INVESTIGATING THE EFFECTS OF TREATMENT ON TRAUMATIC BRAIN INJURY PATHOLOGY AND RECOVERY

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BY

Macy Rae Komnick

APPROVED :

Katelyn Black, Ph.D.
Director of Thesis

James Zadina, Ph.D.
Second Reader

James Cronin, Ph.D.
Third Reader

Abstract:

Traumatic brain injury (TBI) results from a sudden bump or jolt of the head. It is a growing public health problem that is responsible for significant neurological impairment. TBI can result in several debilitating side effects, including motor dysfunction, cognitive deficits, behavioral changes, and persistent pain. It also causes changes at the cellular level that can affect brain function long-term. Tissue damage initiates a variety of secondary injury pathologies, such as excitotoxicity, neuroinflammation, and oxidative damage. While there are very few pharmacological treatment options for TBI, opioids are commonly used to treat post-injury pain. While opioids are powerful analgesics, their clinical utility is hindered by side effects such as tolerance, addiction, respiratory depression, motor dysfunction, and constipation. Also, opioids alone are responsible for causing many of the same cellular pathologies as TBI, specifically oxidative damage. An increase in reactive oxygen species can deplete neurons of important antioxidants and promote apoptosis. Recently, peroxynitrite (PN) has been implicated in both analgesic tolerance and TBI-related damage. By stimulating neuroinflammation and apoptosis, PN significantly impairs neurological function following both TBI and chronic opioid administration. Therefore, this thesis explores the interaction between opioids and brain injury. It was hypothesized that animals treated with morphine after TBI or sham procedure will exhibit greater motor dysfunction and oxidative damage than those treated with a vehicle solution. While the results of this study are inconclusive, they provide a platform for further research on the subject.

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Chapter One: Introduction

1.1 Traumatic Brain Injury

Traumatic brain injury (TBI) is defined as a head injury that disrupts normal brain functioning [1]. It can be caused by a blow or jolt of the head as well as a penetrating head injury [1]. TBI is a leading cause of disability in the United States, affecting approximately 1.7 million people per year [2]. Disability resulting from injury is primarily cognitive, but deficits can be behavioral, emotional, or motor-related (**Table 1.1**) [1]. Other health effects include psychiatric disorders, such as anxiety and depression, which result from damage to the frontotemporal lobe [2]. In addition, TBI has been shown to increase the risk of neurodegenerative disorders such as Alzheimer's and chronic traumatic encephalopathy (CTE) [1].

TBIs can be broken down based on the type of damage and phase of injury. The primary injury is the result of mechanical force applied to the skull at the time of impact. Characterized by skull fractures, axonal shearing, and intracranial microhemorrhages, it is believed to be an irreversible consequence of TBI [3]. The secondary injury develops days to weeks after the initial insult, causing edema, free radical and oxidative damage, disruption of the blood-brain barrier, and neuroinflammation [1, 3]. The secondary injury can be further divided into the early, intermediate, and late phases. The early phase develops within twenty-four hours of the primary injury, and notable pathologies include glutamate excitotoxicity, disruption of calcium homeostasis, and oxidative stress [4]. Oxidative damage following TBI will be the principle interest of this thesis as it is indicative of early phase cellular dynamics and is responsible for TBI-related brain damage [5]. The administration of free radical scavengers and nitric oxide synthase

(NOS) inhibitors resulted in improved neurological recovery from TBI, implicating a role for reactive oxidative species [6] and reactive nitrogen species (RNS) in impaired recovery [7]. Currently, there are no pharmacological therapies that improve the neurological deficits resulting from TBI [8]. Consequently, the need for therapeutic interventions that can attenuate secondary injury pathologies is significant.

Category	Description
Cognitive	Deficits in: *attention; learning and memory; executive functions like planning and decision-making; language and communication; reaction time; reasoning and judgment*
Behavioral/ Emotional	*Delusions; hallucinations; severe mood disturbance; sustained irrational behavior; agitation; aggression; confusion; impulsivity; social inappropriateness*
Motor	*Changes in muscle tone; paralysis; impaired coordination; changes in balance, or trouble walking*
Sensory	*Changes in vision and hearing; sensitivity to light*
Somatic signs and symptoms	*Headache; fatigue; sleep disturbance; dizziness; chronic pain*

Table 1.1. Neurological deficits resulting from TBI. TBI can affect functioning in the cognitive, behavioral/emotional, motor, sensory, and somatic domains. [1].

1.2 TBI Cellular Pathologies

TBI-related tissue damage can initiate a cascade of molecular events that impair functional recovery. TBI applies strong forces to the brain parenchyma, often resulting in diffuse axonal injury (DAI) [9]. The mechanical impact disrupts membranous ion channels and causes immediate neuronal depolarization [10]. DAI and other TBI related tissue damage stimulates a variety of secondary injury pathologies, including calcium ion dysregulation, depletion of ATP, neurotoxicity, and cell death [4, 9]. Necrotic cell death is common near the injury site, whereas programmed cell death is initiated in the surrounding tissue [10]. Damage-associated molecular patterns (DAMPs) released in response to tissue damage are key mediators of neuroinflammation. DAMPs are sensed by nucleotide binding leucine-rich region (NLR) receptors, which mediate the release of pro-inflammatory molecules that potentiate the actions of oxidative stress-causing agents [11, 12]. Ischemic events resulting from injury can also disrupt brain functioning by impairing cerebral metabolism [4]. Reduced metabolism is the basis for several of the cellular pathologies resulting from TBI, especially oxidative stress.

Pathologies resulting from tissue damage are highly interactive, feeding back on each other to disrupt neuronal homeostasis [10]. Excess glutamate accumulates in the extracellular space as a result of increased release from the synapse and decreased reuptake, causing an increase in calcium influx through the NMDA receptor and ion channels [4]. NMDA receptor activation also increases the release of calcium from intracellular stores [10]. Glutamate can diffuse through the extracellular space to cause neurotoxicity in brain regions distant from the injury site [11]. Calcium ions exerts toxic effects by inducing the excessive release of neurotransmitters and disrupting

mitochondrial function [9]. Furthermore, calcium can initiate oxidative stress and activate apoptotic pathways [10]. Therefore, calcium plays an important role in the development of phase early secondary injury pathologies.

The mitochondrion is the major organelle responsible for calcium sequestration during cytosolic overload [13]. Excess calcium entry into the mitochondria is associated with the uncoupling of oxidative phosphorylation and a reduction in ATP synthesis [4, 5]. The activity of mitochondrial nitric oxide synthase (NOS) is calcium dependent, and activation of this enzyme plays a role in the synthesis and release of reactive oxygen species (ROS) [6] and reactive nitrogen species (RNS) from the mitochondria [7]. The endogenous antioxidant system within the mitochondria, composed primarily of glutathione (GSH), manganese superoxide dismutase (MnSOD), and catalase, is depleted due to an overwhelming increase in ROS production [8]. GSH is the most important scavenger of ROS, reacting with nitric oxide (NO) to form s-nitroglutathione (GSNO) [8, 14]. GSNO exerts neuroprotective effects by suppressing the activity of iNOS and pro-inflammatory cytokines, ameliorating neuroinflammatory processes [14, 15]. GSNO also decreases levels of deleterious nitro-oxidative species, such as peroxynitrite (PN), to suppress oxidative damage [15]. MnSOD catalyzes the decomposition of superoxide (SO) into hydrogen peroxide and oxygen [16]. Under normal conditions, these antioxidants control the levels of ROS within the mitochondria; however, the large amount of ROS generated as a result of TBI exceeds the capacity of the endogenous antioxidants [8]. For example, the reaction between SO and NO to form peroxynitrite, another oxidative stress-causing agent, is highly favorable and occurs at a higher rate than the dismutation of superoxide by MnSOD [16]. This reaction also decreases the amount

of nitric oxide that can react with GSH to form the neuroprotective agent GSNO [17]. The depletion of endogenous antioxidant systems results in excess ROS activity and subsequent neuroinflammation.

Once calcium enters the mitochondrial matrix, it induces a mitochondrial permeability transition (MPT), allowing the non-selective movement of substances through the inner mitochondrial membrane [13]. Decreased cellular metabolism along with MPT leads to an increase in the production and release of ROS, such as NO and SO, and calcium ions from the mitochondria [13, 18]. In calcium-overloaded mitochondria, SO can more easily diffuse through electron transport chain proteins in the inner mitochondrial membrane into the cytoplasm [16]. MPT can also result in the release of pro-apoptotic factors that normally reside within the mitochondrion to promote cell death. One such factor is cytochrome c, an inner mitochondrial membrane protein. In the cytosol, cytochrome c binds to apoptotic protease activating factor (Apaf-1) to form a complex called an apoptosome [19]. The apoptosome cleaves procaspases to generate active caspases [19]. Caspases promote cell death by breaking down cellular components necessary for cell survival and inactivating apoptotic inhibitors [19]. ROS and calcium ions released from the mitochondria can also activate calpain and caspase-independent apoptotic pathways to initiate programmed cell death [18]. Calpains stimulate apoptosis by breaking down components of the neuronal cytoskeleton [18]. The overproduction of ROS within the mitochondria is responsible for the disruption of mitochondrial membrane potential and the activation of caspases [20]. While mitochondria are responsible for the production and release of ROS, they are also susceptible to oxidative damage [20]. ROS can exacerbate mitochondrial dysfunction by disrupting oxidative

phosphorylation and compromising the calcium buffering capacity of the mitochondria [10, 16]. Glia are also affected by oxidative stress following TBI; oligodendrocytes are particularly susceptible to oxidative damage as they produce large amounts of ROS and have low antioxidant capacity [10]. Oxidative stress is indicative of injury severity, and damage resulting from ROS activity is implicated in injury-induced neurodegeneration [10].

The increase in ROS both in the mitochondria and the cytosol favors the formation of peroxynitrite (PN), a free radical formed by a reaction between NO and SO [18]. NOS uses L-arginine as a substrate to produce NO, which can then combine with SO to form PN [11]. While PN alone can induce cellular damage, its decomposition produces several potent free radical species. PN can combine with a proton to form peroxynitrous acid (ONOOH), which decomposes into reactive nitrogen dioxide and hydroxyl free radicals [18, 21]. An additional source of the hydroxyl free radical is the reaction between free iron and hydrogen peroxide [16, 21]. Alternatively, PN can react with carbon dioxide and subsequently decompose into the carbonate free radical nitrosoperoxocarbonate ($ONOOCO_2-$) [18]. PN is a proinflammatory and proapoptotic species that contributes heavily to oxidative damage [22]. It has also been implicated in delayed neurodegeneration, blood brain barrier leakage, metabolic dysfunction, and neurobehavioral deficits following injury [15, 21, 23]. MtNOS, an isoform of NOS located in the mitochondria, is largely responsible for the production of PN [7]. However, all isoforms of NOS have been shown to be upregulated within 24 hours of TBI and contribute to oxidative stress [7, 21]. Epithelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) are post-translationally modified to their active

conformations by calcium ions that influx through the NMDA receptor [11]. Inducible nitric oxide synthase (iNOS) is upregulated in response to DAMPs, which increase following brain injury [11]. The rate of formation of PN occurs faster than the manganese super oxide dismutase (MnSOD) enzyme can convert SO to hydrogen peroxide, allowing PN to accumulate in the mitochondria [16]. PN has a relatively long half-life, allowing it to diffuse out of the mitochondria and cause widespread damage to the neurons [18].

PN activity can affect many cellular components to disrupt cellular homeostasis (**Figure 1.1**). Lipid peroxidation is a likely consequence of PN activity due to the high concentration of poly-unsaturated fatty acids in neuronal membranes [10, 18]. Lipid peroxidation can be observed as early as thirty minutes post-injury, and it is correlated with an increase in the PN metabolite hydroxyl free radical [21]. The vascular endothelium is targeted by lipid peroxidation, leading to the compromise of the blood-brain barrier (BBB). The compromise of neuronal membrane components ultimately results in BBB dysfunction and cerebral edema [21]. The products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxylnonenal (4HNE), can further act on cellular proteins to alter their structures and render them dysfunctional [21]. Lipid peroxidation can also cause damage to the Na+/K+ and Ca2+ ATPases located on the plasma membrane, contributing to the cycle of oxidative damage and glutamate excitotoxicity by increasing intracellular concentrations of calcium and sodium [16].

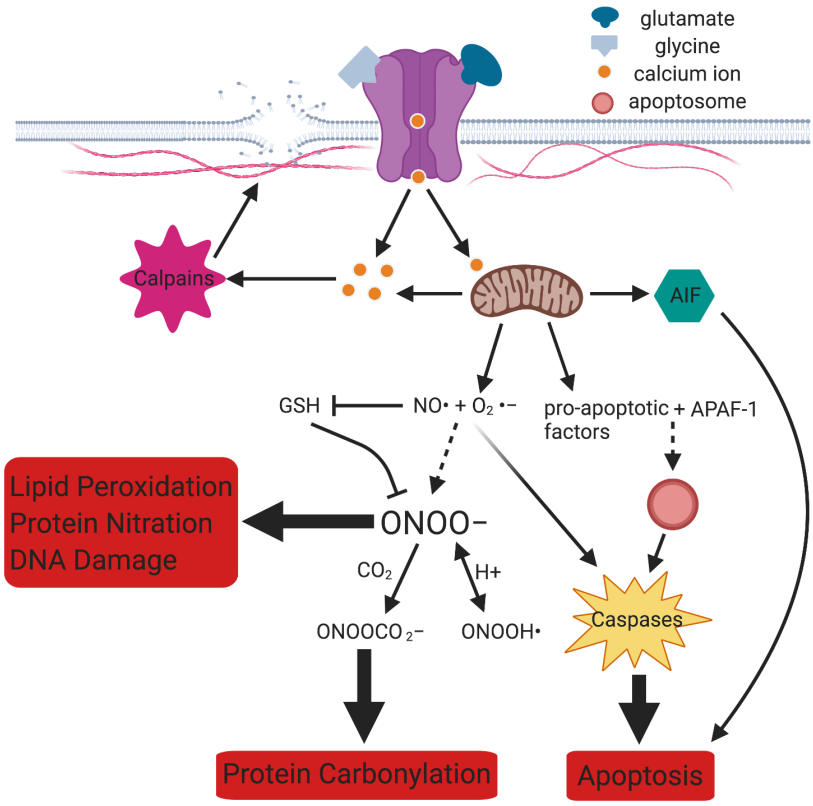


Figure 1.1. Schematic of nitro-oxidative damage following traumatic brain injury. Tissue damage induces excessive glutamate release and increases the activity of the NMDAR, causing cytosolic calcium overload. Calcium ions taken up by the mitochondria induce MPT, allowing reactive oxygen species (ex. NO• and O_2-•) to overwhelm mitochondrial antioxidants and be released into the cytosol. MPT also allows pro-apoptotic factors to be freed from the mitochondrial matrix. These factors combine to form the apoptosome, which activates caspases to promote apoptosis. Intracellular calcium can also activate calpains, which stimulate apoptosis by disrupting the integrity of the neuronal membrane. Apoptotic inducing factor (AIF) is also freed from the mitochondria, allowing it to enter the nucleus and cause DNA damage. The presence of NO• and O_2-• in the cytosol promotes the formation of PN (ONOO-). PN can decompose

into highly reactive free radicals or cause direct cellular damage through lipid peroxidation, protein nitration, and DNA damage. The pathways initiated by increased intracellular calcium work together to potentiate free radical formation and neuronal damage (Created in BioRender.com).

PN can target a variety of proteins in a neuron to disturb cellular metabolism and homeostatic conditions (**Figure 1.2**). PN has been shown to cause large-scale DNA fragmentation by inducing single strand breaks and altering base pairs [11, 24]. By promoting MPT, PN allows apoptosis-inducing factor (AIF) to exit the mitochondrial membrane and diffuse into the nucleus [24]. There, it can act either as an endonuclease or as a cofactor to a pre-existing endonuclease, leading to DNA damage and apoptosis [24]. PN can also modify base pairs to increase mutagenesis [25]. This mechanism represents an additional pathway for cell death that is caspase-independent. DNA single strand breaks can also activate PARP, a nuclear and mitochondrial enzyme that can slow the rates of glycolysis and ATP synthesis to promote cell death [25]. PARP further contributes to neuroinflammation by increasing the expression of pro-inflammatory agents [11].

PN elicits many of its deleterious effects through protein nitration and carbonylation. The protonation of PN yields peroxynitrous acid (ONOOH), believed to be the active agent responsible for protein nitration at tyrosine residues [26]. Protein nitration results in the structural alteration of proteins, rendering them inactive or dysfunctional. Actin and neurofilaments are targets of nitration, leading to disruption of the neuronal cytoskeleton [26]. Enzymes, such as MnSOD and mitochondrial ATP synthase, can also be inactivated by nitration, compounding mitochondrial dysfunction initiated excessive calcium influx [26]. Nitration of the NMDA receptor causes constitutive calcium influx and contributes to prolonged glutamate excitotoxicity [27]. Furthermore, glutamate transporters located on astrocytes have intracellular domains that are rich in amino acids susceptible to nitration [27]. Dysfunctional glutamate transporters further potentiate neurotoxicity by

decreasing the reuptake of extracellular glutamate. Glutamine synthetase, the enzyme responsible for the enzymatic degradation of glutamate in astrocytes, can also be inactivated by nitration [27]. In addition, nitrosoperoxocarbonate, a metabolite of PN, mediates the carbonylation of proteins through its decomposition into the carbonate free radical [16]. PN-mediated protein alterations play an important role in TBI secondary pathologies by inactivating proteins necessary for neuronal homeostasis.

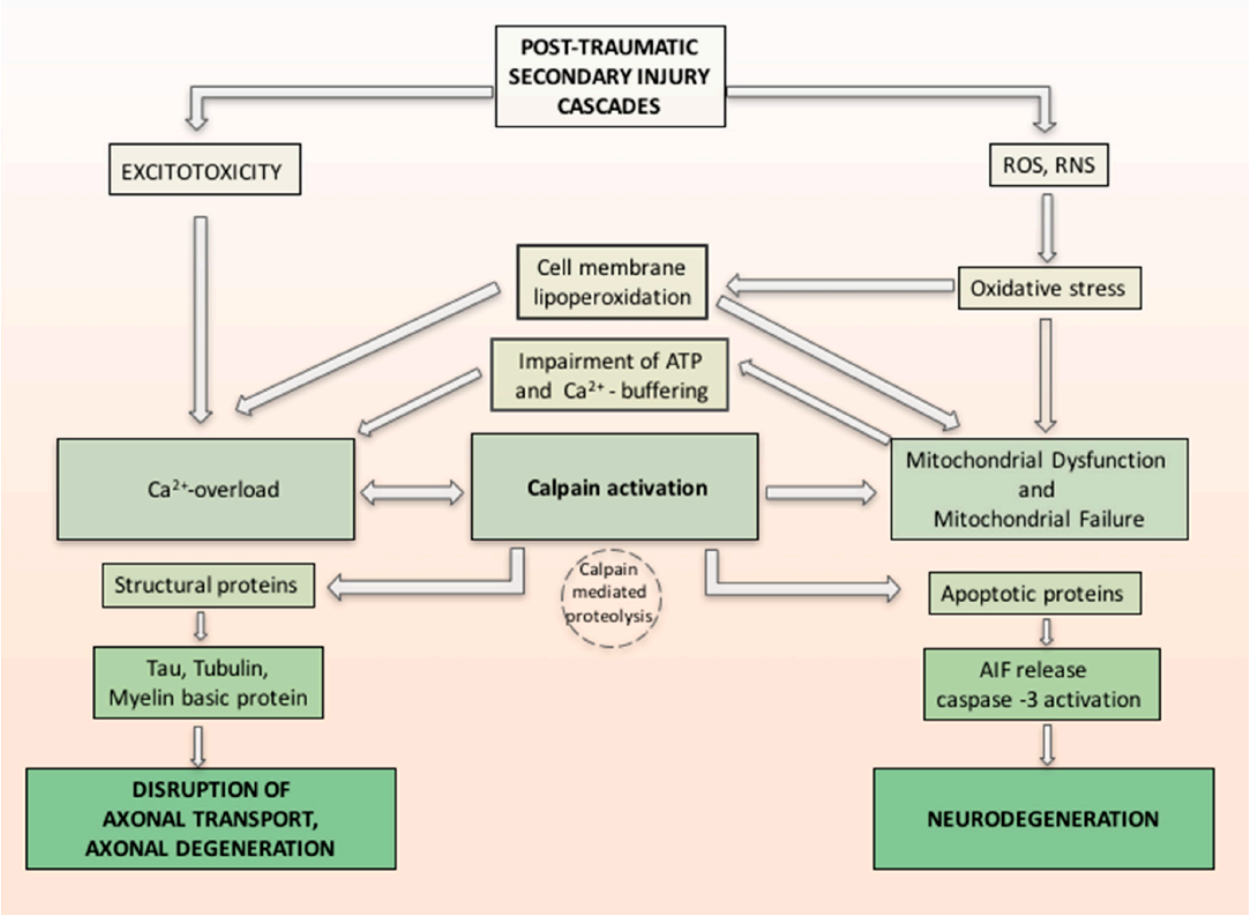


Figure 1.2 Extensive interactions between various mechanisms of TBI secondary injury pathologies. Glutamate excitotoxicity and the production of ROS and RNS initiate the cascade. ROS/RNS increase the release of calcium ions from intracellular stores, while excitotoxicity increases the influx of calcium through the NMDA receptor. Calcium overload activates calpains, which further exacerbate mitochondrial damage and calcium dysregulation. A positive feedback loop occurs due to the excessive entry of calcium ions into the mitochondria, disrupting its calcium buffering capacity. Mitochondrial dysfunction further contributes to calcium overload as well as oxidative damage. Calpains also mediate neurodegenerative processes such as the activation of apoptotic proteins and the proteolysis of cytoskeletal components. These processes lead to neurodegeneration and axonal dysfunction. (Frati et al., 2017)

Attenuation of oxidative-stress causing agents has been shown to improve the functional outcome of TBI. Inhibition at several points along the nitro-oxidative damage cascade have been utilized to assess the contribution of individual factors to TBI-related brain damage. Acute administration of NOS inhibitors improves neurological recovery from injury [28]. SS-31, a novel mitochondrial-targeting peptide, destroys excess ROS in the mitochondria and has been shown to alleviate cerebral edema, oxidative stress, and motor deficits, as well as promote neuronal survival [5]. Another antioxidant, diphenhydramine, has exhibited similar neuroprotective effects; administration of diphenhydramine decreased edema, markers of neurodegeneration, and the release of proinflammatory cytokines [29]. Addition or increased expression of endogenous antioxidants has also been shown to attenuate oxidative damage. Administration of GSNO decreased PN levels, lipid peroxidation, BBB leakage, and neuroinflammation with short-term treatment [15]. Treatment with dimethyl fumarate, a compound that increases the synthesis of GSH, attenuates neurological deficits and prevents injury-induced depletion of endogenous antioxidants [8]. Transgenic animals that overexpress antioxidant enzymes have demonstrated a decrease in post-traumatic brain damage compared to the wild type [21]. Several studies have found an increase in levels of 3-nitrotyrosination (3-NT), a molecular marker of PN-mediated protein nitration, following TBI (**Figure 1.3**) [16, 28]. A decrease in 3-NT was correlated with a decrease in calcium-mediated calpain activation and cytoskeletal degradation [16]. Administration of tempol, a PN scavenger, decreased levels of 3-NT in cortical tissue, improved mitochondrial respiration, and inhibited calpain-mediated cytoskeletal degradation [23]. These effects correlated with improved motor function following TBI [23]. The NOS inhibitor N-nitro-

L-arginine-methylester (L-NAME) has been shown to decrease 3-NT as well improve neurological function [28, 30]. Treatment with mildronate, an anti-ischemic agent found neuroprotective in models of stroke and degenerative diseases, decreased caspase-3 activity and increased MnSOD activity in a controlled cortical impact TBI model [31]. Hall et al. (2004) found that inhibitors of lipid peroxidation are neuroprotective in rodent TBI models [21]. While attenuating oxidative damage has proven to be neuroprotective in animal models of TBI, the complex nature of the secondary injury limits the potential for any one factor to prevent TBI-related brain damage [32].

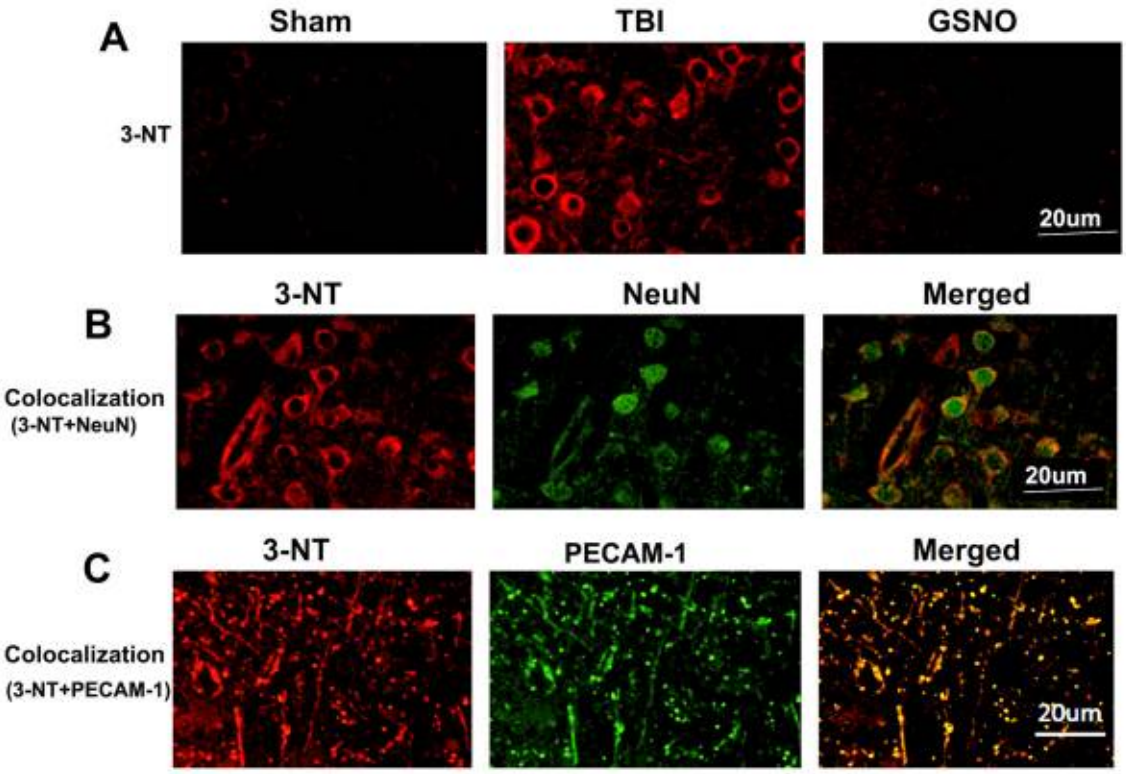


Figure 1.3. Effect of GSNO treatment on 3-NT expression 14 days after TBI. (A) Immunohistochemistry shows increased expression of 3-NT (red) in the TBI group compared to the GSNO group. Shams do not exhibit 3-NT expression. (B,C) Colocalization of 3-NT with the neuronal marker NeuN (green) and the endothelial cell marker PECAM-1 (green) indicates that both neurons and endothelial cells are affected by nitro-oxidative damage even 14 days after TBI. (Khan et al., 2011).

1.3 TBI and Chronic Pain

Brain injury initiates a series of molecular events, both local and global, that may lead to chronic pain. Damage to the skull, meninges, or cerebrovascular system can play a role in chronic pain development [33]. Chronic pain is reported in over 50% of individuals who have sustained a TBI, with the primary concern being headaches [2]. Post-traumatic headache (PTH) develops within 7 to 31 days post-injury, and symptoms often resemble migraine or tension-type headaches [33]. Other pain syndromes include complex regional pain syndrome (CRPS), peripheral neuropathic pain, neuromuscular spasticity, and neurogenic ossification [2, 33, 34]. Pain-sensitive areas often exhibit allodynia and hyperalgesia [33]. Several hypotheses have been proposed as to the mechanism of TBI-induced dysfunctional pain signaling, especially in PTH. Reduced activation of descending modulatory pain signaling, alterations in brain structures that play a role in pain perception, and sensitization of the trigeminal system have all been implicated in pain onset and persistence [2, 33]. Furthermore, TBI-related pain has shown correlation to psychiatric co-morbidities such as depression, anxiety, and paranoia [2]. Chronic pain represents a significant impact on the quality of life of individuals who have suffered TBI.

1.4 Opioid Analgesics

μ-opioid receptor (MOR) agonists remain the primary treatment for chronic pain, including the types resulting from TBI [33, 34]. While opioids such as morphine are potent analgesics, their high abuse potential makes them extremely dangerous. Opioid abuse has become epidemic in the United States, causing approximately 150,000 deaths

every year [35]. The use of prescription opioids increases the likelihood of a transition to more potent opioids such as heroin [36]. Furthermore, chronic opioid treatment has been shown to result in motor dysfunction, addiction, tolerance, and respiratory depression [37]. The clinical use of opioids is limited by their potential to quickly cause analgesic tolerance and dependence as well as hypersensitivity to both painful and innocuous stimuli [38]. The pathways involved in opioid dependence also play a role in learning and memory, causing morphine-induced cognitive deficits in chronic users [39].

Morphine initiates a cascade of cellular events that impede its analgesic potential. Morphine has a high affinity for the MOR; upon binding, an inhibitory G protein is activated leading to a decrease in cyclic AMP production [40]. However, morphine has been shown to interact with a variety of different receptors to affect cellular dynamics. It has been hypothesized that morphine administration can increase neuroinflammation by binding directly to toll-like receptors (TLR) on glial cells [37, 41]. Glial activation counteracts opioid analgesia and plays an important role in tolerance and dependency [41]. Additionally, morphine has been shown to participate in the development of oxidative damage by both initiating an oxidative stress response and depleting the brain's endogenous antioxidant system (**Figure 1.4A**) [40]. Neurons contain a variety of powerful antioxidants that inhibit ROS and RNS, including superoxide dismutase, GSH, and catalase [40]. However, the proliferation of ROS and RNS can exceed the capacity of these antioxidants, resulting in oxidative damage. Oxidative stress has been implicated in the development of opioid tolerance and physical dependence as well as synaptic alterations resulting from chronic opioid use [38, 40, 42, 43]. Therefore, treatment of TBI

with opioid analgesics may potentiate the cellular pathologies associated with injury to impair functional recovery.

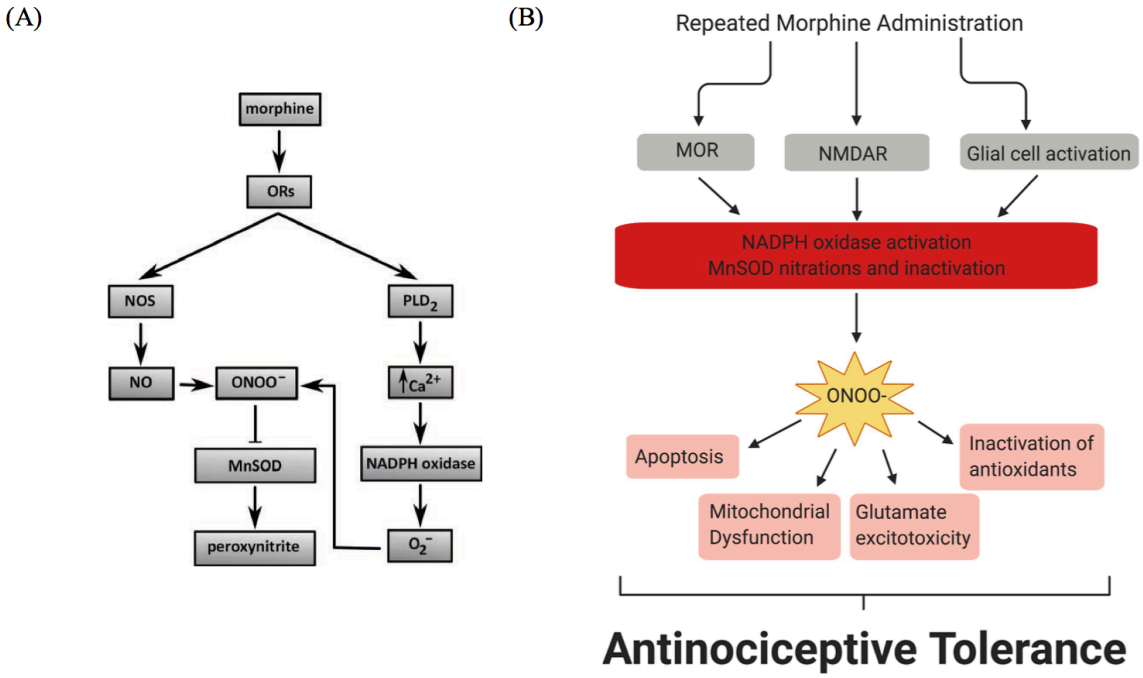


Figure 1.4 Morphine as a potential oxidative stress-inducing agent. A schematic of the possible mechanisms by which morphine potentiates oxidative stress. (A) Morphine can act directly at opioid receptors (ORs) to initiate oxidative stress by activating NOS and phospholipase pathways that lead to the formation of ROS and RNS. It has also been shown to interfere with the endogenous antioxidant system, further contributing to increased nitro-oxidative damage (Skrabalova et al., 2013). (B) Morphine affects several additional systems, including NMDA receptor (NMDAR) function and glial cells to increase peroxynitrite and subsequent processes including antinociceptive tolerance (Adapted from Salvemini, 2009. Created in BioRender.com).

Opioid tolerance and dependence are significant consequences of chronic morphine administration. Tolerance is indicated behaviorally by a progressive decrease in analgesic latency [44]. With chronic exposure to morphine, opioid receptor genes can be downregulated in order to desensitize neurons to the agonist [40]. Dependence can be demonstrated by the precipitation of withdrawal symptoms following administration of a MOR antagonist such as naloxone [45]. There are several mechanisms by which morphine is proposed to induce tolerance and dependence, many of which involve the formation of ROS and RNS. As previously stated, the brain is particularly susceptible to oxidative damage as it sustains high levels of oxygen metabolism and contains membranes with a high content of polyunsaturated fatty acids [46]. Therefore, oxidative stress can have detrimental effects on brain functioning while diminishing the efficacy of opioids. Similar to TBI, the co-administration of nitric oxide scavengers or antioxidants can attenuate antinociceptive tolerance and dependence [47].

Morphine induces a variety of cellular changes to induce its analgesic effect as well as untoward side effects. Even acute doses of morphine can alter cellular dynamics, including decreasing activity of the Na+/K+ ATPase and reducing concentrations of GSH [48]. Morphine administration causes a progressive increase in oxidative stress-causing agents while depleting the brain's endogenous antioxidant systems [44]. Opioid-induced oxidative stress, similar to TBI, is believed to be the result of mitochondrial dysfunction [49]. ROS can initiate inflammation by activating NF-κB, a redox-sensitive transcription factor that mediates pro-inflammatory signaling pathways [50]. After transportation into the nucleus, NF-κB induces transcription of pro-inflammatory agents including chemokines and cytokines [50]. Opioids have also been shown to upregulate the

transcription of iNOS, potentiating the increase in harmful oxidative species [50]. Additionally, the increase in free radicals associated with chronic opioid administration promotes the nitration and inactivation of enzymes, such as MnSOD, involved in free radical homeostasis [38]. Furthermore, morphine can have detrimental effects on cellular components and organelles. It has been shown that morphine administration leads to an increase in MDA levels, a major metabolite of lipid peroxidation [44, 50]. Lipid peroxidation is a consequence of increased ROS production following morphine administration and ultimately results in cell death [50, 51]. An additional consequence of chronic morphine exposure is apoptosis. The proliferation of nitro-oxidative species can damage DNA and increase the presence of apoptotic markers [50]. The combinatorial actions of inflammation and oxidative damage increase the expression of pro-apoptotic proteins such as Bax and p53 while simultaneously decreasing the expression of protective proteins, particularly Bcl-2 [50]. Bcl-2 prevents apoptosis by retaining pro-apoptotic agents inside the mitochondria. Caspase-3, another protein found to be upregulated following chronic morphine exposure, can activate caspase activated deoxyribonuclease (CAD) which enters the nucleus to cause DNA fragmentation [52]. Neuronal apoptosis is a significant consequence of opioid addiction due to the ability of opioids to disrupt the regulation of proteins involved in apoptosis [52].

Morphine can manipulate neurotransmitter systems, such as the monoamines, that are important for cognitive functioning [50]. The N-methyl-D-aspartate (NMDA) receptor has been implicated in morphine tolerance as the downstream pathways activated by the receptor lead to the activation of NOS [44, 53]. While acute morphine causes a reduction in glutamate levels, chronic morphine is associated with increased glutamate transmission

and antinociceptive tolerance [51]. Over-activation of the glutamate system results in increased nitro-oxidative stress. Morphine administration results in over-activation of the glutamate system and subsequent oxidative damage through the potentiation of NMDA receptor activity (**Figure 1.4B**) [44, 51]. NMDA activity, as well as glial cell activation, leads to the formation of NO and SO, which combine to form PN [38]. The formation of the PN free radical in the dorsal horn of the spinal cord is associated with the development of hyperalgesia [38]. PN activity resulting from NMDA receptor activation also contributes to central sensitization following morphine administration [54]. Additionally, naloxone-precipitated physical withdrawal symptoms are associated with a significant increase in extracellular glutamate in several brain regions as shown by microdialysis studies [55]. It has previously been found that chronic morphine administration changes NMDA receptor subunit expression in the cortex, hippocampus, and nucleus accumbens [53]. Specifically, the expression of the NR1 and NR2A proteins was significantly increased in the nucleus accumbens, while it was decreased in the cortex and hippocampus [53]. These results suggest the potential role of the NMDA receptor in morphine-induced tolerance and physical dependence; the change in subunit expression in the nucleus accumbens may manipulate the mesolimbic dopamine signaling, ultimately resulting in opioid dependence [53]. The involvement of the glutamate system in morphine induced-oxidative stress is further supported by pharmacology studies. The co-administration of both competitive and non-competitive NMDA antagonists attenuates morphine tolerance and dependence [51].

Similar to TBI, morphine induces the formation of the PN free radical (**Figure 1.4,5**) [40]. PN has been shown to play a role in opiate tolerance and dependence in addition to

its ability to induce hyperalgesia [38]. As previously described, PN is formed by the reaction between NO and SO. NO is derived from molecular oxygen and the amino acid L-arginine, and the reaction is carried out by NOS. Morphine is believed to increase NOS activity and NO production through its action on the NMDA receptor [51]. Morphine promotes SO production by activating the phospholipase D pathway, involved in intracellular signaling, and increasing the concentration of calcium ions [42]. SO is ultimately formed by both the uncoupling of the electron transport chain and the activation of oxidases such as NADPH oxidase, both consequences of phospholipase D activity and increased calcium (**Figure 1.5B**) [40, 56]. NADPH is expressed in neurons, microglia, and astrocytes [38]. High concentrations of NO and SO overwhelm the brain's antioxidant defenses and favor the formation of PN [54]. Overall, morphine-induced PN production results from NOS activity, the nitration and inactivation of MnSOD, and the activation of NADPH oxidase [38]. Morphine-derived PN as well as its metabolites can cause damage to cellular components and compromise homeostasis [56]. As previously stated, PN activity results in DNA damage, mitochondrial dysfunction, and the inactivation of antioxidant enzymes and neurotransmitters [56]. PN stimulates apoptosis by nitrating heat shock proteins, cytoskeletal components, and mitochondrial proteins [57]. Specifically, PN inactivates mitochondrial MnSOD, resulting in the proliferation of SO and additional production of PN [38]. Inflammation commonly results from PN activity as it has the ability to activate transcription factors involved in the synthesis of pro-inflammatory cytokines [38]. PN promotes the disruption of glutamate homeostasis through the nitration and inactivation of NMDA receptors, glutamate transporter, and glutamine synthase [38]. Morphine-derived PN also depletes the brain of endogenous

antioxidants such as GSH by inactivating enzymes that participate in antioxidant synthesis and homeostasis [38]. Several studies have demonstrated the role of both spinal and supraspinal PN in morphine tolerance and dependence [22, 54].

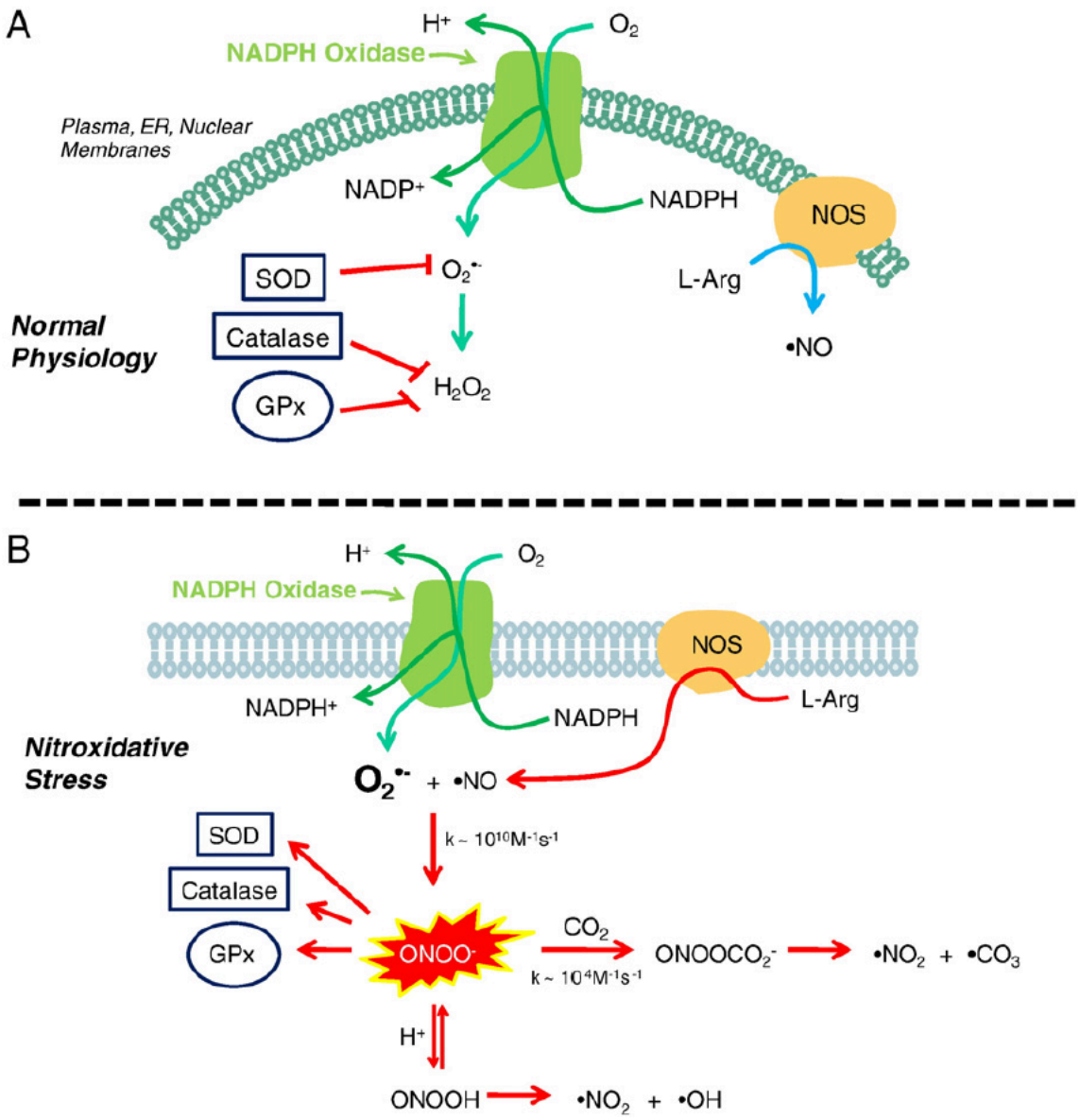


Figure 1.5 The formation of peroxynitrite following chronic morphine exposure. (A) Under normal physiological conditions, the free radicals produced by NADPH oxidase and NOS are neutralized by endogenous antioxidant enzymes including SOD, catalase, and glutathione peroxidase (GPx). (B) With nitro-oxidative stress from chronic morphine administration, the increased production of free radicals overwhelms the antioxidant system. This results in the production of PN and other oxidative-stress causing agents as well as the inactivation of antioxidant enzymes. (Salvemini and Neumann, 2010).

A variety of NOS inhibitors and free radical scavengers have been shown to reduce morphine tolerance and dependence, indicating oxidative stress as a potential molecular mechanism of such side effects (**Figure 1.6**). For example, repeated administration of VLF, an antidepressant shown to have antioxidant properties, was found to attenuate morphine-induced antinocieptive tolerance [47]. Furthermore, evidence for the role of PN in tolerance, dependence, and hyperalgesia has developed in recent years. It has been hypothesized that morphine contributes to the increase in spinal PN through several different mechanisms: (1) post-translational protein nitration (2) production and release of pro-inflammatory cytokines and (3) apoptosis [56]. The development on nitro-oxidative damage can be targeted pharmacologically at several steps in the biosynthesis on PN and other destructive free radicals. MnSOD and NOS mimetics have been shown to inhibit tolerance while decreasing the formation and release of pro-inflammatory cytokines [38]. Phospholipase D and NADPH oxidase inhibitors decreased morphine-induced macrophage apoptosis in vitro [42]. Pharmacologically inhibiting PN also prevents tolerance, further indicating a role for PN in antinociceptive tolerance [38]. Recently, synthetic antioxidant enzymes have been developed to attenuate the production of PN after chronic morphine exposure, mimicking the activity of SOD [56]. Administration of these compounds as well as other antioxidants has been shown to prevent or diminish antinociceptive tolerance and dependence [56]. Thus, targeting the SO, NO, and PN free radicals represents a novel therapy for chronic opioid users.

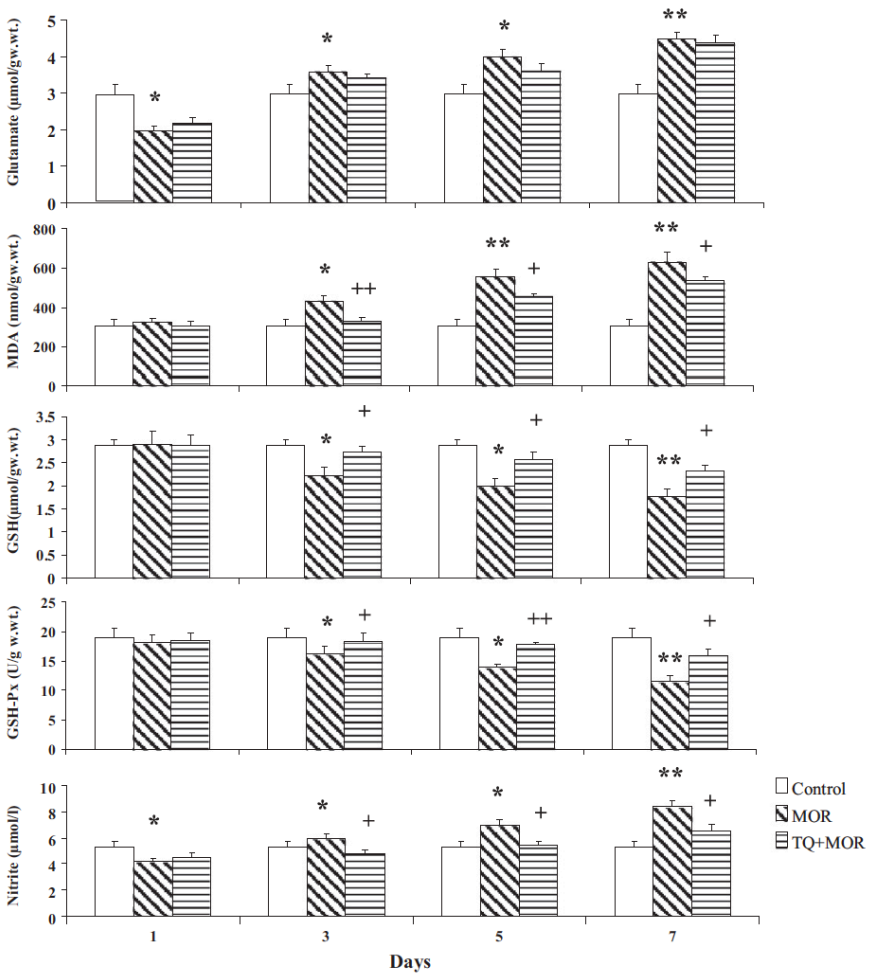


Figure 1.6. Time course of oxidative damage with repeated morphine administration and co-administration of thymoquinone antioxidant. Morphine (5 mg/kg) was administered (s.c.) to male adult mice twice daily for seven days. Glutamate, MDA, and nitrite concentrations increased, while GSH and GSH-Px concentrations decreased during the treatment period. These results demonstrate the ability of morphine to both potentiate oxidative stress and deplete antioxidant reservoirs. When thymoquinine, a suppressor of NOS activity, was co-administered with morphine, the markers of oxidative damage decrease while normal antioxidant concentrations were restored. $P < 0.05, 0.01$ compared to baseline: *, **. $P < 0.05, 0.01$ compared to morphine values: +, ++. (Abdel-Zaher et al., 2013).

The experiments outlined in this thesis will test the hypothesis that treatment with morphine following TBI will negatively impact early phase pathologies and impede recovery mechanisms. Recognizing the effects of opioid treatment on injury pathologies is important for the development of novel pharmacological treatments and improved long-term outcomes. This study aims to further understand morphine-trauma interactions and contribute to the growing body of research on the subject.

Chapter Two: Methods

2.1 Chemicals

Animals were treated with either morphine sulfate or a vehicle solution. Morphine sulfate (MS) was supplied by National Institute on Drug Abuse (NIDA). The vehicle was 20% polyethylene glycol in saline solution. Drug solutions were coded, and experimenters were blind to the treatment.

2.2 Animals

Male Sprague-Dawley rats (250-250 g, Charles River; Wilmington, MA) were housed in a 12-hour light/dark cycle with access to food and water ad libitum. Animals were habituated to noodles and trained on the balance beam prior to injury. There were four experimental groups: 1) sham + vehicle 2) sham + MS 3) TBI + vehicle and 4) TBI + MS. Morphine dose was 5.6 mg/kg per injection. The first drug injection occurred 2 hours after TBI induction, and subsequence injections were given twice daily for 7 days. Injection volume (0.1 mL/g) was determined by the weight of the animal at the time of injection.

2.3 Surgery

2.3.1 Intravenous Catheterizations

In the week prior to TBI induction, intravenous catheters were implanted for drug injection. Animals were anesthetized at 4% isoflurane for induction and 1.5-2.5% for maintenance. Patches of skin behind the shoulders and above the left jugular vein were shaved. A local anesthetic (lidocaine:bupivacaine, 7 mg/kg/mL:8 mg/kg/mL) was injected subcutaneously into the back prior to incisions. A small incision was made below the neck, and the jugular vein was exposed with blunt dissection. Surgical sutures were

used to isolate the vein from the underlying tissue. An incision was made in the back to carefully thread the catheter through the surrounding tissue to exit within the ventral incision. A small incision was made in the vein to insert the catheter. Once inserted, blood was drawn into the catheter using a streptokinase syringe to ensure that the catheter was adequately placed in the jugular vein. 0.1 mL streptokinase was then injected to maintain the patency of the catheter. The catheter was tied off with surgical sutures to secure its placement, and the ventral incision was closed. The catheter hub was placed under the skin on the animal's back before closing the dorsal incision. All incisions were closed with 3-0 sutures and covered with antibiotic ointment. Streptokinase was injected every other day until TBI induction to check the integrity of the catheters.

2.3.2 Craniectomies

Craniectomies were performed following catheter insertion to expose the left primary motor cortex (4 mm rostral bregma, 3.5 mm lateral midline) for injury. As with catheterization surgeries, animals were anesthetized at 4% isoflurane for induction and 1.5-2.5% for maintenance. 0.1 mL lidocaine was injected subcutaneously prior to scalp incision. A small incision was made to visualize the coronal suture. A screw was placed approximately 5 mm posterior to bregma. Using a trephine, a 5 mm circular portion of the skull was removed above the left primary motor cortex. If the dura was breached, animals were euthanized and excluded from the study. A modified syringe hub was glued on top of the exposed skull and held in place with dental acrylic. The hub was flushed with saline and covered with bone wax to seal. Animals were given 2-5 days to recover from surgeries before TBI induction.

2.4 TBI Induction

Animals were anesthetized at 4% isoflurane for 3.5 minutes. The syringe hub was flushed with saline to remove the bone wax and expose the brain. TBI was induced using the lateral fluid percussion (LFP) device (AmScien Instruments; Richmond, VA). Animals received a moderate brain injury (1.8-2.2 atm) and were monitored for apnea, breaths per minute, and latency to righting reflex. Animals who were not within the latency to righting reflex parameters of 5-9 mins were excluded from the study. After being returned to their home cages, animals were allowed to rest for 1 hour before evaluation with the NSS followed immediately by the first drug injection.

2.5 Behavior

Both TBI and chronic morphine administration are associated with motor deficits [1, 38, 58]. Behavioral tests were used to assess motor function before and after TBI and evaluate the influence of morphine treatment on several recovery parameters. A group of sham animals were treated with morphine to investigate the role of morphine in motor dysfunction.

2.5.1 Neurological Severity Score

The NSS contains motor, sensory, and balance tests for a comprehensive evaluation of neurological function [59]. The test used in this thesis was performed following the procedures outlined in Chen et al. (2001) [59]. Neurological function was scored on a scale of 0 to 14 (normal score, 0; maximal deficit score, 14). One point was given for each incorrectly performed test. Therefore, a higher score indicates greater neurological dysfunction. For the motor tests, 1 point was awarded for each limb that demonstrates flexion, giving a total of 5 points possible. Table 2.1 includes the scoring protocol used to evaluate the animals. Prior to injury, animals were trained on the balance

beam, a component of the NSS. A baseline value was taken before TBI induction. The NSS was then performed 1 hour after injury to evaluate initial neurological dysfunction. Animals were evaluated again with the NSS on 2 and 7 days post-injury (DPI).

Behavior	Points
Motor	
Flexion of front paw (2) Flexion of hind leg (2) Head flexion (head moved >10°)	1 1 1
Sensory	
Pinna reflex Cornea reflex Startle reflex	1 1 1
Balance	
Balances with steady posture Grasps the side of the beam One limb falls off the beam Two limbs fall off the beam or no attempt to walk on the beam Attempts to balance but falls off (<40 sec) Attempts to balance but falls off (<20 sec) Falls off the balance beam with no attempt to balance	0 1 2 3 4 5 6
Maximum points possible	**14**

Table 2.1 Neurological Severity Score. The point system for the NSS modified from Chen et al. (2001). For the motor tests, each limb that demonstrates flexion counts as one point. A higher score indicates greater neurological dysfunction.

2.5.2 Vermicelli Handling Test

The vermicelli handling test was performed as described by Allred et al [60]. Animals were habituated to the uncooked vermicelli noodles (approx. 7 cm in length) in the week prior to injury and were food deprived (80% reduction) at least 2 hours prior to testing. To evaluate paw dexterity, animals were given 3-5 pieces of pasta that were marked at 1.5 cm intervals for visualization. The animals were given 15 minutes to eat the noodles. If an animal did not eat the noodles or exhibited freezing behavior for a majority of the testing period, it was excluded from the study. Animals were recorded with a digital camera, and handling patterns or deficits were analyzed after testing. Each animal was given a total score based on how many abnormal behaviors, as outlined in Allred et al. (2008), were exhibited during the testing period (**Figure 2.5.1**).

When eating a vermicelli noodle, rats typically hold the piece of pasta with both forepaws and move it towards the mouth as the piece becomes smaller. The "grasp" paw is held farther away from the mouth at the start of eating. The "guide" paw is held closer to the mouth to guide the pasta piece into the mouth. The paws gradually move together as the pasta piece becomes shorter until the two paws are on top of each other. The "guide" and "grasp" roles are relative and vary between and within subjects.

The abnormal behaviors that were used to evaluate forepaw dexterity are listed in Table 2.5.1. These behaviors were found to be absent in the majority of trials with uninjured rats and tended to increase after brain injury, so they were used for quantification of loss of forepaw dexterity and function [60].

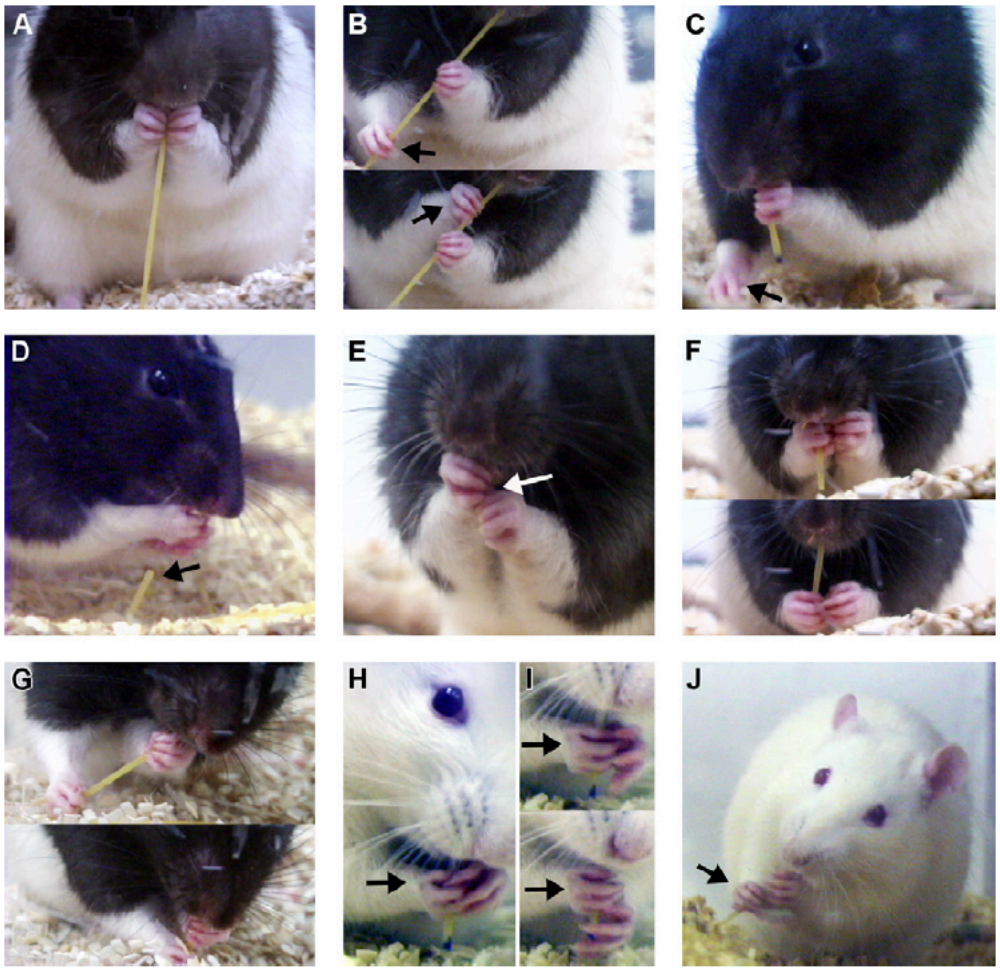


Figure 2.5.1. Abnormal noodle handling behaviors. Visual representation of the abnormal behaviors observed during eating. The descriptions of each behavior can be found in Table 2.5.1. (A) Paws together when long. (B) Guide and grasp switch. (C) Failure to contact. (D) Drop. (E) Paws apart when short. (F) Mouth pulling. (G) Hunched posture. (H) Iron grip. (I) Guide around grasp. (J) Angling with head tilt. (Allred et al. 2008).

Abnormal Behavior	Description
Paws together when long	Holding the paws in symmetrical apposition without a clear guide or grasp paw when the noodle was 3.5 cm or greater in length
Guide and grasp switch	The roles of the guide limb and grasp limb are changed during eating
Failure to contact	The forepaw does not contact the noodle during eating, excluding adjustments
Drop	The noodle is dropped after the animal has started eating
Paws apart when short	The forepaws fail to move to a symmetrical position before the noodle is completely eaten
Mouth pulling	The animal uses its mouth to pull the noodle through its forepaws
Hunched posture	The animal hunches over the noodle and moves its head down as the noodle gets smaller rather than bringing the noodle to its mouth
Iron grip	One forepaw is held in an unmoving and rigid position while eating
Guide around grasp	The guide limb is repeatedly repositioned rapidly in a compensatory manner to manipulate the noodle
Angling with head tilt	The noodle is held in the mouth as the head is tilted sideways

Table 2.5.1. Noodle Handling Quantification. A list of the abnormal behaviors that were used to quantify forepaw dexterity. The following behaviors were only counted once per trial: paws together when long, paws apart when short, iron grip, guide around grasp, and angling with head tilt. The remaining behaviors were counted each time they were observed. (Allred et al. 2008)

2.6 Protein Analysis

2.6.1 Western Blot

Western blot was used to evaluate nitro-oxidative damage of proteins and the integrity of antioxidant defenses. Animals were deeply anesthetized with ketamine/xylazine (85/10 mg/kg) prior to sacrifice by decapitation. Tissue samples from the spinal cord, thalamus, and cortex were obtained for western blot analysis and ELISA assays. Tissue was extracted and placed in an ice cold Petri dish during slicing. The cortical and thalamic regions were separated following the protocol described in Erondu et al [61]. The cerebellum and brain stem regions were separated from the rest of the brain. A midsagittal cut was made to separate hemispheres and expose the midbrain for better extraction of the thalamus. Sections from both hemispheres were combined after dissection and analyzed as a single sample. The hypothalamus was removed and discarded using the anterior commissure as a reference point. The thalamus/midbrain region was separated and placed in the buffer, and the rest of the sample was taken as the cortex. Spinal cord tissue was taken from the thoracic and lumbar regions. Upon separation, tissue samples were placed in ice cold RIPA buffer (Cell Signaling, Danvers MS) and sonicated to homogenize. RIPA buffer volumes were determined based on the weight of the sample (1 mL of RIPA buffer per mg of tissue). Homogenates were kept on ice until centrifuged at 35,000 rpm at 4°C for 30 mins. The supernatant was transferred to a new tube and stored at -80°C until analysis. The total protein concentration was determined with the NanoDrop 2000 (Thermo Fischer Scientific, Waltham, MA), and the concentration was held between 5-10 μg/μL. When necessary, samples were diluted with RIPA buffer to obtain the optimal protein concentration. Proteins were separated using a

precast 4-20% SDS-PAGE gel (Bio-rad) submerged in running buffer (Bio-rad). The sample was heated at 90-100°C for 5 mins prior to loading. 20-30 µg of total protein from supernatant was distributed into each well along with a molecular weight marker. The gel was run at 200 V for approximately 1 hour. Proteins were transferred to a nitrocellulose membrane at 70 V for 1.5 hours while submerged in transfer buffer. The membrane was blocked with 5% milk in TBST (10x tris/glycine/SDS) for 1 hour. Proteins were probed with an anti-nitrotyrosine primary antibody (Cell Signaling, Danvers MS) overnight, then washed 3 times with TBST for 5 mins. The proteins were incubated in mouse anti-rabbit secondary antibody conjugated to Alexaflur488 for 1 hour, followed by washing with TBST. The primary and secondary antibodies were diluted with TBST containing 5% milk. Proteins were imaged with fluorescent microscopy and quantified using ImageJ.

2.6.2 ELISA Assays

Animal sacrifice, tissue extraction, and homogenization occurred as previously described. Homogenates from the spinal cord, thalamus, and cortex were analyzed with ELISA assays for MDA, superoxide dismutase, and catalase (Cell Biolabs, San Diego, CA). All solutions were provided in the ELISA kit. 50 µL of tissue homogenate was loaded into each well along with 50 µL of primary antibody and incubated at room temperature for 1 hour on an orbital shaker. The sample was washed 3 times with wash buffer, then incubated in HRP-conjugated secondary antibody at room temperature for 1 hour on an orbital shaker. 100 µL of sample solution was added to each well and incubated for 20 mins. The enzymatic reaction was stopped with 100 µL of stopping solution. Absorbance of each well was read using 450 nm as the primary wavelength.

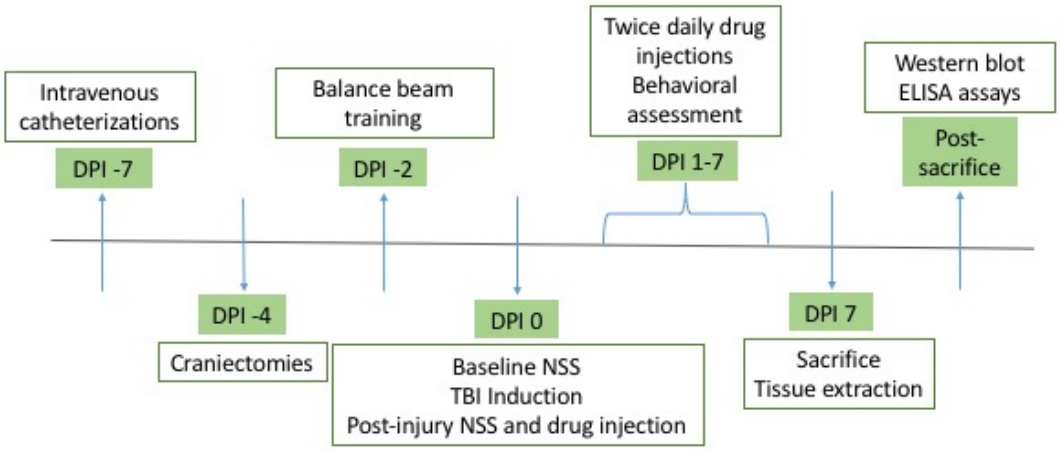


Figure 2.6.1. Experimental Timeline. A timeline indicating surgeries, training, TBI induction, drug injections, and tissue extraction. Specifically, the NSS and noodle handling tests were performed on DPI 2. The NSS was performed again on DPI 7 before sacrifice and tissue extraction.

2.7 Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) and mixed-effect repeated measures analysis followed by an appropriate post hoc test using GraphPad Prism (Graphpad, San Diego, CA). Values within treatment groups were analyzed with respect to baseline by Dunnett tests. Differences among treatment groups were analyzed by comparing the mean of each column to the mean of every other column with ANOVA followed by Bonferroni tests. All data is presented as mean +/- standard error of mean (SEM), and differences were considered statistically significant when $p < 0.05$.

2.8 Expected Results

A significant decrease in motor function and increase in oxidative damage is expected with both TBI and chronic morphine treatment. I hypothesize that the sham + morphine group will perform worse on behavioral assessments and exhibit greater oxidative damage than the sham + vehicle group. Similarly, I expect results the TBI + morphine group to show more adverse effects than the TBI + vehicle group. As morphine treatment alone increases ROS and RNS in neurons, I hypothesize that treating TBI-related pain with morphine will exacerbate cellular damage caused by free radicals and further impair functional recovery.

Chapter 3: Results

3.1 Injury Severity Recovery

3.1.1. Neurological Severity Score

The NSS was used to comprehensively evaluate neurological function. The test consists of motor, sensory, and balance components, and it was performed at 0.042, 2, and 7 days post-injury (DPI) to monitor recovery (**Figure 3.1.1**). A higher NSS indicates greater neurological dysfunction. A significant effect of time ($p < 0.0001$) and treatment group ($p < 0.0011$) was found using ANOVA (**Figure 3.1.1A**). This was confirmed by a mixed-effects repeated measures analysis. Both groups that received TBIs showed an increase in NSS following the injury, while the sham groups remained at baseline. Both TBI groups differed significantly from baseline (TBI + Veh: $p < 0.0436$; TBI + MS: $p < 0.0021$) at 1 hr post injury, indicating similar injury severity before drug injections. Using a Dunnett post-test, it was found that the TBI + MS group, but not the TBI + vehicle group, significantly differed from baseline at the time points following drug injection (2 DPI: $p < 0.0041$; 7 DPI: $p < 0.0019$). The sham treatment groups did not differ significantly from baseline at any time points. There were no differences between treatment groups when analyzed with the Bonferroni post-test.

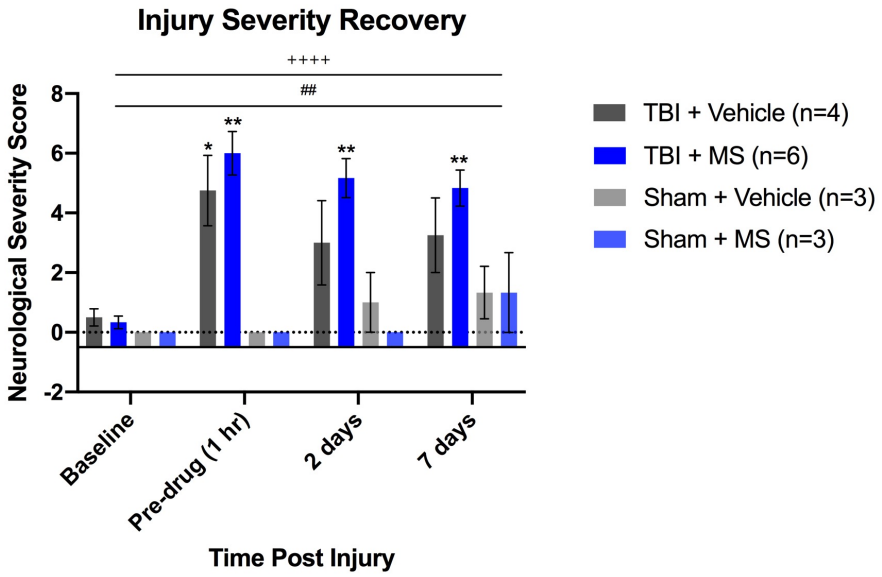
When comparing only the treatment groups that received TBI, there was a significant effect of time ($p < 0.0001$) that was confirmed with a mixed-effects repeated measures analysis. Again, both groups differed significantly from baseline before drug injections (TBI + Veh: $p < 0.0436$; TBI + MS: $p < 0.0021$). The TBI + MS group again differed significantly from baseline at both time points taken after drug injections (2 DPI:

$p < 0.0041$; 7 DPI: $p < 0.0019$). No significant differences between the average NSS of each treatment group were found (**Figure 3.1.1B**).

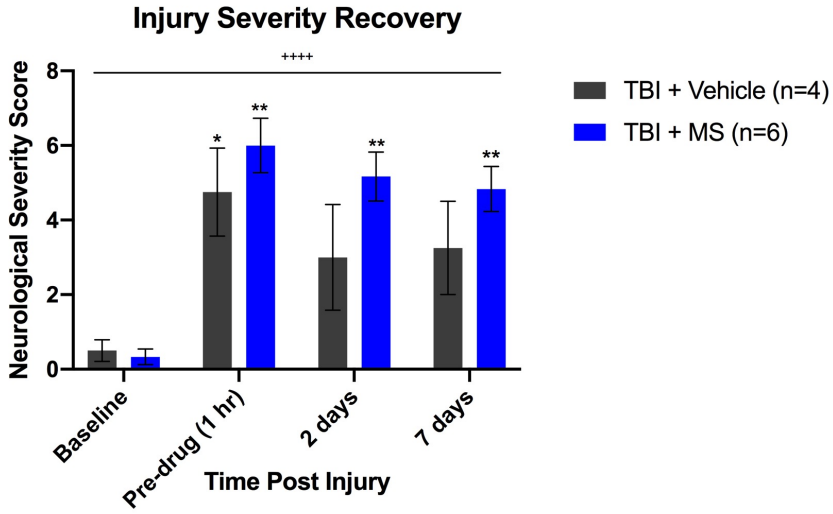
To further understand the effect of morphine treatment on functional recovery, the time points taken during the injection period were analyzed individually. On DPI 2, a significant difference among the means of each treatment group was found using one-way ANOVA ($R^2 = 0.6097$, $p < 0.0084$). However, the Bonferroni multiple comparisons test did not reveal differences between treatment groups (**Figure 3.1.1C**). A difference in the average NSS of each treatment group was not observed on DPI 7 (**Figure 3.1.1D**).

Figure 3.1.1. Injury severity recovery. Time course of injury severity recovery as measured by the NSS. (A) Both TBI groups differ significantly from baseline before drug injections, but only the TBI + MS (5.6 mg/kg) treatment group differs significantly from baseline at 2 and 7 DPI. Other treatment groups did not differ significantly from baseline. Differences between groups were not found at any time points. There was a significant effect of time ($p < 0.0001$) and treatment group ($p < 0.0078$) found with ANOVA. (B) When only comparing groups who received TBI, the TBI + MS group different significantly from baseline at 2 and 7 DPI (C,D). A significant effect of treatment on average NSS was observed on DPI 2 but not DPI 7. $P < 0.05$, 0.01, 0.001, 0.0001 relative to baseline: *, **, ***, ****. Significant effect of treatment group: #, ##, ###, ####. Significant effect of time: +, ++, +++, ++++.

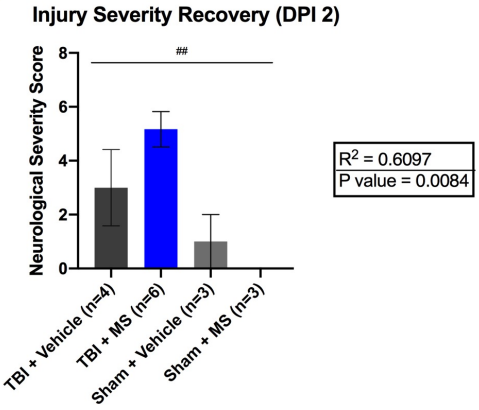
A)


Injury Severity Recovery
++++
##
Neurological Severity Score
8
6
4
2
0
-2
*
**
**
**
Baseline
Pre-drug (1 hr)
2 days
7 days
Time Post Injury
TBI + Vehicle (n=4)
TBI + MS (n=6)
Sham + Vehicle (n=3)
Sham + MS (n=3)

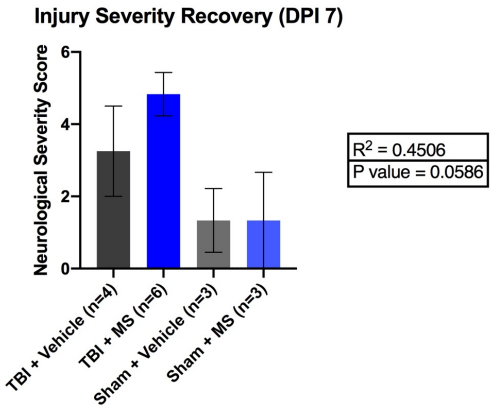
B)


Injury Severity Recovery
++++
Neurological Severity Score
8
6
4
2
0
*
**
**
**
Baseline
Pre-drug (1 hr)
2 days
7 days
Time Post Injury
TBI + Vehicle (n=4)
TBI + MS (n=6)

C)


Injury Severity Recovery (DPI 2)
##
Neurological Severity Score
8
6
4
2
0
TBI + Vehicle (n=4)
TBI + MS (n=6)
Sham + Vehicle (n=3)
Sham + MS (n=3)
R2 = 0.6097
P value = 0.0084

D)


Injury Severity Recovery (DPI 7)
Neurological Severity Score
6
4
2
0
TBI + Vehicle (n=4)
TBI + MS (n=6)
Sham + Vehicle (n=3)
Sham + MS (n=3)
R2 = 0.4506
P value = 0.0586

3.1.2 Balance Recovery

The balance component of the NSS was isolated from the total score to determine if treatment specifically affected balance recovery (**Figure 3.1.2**). A significant effect of time ($p < 0.0009$) and treatment group ($p < 0.0015$) was found. Both TBI groups differed significantly from baseline at 1 hr post-injury (TBI + Veh: $p < 0.0026$; TBI + MS: $p < 0.0131$). The TBI + MS group was the only group to differ significantly from the baseline balance score at 2 and 7 DPI (at 2 DPI: $p < 0.0051$; at 7 DPI: 0.0204). There were no distinctions between treatment groups at any time points ($p > 0.05$).

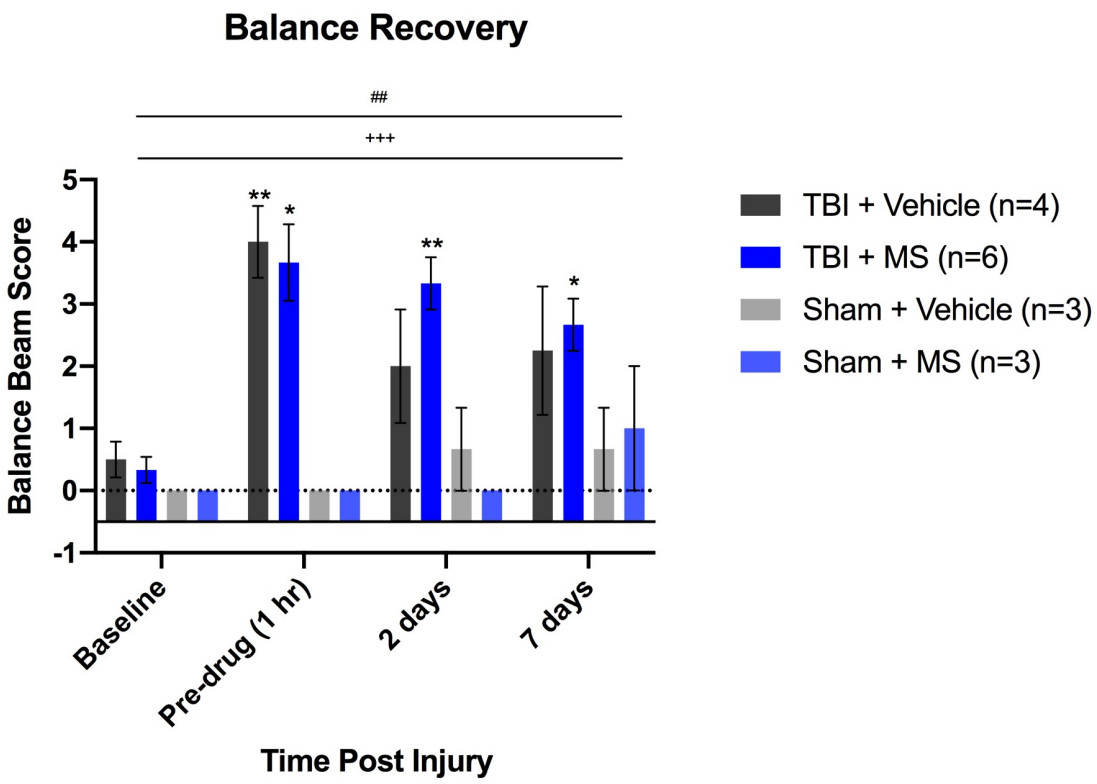


Figure 3.1.2. Balance recovery. Animals treated with MS after TBI had greater balance impairment at 2 ($p < 0.0051$) and 7 DPI ($p < 0.01204$) than those treated with the vehicle solution. Treatment did not affect the balance performance of sham animals. $P < 0.05$, 0.01, relative to baseline: *, **. Significant effect of treatment group: #, ##. Significant effect of time: +, ++, +++.

3.2 Forepaw Dexterity (Noodle Handling)

The vermicelli noodle test served as an indicator of forepaw dexterity. Total score was calculated as described in Table 2.5.1. Similar to the NSS, a higher score indicates greater dysfunction in forepaw dexterity. For reasons described in Discussion, the test was only given to animals who sustained a TBI and took place on DPI 2. An unpaired t-test was used to analyze the data set, and the results are presented in **Figure 3.2.1**. There was not a significant difference in forepaw dexterity between treatment groups.

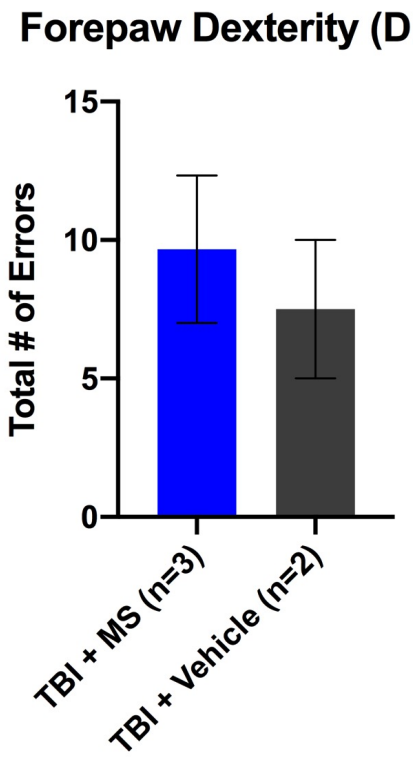


Figure 3.2.1. Forepaw dexterity recovery. No significant difference was found between morphine and vehicle groups in the vermicelli noodle test ($p < 0.05$).

3.3 Western Blot

The Western blots were not completed due to Tulane's closure.

3.4 ELISA Assays

The ELISA assays were not completed due to Tulane's closure.

Chapter 4: Discussion

4.1 Injury Severity Recovery

The present study investigated the effect of morphine treatment on functional recovery following TBI. The results provide preliminary evidence for our hypothesis that animals treated with morphine after TBI will exhibit greater motor dysfunction than those treated with vehicle. While both TBI groups showed increases in the NSS and the balance recovery subcomponent of the NSS, only the increases in the morphine-treated group reached statistical significance at the time points taken after drug administration. This indicates that morphine exacerbated the effects of TBI. The animals that received morphine after sustaining a TBI showed a significant increase in total NSS at 2 and 7 DPI (**Figure 3.1.1A**). A similar result was found when the balance score was isolated from the total NSS (**Figure 3.1.1B**). However, there are caveats to this conclusion. When the average scores of each treatment group were compared at a given time point, no significant differences were found. Also, morphine treatment did not impair neurological function in sham animals (**Figure 3.1.1A-B**). The sample size was small in this initial study, particularly in the sham groups, and the results need to be confirmed with larger group sizes. More cohorts should be tested to further evaluate the potential role of opioid treatment in TBI-related motor dysfunction.

4.2 Forepaw Dexterity Recovery

The vermicelli noodle test was used as an additional assessment of motor impairment after TBI. It specifically focused on forepaw dexterity and fine motor movements. There was not a significant difference from baseline or between treatment groups in animals who sustained a TBI.

The vermicelli noodle test produced several problems that may have affected results. For one, an accurate baseline was difficult to achieve. Although animals had been food deprived for several hours, some were unwilling to eat the noodle or showed no interest in it at all. For this reason, noodle handling data was collected from only 5 animals. It is likely that the animals were not sufficiently food deprived. Freezing behavior was common when the animal was first brought into the testing room despite being habituated to the room prior to injury and drug administration. Also, morphine treatment may have affected appetite of some animals, deterring them from eating the noodle piece even after food deprivation. Constipation is a serious side effect of opioid treatment [38], and it may have played a role in willingness to eat and appetite. The article from which the vermicelli noodle test was modeled after did not examine the effects of pharmacological treatments on eating behavior. When animals did eat the noodle, it was often difficult to detect abnormal behaviors as the camera could not accommodate all angles. Moving the camera closer to the test cage often startled the animal and caused them to stop eating. Furthermore, data was not collected for sham animals as only a small number of them chose to eat the noodle. These variables may have affected the data.

Modifications to the testing protocol may eliminate errors and improve accuracy. Particularly important, a longer food deprivation (e.g. overnight) would likely increase the behavior. Giving animals more time to habituate in the testing room may decrease freezing behavior and startle responses to a moving camera. Optimizing the time of day in which animals undergo testing could increase appetite in animals treated with morphine. Other evaluations of fine motor function, such as the grooming assessment or grasp test, could be used to supplement the vermicelli noodle test. Additional tests that

look specifically at hind limb function may provide a more comprehensive understanding of the effects of both TBI and morphine administration. Although they are primarily used after spinal cord or nerve injury, the diffuse nature of TBI-related damage warrants their use. According to Nichols et al. (2005), hind limbs may be more vulnerable to dysfunction following brain injury and post-operative analgesics [62]. While hind limb function is necessary for rats to walk on a balance beam, it does not isolate the hind limbs. Incorporating tests such as gait analysis or the inclined plane test into this treatment paradigm may provide a more complete representation of morphine-trauma interactions [62].

In summary, this study provides preliminary evidence for the hypothesis that animals treated with morphine after TBI will exhibit greater motor dysfunction than those treated with vehicle. Additional studies with larger groups, however, will be needed to confirm these findings.

Chapter 5: Conclusion

TBIs initiate a number of cellular pathologies that can disrupt normal brain functioning. Damage to brain tissue causes uncontrolled neurotransmitter release and a drastic increase in intracellular calcium ions. Calcium is responsible for stimulating a variety of signaling pathways that can interrupt cellular functions. As the mitochondria are primarily responsible for calcium sequestration, they are most susceptible to ion-induced damage. Excess calcium favors the formation and release of ROS and RNS from the mitochondria, resulting in oxidative damage. While TBIs have been identified as a growing public health problem, there are very few treatment options available for these injuries. Opioids such as morphine are commonly used to treat chronic pain that develops after TBI, but the consequences of morphine-trauma interactions have not been fully elucidated. Morphine has been shown to cause many of the cellular pathologies seen with TBI, including oxidative damage. It is also associated with many negative side effects, such as motor dysfunction, that could hinder injury recovery. Therefore, morphine may not be an ideal treatment for post-injury pain conditions. In this thesis, the role of morphine in the development of oxidative damage and motor dysfunction after TBI was investigated. I hypothesized that treatment with morphine after injury or sham procedure would increase oxidative damage and motor dysfunction compared to vehicle controls. The results provide preliminary evidence that morphine exacerbates motor dysfunction after TBI. Future directions could include testing more subjects to minimize variation in motor performance and adding supplemental behavioral assessments to better understand the effect of morphine treatment on motor function.

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