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Modulation of Glutamate-mediated Neuronal Cell Death by Neurosteroids

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Senior Honors Project

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Abstract:

Traumatic brain injury (TBI) is a major cause of death and permanent disability in the United States. Approximately 1.7 million cases of TBI are reported annually. After an injury to the head, excessive glutamate, an excitatory neurotransmitter, is released into the extracellular fluid resulting in the excitotoxic death of neuronal tissue. Recent studies have suggested neurosteroids, may serve as an effective means by which to modulate excitotoxicity via the excitatory neurotransmitter alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Using mixed astrocyte-neuronal cell cultures (14-16 DIV) exposed to increasing concentrations of AMPA as the model for TBI, the experiments examined the effect of the neurosteroids, pregnenolone sulfate (PS) and dehydroepiandrosterone sulfate (DHEAS), on the AMPA-mediated cell death using the lactate dehydrogenase (LDH) assay. In order to observe reactivity of neurosteroids on the specific receptor, AMPA, MK-801, an NMDA antagonist was incorporated into the experimental design. Experimental results concluded pregnenolone sulfate attenuates AMPA-mediated excitotoxicity in a dose-dependent manner, while dehydroepiandrosterone sulfate provides a lesser degree of protection from excitotoxicity at higher concentrations in vitro. Neurosteroids may serve as a viable means by which to limit excitotoxic effects on the brain of an individual that has suffered from a TBI.
Introduction:

Traumatic brain injury (TBI) is the leading cause of death and permanent disability in individuals under 45. Approximately 1.7 million cases of TBI are reported within the United States annually and, of those affected, over 50,000 die, 235,000 are hospitalized and 1.1 million are treated and released from an emergency room. (Polito et al., 2010) Due to the significant number of affected individuals, it is imperative to study and understand the mechanisms and impacts of TBI as a way to prevent, diagnose, and treat this condition. The recent realization of the prevalence of TBI associated with other diseases and symptoms has increased the study and development of treatment methods to decrease the severity of injury. Head injury accounts for 2% of all deaths from all causes in the United States alone (McIntosh et al., 1996). Traumatic brain injury is caused by a blow or jolt to the head which results in the brain impacting into the skull or a penetrating head injury in which the function of the brain is disrupted. There are a variety of brain injury mechanisms which depend on the location and severity of the mechanical distortion applied to bone, blood vessels, and brain tissue. The initial trauma to the brain leads to immediate injury effects, including the death of brain tissue, and is later accompanied by secondary delayed injury including inflammation, receptor dysfunction, and negative free radical effects on surrounding tissue (Graham et al., 1995). The excitotoxin hypothesis of TBI proposes that secondary injury is mediated by elevated interstitial concentration of excitatory amino acids, such as glutamate and aspartate (Palmer et al., 1993). These delayed effects, causing ischemia, high intracranial pressure, and edema, are the targets for several treatment methods to reduce the severity of injury to the brain.
During the period from 1995-2001, the leading causes of TBI were falls, accounting for 28% of all reported cases, followed by motor vehicle accidents at 20%, and individuals struck by an object accounted for 19% (Polito et al., 2010). The repercussions of brain injury may be temporary or may result in profound disability. Individuals affected by TBI are likely to experience signs or symptoms that appear in unpredictable ways throughout their lifetime.

Long-term effects of TBI include altered cognitive function, personality, behavior, and sensory and motor impairment. Studies regarding the biochemical mechanisms of injury to brain tissue have provided a great deal of insight into the pathology underlying patterns of post-traumatic tissue death and damage and may lead to developments reducing the mortality and morbidity of those affected with TBI (McIntosh et al., 1998; Leskiewicz et al., 2008; Shirakawa et al., 2005).

Research into TBI has significant implications in the treatment of veterans of the military operations in Iraq and Afghanistan. In a study conducted by Hill (2009), researchers assessed the diagnostic technique used by the Veterans Affairs (VA) association and applied the screening tool at the Walter Reed Medical Center to routinely test for TBI among soldiers injured as a result of explosive blasts, motor vehicles, falls, other explosions and gunshot wounds to the head. Traumatic brain injury was diagnosed in 62% of admitted soldiers in the three month period the test was conducted. Thirty-five percent of veterans that screened positive for TBI were diagnosed with service-connected, post-traumatic stress disorder (PTSD) in the polytrauma program. (Hill et al., 2009) The study concluded that soldiers suffering from mild to severe TBI via the VA TBI screening tool have higher rates of post-traumatic stress disorder. In a similar article by Polito et al. (2010), of the 1.64 million troops deployed as part of Operation Enduring Freedom and Operation Iraqi Freedom, 320,000 have sustained a brain injury, costing billions of dollars in
health care (Polito et al., 2010). The paper also describes a diagnostic technique developed by the International Brain Research Foundation to more accurately diagnose TBI in affected individuals.

There are several mechanisms that cause trauma to the soft tissue of the brain. Traumatic brain injury is caused by a blow or jolt to the head and subsequent soft tissue damage caused by the physical strain of the impact of an object or of the brain against the skull. Regardless of the diverse means by which TBIs may occur, they all involve the nearly instant transfer of kinetic energy. Such impact will require either an absorption (acceleration) or release (deceleration) of kinetic energy by the head and brain (Shaw, 2002). The two main mechanisms of TBI include local skull distortion which propagates stress waves though the brain from the point of impact, and movement or distortion of the brain as a result of inertial or acceleration forces (McIntosh et al., 1996). Both of these mechanisms occur when the head is struck with an object or the brain moves within the skull as an indirect effect of contact or inertia, as in a motor vehicle accident (McIntosh et al., 1996)

Both types of mechanical force, contact and inertia, lead to soft tissue stress and strain which, in turn, causes focal and diffuse lesions to the brain. Focal lesions are injuries that are localized or restricted to specific areas. The most common causes of this type of injury include skull fracture, epidural hematoma, or bleeding between the inside of the skull and the dura layers of protective tissue covering the brain, cerebral contusions, and intracerebral hematoma, or bleeding within the macro or microvasculature throughout the brain (McIntosh et al., 1996). Diffuse lesions, or widespread injury of brain tissue, occur as a result of numerous focal injuries as well as diffuse axonal injury, caused by waves of physical stress that pass through the soft tissue as a result of the contact or inertial forces (McIntosh et al., 1996).
A large force applied to a relatively small area on the surface of the skull will cause the skull to fracture and subsequently impact the underlying soft brain tissue. This type of injury, penetrating or depressed fracture, causes localized damage to the brain. Local distortion of the skull without fracture can deform the underlying brain tissue and create pressure gradients throughout the brain (McIntosh et al., 1996). Elevated intracranial pressure has been hypothesized to be significant factors in production of cerebral contusions at the site of impact (McIntosh et al., 1996).

The inertial effects on the brain during injury occur via different mechanisms. The two major forms of acceleration injuries are rotational and translational (McIntosh et al., 1996). Rotational inertial injury, in which the brain is rotated and impacted into the skull, produces significant widespread tissue damage and strain throughout the brain relative to the direction of motion and location and presence of intracranial dural compartments. Translational injuries, in which the brain is directly impacted into the skull, produce intracranial pressure gradients and movement of the brain in relation to the skull. This type of injury is closely linked to cerebral contusions, hematomas, and histological lesions in the brainstem regions (McIntosh et al., 1996). Regardless of the precise role played by translation or rotation, it is clear that the energy imparted by acceleration of the head sets the brain in motion (Shaw, 2002). The brain is suspended in a protective layer of cerebrospinal fluid within the subarachnoid space which allows it some freedom to move. Due to its gelatinous and viscoelastic properties, it is relatively incompressible but readily distortable (Shaw, 2002).

Brain damage occurs in various areas throughout the soft tissue. Surface contusions and lacerations affect the gyri and have characteristic distribution affecting frontal poles, the ventral areas of frontal lobes, various areas of the cortex and, more specifically, the orbital gyri,
temporal lobes, and ventral aspect of the cerebellum (McIntosh et al., 1996). Surface contusions are both problematic and life-threatening. Injuries to the prefrontal cortex of the brain may cause significant cognitive, behavioral, or executive function problems, because this area is responsible for higher-thought processes, decision making, and aspects of personality. Initially, the injured tissue is hemorrhagic and swollen which results in delayed effects of the injury (Shaw, 2002).

There are several types of surface contusions. Fractural contusions occur at the site of fracture, coup contusions occur at the site of injury without fracture, countercoup contusions occur in the brain tissue diametrically opposite of the point of impact, herniation syndrome usually involves medial elements of the temporal lobes when brain tissue forces the deformation of other brain tissue, and gliding contusions describe hemorrhagic lesions in the cerebral cortex which characterize some of the vascular damage associated with TBI (McIntosh et al., 1996).

Intracranial hematoma is reportedly the most common cause of clinical morbidity and death in patients who suffer from TBI. The effects of this type of injury are often delayed because the associated brain swelling is responsible for the secondary events that lead to death in most cases (McIntosh et al., 1996). Following TBI brain damage due to ischemia is an important aspect underlying brain tissue destruction and patient death. Several factors contribute to decrease in brain blood flow including stretching and distortion of blood vessels, atrial hypotension, vasospasm, and post-traumatic changes in the cerebral microvasculature (McIntosh et al., 1996). Diffuse axonal injury (DAI) is caused by extensive diffuse lesions in white matter tracts. This type of injury is identified in 30% of all cases of TBI and is characterized by the diffuse degeneration of white matter (McIntosh et al., 1996). Diffuse axonal injury is particularly related to motor vehicle accidents and is often characterized after skull fracture, surface
...contusion or intracerebral hematoma and is one of the major causes of loss of consciousness and coma (McIntosh et al., 1996).

After sustaining an injury to the head, patients suffering from concussion symptoms and most severely injured patients continue to have residual impairments consisting of physical limitations, as well as cognitive, social, and behavioral limitations (Benedictus et al., 2010; Hartlage, et al., 2001). The most frequent cognitive impairments include mental slowness, attention deficits, memory restrictions, and problems with executive functioning. The main behavioral change after injury involves the increased frequency of aggression. (Chevignard et al., 2009). In a study conducted by the American Congress of Rehabilitation Medicine, behavioral and cognitive impairments after traumatic brain injury were studied and related to outcome and time needed to return to work. This study concluded that almost half of the patients observed experienced cognitive and behavioral problems one year after injury. Even in patients with mild TBI, 43% experienced cognitive impairment, and one in three experienced behavioral problems (Benedictus et al., 2010).

Memory and learning disturbances are among the most frequently reported cognitive symptoms after TBI (Ariza et al., 2006). This damage is caused by a combination of direct injury involving diffuse axonal injury, various contusions, ischemia, as well as the neuronal tissue loss associated with the excitotoxic cascade. These symptoms are often hard to predict after injury because most memory impairments in patients with TBI are non-specific due to the diffuse damage incurred by the injury (Shaw, 2002). In a study conducted by the Department of Psychiatry and Clinical Psychobiology at the University of Barcelona, Ariza and colleagues analyzed experimental evidence concerning the specific location of focal lesions on the brain and their relationship to a specific pattern of memory impairment. The study suggests material-
specific memory impairment, such as facial recognition problems, may occur after trauma involving focal lesions, but non-specific memory impairment is prevalent after diffuse injuries that affect multiple areas in the brain (Ariza et al., 2006).

Executive function deficits are also a common consequence of TBI. This type of functioning and higher-thought processing is controlled and regulated by the frontal lobes, which are more commonly injured during a closed brain injury. The prefrontal cortex handles the processing of information in decision making, reasoning, as well as derives aspects of an individual’s personality (Chevignard et al., 2009; Dikmen et al., 2003). Focal damage to any of the cortical structures may cause development of specific disabilities; however, diffuse injury effects are more widespread. Motor impairment usually involves damage to the cerebellum or the somatosensory cortex at the pre- and postcentral gyrus. This system combines signals from the periphery to produce sensory modalities such as touch, temperature, and proprioception (Lotze et al., 2006).

Following the mechanical injury, secondary neuronal and cellular injury occurs hours to days after the initial trauma. This delayed injury is associated with neurochemical alterations which affect brain blood flow, ion homoeostasis, and metabolism (McIntosh et al., 1996). The initial injury also has direct toxic effects on localized neurons and glial cells.

Several types of cellular changes occur upon the impact of injury. Increased acetylcholine release is associated with impairment of cholinergic neurons and loss of consciousness and coma following TBI (McIntosh et al., 1996). The arachidonic acid cascade caused by elevated levels of intracellular calcium activates proteins such as phospholipase-A2, lypoxygenase and cycloxygenase which break down arachidonic acid into metabolites which, in turn, activate proteases and lipases that degrade the cell membrane of affected neurons and glial cells.
Cytokines may also have a significant function in the delayed effects of TBI. Congregation of leukocytes at the site of injury via cytokine signaling and possible disruption of the blood-brain barrier is associated with cerebral edema. Leukocytes, including macrophages, may have degrading affects on damaged neurons (McIntosh et al., 1996).

The most dramatic neurochemical change following trauma is the uncontrolled release of excitatory neurotransmitters. This efflux of chemical messengers is associated with secondary or delayed injury processes that develop over a period of minutes to hours and cause irreparable damage to sensitive neuronal tissue. This secondary injury is mediated by increased interstitial concentrations of excitatory amino acids, specifically glutamate and aspartate (Kim et al., 2010). The excessive release of these neurotransmitters causes a cascade of cellular signaling that eventually leads to glial swelling and neuronal cell death. In a recent study, brain injury induced on animal models using fluid percussion caused a marked increase in extracellular glutamate and aspartate surrounding the site of trauma. The concentration of these excitatory amino acids was relative to the severity of experimental injury (Kim et al., 2010). The uncontrolled release of these neurotransmitters contributes to the delayed tissue damage following injury to the brain.

Glutamate is the most abundant amino acid in the central nervous system. Sixty percent of all neurons utilize glutamate as their primary excitatory neurotransmitter. (Watkins and Jane, 2006) Toxic effects caused by excessive release of glutamate, known as glutamate-induced excitotoxicity, cause marked changes in brain tissue physiology and result in a significant number of cellular disruptions including interference of ion gradients, increase in intracellular calcium levels and over-activation of secondary messenger systems. This massive progression of cellular events leads to neuronal cell death due to a variety of processes that end in apoptosis or necrosis. (Watkins and Jane, 2006)
The regulation of specific ion gradients is essential to the function and continuation of cellular processes. After injury, ionic gradients and their respective concentrations are significantly altered and, consequently, a variety of harmful events proceed until the gradients can be restored (Hilton et al., 2006). The maintenance of intracellular calcium levels in neuronal tissue is an essential factor in the regulation of cell signaling events and activation of intracellular enzymes (Hilton et al., 2006). Total brain tissue calcium levels appear to be significantly elevated post-injury in experimental models of TBI (McIntosh et al., 1996). The subsequent over-activation of the N-methyl-D-aspartic acid (NMDA) receptor by glutamate causes increased expression of various genes through activation of calcium-dependent transcription factors and calcium-activated neutral proteases, such as calpain, which contributes to significant cytoskeletal degradation (McIntosh et al., 1996).

Other ions associated with neuronal cell function include magnesium, Mg$^{2+}$, and sodium Na$^+$. Magnesium is an essential element for several processes within the central nervous system, as well as glycolysis, synthesis of DNA and RNA, and maintaining integrity of mitochondrial and cell membranes (Hilton et al., 2006). Recent experiments have shown that the concentration of magnesium declines after brain trauma, causing a significant decrease in glucose metabolism and protein synthesis. Magnesium has also shown some effect on regulation of calcium levels within the cell (McIntosh et al., 1996). Sodium ions are essential for cell signaling and the maintenance of critical concentration gradients. After injury the excitotoxic cascade causes an increase in the release and concentration of excitatory amino acids into the synaptic gap, which, in turn, activate specific ionotropic receptors located on the cell itself or adjacent cells (McIntosh et al., 1996). Sodium influx through excitatory receptors causes the widespread depolarization of
neurons, and thus contributes to the influx of calcium, uncontrolled firing of action potentials, and activation of various cell signaling pathways (McIntosh et al., 1996).

Glutamate-induced excitotoxicity provokes uncontrolled calcium influx resulting in an intracellular cascade of cytotoxic events. The activated intracellular enzymes include the neuronal nitric oxide synthase with subsequent nitric oxide production and formation of radical oxygen species, damaging surrounding cells and leading to further cell death (Kim et al., 2010). A number of in vitro studies indicate that at high concentrations, glutamate is a potent neurotoxin capable of destroying neurons by apoptosis (Zhang and Bhavnani, 2005). The influx of extracellular ions leads to the activation of enzymes, neuronal depolarization and uncontrolled firing, and the failure of ion pumps and subsequent depletion of essential concentration gradients. In a study conducted at the University of Pittsburgh School of Medicine, researchers compared results of fluid percussion injury on rat cortical slices in vivo to effects of direct injection of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) excitatory amino acids on the same areas studied in vivo. Researchers concluded that both AMPA and NMDA produced a cavitary lesion with an anatomical extent similar to that caused by severe TBI, as replicated by in vivo fluid percussion models (Palmer et al., 1993). The experimental model in my study utilizes the specificity of the AMPA excitatory amino acid to replicate TBI.

Glutamate acts on many different types of receptors. The classes of ionotropic glutamate-binding receptors include the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, N-methyl-D-aspartic acid (NMDA) receptor, and kainate receptor, named for chemical agonists of the receptor proteins. Ionotropic receptors contain associated ion-gated channels that are regulated by agonist binding. Together, these receptors control and modulate neural circuits
in the brain and underlie aspects of cognitive function (Morimoto-Tomita et al., 2009). The receptors mediate excitatory transmission between neurons. Binding of glutamate to the AMPA or NMDA receptor opens post-synaptic cation channels and initiates excitatory post-synaptic current. Considerable experimental evident shows that AMPA and NMDA receptors co-localize at most functional excitatory synapses, however the ratio of AMPA to NMDA receptors varies greatly between different neural synapses. Kainate receptors appear in smaller numbers throughout regions in the central nervous system (Nestler, 2009).

The AMPA receptor is a 4-subunit protein which allows the influx of Na\(^+\) cations into the postsynaptic cell. Some of these receptors, specifically those that lack important features of the GluR-2 subunit, are also permeable to Ca\(^{2+}\) which allows the flow of calcium into the cell and subsequent activation of secondary messenger systems. These types of receptors are commonly found on inhibitory interneurons throughout the brain (Nestler, 2009). Allosteric modulation of the AMPA receptor is potentiated by binding of the specific molecule to the GluR-2 subunit, which in turn, regulates activity and influx/efflux of specific ions (Fukata et al., 2005). This receptor functions as an ion channel and is inhibited by 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), a drug that competitively antagonizes AMPA receptor function. This type of drug permits unequivocal, experimental-separation of AMPA receptors from other categories of glutamate receptors (Fukata et al., 2005).

The NMDA receptor is likely to provide the most significant means by which synaptic activity may increase the level of postsynaptic intracellular Ca\(^{2+}\). Activated by glutamate or NMDA binding, this receptor is the predominant molecular device for synaptic plasticity and memory function (Nestler, 2009). This receptor is composed of a heterotetrameric of two sets of the subunits, NR1 and NR2. The ionotropic receptor provides a channel for K\(^+\), Na\(^+\), and is
highly permeable to Ca\(^{2+}\), however, channel function is voltage-gated by the presence of a Mg\(^{2+}\) cation that blocks the channel’s pore and corresponding movement of ions across the membrane upon activation (Mony et al., 2009). As the postsynaptic membrane potential depolarizes, the Mg\(^{2+}\) is released, and depolarization is expedited by influx of Na\(^{+}\) and Ca\(^{2+}\) (Siegel, 1989). The NMDA receptor has several target sites for allosteric modulation by molecules resembling polyamines and aminoglycosides. Dizocilpine (MK-801) acts as a non-competitive antagonist of NMDA receptor function. N-methyl-D-aspartic acid (NMDA) receptors have been prominent drug targets for conditions such as stroke and head trauma in which excitotoxicity has a significant role (Nestler, 2009).

The kainate receptor is a glutamate-induced ionotropic receptor that provides the channel for influx of sodium and potassium and is located on the presynaptic cell (Siegel, 1989). The structure of the receptor is composed of 5 subunits which arrange to form a functioning membrane channel protein. This receptor provides modification and regulation of a specific neurotransmitter release but has a more limited distribution throughout the brain as compared to AMPA and NMDA receptors (Siegel, 1989).

Metabotropic glutamate receptors (mGluR) are a type of membrane protein activated in the presence of glutamate to initiate indirect metabotropic processes. This type of receptor protein is a member of the G-protein-coupled receptors, which can simultaneously activate multiple signaling pathways (Skeberdis et al., 2001). Activation of mGluRs initiates a variety of intracellular functions throughout the central nervous system. The eight different types of mGluRs have seven transmembrane regions which span the cell membrane and, upon activation, stimulate a variety of biochemical cascades which modulate the activity of other proteins, including the NMDA ionotropic receptor (Skeberdis et al., 2001).
The excitotoxic cascade is the most prevalent cause of delayed injury in cases of TBI. The excessive stimulation of ionotropic glutamate receptors is implicated in the pathogenesis of various neurodegenerative conditions (Shirakawa et al., 2006). However, extensive experimental evidence supports that neuroactive steroid compounds may modulate effects of glutamate-induced excitotoxicity. Although NMDA receptors have an important role in excitotoxicity due to their high permeability to Ca$^{2+}$, over-activation of non-NMDA receptors may also contribute significantly to the overall effects of excessive glutamate release on neuronal cells (Shirakawa et al., 2006). Neurosteroids are synthesized in the central nervous system in glial support cells and neurons as well as throughout the periphery from cholesterol or other steroidal precursors imported from peripheral sources. (Baulieu, 1998) The focus of my study is on the 3β-hydroxy-delta-5-compounds, pregnenolone sulfate (PS) and 5-dehydroepiandrosterone sulfate (DHEAS) and their effects on the AMPA receptor in vitro during an AMPA-induced excitotoxic cascade. These steroids have been shown to modulate the inhibitory receptor, γ-aminobutyric acid A (GABA$_A$), NMDA, and sigma-1 receptors (Baulieu, 1998) however, there effect on the AMPA receptor is relative unknown.

These neuroprotective steroids are synthesized from cholesterol and similar compounds. Cholesterol is synthesized locally in the brain and peripheral nerves as a source of maintenance for fatty myelin sheaths. Cholesterol is converted to pregnenolone by the cytochrome P450scc (side chain cleavage) protein (Fig. 1), which is a specific hydroxylase involved in cleaving side chains of cholesterol with other enzymes andrenodoxin and adrenodoxin reductase by neurons and glial cells (Baulieu, 1998). The regulatory mechanisms for cytochrome P450scc are still unknown. 5-dehydroepiandrosterone (DHEA) and PREG are converted to PS and DHEAS by a sulfotransferase enzyme within neurons and glial cells (Baulieu, 1998).
Figure 1: The metabolism of neurosteroids.
(Modified from Baulieu, 1998)

Pregnenolone and PS are the most abundant neuroactive steroids in the brain. These neurosteroids act as specific and potent allosteric modulators of the GABA\textsubscript{A} receptor (Shirakawa et al., 2005). The GABA\textsubscript{A} receptor is the primary inhibitory receptor in the brain. This ionotropic receptor has several allosteric sites which allow modulation of its activity upon ligand binding. This receptor allows the influx of chloride ions, both pre- and post-synaptically into the cell, causing a repolarization effect. Activation of this specific receptor has shown to have anesthetic and anticonvulsant effects. Experimentally, PS and DHEAS have been shown to be negatively modulate GABA\textsubscript{A} receptor function, decreasing current across the post-synaptic membrane (Shirakawa et al., 2005).

In a similar experiment researchers at Kyoto University showed that PS allosterically potentiates the NMDA receptor activity \textit{in vitro} but has an inhibitory effect on non-NMDA
receptors. Evidence suggests that PS exacerbates neuronal cell death associated with NMDA excitotoxicity (Shirakawa et al., 2005). The researchers also evaluated the effect of PS and DHEAS on the sigma-1 receptor, an intracellular transmembrane chaperone protein that modulates the release of Ca^{2+} from the endoplasmic reticulum (Kimonides et al., 1998). It has been shown that DHEAS augments the effect of NMDA excitotoxicity by increasing the levels of intracellular Ca^{2+} and, thus, causes further depolarization of the membrane and over-activation of secondary messenger systems which lead to cell death (Shirakawa et al., 2005). The impact of pregnenolone sulfate (PS) and 5-dehydroepiandrosterone sulfate (DHEAS) on the NMDA, \( \text{GABA}_A \), and sigma-1 receptor may give insight as to how these drugs may affect the modulation of AMPA excitotoxicity.

Although the neurosteroids PS and DHEAS enhance NMDA receptor function during excitotoxicity, the effects of these compounds on the AMPA receptor are still relatively unknown. The AMPA receptor plays a key role in the progress of neurotoxicity during the cascade of glutamate release and excitatory signaling. In a study conducted at Kyoto University, the effect of PS, PREG, and another, similar compound, progrenolone hemisuccinate on AMPA-induced excitotoxicity was observed using rat cortical slice cultures. The study concluded that PS may attenuate AMPA excitotoxicity by inhibiting activity of the AMPA-associated channel protein (Shirakawa et al., 2005). In electrophysiological study, PS has been shown to decrease membrane depolarization caused by AMPA receptor function (Wu et al., 1998).

The purpose of this investigation was to assess the neuroprotective effects of PS and DHEAS on mouse cortical cell cultures \textit{in vitro} by exposing cultures to increasing levels of AMPA-induced excitotoxicity and measuring the level of neuronal cell death by utilizing the lactate dehydrogenase (LDH) assay. Based on previous electrophysiological data, it was
hypothesized that PS and DHEAS would decrease the level of neuronal cell death induced by AMPA receptor over-activation after exposure to increasing levels of AMPA.

**Materials and Methods:**

*Drugs and Chemicals*

Trypsin, cytosine β-D-arabinofrunasonside (Ara-C), N-methyl-D-aspartic acid (NMDA), dizocilpine maleate (MK-801), pregnenolone sulfate (PS), 5-dehydroepiandrosterone sulfate (DHEAS), Nicotinamide adenine dinucleotide (NADH), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Fetal Bovine Serum (FBS), Fetal Calf Serum (CS), Fetal Growth Serum (FGS), Hanks Balanced Salt Solution (HBSS), Modified Earle’s Media (MEM), Epidermal Growth Factor (EGF), penicillin and streptomycin were obtained from Fischer Scientific (Rocksford, IL, USA). (S)-2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from TOCRIS Bioscience (Ellisville, MO, USA). LDH standard solution consisted of Level II Control Serum (400U/L, Cat. # C7591-50) from Pointe Scientific, purchased through Fischer Scientific (Rocksford, IL, USA). Drug stocks were made using PS and DHEAS in dimethyl sulfoxide (DMSO) at 10mM.

*Animal Care*

Swiss mice (Hilltop Lab Animals, Scottdale, PA) were housed at room temperature in a 12/12 light-dark cycle access to food (Harlan Teklad, 22/5 Rodent Diet (8640), Madison, WI) and water provided *ad libitum*. Animals were housed in accordance with Lynchburg College guidelines and expectations.
**Experimental Model**

The experimental model in this study involved the application of varying concentrations of AMPA to mouse cortical cell cultures to replicate an AMPA-mediated excitotoxic event and comparing the affected groups with those treated with the specific drug, either PS or DHEAS at varying concentrations. Twenty-four hours prior to each experiment, 250µL of media was removed from each well and replaced with media stock (500mL MEM media with 45mL sterile glucose bicarbonate solution (20mM)) supplemented with 10µM glycine (MS/gly solution). Days in vitro (DIV) 14-16 mixed cortical cell cultures, used in each of the experiments, were washed by removing 250µL of media from each well and replacing with 500µL MS/gly solution twice. After wash, experimental drug solutions were added to the corresponding culture wells. Each experimental group was compared and normalized to a blank test group (0µM AMPA) and a complete excitotoxic death group (300µM NMDA solution). The blank test group accounted for sham injury caused without exposure to the experimental conditions, as when changing culture media or moving cultures. Exposure to 300µM NMDA accounted for total amount of neuronal LDH (=100%). Neuronal cell death was measured quantitatively by the lactate dehydrogenase assay at 20-24 hours post-injury as described in Kho and Choi, 1987. The use of Swiss mouse cortical cell cultures and subsequent analysis of cell death by the LDH assay is a well-established technique to test induced-excitotoxicity in vitro.

**Isolation and Culture of Astrocytes**

Astrocytes were isolated from 1-3 postnatal day mice, obtained from adult (2-5 month) females (Hilltop Lab Animals, Scottdale, PA) mated at Lynchburg College. Animals were sacrificed by rapid decapitation, the heads were washed twice in 70% ethanol solution to remove
debris and transferred to a dissection dish containing a volume of dissection media (DM) (HBSS supplemented with sucrose, dextrose, and HEPES). The dish was kept on ice in a sterile horizontal flow hood. Using micro-dissection forceps, the skin and skull of subject heads were removed exposing the soft brain tissue. Brains were then aseptically removed from the skull and placed into a separate dish containing DM, meninges were removed, and the cortical tissue from each hemisphere was isolated from the basal forebrain, hippocampus and brainstem. Each hemisphere was transported to 1mL DM, mechanically minced, and placed in tube containing 5mL dissection trypsin (0.025% trypsin in DM). The tube incubated at 35°C for 20 minutes and centrifuged at 1000 rpm for 5 minutes. The pellet was then triturated in a small volume of 10+10 plating media (PM) (MEM supplemented with 20mM glucose, 10% Fetal Bovine Serum, 10% calf serum, 2mM L-glutamine, 50 IU/mL penicillin, 50μg/mL streptomycin and epidermal growth factor (10ng/mL)) and was added to a specific volume 10+10 PM (1.2 hemispheres per 10mL PM). Four-hundred μL of the plating solution was added to each well on a 24-well plate and incubated at 32°C at 5%CO₂. Astrocytes were fed 2-3 days after plating with 10+10 PM, and when cultures became confluent; the cells were inhibited with 10μL of 9μM cytosine β-D-arabinofuranoside (Ara-C solution) for 4-7 days. After inhibition, inhibition chemical treatments were removed and replaced with growth media (GM) (Modified Earle’s Media supplemented with 10% calf serum, 2mM L-glutamine, and 50 IU/mL penicillin, 50μg/mL streptomycin).

Isolation and Culture of Neurons

Neurons were isolated from fetal cortices from 15-17 prenatal day timed-gestation Swiss mice (Hilltop Lab Animals, Scottdale, PA). Animals were anesthetized using an isoflourane chamber and sacrificed by cervical dislocation. The uterus of the adult female was aseptically removed and transferred to a dissection dish containing a volume of DM and placed in the sterile
horizontal flow hood. Fetuses were aseptically removed and decapitated into a new dish of DM. Using techniques described previously, cortices were isolated, incubated in dissection trypsin at 35°C for 20 minutes, centrifuged and triturated in 5+5 plating media (Modified Earle’s Media (MEM) supplemented with 20mM glucose, 5% Fetal Bovine Serum, 5% calf serum, 2mM L-glutamine, 50 IU/mL penicillin, 50μg/mL streptomycin and epidermal growth factor (10ng/mL) and added to a specific volume 5+5 plating media (~3 hemispheres per 10mL 5+5 PM). Media from each well on astrocyte culture plate was replaced with the 400μL 5+5 plating media containing cells and incubated at 32°C at 5%CO₂. Four to five days after plating, ~250μL of media was removed from each well of mixed astrocyte/neuron culture and replaced by 250μL of GM. Two days after mixed cultures were fed in 5+5 PM, the cultures were inhibited with the addition of 10μL Ara-C solution for two days. After that, 250μL of cell culture media was removed and replaced with 250μL GM. Twenty-four hours before each experiment, 250μL cell culture media was replaced with 250μL MS/gly solution. (See Appendix C)

**AMPA and CNQX Dose Response**

Drug stock solutions (4.5mL) were prepared with increasing concentrations of AMPA (3μM, 6μM, 9μM, 12μM) supplemented with 10μM MK-801 (Eq. 1; See Appendix A). The experiment was conducted by removing 500μL of media from each well and replacing media with 250μL of corresponding drug stock.

Drug stock solutions (3.5mL) were prepared using a constant concentration of AMPA (10μM) supplemented with 10μM MK-801 and increasing concentrations of CNQX (3μM, 10μM, 30μM). Using selected mixed culture plates, each well was washed by removing 250μL
of media and replacing with 500μL MS/gly twice. Each experiment was conducted by removing 500μL of media from each well and replacing media with 250μL of corresponding drug stock.

**AMPA Dose Response with and without PS**

Drug stock solutions (2.5mL) were prepared with increasing concentrations of AMPA (1μM, 3μM, 9μM, 27μM). Each drug stock was supplemented with 10μM MK-801 and either 10μM, 50μM or 100μM PS depending on the specific experiment being conducted. Pregnenolone sulfate was diluted from a 10mM stock solution and added to drug stocks. Drug stock solutions were supplemented with corresponding volumes of DMSO in absence of PS. The experiment was conducted by removing 500μL of media from each well and replacing media with 250μL of corresponding drug stock.

**AMPA Dose Response with and without DHEAS**

Drug stock solutions (2.5mL) were prepared with increasing concentrations of AMPA (1μM, 3μM, 9μM, 27μM). Each drug stock was supplemented with 10μM MK-801 and either 10μM, 50μM or 100μM DHEAS depending on the specific experiment being conducted. DHEAS was diluted from a 10mM stock solution (Eq. 2) and added to drug stocks. Drug stock solutions were supplemented with corresponding volumes of DMSO in absence of DHEAS. The experiment was conducted by removing 500μL of media from each well and replacing media with 250μL of corresponding drug stock.

**Evaluation of Cell Death**

The lactate dehydrogenase (LDH) cytotoxicity assay (Kho and Choi, 1987) was used to assess cell death 20-24 hours after experiment. Lactate dehydrogenase buffer was diluted from a
sterile 10M stock solution. Nicotinamide adenine dinucleotide (NADH) solution was made using crystalized NADH dissolved in 1M LDH buffer at 3mg/10mL. Pyruvate solution was retrieved from a sterile stock solution (22.7mM). Lactate dehydrogenase buffer, NADH solution, and pyruvate solution were transferred to 50mL centrifuge tubes labeled correspondingly in a sterile environment provided by a laminar-flow hood. Supernatant (50μL) was removed from each experimental well on the 24-well mixed-culture plate and placed in corresponding wells on a 96-well microtiter plate (LDH plate). A standard curve was also plated onto the 96-well plate on rows G and H, columns 1-9 using corresponding volumes of the Level II control serum (400U/L) supplemented with PBS buffer. (Appendix B) Using a multipipette, 125μL LDH solution and 100μL NADH solution were added to each row on the LDH plate. Pyruvate solution (50μL) was expediently added to each of the wells on the LDH plate, and the plate was placed in the spectrophotometer. Changes in optical density at 490 nm were measured using the Microquant by Bio-Tek Instruments. (Appendix B)

Results:

AMPA Dose Response with CNQX

Mixed cell cultures, 14-16 DIV, were exposed to increasing concentrations of AMPA (3μM, 6μM, 9μM, 12μM) supplemented with 10μM MK-801 to determine the effect of AMPA on neuronal cell death. The non-competitive NMDA antagonist MK-801 was included to block NMDA receptor activation by glutamate released from dead or dying neurons (Figure 2).
Figure 2: AMPA induces a dose-dependent increase in neuronal cell death. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=12 cultures per condition pooled from 3 experiments).

Application of increasing AMPA concentrations to mixed cortical cell cultures showed a concentration-dependent increase in neuronal cell death determined at 20-24 hours later using the LDH assay. This experiment served as a standard for comparison of other, similar experiments as well as demonstrated the effectiveness of our experimental model. The data represented in Figure 2 shows 20% neuronal cell death after exposure to 3μM AMPA and as much as 65% neuronal cell death after exposure to 12μM AMPA.
To demonstrate that this injury was AMPA-mediated, 14-16 DIV mixed cell cultures were exposed concurrently 10μM AMPA and an increasing concentration of CNQX (3μM, 10μM, 30μM). Application of increasing concentrations of CNQX, a non-competitive antagonist of the AMPA receptor, caused a marked decrease in the neuronal cell death after exposure to 10μM AMPA and completely inhibited injury at 30μM CNQX.

Figure 3: CNQX blocks AMPA-mediated neuronal cell death in a dose-dependent manner. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=12 cultures per condition pooled from 3 experiments).

Inhibition of the AMPA receptor by the competitive antagonist CNQX, (Fig. 3) displayed the specificity of modulation of excitotoxicity via the AMPA receptor. The level of cell death after
administration of 10μM AMPA was 74.6%, and when cell cultures were concurrently exposure with 10μM AMPA and increasing concentrations of CNQX, neuronal cell death noticeably decreased. AMPA-mediated neuronal cell death was decreased by nearly 50% after exposure to 3μM CNQX. These experiments displayed the specificity of the experimental design for modulation of neuronal cell death through the AMPA receptor.

**Effect of Pregnenolone Sulfate on AMPA-mediated Cell Death**

Mixed neuronal cell cultures (14-16 DIV) were exposed to increasing concentrations of AMPA (1μM, 3μM, 9μM, and 27μM) with or without PS (at 10μM, 50μM, or 100μM). All experimental conditions were concurrently treated with 10μM MK-801 to block activation of NMDA receptors. In addition, experimental conditions without PS treatment were supplemented with an equal amount of DMSO. Exposure to increasing concentrations of AMPA caused a dose-dependent increase in the amount of neuronal cell death. Inhibition of excitotoxicity induced by exposure to increasing concentrations of AMPA was evident in experimental results (Fig. 4, 5, 6). Data from AMPA dose response experiments with 10μM, 50μM and 100μM PS showed apparent reduction of injury as determined by LDH release provided by the addition of various concentrations of the selected neurosteroid, PS. The effects of PS seem to be concentration dependent, in that 100μM PS decreased the level of excitotoxicity to a greater extent as compared to 10μM and 50μM concentrations of PS.
Figure 4: Neuronal cell death induced by AMPA is reduced by concurrent exposure to 10μM PS. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=11-12 cultures per condition pooled from 6 experiments).

The AMPA dose response with 10μM PS displayed slight inhibition of AMPA-induced excitotoxicity. Experimental groups treated with 10μM PS decreased the level of cell death at 3, 9, and 27μM AMPA by 12.6, 8.9, and 9.2% respectively. The groups at 1μM AMPA without PS displayed 6.2% cell death, while the group treated with 10μM PS at 1μM AMPA showed a decrease in cell death as compared to the blank (-3.5% cell death). This is attributed to the amount of cell death in the sham injured groups, which was 18.7%.
Figure 5: Neuronal cell death induced by AMPA is reduced by concurrent exposure to 50μM PS. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=26-27 cultures per condition pooled from 14 experiments).

The AMPA dose response with 50μM PS showed the inhibition of AMPA-induced excitotoxicity. The groups treated with 50μM PS showed modulation of the AMPA receptor resulting in 17.1, 22.5, and 29.3% decrease in cell death at 3, 9, and 27μM AMPA respectively. The level of cell death incurred by 1μM AMPA was reduced to 0% with 50μM PS.
Figure 6: Neuronal cell death induced by AMPA is reduced by concurrent exposure to 100μM PS. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=22-24 cultures per condition pooled from 12 experiments).

The test groups administered with the AMPA dose response with and without 100 μM PS showed an apparent decrease in cell death at 3μM AMPA (36.1%), 9μM AMPA (52.6%), and 27μM AMPA (43.4%). Increasing concentrations of AMPA caused subsequent increase in neuronal cell death in each of the experimental groups but in the presence of PS the amount of cell injury was reduced. The greatest reduction of neuronal cell death was in groups exposed to 100μM PS.
Effect of Dehydroepiandrosterone Sulfate on AMPA-mediated Cell Death

Mixed neuronal cell cultures (14-16 DIV) were exposed to increasing concentrations of AMPA (1μM, 3μM, 9μM, and 27μM) with or without DHEAS (at 10μM, 50μM, or 100μM). All experimental conditions were concurrently treated with 10μM MK-801 to block activation of NMDA receptors. Experimental conditions without DHEAS treatment were supplemented with an equal amount of DMSO. Exposure to increasing concentrations of AMPA caused a dose-dependent increase in the amount of neuronal cell death. Figure 7, 8, and 9 represent the results of the LDH assay for the AMPA dose response with 10μM, 50μM, and 100μM DHEAS, respectively. Data from AMPA dose response experiments with 10μM DHEAS showed a no apparent reduction of neuronal cell death provided by the addition of 10μM of the selected neurosteroid, DHEAS (Fig. 7). However at higher concentrations of DHEAS, the reduction of neuronal cell death as determined by LDH release was more apparent. There is a slight decrease in AMPA-induced excitotoxicity in groups exposed to 50μM DHEAS (Fig. 8), and a more noticeable decrease in the percentage of cell death in groups administered with 100μM DHEAS (Fig. 9).
Figure 7: Low concentrations of DHEAS (10μM) have no effect on AMPA-induced neuronal cell death. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=6 cultures per condition pooled from 3 experiments).

The results of this experiment (Fig. 8) display a slight increase in cell death at 10μM DHEAS. The occurrence of cell death in each test group increased by at least 5% at every concentration tested, suggesting that lower concentrations of DHEAS has no affect on reduction of AMPA-induced excitotoxicity. The cell death caused by 1μM AMPA solutions increased 8.2% with exposure to 10μM DHEAS.
Figure 8: DHEAS (50μM) concurrent with AMPA caused minimal reduction in neuronal cell death. Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=8 cultures per condition pooled from 4 experiments).

The results of this experiment (Fig. 9) display a decrease in AMPA-induced cell death with concurrent exposure to 50μM DHEAS. This concentration of DHEAS appeared to slightly decrease the percentage of cell death induced by exposure to increasing concentrations of AMPA. A decrease in cell death by at least 10% was observed in test groups administered with 1μM and 3μM AMPA, with 7.8% decrease in cell death noted at 9μM AMPA, and 12.2% decrease in cell death at 27μM AMPA.
Figure 9: AMPA-induced neuronal cell death is reduced by DHEAS (100μM) Supernatant of corresponding cell cultures were collected 20-24 hours following injury and the cell death was quantified spectrophotometrically by measurement of LDH release. Values represent the mean ± S.E.M. expressed as a percentage of the total neuronal LDH (=100%), which was determined by exposure of sister cultures to 300μM NMDA (n=10 cultures per condition pooled from 5 experiments).

The results of this experiment (Fig. 10) display the decrease in AMPA-induced cell death with concurrent exposure to 100μM DHEAS. Higher concentrations of DHEAS (100μM) appeared to show a larger decrease in the amount of cell death at all concentrations of the AMPA dose response. The level of cell death decreased 4.3% at 1μM AMPA, and 6.3% in groups exposed to 27μM AMPA. Neuronal cell death was decreased by at least 15% in groups tested with 3μM AMPA and 9μM AMPA.
Discussion:

Traumatic brain injury is replicated in vitro utilizing cortical cell cultures in this study to isolate the activity of the specific glutamate receptor, AMPA. Using cortical cell cultures as opposed to in vivo tests allows more control of experimental conditions and consequently increased data specificity with reference to the modulation of AMPA receptor function when concurrently exposed to agonist and neurosteroid. AMPA-receptor specific modulation by the neurosteroid PS appears to be dose-dependent, as the increase in protection corresponds with the increase in concentration of PS. Pregnenolone sulfate provides protection from AMPA-induced toxicity via the modulation of the AMPA receptor in all trials (Fig. 5, 6, and 7). At higher concentrations, the neurosteroid dehydroepiandrosterone sulfate displayed a similar, but smaller effect. Both 50μM and 100μM concentrations of DHEAS decreased neuronal cell death after concurrent exposure of cell cultures to the neurosteroid and AMPA dose response (Fig. 9 and 10). At lower concentrations of DHEAS, no noticeable decrease in neuronal cell death elicited by increasing concentrations of AMPA was observed. The original hypothesis proposed in this study, based on previous electrophysiological data (Wu et al., 1998), was that PS and DHEAS would decrease the level of neuronal cell death induced by AMPA receptor over-activation and according to experimental evidence gathered from this study, PS and higher concentrations of DHEAS decreased the level of cell death caused by increasing concentrations of AMPA.

As compared to PS, DHEAS is not as effective in providing protection from excitotoxicity via AMPA-receptor modulation. In conclusion, our experimental data indicate the receptor-specific modulation of AMPA by increasing levels of PS decreases the amount of neuronal cell death in mixed cell cultures and the neurosteroid, DHEAS provides a lesser degree of excitotoxic protection at higher concentrations. The neuroprotective effect of PS against
AMPA neurotoxicity suggests that this neuroactive steroid acts as an endogenous regulatory factor in the central nervous system pathology involving AMPA receptor-mediated excitotoxic events (Shirakawa et al., 2005). Further research into the exact mechanism of the neurosteroid is suggested in order to develop a treatment method for detrimental excitotoxic events following TBI.

The first experiment conducted observed how increasing the concentration of AMPA induced neuronal cell death (Fig. 3). The next experiment involving inhibition of the AMPA receptor by the non-competitive antagonist CNQX, (Fig. 4) displayed the specificity of modulation of excitotoxicity via the AMPA receptor. Specific inhibition of the receptor caused a marked decrease in levels of LDH and thus a decrease in the level of cell death. The level of cell death after administration of 10μM AMPA was 74.6%, and when paired with increasing concentrations of CNQX, cytotoxicity noticeably decreased in a dose-dependent manner and at 30μM CNQX, neuronal cell death caused by 10μM AMPA was completely inhibited. The effect of CNQX and increasing concentrations of AMPA on mixed cortical cell cultures is evident in literature. (Morimoto-Tomita et al., 2009; Fukata et al., 2005) These experiments exemplified the specificity of our experimental model in the modulation of AMPA-regulated events.

The lactate dehydrogenase (LDH) cytotoxicity assay was used to assess cell death 20-24 hours after experimental treatments. The prevalence of LDH in the supernatant is directly comparable to the level of cell death. Lactate dehydrogenase is a cytosolic enzyme that is released upon cell death and lysis of the cell membrane. Extracellular LDH is both chemically and biologically stable; the magnitude of LDH efflux in the cultures correlates in a linear fashion with the number of neurons damaged by glutamate exposure (Kho and Choi, 1987). The LDH
assay is a well-established procedure for quantification of excitotoxicity in vitro (Kho and Choi, 1987; Minor, 2006).

This study focuses on observing modulation of the neurosteroids PS and DHEAS on the AMPA receptor; however the AMPA receptor is only one part of a very complicated system affected by glutamate excitotoxicity. The function of the AMPA receptor is shown to be negatively modulated upon exposure to the neurosteroid PS; however the NMDA receptor, another major ionotropic receptor activated by glutamate, is inversely affected. Electrophysiological studies have suggested NMDA activation is increased upon exposure to the neurosteroid PS, allowing further depolarization of the neuronal cell membrane (Wu et al., 1998) and consequently increasing levels of neuronal cell death. Higher concentrations of PS (100μM) have been shown to increase neuronal cell death in vitro 10-15% after a 24-hour concurrent exposure with NMDA (Melchior and Muir, 2010). Electrophysiological studies of the affect of PS on the kainate receptor also suggest that activity of the kainate receptor is inhibited upon exposure to the neurosteroid PS, causing a decrease in the depolarization of the membrane incurred by agonist binding (Wu et al., 1998). There is a wide range of glutamate receptors activated by the uncontrolled release of the excitatory neurotransmitter during TBI that elicit a combination of downstream events including modulation of membrane potentials, disruption of specific ionic gradients, and activation of secondary messenger systems. This experimental evidence exemplifies the complexity of neuronal communication and modulation of the events following trauma to the brain.

Concentrations of PS required to attenuate AMPA neurotoxicity in vitro were much higher than those reported in adult-normal rat brain, in human plasma and in aged human brain tissue. However, synthesis and release of neuroactive steroids may be reinforced under several
pathological conditions including cerebral ischemia, epilepsy and depression, and prove to be a viable target for drug synthesis (Shirakawa et al., 2005). Further research in the field of TBI is required to understand the specific mechanisms that lead to disabilities and death in order to develop both therapeutic action and preventative treatment at the cellular level. Continued research in the study should include different models of measuring the level of cell death including cortical slice cultures and Nissl staining, as is evident in similar research. In addition other, prospective neurosteroids should be included in hope of providing a substantial evidence of neuroprotection to elicit the development of a drug treatment method to decrease delayed injury following TBI. The time course of excitotoxicity may also be a subject of future investigation, as suggested by this study. Delayed injuries continue to cause problems with cognitive function and mental abilities following an injury to the brain, consequently the characterization of secondary injury factors and specific biomechanical operations will further provide insight into treatment and therapy for those who suffer from TBI.

Animal research is an essential part of biomedical research and has a vital role in discovery and development of cures and therapeutic treatment for human diseases. The use of rodent, specifically mouse, models in biomedical research is ideal due to the anatomical and physiological similarities between rodents and humans. Virtually every major medical advance involving human disease and disability has been achieved through biomedical research involving animal models. Continued basic research is needed to fully understand the incredibly complicated mechanisms underlying pathology of traumatic brain injury in order to develop safe and effective treatment methods of patients who suffer from TBI.
Literature Cited:


Appendix A:

Experimental Calculations and Details:

Equation 1:

Calculation final volumes of corresponding drug concentrations from stock solutions.

\[ M_1 V_1 = M_2 V_2 \]

\[ 4500 \mu L \times 10 \mu M \text{ MK-801} = 2000 \mu M \text{ MK-801} \times V_2 \]
\[ V_2 = 22.5 \mu L \text{ MK-801 solution} \]

\[ 4500 \mu L \times 3 \mu M \text{ AMPA} = 10000 \mu M \text{ AMPA} \times V_2 \]
\[ V_2 = 1.35 \mu L \text{ AMPA solution} \]

<table>
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<tr>
<th>Concentration of AMPA</th>
<th>Volume AMPA Stock (10mM)</th>
<th>Volume MK-801 Stock (2mM)</th>
<th>Volume of PS Stock (10mM)</th>
<th>DMSO (w/o PS Treatment)</th>
<th>Total Volume of Drug Stock</th>
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<td>0 \mu L</td>
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Chart of respective volumes in each drug solution for the AMPA DR with 100 \mu M PS experiment
Appendix B:

LDH Assay Details:

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Enzyme used was Level II Control Serum

LDH standard curve was plotted on a chart. R-squared value maintained above 0.94 and recorded to ensure accuracy of results as compared to the standards.

LDH Results from 10/31/10: AMPA DR with and without 50μM PS.

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<td>27AMPA</td>
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<td>+ 50 PS</td>
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<td>BK, 50PS</td>
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The data derived from each test well was averaged and normalized with the full kill solution.

After normalizing the data, the blank, accounting for sham injury, or LDH release not associated with exposure to experimental conditions, was subtracted from each test group so that the blank tests represented 0% cell death, and the 300μM NMDA, full kill, test group (100% cell death).

<table>
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<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
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The normalized data was plotted on a column graph and analyzed following each experimental procedure.
Appendix C:

Pictured are mixed astrocyte-neuron cell cultures viewed under a light microscope. The photograph left is a grouping of neuronal cell bodies (grey surrounded by a white halo) with neuritis (dark, branch-like extensions) extending in different directions. This picture is at 40x magnification. Right is several groupings of neuronal cell bodies, with a good view of the underlying astrocyte bed (darker grey, resembling cobble stones). The neurons require the plating of astrocytes in this experimental model to support the livelihood of the neurons as well as regulate their external environment. This picture is at 20x magnification. The induction of injury by selected concentration of AMPA causes the noticeable degeneration and destruction of neuritis and neuronal cell bodies through visual observation under a light microscope in a dose dependent manner.