995; Feeney et al., 1982; Plenger et al., 1996), neurotrophic factors (Dietrich et al., 1996; Dixon et al., 1995; Sinson et al., 1995; Saatman et al., 1997; Hatton et al., 1997), stem cells (Park et al., 1999; Tzeng and Wu, 1999). Additional studies remain to be completed in the area of chronic posttraumatic therapy, and if there was a drug that was still effective up to eleven days after brain trauma, then many cognitive difficulties could be alleviated in thousands of TBI victims every year. Acute or chronic treatments on their own may be efficacious if administered at the appropriate time. However, a combination approach of acute and chronic pharmacological treatment together with rehabilitation therapy may provide most successful management of traumatic brain injury deficits.

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