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# Current Therapeutic Modalities, Enzyme Kinetics, and Redox Proteomics in Traumatic Brain Injury

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Additional information is available at the end of the chapter

## 1. Introduction

Traumatic brain injury (TBI) is an event that occurs suddenly and has long lasting effects to the brain that are dependent on the severity of insult. Symptoms can range from mild to severe. It is a well-established notion that imbalances in the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and native antioxidant mechanisms have been shown to increase oxidative stress. Multiple research efforts have evaluated the use of pre-injury therapies on moderate TBI; however since traumatic brain injuries are sudden, treatment should begin post-injury. There is no known cure for TBI, although flavonoids, neurosteroids, statins, gamma-glutamylcysteine ethyl ester, and novel histone deacteylase inhibitors show therapeutic promise. Hence, therapeutic strategies that improve outcomes following injury and the time at which treatment is most beneficial is paramount. As traumatic brain injury has been shown to alter energy metabolism and the use of redox proteomics and enzyme kinetics has been used to investigate the oxidative modification of proteins that may lead to reduced cognition observed in TBI patients. This chapter will evaluate the current therapeutic modalities for traumatic brain injury in various animal models as well as affected patients. It will also discuss the role of redox proteomics and enzyme kinetics in traumatic brain injury, most importantly how these oxidatively modified proteins play a role in learning and memory, both of which are effected in those affected by traumatic brain injury.

## 2. Current models

The field of traumatic brain injury research employs multiple animal models to investigate consequences of this devastating injury. Glutathione (GSH) is the major antioxidant in the brain, therefore treatment of neurodegenerative diseases including traumatic brain injury (TBI) with GSH is of great interest. Alzheimer's disease and other classically diagnosed neurodegenerative diseases have similar patterns of decreased GSH as found in blast induced TBI by fluorescence assay [1]. Proposed studies utilizing rat models incorporate prevention of secondary insults post-TBI with a glutathione mimetic. Omega-3 fatty acids from fish oils as a dietary supplement are used in multiple mild TBI rat models as a preventative measure [2]. This study was found to show cognitive improvement and weight loss recovery post-injury when a 6% fish oil supplement containing omega-3 fatty acids was given as a regular dietary supplement prior to the injury [2]. Axonal damage can cause detrimental effects on the brain. Axonal damage due to TBI results in loss of elasticity in axons making them brittle, swollen, and dramatically stretched allowing calcium to enter the axons leading to extensive damage by initiating a protease cascade [3]. In order to determine the efficiency and safety of future human TBI treatment for restoration of axonal damage, recently rat models have been used as a preliminary study for stem cell treatment for TBI [4]. While understanding axonal damage is important when researching TBI, metabolic rat model studies are exploring cerebral glucose uptake in TBI metabolic pathways [5]. Results show that a decrease in cerebral glucose uptake occurs in TBI rat models with axonal damage and glial activation [5]. Therefore, glucose is unable to be transported out of the cell for use as fuel to the body, much like insulin resistance in Type-2 diabetes. Additionally, rat models are utilized to determine histone deacetylase inhibitors (HDACi) in post injury treatment as a neuroprotectant in acute TBI [6]. HDAC inhibitors activate neurotrophic tyrosine kinase receptor type 1 (TrKA) and nerve growth factor (NGF) expression present significant results in preventing cell apoptosis [6]. Consequently, HDAC inhibitors are able to prevent cell death post-TBI. Findings support a decrease of glial fibrillary acidic protein (GFAP), a biomarker for neurotoxicity in damaged tissues [6, 7]. As findings in rat studies are gaining positive results, progressive animal models with physiology closer to humans are being incorporated into TBI research.

TBI is a growing issue among military personnel, athletes, and victims of all ages and backgrounds; therefore, it is of great importance to include a variety of animal models with physiological properties close to humans. Use of a neonatal piglet TBI model has increased due to their similarities in human myelin sheath, tissue morphology, cerebral structure, and development [8]. Neonatal piglet TBI research allows for future research in behavioral and cognitive assessment and is presumed to be utilized as a tool for preventing secondary injury, such as stroke and resuscitation strategies [9]. Canine epilepsy due to traumatic brain injury has brought insight into therapeutic strategies for posttraumatic epilepsy (PTE) as secondary insult of human TBI [10]. Researchers hope to find treatments in the canine model that will greatly benefit clinical research of TBI as epilepsy and seizures are a severe secondary insult that can lead to detrimental cognitive issues, specifically due to the seizures increasing weeks after the initial injury [10, 11]. Hypothermia treatment has been considered for post-TBI therapy, although clinical trials have not been completed at this time [12]. Previous research

demonstrated that rat and swine models have shown positive results with hypothermia experiments as preventative measures against neurodegeneration and currently non-human primates are the prime model for this study [12-14]. A product known as the "chiller-pad" has been used in trials to determine its effectiveness on neuroprotection. Results are encouraging, although the need for a craniotomy to allow hypothermia to be addressed at an exact locality is a perceived downside [12, 13]. It is unclear how hypothermia protects neurodegeneration; however it is necessary to localize cooling, as to not reduce overall body temperature [12]. Rhesus monkey models ranging from ages 3.9 to 14 years old have been used in a long-term study to determine the effects of microglia as defense against injured neurons in the brain and spinal cord after TBI [15]. Most studies appear to monitor the crucial timeframe of TBI, which typically falls immediately after the injury's occurrence [15, 16]. This study allows scientists to view the effects of progression or degression of each monkey for one year [15, 17]. The results encourage further study into therapy with genetic manipulation of microglial phenotype to determine the functionality of impairment on the recovery process as well as anti-inflammatory treatment [15, 17].

Overall, the previous and current studies for traumatic brain injury have been beneficially positive for future studies and treatment for TBI. Researchers are aware that there is still an informational gap in understanding the mechanisms behind TBI and therapeutic strategies, but with future studies and the scientific, as well as public communities' increasing awareness, there will be a bridge to close that gap of uncertainty [18].

## 3. Pre and post injury strategies

Pre-injury traumatic brain injury (TBI) strategies occur prior to the injury as preventative measures, while post-injury TBI strategies are therapeutic measures taken to prevent secondary insult from occurring after the injury, as there is no way to reverse the initial injury, but further damage can be prevented. With the increasing awareness of TBI, research for preventative and post injury measures is on the rise. There are numerous studies with a variety of computational and animal models that depict a range of TBI severities. There are definite correlations between past and present studies as well as connections between different animal models, with a hopeful finality for positive treatment of humans with TBI.

Researchers are developing statistical models to compare different TBI patient populations in order to determine which new therapies would be most beneficial. The Traumatic Brain Injury National Data and Statistical Center has developed a longitudinal prospective cohort study utilizing Extended Glasgow Outcome scale (GOS-E) scores to determine the furtherance of secondary insult over a period time after the TBI has occurred [19]. The parameters for this model include initial (GOS-E) scores, Functional measure of independence (FIMTM), race, gender, and reusable learning objects (RLOS) [19, 20]. In addition to statistical data, many of the testing strategies require the use of psychological testing to determine pre and post coping models of TBI in humans. The testing mechanism for these models include Coping Scale for Adults (Short Version), Quality of Life Inventory, Sydney Psychosocial Reintegration Scale,

and Hospital Anxiety and Depression Scale during 36 months post-injury [21]. In 2008, the Department of Veterans Affairs Polytrauma System of Care partnered with the Department of Education to perform ongoing research with longitudinal patterns using the Traumatic Brain Injury Model Systems of Care (TBIMS) project in order to keep track of rehabilitating veterans with TBI [22].

Where some studies investigate computational approaches to evaluating traumatic brain injury progression in patients, others are utilizing various animal models and combinatory models to determine the most effective way to treat TBI in humans. Patients with a combination of TBI and acute respiratory distress syndrome (ARDS) have symptoms of intracranial pressure, cerebral perfusion pressure, partial pressure of carbon dioxide in the blood ($PaCO_2$), and fractional inspired oxygen [23]. High-frequency oscillation (HFO) and tracheal gas insufflation (TGI) were found to ameliorate gas-exchange and alleviate the pressures caused by these symptoms [23]. Studies suggest monitoring beyond intracranial pressure symptoms by using the brain tissue oxygenated pressure ($PbtO_2$) targeted therapy, transcranial doppler, and cerebral microdialysis to individualize treatment plans of TBI patients [24]. A link between Alzheimer's disease (AD) development and post-TBI in humans is being hypothesized due to ongoing research. Cleavage of amyloid precursor protein (APP), which produces amyloid-β (Aβ) peptide, is a common hallmark in AD. At autopsy, TBI patients have reported Aβ peptides and neurofibrillary tangles of hyperphosphorylated tau proteins, thus leading to the hypothesis that AD can possibly be a secondary effect of TBI [25]. A past study showed that an increase of neuronal C5a receptors and C5b-9 terminal complex, a promoter of the cell cycle and cell lysis, in diffuse axonal injury (DAI) of TBI, could lead to possible secondary neuronal cell death [26, 27]. New studies show that the C5a receptors and C5b-9 terminal complex should be used as targeted treatment areas, but are not necessarily a source of secondary axotomy in DAI [28].

Alternate studies have utilized medication to treat and prevent secondary insults due to the TBI. Progesterone, a cholesterol derived hormone, has been used in rat and human models as a neuroprotectant [29]. Progesterone (PROG) is converted into allopregnanolone (ALLO), which is beneficial to an injured brain. Although it is unknown how the PROG metabolic process assists in the recovery of TBI patients; progesterone is one of the first applicable medicinal treatments for TBI in humans [29]. The ProTECT III study has reached human clinical trials for progesterone treatment in traumatic brain injuries [30]. The ProTECT program has shown great benefits of progesterone as a neuroprotective agent due to its ability to enter the blood brain barrier quickly to decrease damage, downregulate inflammation, and limit inclusive cellular necrosis, apoptosis, and cerebral edema [31, 32]. This program has been carried out in several medical institutions including Emory University, University of Kentucky, and The Ohio State University. A useful tool for monitoring TBI in human patients is the time-resolved optical method [33]. This method is used to obtain data regarding reflected photons and fluorescence signals with indocyanine green (ICG) to determine brain perfusion in TBI patients [33]. During this optical method study researchers were able to differentiate between healthy patients with TBI [33]. As the science behind human studies of TBI becomes progressively clearer, increasing animal model studies are developing promising results. With

the collaboration of scientists, clinical studies representatives, statisticians, and induced TBI studies, therapies used to fight TBI will continue to move in a positive direction.
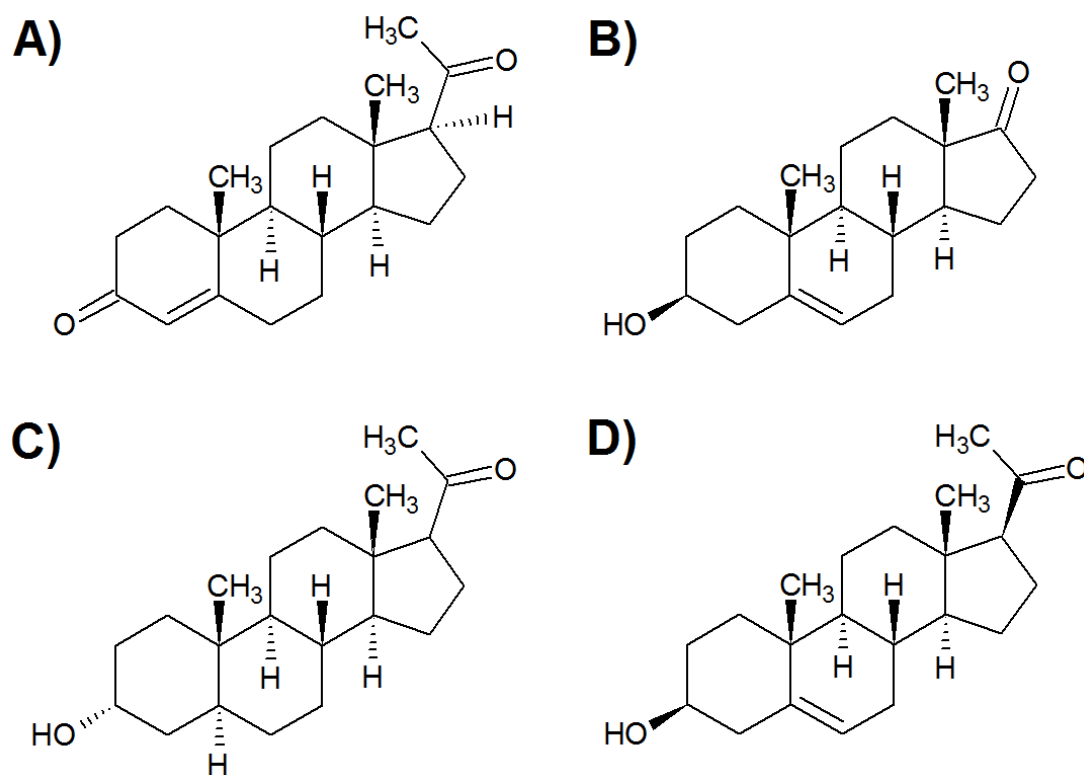
# 4. Neurosteroids

The nervous system is capable of synthesizing its own reservoir of steroids, termed neurosteroids due to their origins, both independently of serum steroid levels as well by utilizing serum steroids. These were first observed by Baulieu in 1981 [34, 35]. Using cholesterol as a starting material, endogenous steroid production in the brain and other organs amounts to a highly involved pathway map of enzymatic action employing a number of cytochrome P450 enzymes among others. Overall, this biosynthesis pathway is called neurosteroidogenesis (Figure 1). The majority of these steroidogenic enzymes are expressed at different locations around the nervous system and at multiple sites within neurons, indicating that a high amount of coordination is involved in neurosteroid production [36]. This amount of coordination highlights the importance of regulating such highly potent compounds. The pervasive functions of neurosteroids involving binding and modulating neurotransmitter receptor, in addition to influencing the genome via nuclear steroid receptors, implicate neurosteroids in a number of neurological processes and thus possible candidates for post-injury treatment of traumatic brain injury (TBI) [29, 36].
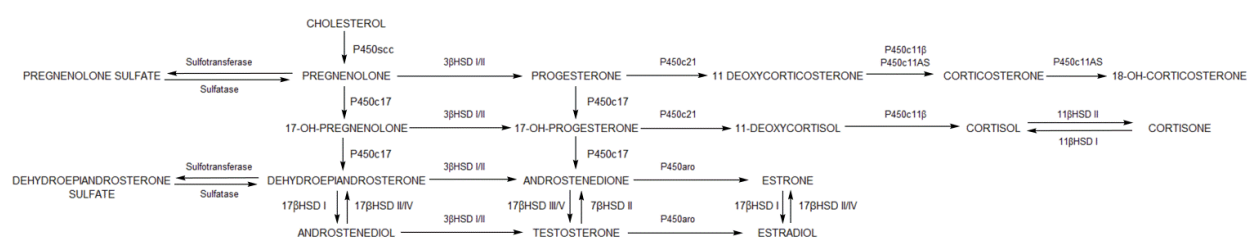


**Figure 1.** Pathway of neurosteroidogenesis and neurosteroidogenic enzymes. Cholesterol is initially converted into pregnenolone. Neurosteroids are then synthesized from pregnenolone through different pathways involving various enzymes. P450scc, mitochondrial cholesterol side chain cleavage enzyme, mediates c20-hydroxylation, 22-hydroxylation, and scission of the c20–22 bond; P450c17, mitochondrial 17-hydroxylase, mediates 17α-hydroxylation and scission between c17-20 bond; P450c 21, mitochondrial 21-hydroxylase, mediates 21-hydroxylation; P450aro, mitochondrial aromatase, mediates two 19-hydroxylations and one 2-hydroxylation; P450c11β, mitochondrial 11β-hydroxylase, mediates 11-hydroxylation; P450c11AS, mitochondrial aldosterone synthase, mediates c11,18-hydroxylation and 18-oxidation; P450c11B3, the third mitochondrial 11β-hydroxylase, mediates 11β and18-hydroxylation; 3β-HSD, 3β-hydroxysteroid dehydrogenases, mediates both 3β -hydroxysteroid dehydrogenase and D5-D4-isomerase activities; 17β-HSD, 17-ketosteroid reductase (KSR), mediates c17β reduction or c17 oxidation; HST, sulfotransferase, mediates 3β-hydroxylation with sulfate groups; STS, sulfatase, hydrolyzes sulfate groups at 3β.

A number of neurotransmitter receptors are modulated by neurosteroids, including the γ-aminobutyric acid (GABA$_A$), N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, glycine, 5-hydroxytryptamine (5-HT$_3$ or serotonin), sigma type 1 (σ$_1$), nicotinic acetylcholine, and oxytocin receptors, and the modulation can be positive or negative depending on the steroid identity [36]. Although some steroids produced in other endocrine organs are still found in the brain, the physiological role

and significance of neurosteroids are different in the brain. Neurosteroids affect the development, structure, and function of the central nervous system. This diverse population of neurotransmitter receptors illustrates how influential neurosteroids are on the brain. The complexity of these interactions is illustrated by considering how similar steroid structures can have different effects on the same receptor and how these minor structure alterations are tightly regulated. In particular, the neurosteroids progesterone, dehydroepiandrosterone (DHEA), pregnenolone, and allopregnenolone (ALLO), illustrated in Figure 2, are potential post-TBI therapeutic candidates due to their roles in various aspects of neurogenesis and the repair and survival of neurons [37].



**Figure 2.** Progesterone (A), dehydroepiandrosterone (B), pregnenolone (C), and allopregnenolone (D) are potentially beneficial neurosteroids for the treatment of TBI.

DHEA promotes axonal growth and functional activity of specific neuronal networks, while ALLO can induce axonal regression in the developing hippocampus. Progesterone can regulate myelin formation, improve the myelination of injured nerves *in vivo*, facilitate myelination *in vitro*, and induce oligodendrocyte maturation *in* vitro. Moreover, neurosteroids can modulate neuronal excitability and activity by interacting with different types of receptors. There are mainly two different action mechanisms that neurosteroids undergo to carry out their biological functions in the brain: genomic actions and nongenomic actions. Genomic actions are mediated by nuclear steroid receptors, such as progesterone, glucocorticoid, and mineralocorticoid receptors, while nongenomic actions are mediated by neurotransmitter receptors, such as $GABA_A$, N-methyl-D-aspartate (NMDA), and sigma receptors. Classical

nuclear steroid receptors regulate gene transcription so it is a long-term process. PROG binds both to progesterone and neurotransmitter receptors. Corticosterone binds to glucocorticoid receptors with a low affinity, while both corticosterone and aldosterone bind to mineralocorticoid receptors with a high affinity. However, the acute effects of neurosteroids are not related to nuclear steroid receptors, but the majority of neurosteroids interact with neuronal membrane receptors and ion channels [38].

As positive modulator(s) of the $GABA_A$ and kainate receptors and negative modulator(s) of the glycine, $5\text{-}HT_3$, nicotinic, acetylcholine, and oxytocin receptors, progesterone and its derivatives could play potential roles in neuroregeneration involving reducing inflammation, helping remyelinate neurons and reducing inflammation [36, 37]. In part by negatively modulating the $GABA_A$ receptor and positively modulating the NMDA and $\sigma_1$ receptors, DHEA and its sulfate derivative (DHEAS) promote dendritic growth and branching and form synapses as well as help with memory [36]. DHEA protects against oxidative damage in hippocampal neurons and glutamate toxicity by inhibiting nitric oxide synthase and G-protein-coupled receptors that activate cell-survival pathways [39-41]. Pregnenolone sulfate is a negative modulator of $GABA_A$, kainate, AMPA, glycine, and $\sigma_1$ receptors and a positive modulator of NMDA receptors that has no effect on $5\text{-}HT_3$ receptors, helping this neurosteroid play an important role in benefiting memory [36]. ALLO, a potent positive allosteric modulator of $GABA_A$, binds to discrete site on the $GABA_A$ receptor, thereby increasing the mean open time and decreasing the mean closed time of the channel, resulting in the increase in the chloride current through the channel and consequently in a reduction of neuronal excitability. NMDA receptors exhibit at least two distinct sites for neurosteroid modulation, mediates the effects of either positive or negative modulators. A significant number of studies have shown the neuroprotective effects of neurosteroids against many pathologic conditions (Figure 3). PROG and ALLO reduce cell damage and improve outcomes of focal ischemia in a stroke model [42]. Allopregnenolone, while contrasting pregnenolone's benefits on memory, does help relieve anxiety [36]. Many studies focus on the neuroprotective role of estrogen specifically in the hippocampus and as it improves cognitive function against disease, aging, and stroke, mitigating cell death and stimulating neuronal proliferation in the hippocampus and other structures [43]. Testosterone also shows a neuroprotective effect, but it is partly due to its conversion to estrogen in the CNS [44]. Testosterone protects primary human fetal neurons against serum-deprivation-induced cell death and oxidative stress through androgen receptors [45, 46].The structures for these described neurosteroids can be seen in Figure 3.

Neurosteroids can affect brain function by not only interacting with neurons, but also acting on glia cells, such as microglia, Schwann cells, oligodendrocytes, and astroglia. By acting on microglia and Schwann cells myelination is modulated, while acting on oligodendroglia and astroglia the response of nerve tissue to pathological insults is altered. Specifically, the effect of neurosteroids on astroglia is crucial because astroglia play critical roles both in the central nerve system (CNS) and neural signaling by regulating extracellular ion concentrations and local cerebral blood flow, and by modulating synaptic transmission and plasticity [35]. Due to their wide variety of influences on important neurotransmitter receptors, neurosteroids have a large potential benefit for neuronal protection and repair following TBI. The various roles of
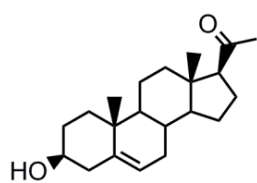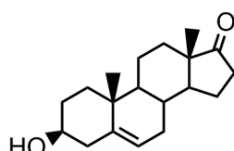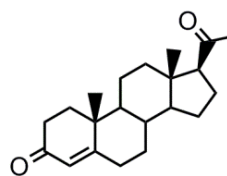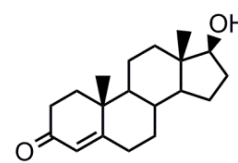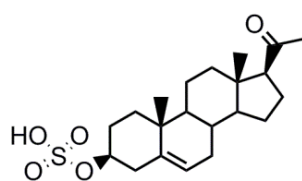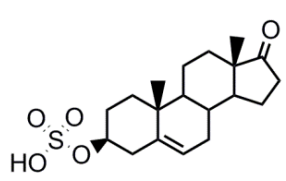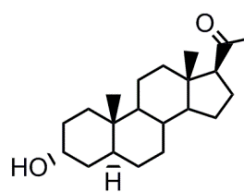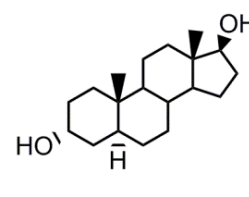
**(A)**
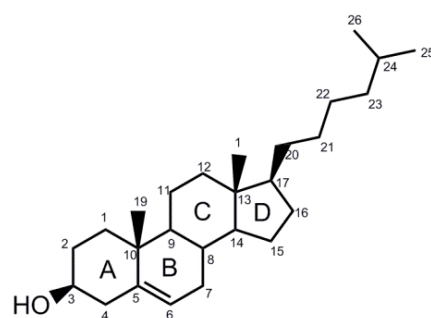


Cholesterol

**(B)**



| Pregnenolone (PREG) | Dehydroepiandrosterone (DHEA) | Progesterone (PROG) | Testosterone |

Pregnenolone sulfate (PREGS)    Dehydroepiandrosterone sulfate (DHEAS)    Allopregnanolone (ALLO)    Androstanediol

**Figure 3.** Chemical structure of cholesterol and neurosteroids. Cholesterol (A) is converted into various neurosteroids (B). Each neurosteroid has significant roles in the brain and nerve system, but these neurosteroids, specifically, have shown neuroprotective properties.

neurosteroids such as progesterone, dehydroepiandrosterone, pregnenolone, and allopregnenolone give them special consideration as post-TBI therapeutics, and progesterone research in particular is offering promising results [29, 37, 47]. Studies in rodents and humans have shown beneficial effects of PROG on both mortality and functional outcomes following TBI by its neuroprotective effect [48]. In addition to PROG, its metabolite, ALLO also has shown beneficial effects equal to or in excess of those of PROG in TBI by modulating the $GABA_A$ receptor, not classic nuclear receptors [29]. Both PROG and ALLO have beneficial effects in reducing blood-brain barrier dysfunction and intracranial pressure following TBI, and also in reducing brain swelling and edema that are associated with TBI [49]. DHEAS and its analog, fluasterone (DHEF), can also improve recovery of function in male rats after TBI [50, 51]. These findings altogether bolster the neuroprotective effects of neurosteroids and their analogs thereby, leading to plausible future therapeutic strategies in the clinical treatment of TBI.
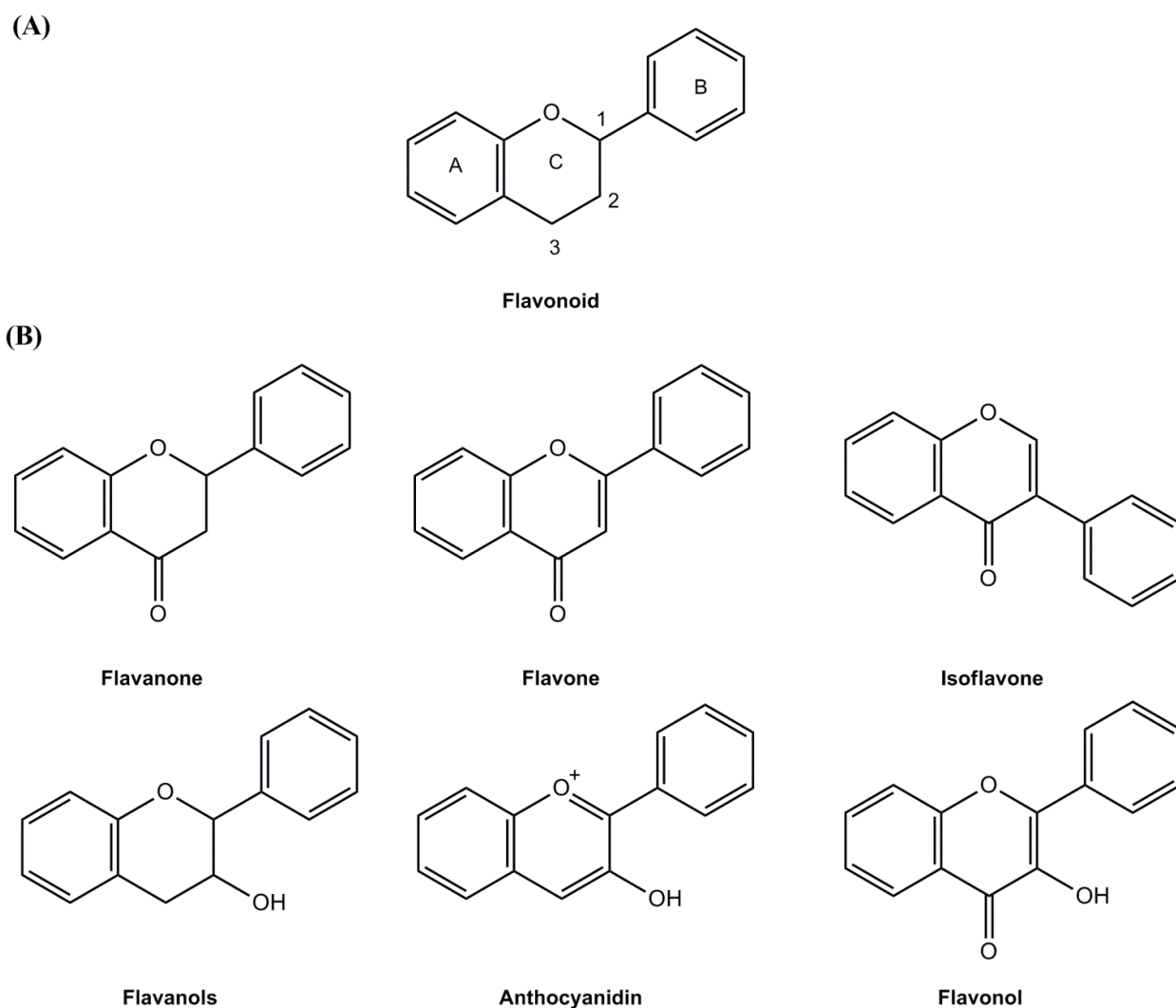
## 5. Flavonoids

Secondary events following a traumatic brain injury (TBI) account for the majority of neuro-degeneration, beginning within the first hour post-TBI and lasting for days [52]. The elevation of ROS levels within neurons has been recognized as an early event following TBI occurring within minutes [53].The overproduction of ROS due to TBI cannot be eliminated by the limited amount of antioxidants, leading to oxidative stress, one of the most common secondary injury mechanisms. These remaining ROS begin to interact with proteins, lipids, carbohydrates, nucleic acids, or signaling molecules. The oxidative modification of neuronal molecules changes their structures and function as well as signaling pathways, subsequently leading to irreversible neuronal inflammation, dysfunction, and death. Due to the initial rush of rapid superoxide ($O_2^-$) production during the first hour and the shift to a pro-oxidant environment that occurs as early as 3 hours post-TBI, free radicals play an integral part in the initial events following a TBI and continue to react with cellular components for several days, with their prevalence peaking between one and two days after the initial injury [52-54]. As revealed by Ansari, et al. there is a narrow window of opportunity for post-TBI therapeutic intervention due to this shift towards an oxidative environment [54]. Thus, supplementing the brain with substances having potent antioxidant capacities, such as flavonoid compounds, as early as possible could be a beneficial treatment for mitigating the otherwise inevitable cascade of secondary damage.

The flavonoid class of compounds describes molecules that share one of the three similar backbones illustrated in Figures 4 and 5. Flavonoids are polyphenolic compounds with a diphenylpropane skeleton which consists of two aromatic rings, (A ring and B ring) bound together by three carbon atoms ($C_1$, $C_2$, and $C_3$) forming an oxygenated heterocycle (C ring) as depicted in Figure 5A. These compounds are divided into six major classes depending on their saturation and the number and arrangement of either the carbonyl group or hydroxyl groups in the C ring (Figure 5B). Flavanones contain a carbonyl group at $C_3$, flavones have a carbonyl group at $C_3$ and a double bond between $C_1$ and $C_2$, isoflavones have a carbonyl group at $C_3$, a double bond between $C_1$ and $C_2$, but the B ring is connected to $C_2$ instead of $C_1$, flavanols contain a hydroxyl group at $C_2$, while anthocyanidins have a hydroxyl group at $C_2$, one double bond between oxygen and $C_1$ and another between $C_2$ and $C_3$, lastly flavonols have a carbonyl group at $C_3$ and a hydroxyl group at $C_2$. Furthermore, there are a variety of flavonoids present within each group due to the differences in the number and arrangement of hydroxyl group in the A and B rings in the structure (Figure 5B). The relative antioxidant ability of individual flavonoids to scavenge ROS is correlated with these different chemical structures [55]. The unsaturation of the C ring enhances the scavenging ability of flavonoids by increasing their stability via electron delocalization. The effects of the number and the arrangement of these hydroxyl groups also affect antioxidant activity. For example, the absence of a hydroxyl group at $C_2$ of the C ring decreases the activity, while the presence of two hydroxyl groups in the *ortho*-diphenolic arrangement in the B ring increases the activity against superoxide anion. In addition, the presence of a catechol group also enhances the ability against peroxynitrite scavengers, because catechol can afford a two-electron reduction of peroxynitrite to nitrite coupled to their oxidation to the corresponding *o*-quinones [56].
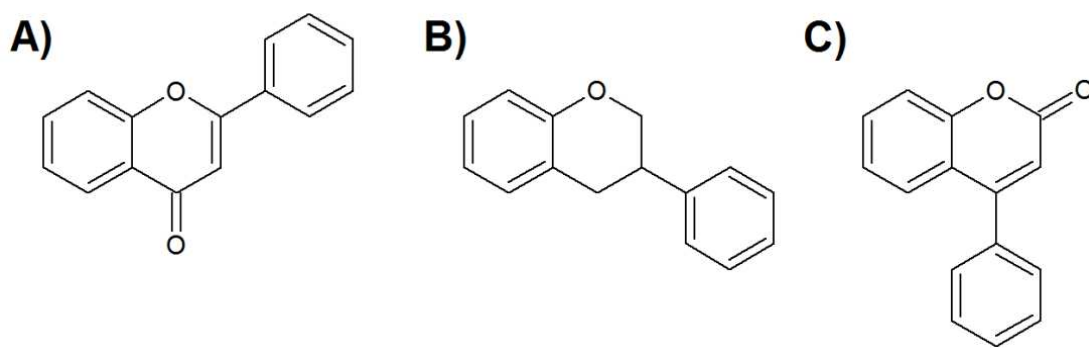
**Figure 4.** The flavone (A), isoflavan (B), and neoflavonoid (C) structures serve as the chemical backbones for flavonoid compounds.



**Figure 5.** Chemical structure of flavonoids. Basic chemical structure (A) and six classes (B) including: flavanones, flavones, isoflavones, flavanols, anthocyanidins, and flavonols.

Many natural flavonoids can be found in plant material such as fruits, vegetables, and plant-derived beverages, such as green or black tea. Once introduced into the body, flavonoids are converted into their metabolites which can have substantially different bioactivity properties. These structures have been well established as antioxidants that reduce oxidative stress not only by directly scavenging reactive oxygen species (ROS), but also by increasing the activity of antioxidant enzymes or decreasing the activity of redox enzymes respectively [57]. More-over, flavonoids can modulate cellular homeostasis helping to reduce inflammation and cell toxicity [57].These compounds have neuroprotective properties in addition to their abilities to scavenge free radicals [34, 58, 59]. Due in part to the these properties and the abilities of certain flavonoids to cross the blood-brain barrier (BBB), flavonoids appear to be excellent treatment opportunities for TBI models [59]. Indeed, extensive therapeutic use of flavonoids and similar polyphenolic compounds post-TBI have proven this to be true, with these compounds serving to remedy many TBI-induced maladies such as edema, neuroinflammation, cognitive and neuronal functions, cell survival, oxidative stress, and cellular energy homeostasis [60-63]. Catechin, epicatechin, and epigallocatechin gallate (EGCG), chemicals all found in green tea (Figure 6A), reduce the production of nitric oxide and hydrogen peroxide, lipid peroxidation, and DNA oxidation induced by ischemia/reperfusion in rats [64]. Several flavones including baicalein, baicalin, and wogonin (Figure 6B) components of the *Scutellaria* plant, reduce lipid peroxidation and nitric oxide production in the cortex and hippocampus of rats as well as inhibit pro-inflammatory cytokines [65]. The down-regulation of the $NF_kB$ pathway confirmed with medicinal extracts rich in flavonoids, such as *Crataegus,* orally administered and protected the brain against delayed cell death by increasing the levels of antioxidants in the brain, Ginkgo biloba extract (EGb 761) [66]. It has been well documented that metabolites of flavonoids also have neuroprotective properties. The death of both cortical and striatal neurons induced by oxidative stress has been shown to be inhibited by the methylated metabolite of epicatechin with a protection capacity similar to epicatechin [67]. Specifically, caffeic acid has shown neuroprotective properties and is a component of a patented mixture of flavonoid compounds, known as Pycnogenol® (PYC), which has produced very promising results in rat models of TBI [58, 68].

Based on studies showing that flavonoids have potent neuroprotective and anti-inflammatory properties, there are several recent studies in which flavonoids were used as post injury therapeutic strategies for TBI. These include epicatechin, wogonin, baicalein, and pycnogenol. Water containing EGCG, a major component of green tea possessing strong antioxidant properties, was given to rats after TBI and demonstrated the ability to inhibit free radical induced degradation that have the potential to differentiate into neurons and glia around the injured area [69]. The effects of wogonin and baicalein, flavonoids possessing potent anti-inflammatory properties, were investigated on mice or rats subjected to controlled cortical impact injury as post-injury treatments [61]. Their neuroprotective effects were suggested because they improve functional and histological outcomes, and reduced induction of pro-inflammatory cytokines. Prior to its use in TBI models, the bioflavonoid, PYC has exhibited various antioxidant properties as well as the abilities to prevent neurotoxicity and apoptosis [54, 70-74]. Scheff, et al. recently reported a number of benefits from post-TBI intraperitoneal

**(A)**



Catechin

Epicatechin

Epigallocatechin gallate (EGCG)

**(B)**

Baicalein
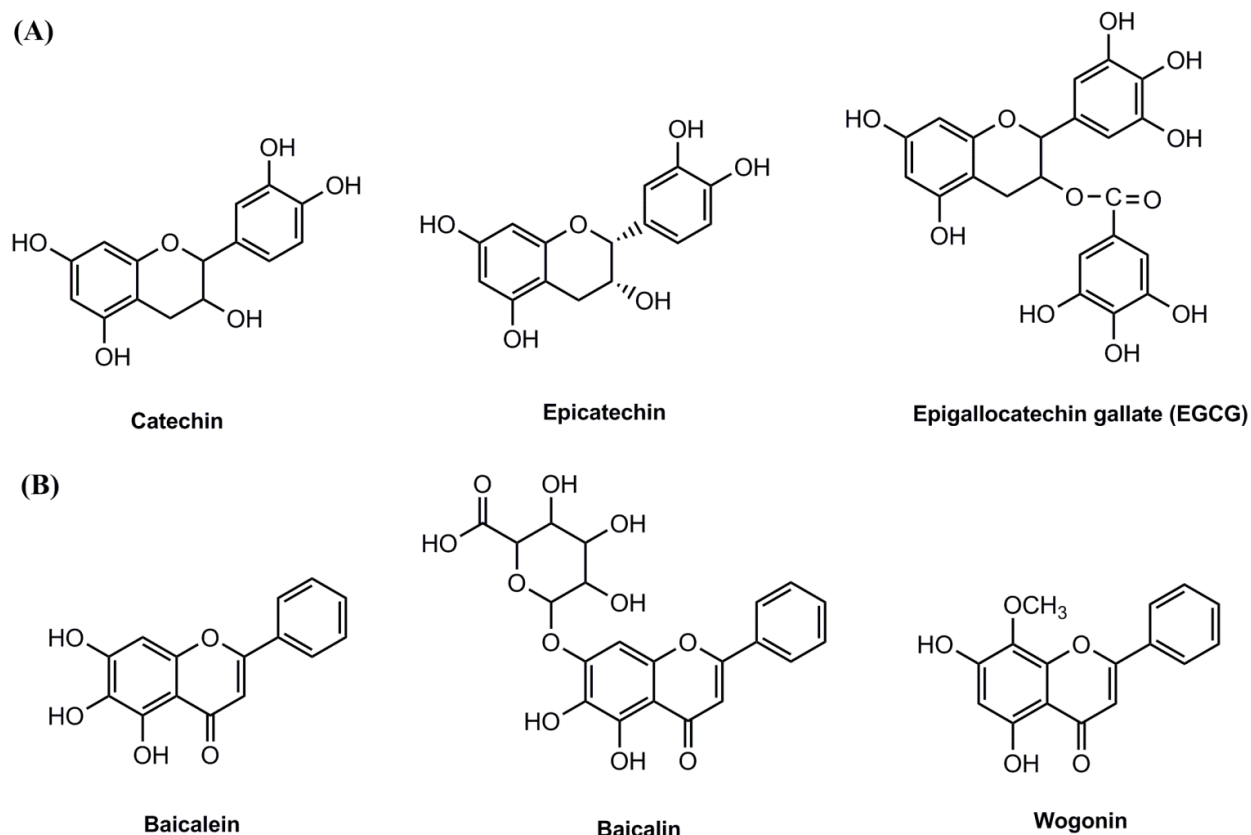
Baicalin

Wogonin

**Figure 6.** Flavonoids possess neuroprotective properties and therapeutic effects in TBI. Flavanols, such as catechin, epicatechin, and epigallocatechin gallate (EGCG), found in green tea (A). Flavones, such as baicalein, baicalin, and wogonin, are found in celery (B).

administration of PYC in rat models, including an increased presence of endogenous antioxidants in the hippocampus and cortex at 48 hours post-TBI, increased GSH:GSSG ratio in both regions at 48 hours post-TBI, and increased synaptic proteins at 96 hours post-TBI [58]. The neuroinflammatory cytokines interleukin-6 and tumor necrosis factor $\alpha$ were also significantly decreased at 48 hours post-TBI using this treatment. Because of the substantial amount of free radical-induced oxidative damage that occurs post-TBI, these increases in antioxidants and decreases in inflammatory markers not only highlight the benefits of the particular mixture of flavonoids in PYC, but also serves to further evidence the abilities of flavonoids in general to help re-establish the balance of antioxidants and oxidants to offset the development and progression of oxidative stress post-TBI.

Flavonoids such as those found in PYC offer a unique approach to TBI treatment, which aims to remedy the initial post-TBI cascade of oxidative damage and early decreases in antioxidant capacity. PYC has helped to prove the potential of flavonoids in mitigating the secondary events of TBI by supplementing the brain with antioxidants that cross the BBB. Because the initial injury of TBI is virtually untreatable, therapeutic intervention within the first three hours post-TBI with compounds such as flavonoids offer promising insight into treatments that will result in future clinical significance.

## 6. Statins

Statins are a class of drugs that inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in the endogenous production of cholesterol, as illustrated in Figure 7. As such, statins are prescribed to lower serum cholesterol levels in patients for the purpose of preventing heart disease. However, a number of benefits of statin use have also been reported for the treatment of traumatic brain injury (TBI) which are not related to lower cholesterol levels, including increasing synaptogenesis and angiogenesis, increasing neuronal survival, improving cognitive abilities, and decreasing lipid peroxidation, among others [75-77]. This versatility of statins gives them unique advantages over other potential treatments for TBI that are specialized for only certain injury-related symptoms [76].
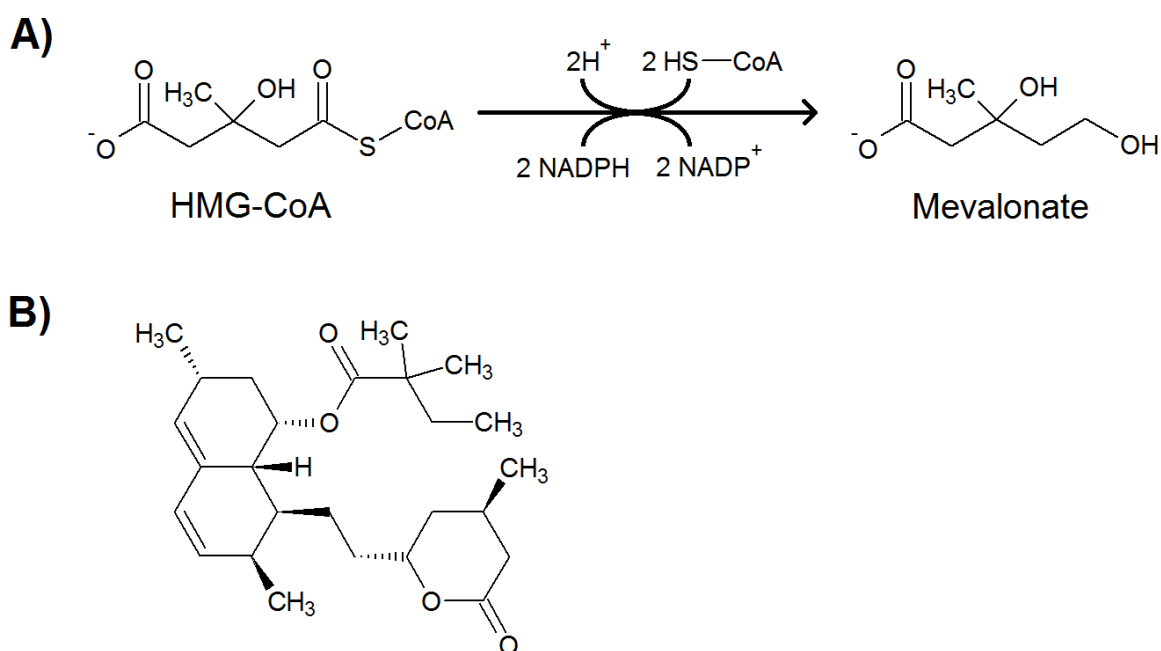


**Figure 7.** HMG-CoA reductase catalyzes the rate-limiting step of cholesterol formation (A). The structure for simvastatin, a statin drug that inhibits HMG-CoA reductase to decrease endogenous cholesterol synthesis (B).

Most of the neurodegeneration that results from a TBI is generated over the days following the initial insult [52]. The events causing this neurodegeneration, collectively referred to as the secondary injury, offer several opportunities for therapeutic intervention. There are currently no approved therapeutic treatments for TBI, so care is limited to non-chemotherapeutic methods of supporting normal cranial pressure and cerebral perfusion [6]. Because the primary injury of TBI creates a contusion around which cerebral blood flow is significantly limited, there is a high potential for ischemia and thus infarction as a secondary injury [78, 79]. In addition, many microscopic inflammatory processes are mediated largely by oxidative stress from excess free radical formation, which occurs in significant amounts within the first hour post-injury [52, 53]. Thus, because of the many benefits of statin use on endothelial function

and the role(s) of statins in preventing oxidative stress, this avenue of research offers significant promise for TBI treatment [80, 81].

Since Lu, et al. first began investigating the therapeutic potential of statins in 2004, a growing body of evidence illustrating the neuroprotective effects of statin use in both pre- and post-TBI treatment has been established [75, 77, 82-85]. However, the exact mechanism(s) by which statins execute this neuroprotection is not completely understood. Statins are known to provide many benefits to endothelial function independent of cholesterol levels, many of which involve nitric oxide (NO), a second messenger molecule deeply rooted in signaling pathways related to endothelial functions. These NO–related functions of statins include both upregulating and triggering activation of the enzyme endothelial NO synthase (eNOS), recovering eNOS function when oxidized low density lipoprotein is present, and recovering eNOS function during hypoxia [86-88]. Studies aimed at elucidating the exact mechanisms underlying the relationship between statins and eNOS have determined that inhibiting the synthesis of mevalonate has downstream inhibitory effects on the isoprenylation of the Rho protein, a member of a multifunctional family of GTPase proteins [89, 90].

As the direct relationships between statins, Rho, and eNOS begin to unravel, the importance of statins in potential post-TBI treatment will likely increase. Statins differ in their lipophilic properties, HMG-CoA reductase binding abilities, and their abilities to cross the blood-brain barrier (BBB), which will make the search for an ideal post-TBI candidate very involved. Simvastatin, depicted in Figure 7B, has proven useful in TBI treatments due in part to its BBB penetration, leading to significant benefits on BBB permeability, cerebral edema, and learning and memory [83, 91].

## 7. Gamma-glutamylcysteine Ethyl Ester

Glutathione (γ-glutamylcysteineglycine), illustrated in its reduced (GSH) and oxidized (GSSG) forms in Figure 8, plays a key role in cellular defense and repair, where it serves to maintain intracellular redox status by conjugating toxic species for export, regulating the activities of redox-sensitive enzymes, and neutralizing oxidative species via antioxidant enzyme mechanisms [92]. To highlight the significance of these responsibilities, glutathione exists at millimolar concentrations in the cell and its reduced state is carefully maintained to keep the GSH:GSSG ratio high [93]. The combination of high polyunsaturated fatty acid content, high oxygen respiration and glucose metabolism, and relatively low antioxidant capacity in the brain establishes an environment that is highly susceptible to oxidative stress. Thus, because the brain is not adequately prepared to defend against the almost immediate surge of reactive oxygen species (ROS) formation that is brought about by a traumatic brain injury (TBI), utilizing the native antioxidant mechanisms of the brain by increasing glutathione availability is a treatment route whose ability to curb initial inflammatory processes in the brain appears very promising [94, 95].
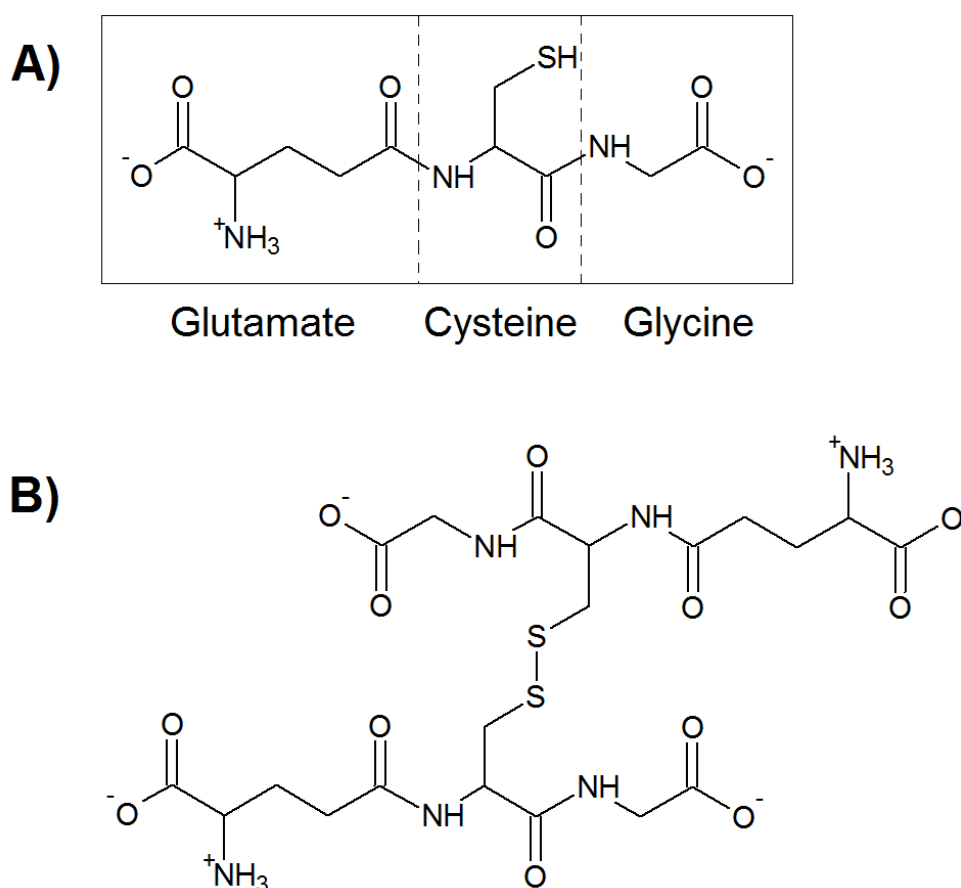
**Figure 8.** Glutathione can exist in its reduced form (A) and oxidized form (B) at physiological pH.

Synthesis of glutathione, illustrated in Figure 9, occurs in a two-step mechanism where glutamate and cysteine are first covalently bound via $\gamma$-linkage to produce $\gamma$-glutamylcysteine ($\gamma$-GC) by the enzyme $\gamma$-glutamylcysteine synthetase, and then glycine is covalently bound to $\gamma$-GC via $\alpha$-linkage to cysteine by the enzyme glutathione synthetase. This mechanism offers a prime opportunity to increase glutathione synthesis since regulation only occurs at the first step. Glutathione is a feedback inhibitor of $\gamma$-glutamylcysteine synthetase, prohibiting excess production of $\gamma$-GC. This step is also rate-limited by the availability of cysteine, whose intracellular concentration is lower than the concentrations of glutamate or glycine. However, glutathione synthetase is not inhibited by glutathione, so glutathione concentrations can be increased when excess $\gamma$-GC is supplemented [93]. In fact, intracerebroventricular adminis-tration of $\gamma$-GC increases brain glutathione content more so than intracerebroventricular administration of GSH ethyl ester or intraperitoneal administration of cysteine in rats [96]. The $\gamma$-GC analog, $\gamma$-glutamylcysteine ethyl ester (GCEE), shown in Figure 9B, has also proven to be an excellent candidate for supplementing intracellular $\gamma$-GC availability by increasing glutathione concentrations, and has been shown to decrease markers of oxidative stress both *in vitro* and *in vivo* [95, 97-99].

Various models of TBI have offered valuable insight into the mechanisms by which the brain incurs substantial damage following traumatic injury. Following the initial TBI injury,
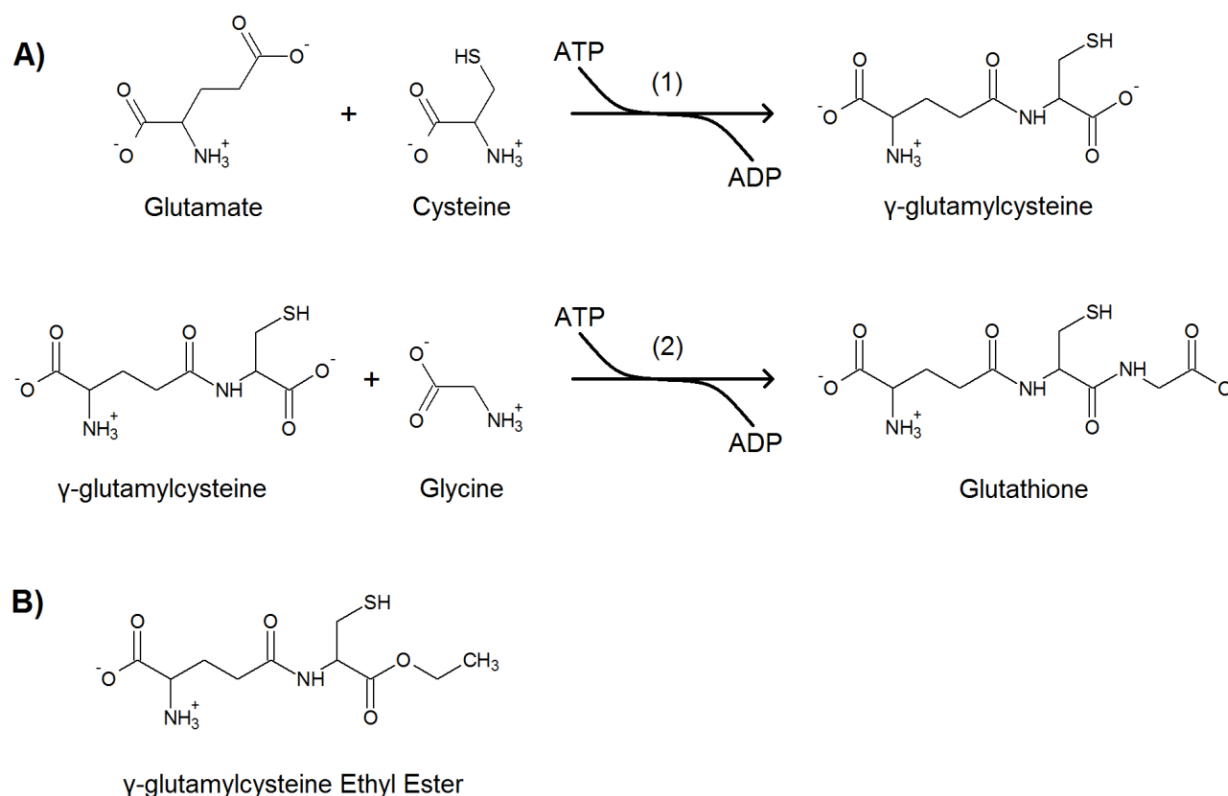
**Figure 9.** The enzymes γ-glutamylcysteine synthetase (1) and glutathione synthetase (2) synthesize glutathione in a two-step mechanism (A). The γ-glutamylcysteine analog γ-glutamylcysteine ethyl ester can increase glutathione synthesis (B).

secondary events can occur for days post-injury and these events account for the majority of TBI-induced neurodegeneration [52]. Due to the role of the superoxide radical ($O_2^-$) in creating an oxidative stress environment, the significant production of $O_2^-$ in cat brain during the first hour following a fluid percussion model of TBI implicates free radical-induced oxidative stress in early mechanisms of TBI secondary injury [53]. The large capacity of GSH for detoxifying reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are produced directly from native reactions of $O_2^-$ with various intracellular components, emphasizes the importance of maintaining adequate GSH availability during such rapid increases in $O_2^-$ production. Indeed, GSH availability is significantly decreased at 2 hours and even 24 hours post-injury using a rat model of blast induced mild TBI [1]. By replenishing GSH reserves via bypassing native feedback inhibition of GSH synthesis, GSH becomes available when it is needed most following TBI, and GCEE treatment post-TBI has so far proven to be very favorable by decreasing protein nitration, decreasing autophagy, and decreasing blood-brain barrier permeability [95, 100, 101].

In contrast to the various beneficial mechanisms of ROS and RNS scavenging and detox by glutathione, it is also worth noting that GSH can react directly with $O_2^-$ to produce the glutathione radical (GS•), which can abstract hydrogen atoms polyunsaturated fatty acids, carbohydrates, and peptides [102-105]. Also, mitochondrial manganese superoxide dismutase

(Mn-SOD) is known to be a better scavenger of $O_2^{\cdot-}$ than GSH and should thus limit the amount of $GS^\bullet$ formed, but the activities of Mn-SOD and copper/zinc-SOD (Cu/Zn-SOD) are at their minimums at 24 hours post injury in the ipsilateral hippocampus of rat brain following a cortical contusion model of mild TBI and stay decreased for several days post-injury [54, 105]. Indeed, the presence of the lipid peroxidation product 4-hydroxynonenal (HNE) is significantly elevated as protein-bound HNE in a TBI rat model, and post-TBI treatment of GCEE does not decrease its presence [95].

Post-TBI treatment and treatment of oxygen-glucose deprivation, a possible effect of ischemia, with GCEE have proven to be very beneficial, offering promising avenues of TBI research to determine how these effects translate into ameliorating clinical symptoms [95, 101]. While glutathione does have the potential to cause lipid peroxidation and models have yet to show a beneficial effect in this regard, increased glutathione availability via post-TBI treatment with GCEE has been shown to significantly decrease the presence of nitrated and carbonylated proteins, which are known to be formed by peroxynitrite ($ONOO^-$), a product of $O_2^{\cdot-}$ reacting with nitric oxide (NO) in the presence of carbon dioxide [95, 104]. *In vitro* models using synaptosomes indicate that up regulated glutathione synthesis via GCEE administration can detoxify $ONOO^-$ directly [97]. However, due to the reaction of GSH and $O_2^{\cdot-}$ in forming $GS^\bullet$, there is also potential for a trade-off of ROS formation that allows unreacted NO to carry out its beneficial endothelial functions: elevated glutathione could potentially out-compete NO for reacting with $O_2^{\cdot-}$, increasing lipid peroxidation but allowing NO to promote blood flow and thus encourage neuron survival [81, 106]. Because glutathione has the potential to cause lipid peroxidation but is also known to export products of lipid peroxidation, such as HNE, out of the cell via multidrug resistant proteins, the lack of a significant increase in protein-bound HNE levels in GCEE-treated rats post-TBI could be a result of this trade-off. While further studies are required to determine the exact mechanisms and therapeutic significance of GCEE treatment post-TBI, the many functions of glutathione and initial positive results indicate a high therapeutic potential for this compound [92, 95, 101].

## 8. Histone deacetylase inhibitors

Treatment of traumatic brain injury (TBI) using histone deacetylase inhibitors (HDACi) is a relatively new therapeutic strategy that has so far provided some very encouraging results for invoking neuroprotective mechanisms via epigenetic changes. By inhibiting histone deacetylase, HDACi suppress the removal of acetyl groups from histone lysine residues, freeing DNA segments for transcription. Like statins, the exact mechanisms by which these compounds provide benefits in TBI models are largely unknown. This could be due in part to the large number of proteins affected by such epigenetic changes as well as the intricate relationships between affected proteins and cognition, although the development of novel HDACis is beginning to shed light on proteins of particular importance such as nerve growth factor (NGF) and heat-shock protein 70 (HSP70) [6].

Epigenetic changes are chemical modifications to the genome changes that result in altered gene regulation. These epigenetic changes occur throughout one's life due in part to environ-

mental interactions [107, 108]. Following such a drastic change to one's internal environment as that which results from a TBI, it should be expected that one's epigenetic profile would change accordingly. Indeed, TBI results in substantial epigenetic changes, resulting in increased transcription of several proteins involved in pro-survival pathways and anti-apoptotic pathways [6]. Epigenetic changes in the hippocampus histone-H3 have been found to occur for the first days after a pediatric rat model of TBI, during the same time frame that the secondary injury of TBI is known to occur [52, 109]. Changes in gene regulation are key responses to TBI, and like statins, HDACis are novel therapeutics that can treat several consequences of a TBI at once [6, 110].

Gene arrays of TBI models indicate that there are substantial changes to the gene expression following injury [1, 111-113]. Post-TBI treatment with the HDACi LB-205 was able to increase histone-H3 acetylation, a key histone that has been found to have reduced acetylation following TBI, at safer therapeutic doses than a previously employed therapeutic. In addition, the up regulation of pro-survival proteins nerve growth factor (NGF), p-TrkA, p-AKT, NF-kB, and Bcl-2, as well as down regulation of pro-apoptotic proteins p-JNK, BAX, and p75(NTR) were results of post-TBI LB-205 treatment [6]. Of particular importance to neuron survival is the up regulation of NGF. This neuropeptide is essential to the survival of specific neurons and could help with regeneration of neurons post-TBI as higher CSF levels of NGF are associated with better clinical outcomes following TBI [114-119]. While further research into the potential therapeutic uses of HDACis for treatment of TBI is necessary to fully understand their clinical value, current understanding of post-TBI gene regulation and initial testing of HDACis such as LB-103 have uncovered a multifunctional treatment opportunity that may prove to be of significant importance in the future [1, 6].

## 9. Enzyme kinetics and TBI

Enzyme kinetics focuses on chemical reactions that are catalyzed by enzymes. Enzymes are proteins that alter other molecules by forming an enzyme-substrate complex, transforming the initial substrate bound to the active site of the enzyme into a product via an enzymatic mechanism in which the enzyme is not consumed within the catalyzed reaction. Enzymes do not alter the equilibrium position of a reaction, but continue to increase the reaction rate as the substrate concentration of the respective enzyme increases until all active sites of the enzyme are saturated. From this point, the reaction rate will depend upon the turnover rate of the enzyme. Numerous enzymes bind to one substrate and produce a single product. However, many enzymes are capable of binding to multiple substrates. Kinetic studies on single-substrate binding enzymes can typically show measurements of enzyme affinity to substrate binding, as well as turnover rate. Kinetic studies on multi-substrate binding enzymes usually can show substrate binding sequences, as well as product release sequences. There is typically one rate-determining step that indicates overall kinetics of the enzyme, which could include a conformational change or chemical reaction. Enzyme kinetics can be used to measure the reaction rate of a diverse range of processes for investigation of varying conditions in an

experimental design. Enzyme kinetics can uncover metabolic roles, control mechanisms, catalytic mechanisms, and how a drug/agonist may inhibit an enzyme.

## 10. Alteration of energy metabolism

Alteration of energy metabolism in specific enzymatic reactions involving varying conditions of oxidative stress placed on the enzymes following moderate traumatic brain injury (TBI) has been investigated as possible biomarkers of moderate TBI, which can be observed by enzyme kinetics. Moderate TBI is a common source of oxidative stress in biological systems, caused by secondary damage due to the presence of reactive oxygen and nitrogen species (ROS and RNS, respectively). This leads to a decrease in overall antioxidant capacity [120, 121]. This oxidative stress can disrupt protein structure, leading to a loss of function. The body can attempt to prevent or overcome this event in numerous ways including the use of antioxidants and epigenetic factors. Antioxidant enzymes that are found to be more active following TBI include catalase and glutathione peroxidase. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen (Figure 10), while glutathione peroxidase reduces hydroperox-idases to their respective alcohols (including the conversion of hydrogen peroxides to water), using reduced glutathione as a cofactor, which will be oxidized to glutathione disulfide (Figure 11). Catalase was found to have a threefold increase, while glutathione peroxidase had a twofold increase in a time course study reflecting temporal increase in nerve growth factor (NGF) following a cortical contusion model [122]. Enzyme activity levels of energy-producing enzymes such as enolase and creatine phosphokinase are of interest as possible indicators of moderate TBI. Enolase and creatine phosphokinase both play significant roles in energy production. Enolase, an integral part of the glycolytic pathway, is highly expressed in neuronal cytoplasm. It converts 2-phosphoglycerate to phosphoenolpyruvic acid (Figure 12). Neuron-specific enolase, found only in the brain, can be used for the possible detection of neuronal cell death with high sensitivity and specificity. Increased cerebrospinal fluid (CSF) and serum levels of enolase are associated with TBI and could be used to gauge the degree of injury. This finding could play a key role in future research, as upregulated proteins after TBI injury are typically identified as possible diagnostic biomarkers [123]. S100β proteins, a component of astroglia and Schwann cells, are known to increase post-injury in CSF and serum. Creatine phosphokinase can act as an energy reservoir for rapid buffering of ATP concentration in cells, as well as an intracellular mode of energy transport via a creatine phosphokinase shuttle circuit [124]. Creatine phosphokinase acts by converting creatine to creatine phosphokinase (Figure 13). Creatine kinase isoenzyme BB (CK-BB), neuron specific enolase, and S-100β proteins have been widely studied as biochemical serum markers of TBI. It has been suggested that CK-BB sensitivity and specificity is inadequate for use as a TBI indicator [125]. It is also suggested that serum levels of neuron specific enolase do not correspond to the extent of TBI damage, most likely due to a long half-life of 20 hours. S-100β serum levels have correlated to injury severity in several studies [125].

Catalase enzyme reaction
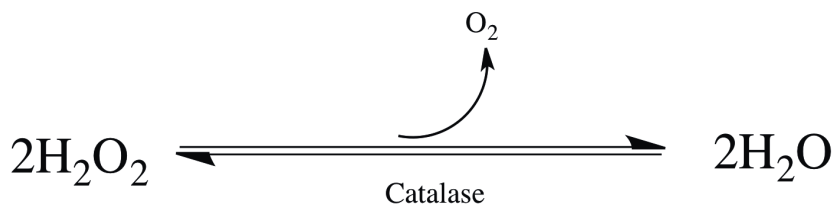


**Figure 10.** Catalase enzymatic reaction.

Glutathione peroxidase enzyme reaction
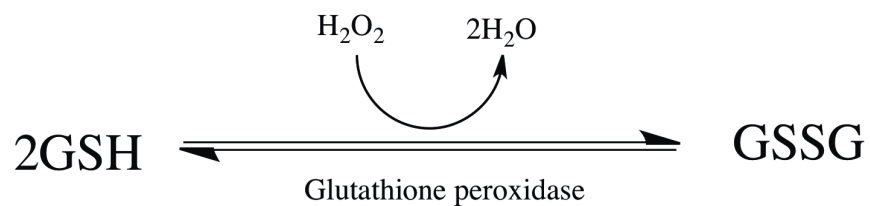


**Figure 11.** Glutathione peroxidase enzymatic reaction.
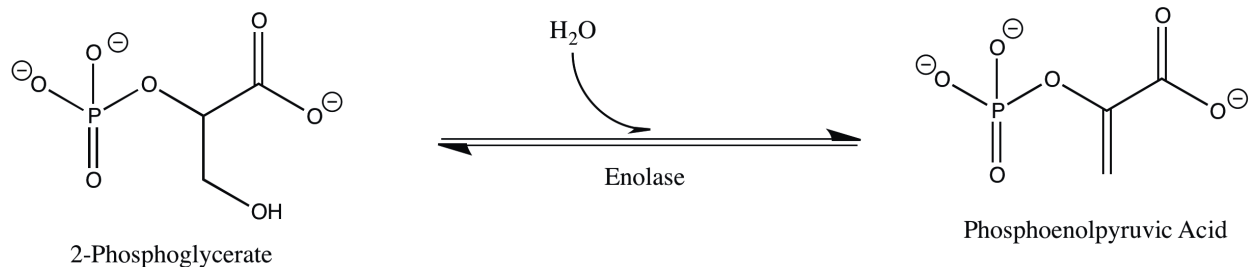
Enolase enzyme reaction



2-Phosphoglycerate

Phosphoenolpyruvic Acid

**Figure 12.** Enzymatic reaction for enolase.

Creatine phosphokinase enzyme reaction
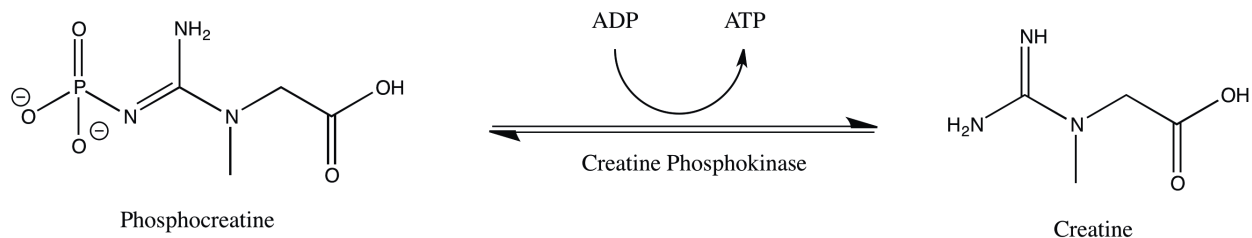


Phosphocreatine

Creatine

**Figure 13.** Enzymatic reaction for creatine kinase.

Numerous other proteins such as enolase are known to be upregulated post-injury including C-reactive proteins, transferrin, and breakdown products of collapsin response mediator proteins (CRMP-2), synaptotagmin, and α2-spectrin. C-reactive proteins bind phosphotidyl-choline on the surface of dead cells, which is a sign of inflammatory response. Transferrin, an iron-binding glycoprotein, transfers iron from transferrin receptors on the surface of cells to cytoplasmic regions. Expressed in the nervous system, CRMP-2 is essential for axon formation from neurites and growth cone guidance. As a result of trauma, the protease calpain targets CRMPs for cleavage. Both calpain and caspase-3 proteases cleave α2-spectrin, indicating necrotic and apoptotic cell death. Synaptotagmin act as a calcium sensor throughout the brain, thereby monitoring calcium homeostasis. In brain-specific systems biology analysis, over 320 proteins have been found to be upregulated 24 hours post-injury in a penetrating ballistic-like brain injury model (PBBI). The majority of these proteins are involved in protein metabolism. These proteins are essential in neurite outgrowth and cell differentiation. Calcineurin, also known as protein phosphatase 3, is a calcium-stimulated phosphatase. It was significantly increased in post-injury hippocampal and cortical homogenates of a moderate, central fluid percussion TBI injury model for 2-3 weeks post-injury. Substrate affinity was not changed, but maximal dephosphorylation rate did increase [126]. The increased rate has many physiological consequences, resulting in a greater possibility of neuronal cell death. Enolase, phosphogly-cerate mutase, and ATP synthase are nitrated in TBI and all are associated with energy metabolism in the brain [127]. Phosphoglycerate mutase, a key glycolytic enzyme, catalyzes a phosphoryl group transfer allowing 3-phosphoglycerate to be converted to 2-phosphoglycer-ate (Figure 14). ATP synthase provides energy to cells through the production of ATP from ADP and inorganic phosphate. Protein dysfunction of these key enzymes can lead to an accumulation of glycolytic intermediates in several metabolic pathways. This ultimately signifies a reduction in pyruvate formation and overall ATP production in both cytoplasmic and mitochondrial regions of the cell. Since ATP is such an important component of cells it would be beneficial for the cells to upregulate ATP producing enzymes, particularly at points where neural communication is established. The acute phase after TBI shows a regionally heterogeneous metabolic state. The glucose transporter protein family and hexokinase activities were studied, showing consistently reduced hexokinase activity throughout the whole brain following contusional TBI. Overall, glucose transporter proteins appeared to only be impaired in the immediate area around the contusion [128].

Many other candidate biomarker proteins are in the process of receiving clinical validation. An abundance of glial fibrillary acidic proteins (GFAPs), acting as intermediate filament proteins, can indicate damaged glia. GFAP, found only in the central nervous system, forms a network support in astroglial cytoskeletons. GFAP serum levels have been found to increase post-injury and rapidly decrease after the first six hours, making GFAP an ideal biomarker due to the specificity of the protein [125]. A recent study found that cerebrospinal fluid levels of neuron specific enolase, brain specific creatine kinase, glial fibrillary acidic protein, and S100β were all significantly increased six hours post-TBI in a swine model [129]. These enzyme levels then decreased 24 hours post-injury in all markers except S100β at 72 hours post-injury. Increased tau and spectrin protein breakdown products indicate axonal damage. Other enzymes consistently showing upregulation in post-TBI injuries include ubiquitin carboxy-

Phosphoglycerate mutase



3-phosphoglycerate                                    2-phosphoglycerate
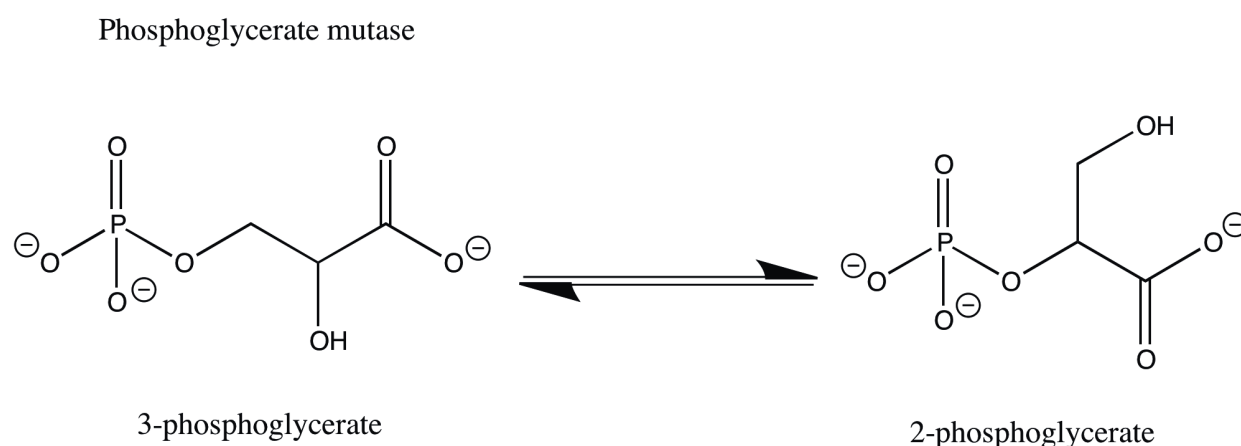
**Figure 14.** Enzymatic reaction for phosphoglycerate mutase.

terminal hydrolase L1 (UCHL1), tyrosine hydroxylase, and syntaxin-6. UCHL1 is a deubiquitinating protease enzyme, hydrolyzing the C-terminal bond of ubiquitin or unfolded polypeptides. It showed significant elevation in TBI patients [130]. Calpain and caspase-3 proteases are intimately involved in necrotic and apoptotic cell death; their respective spectrin breakdown products appear in significantly elevated levels in mortality and severe cases involving TBI [123]. Additional mRNA expression testing has been done on caspase-1 and caspase-3, both members of the cysteine-aspartic acid protease family. The mRNA expression of caspase-3 was increased fivefold in the injured zone of the cortex and twofold in the injured zone of the hippocampus on fluid percussion-induced TBI rat models at 24 hours post-injury; caspase-1 mRNA expression is also increased to a lesser extent [131]. This suggests elevated caspase protein activity during neuronal apoptosis.

In mitochondria, a crucial location for metabolic enzymes, moderate TBI has negative effects on function including mitochondrial glucose utilization and oxidative phosphorylation. Dysfunction of the cytochrome oxidase complex in mitochondrial metabolism has been observed including cytochrome c oxidase, which is essential for oxidative phosphorylation [121]. Cytochrome c oxidase mRNA expression was investigated in rat impact accelerator models of diffuse TBI (DTBI), also known as "Marmarou" weight-dropping trauma models, followed by real-time quantitative PCR assessment of tissue sections from ipsilateral and contralateral cortex zones. Reductions of cytochrome c oxidase I, II, and III were detected in injured cortex zones. The lowest expression was seen three days post-injury. Contralateral cortex zones were detected to have slightly increased mRNA expression at times ranging from six hours to seven days [121]. *In vivo* structural damage to the mitochondria stimulates free radical generation, causing mitochondrial population depletion depending on injury severity. The remaining mitochondrial population provides metabolic support that the body needs for survival and repair [132]. This appears to be one of many complex multifunctioning mechanisms the brain is capable of in terms of neuroregeneration. It is important that nitrated and oxidized enzymes that can be used as biomarkers for TBI, as well as upregulated or increasingly active enzymes during post-injury, continue to be explored for expansion of neurological insight and therapeutic strategies for harmful brain injuries.

## 11. Redox proteomics and TBI

The field of proteomics encompasses the study of the proteome that provides specific details regarding its structure and function. Proteins are vital aspects of living organisms, as they are the primary factors of the metabolic pathways of cells. Proteomics assesses the expression level of proteins, posttranslational modifications, and interactions of proteins within a tissue, cell, subcellular compartment, or biofluid [123]. The objective of proteomics is to observe and thoroughly understand the physiological processes that are occurring in normal and diseased cells.

## 12. Proteins oxidatively modified by TBI

Remarkable efforts have been invested into the discovery of biomarkers of specific neurological disorders. Although there are no clinically validated biomarkers to diagnose TBI, several candidate biomarkers of TBIs are being investigated and multiple studies are being conducted to confirm these protein biomarkers as clinically accurate. Protein oxidation is the modification of a protein induced either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress [133]. Because proteins have many different and unique functions, oxidative modifications of proteins can lead to diverse functional consequences. Modifications of proteins can lead to a loss of function, inhibition of enzymatic activity, or potential risk of diseases. Kobeissy et al. identified several proteins decreased in abundance in traumatic brain injury including, but not are limited to, collapsin response mediator protein-2 (CRMP-2), glyceraldehyde-3-phosphate dehydrogenase, microtubule-associated proteins MAP2A/2B, and hexokinase [134]. Upregulated proteins included C-reactive proteins, transferrin, and breakdown products of CRMP-2, synaptotagmin, and $\alpha$2-spectrin [134]. Furthermore, ubiquitin carboxy-terminal hydrolase L1 protein (UCHL-1), spectrin breakdown products (SBDPs), and neuron-specific enolase (NSE) are presented as the most studied candidate protein biomarkers for TBI as neuronal and axonal protein-specific, while glial protein-specific markers include glial fibrillary acidic protein (GFAP) and S100$\beta$ [123].

UCHL-1, a cysteine protease, hydrolyzes the C-terminal bond of ubiquitin or unfolded polypeptides [135]. UCHL-1 is predominantly expressed in neurons, although it is also expressed in small amounts in neuroendocrine cells [123]. Compared to control, the serum UCHL-1 levels of TBI patients were significantly elevated after the acute phase of TBI and beyond (> 1 week) [136]. Mondello et al., 2012 demonstrated that the parameters of UCHL-1 could be used as a biomarker for severely injured TBI patients.

$\alpha$2-spectrin, primarily found in neurons, is concentrated in axons and presynaptic terminals [137]. Upon activation in TBI, calpain cleaves the protein to SBDPs of molecular weights 145 kDa (SBDP145), 150 kDa (SBDP150), while casapse-3 cleaves $\alpha$2-spectrin to a 120-kDa (SBDP120) product. Calpain and caspase-3 are major executioners of necrotic and apoptotic cell death, respectively, during ischemia or TBI [138-140]. SBDPs indicate calpain and caspase-3

proteolysis of $\alpha$2-spectrin, providing crucial information on the underlying cell death mechanisms [123]. Elevated levels of SBDPs in CSF from adults with severe TBI were reported and their significant relationships with severity of injury and outcome [139]. Increased CSF SBDP levels were found to be significantly associated with mortality in patients with severe TBI. The temporal profile of SBDPs in non-survivors was also found to be different compared to those of survivors [140]. Taken together, these findings suggest that SBDPs may provide crucial information not only on severity of brain injury, but also on underlying pathophysiological mechanisms associated with necrotic and apoptotic cell death [123].

Neuron-specific enolase (NSE), a glycolytic pathway enzyme, is highly expressed in neuronal cytoplasm. NSE has been shown to have the sensitivity and specificity to detect neuronal cell death [141]. Increased CSF and serum levels of NSE have been reported after TBI. NSE concentrations were also associated with severity of injury, CT scan findings, and outcome [141-143]. Of the numerous candidate biomarkers for TBI, GFAP holds the most promise. One of the strengths of GFAP as an ideal biomarker for TBI is that this protein is not found outside the central nervous system [144]. GFAP, an intermediate filament protein that forms networks that support the astroglial cells, is found only in astroglial cytoskeleton. Damage to the astroglial cells (astrogliosis) shows subsequent upregulation of GFAP [123]. Even if the body is subjected to multiple forms of trauma, GFAP doesn't rise without brain injury [145, 146]. The specificity of GFAP as a biomarker makes it a formidable indicator of glial injury.

S100$\beta$ is mainly found in astroglia and Schwann cells and is one of the most well established biomarkers of brain damage [147-149]. The concentration of S100$\beta$ is known to increase in CSF and serum after injury making this protein a potential biomarker for TBI [150]. However, S100$\beta$ is not specific to the brain, as it is observed in non-nervous cells such as adipocytes, epidermal, chondrocytes, melanoma, and Langerhans cells [151]. Furthermore, general trauma without brain injury can increase S100$\beta$. Although a possible candidate as a biomarker for TBI, it seems S100$\beta$ is not independently accurate to determine brain damage and prognosis, but rather in comparison to other biomarkers.

3-Nitrotyrosine (3-NT) is one of the most frequently observed byproducts from reactive nitrogen species (RNS) reacting with proteins. As a biomarker of nitrosative stress, elevated levels of 3-NT signify the presence of oxidative stress and decreased levels of antioxidant enzymes [152]. The formation of RNS from oxidative stress is assumed to play a major role in neuronal death and 3-NT is a marker for this biochemical event. Therefore, 3-NT can be utilized as an *in vivo* marker of oxidative nitric oxide damage following TBI [153]. 3-NT is formed *in vivo* by the reaction of tyrosine with nitrating oxidants, superoxide and nitric acid [154]. Research has shown that elevated 3-NT levels are directly related to traumatic brain injury (TBI), protein nitration, and oxidative stress. A listing of proteins identified as being nitrated can be seen in Table 1. The lack of consensus in the definition of mild TBI further complicates the matter and the challenge lies in accurate diagnosis in managing post-injury [123]. The role of 3-NT formation as an intermediate will predict the involvement of protein nitration and oxidative stress in the brain.

| Protein | Function |
|---|---|
| Synapsin 1 | Regulation of axonogenesis and synaptogenesis; Encode neuronal phosphoproteins which associate with the cytoplasmic surface of synaptic vesicles |
| Gamma-enolase | Neurotrophic and neuroprotective properties on a broad spectrum of central nervous system (CNS) neurons |
| Guanosine diphosphate dissociation | Regulate the GDP-GTP exchange reaction of members of the Rab family, small GTP-binding proteins of the Ras superfamily, that are involved in vesicular trafficking of molecules between cellular organelles |
| Phosphoglycerate mutase (PGM) | Catalyzes the conversion of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) through a 2,3-bisphosphoglycerate intermediate in glycolysis |
| Heat shock protein 70 (Hsp70) | Molecular chaperone and folding catalyst that assist in a variety of protein folding processes in the cell by transient association of their substrate binding domain with short hydrophobic peptide segments within their substrate proteins |
| ATP synthase | Provides energy for the cell to use through the synthesis of adenosine triphosphate (ATP) |
| Alpha-spectrin | Primary spectrin in blood that supports cell structure and critical for platelet activation |

**Table 1.** Nitrated proteins in TBI

Proteomics and the analysis of potential biomarkers for TBI have provided insight into the mechanism and biochemistry of TBI, which have enabled opportunities to elucidate protein behavior. The field of proteomics has assisted in yielding better insight to the progression of injury, assessment of accurate diagnostic criteria for TBI, as well as the development of possible therapies for TBI. The investigation and discovery of many candidate biomarkers for TBI will continue to increase targeted proteomic experiments in the future.

# 13. Learning and memory

Traumatic brain injury (TBI) is a costly medical crisis for which no clinically proven pharmacological therapies currently exist. TBI, also known as the "silent epidemic", is profoundly related to oxidative stress, which has been indicated as a mechanism of secondary neuronal injury in TBI that can ultimately result in numerous related neurological maladies [155]. Oxidative stress has been associated in the pathogenesis of numerous neurological disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke. TBIs could impair coordination of respiratory muscles, pharyngeal function, and tongue/lip movement involved with speech, which is one of the most complex functions humans can perform. Incoordination of these precise processes can be detrimental and cause slurred or disjointed speech. Problems can arise with expressive and receptive language, literacy abilities, and diminished social communication skills. Furthermore, TBI in humans can cause an extensive

range of cognitive impairments including, but are not limited to, a variety of deficits in learning, memory, retrograde amnesia, anterograde amnesia, impairments in new learning or acquisition, and deficits in working memory, reference memory, and spatial memory.

Impairments in memory are a core element of the cognitive deficits associated with TBI and are likely related to disruption of cholinergic functioning in the hippocampus. Under oxidative stress conditions, antioxidant levels fluctuate significantly. Certain environmental or external factors may be the cause of an imbalance and as a result, reactive nitrogen species (RNS) are created. RNS have the ability to react with biomolecules including carbohydrates, proteins, lipids, DNA and RNA which leads to oxidative damage and ultimately cellular dysfunction [156]. The brain is vulnerable to oxidative stress due to the high content of peroxidizable unsaturated fatty acids, increased consumption of oxygen, elevated levels of free radicals, and moderately low levels of antioxidant defense systems [157]. Damage to the brain from a traumatic event can have life-long, lingering effects resulting in the deformation of normal physiological processes. As a result of these biochemical events results, TBIs serve as a source of significant and persistent cognitive dysfunction.

## 14. Conclusion

Traumatic brain injuries are a sudden, severe trauma to the brain. Presently, there is no cure for these occurrences. These harmful injuries are the causative factors in a multitude of events including protein dysfunction, altered energy metabolism, loss of enzymatic activity, and increases in ROS and RNS. The field of proteomics has demonstrated the importance of how oxidatively modified proteins play a pivotal role in both learning and memory, two key features effected in traumatic brain injury. Current research efforts are introducing new animal models and pre and post therapeutic strategies which show promise in delaying and possibly preventing further neuronal damage for primary and secondary injuries associated with TBI, making a clinical treatment for TBI a strong prospect in the near future.

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