Graduate School ETD Form 9
(Revised 01/14)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Glen Howel G. Acosta

Entitled
SUSCEPTIBILITY OF PARKINSON'S DISEASE FOLLOWING MILD BLAST TRAUMATIC BRAIN INJURY

For the degree of Master of Science

Is approved by the final examining committee:

Dr. Riyi Shi

Dr. Jean-Christophe Rochet

Dr. Daniel Suter

To the best of my knowledge and as understood by the student in the *Thesis/Dissertation Agreement. Publication Delay, and Certification/Disclaimer (Graduate School Form 32)*, this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Dr. Riyi Shi

Approved by Major Professor(s): _____

Approved by: Dr. Laurie Jaeger 07/28/2014

Head of the Department Graduate Program Date

SUSCEPTIBILITY OF PARKINSON'S DISEASE FOLLOWING MILD BLAST TRAUMATIC BRAIN INJURY

A Thesis

Submitted to the Faculty

of

Purdue University

by

Glen Howel G. Acosta

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

August 2014

Purdue University

West Lafayette, Indiana

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr.Riyi Shi, for his guidance on this novel and exciting topic of my thesis research. I am grateful for taking me in as his student and I appreciate his trust in allowing me to work on this project. In addition, I would like to thank Dr. Daniel Suter and Dr. Chris Rochet for their invaluable assistance during this process. I appreciate all your academic teachings and patience.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

3-HPMA – 3-Hydroxypropylmercapturic Acid; 6-OHDA – 6-Hydroxydopamine; BBB – Blood Brain Barrier; BINT – Blast Injury-Induced Neurotrauma; CNS – Central Nervous System; GHS – Glutathione; HNE – 4-Hydroxynonenal IED – Improvised Explosive Device; IP – Intraperitoneal; LB – Lewy Body; LPO – lipid peroxidation; MAOI – monoamine oxidase inhibitor; mBINT – mild Blast Induced Neurotrauma; mTBI – mild Traumatic Brain Injuryy; PD – Parkinson's disease; PLZ – Phenelzine; ROS – Reactive Oxygen Species; SCI – Spinal Cord Injury; TBI – Traumatic Brain Injury; TH – Tyrosine Hydroxylase

ABSTRACT

Acosta, Glen Howel. M.S., Purdue University, August, 2014. Susceptibility of Parkinson's Disease Following Mild Blast Traumatic Brain Injury. Major Professor: Riyi Shi, M.D., Ph.D.

Blast injury-induced neurotrauma (BINT) is steadily increasing in prevalence due to escalated terror activity and constitutes the signature injury associated with current military conflicts. BINT produces significant neurological deficiencies and there is a growing concern that the injury may produce long-term consequences that affect the resilience and the performance of soldiers. One of the potential consequences is an increased susceptibility to Parkinson's disease (PD). A vital goal aimed at curtailing the post-deployment long-term consequences of blast injury-induced neurotrauma is to further our knowledge of pathogenic mechanisms responsible for the escalation of post injury diseases. *The purpose of this project is to investigate the molecular mechanism underlying the susceptibility of PD in post-blast rats.* We have identified acrolein, a highly reactive aldehyde that persists days to weeks following brain-injury and perpetuates oxidative insult, as a potential therapeutic target to curtail chemically-mediated damage, a common feature of BINT and PD. **Our hypothesis is that acrolein is a key pathological factor linking BINT and the development of PD in our rat model.**

CHAPTER 1 - INTRODUCTION

1.1 Research Relevance

Blast injury-induced neurotrauma (BINT) has been a frequent mode of injury associated with increasing efforts against the global war on terrorism and other war-related conflicts [1-3]. Its prevalence has gradually increased in the past decade and has been deemed the "signature wound of the military" [1, 4-6]. Exposure to the primary pressure wave produced by explosive devices is responsible for many of the war-related pathologies during Operation Iraqi Freedom and the Global War on Terror [1]. Recent data have indicated that sixty-five percent of all combat injuries are from explosive blast events. Sixty percent of warfare casualties sustained during current military endeavors in Iraq can be attributed to improvised explosive devices (IEDs) [1, 5-9]. Accordingly, the U.S. Department of Defense has invested a yearly budget of 3.5 billion dollars to improve prevention and treatment of blast injuries [10-13].

Epidemiological studies have shown that BINT is associated with subsequent neurological deficiencies including traumatic brain injury (TBI) and spinal cord injury, which can lead to dysregulation of neuronal processes resulting in decreased function [4, 9, 14-17]. Particularly, 15% of troops serving in Iraq show some level of neurological impairment due to blast exposure, and their symptoms are highly correlated with mild TBI (mTBI) [18]. There is an increased prevalence of blast-induced mTBI (referred to here as mBINT to avoid confusion with conventional impact-acceleration TBI) [2, 19], fostering growing concern that blast-related injury may produce long term consequences and affect the resilience and performance of active duty groups [5]. These consequences include depression [20], memory loss [21], dementia [22, 23], and increased

susceptibility to Parkinson's disease (PD) [24-26], among others. The risk underlying this pathology is exacerbated by its subclinical nature that can delay treatment past a prime window for intervention. *A vital goal aimed at curtailing post-deployment long-term consequences of mBINT is to further our knowledge of pathogenic mechanisms responsible for the escalation of post-mBINT consequences.*

1.2 Definition and Pathophysiology of Blast Injury

'Blast injury' refers to the clinical syndrome describing the pathophysiological effects of an organism exposed to a high explosive detonation [27]. The Center for Disease Control and Prevention has divided blast injury into four distinct categories: primary, secondary, tertiary, and quaternary blast injury. Secondary, tertiary and quaternary blast injuries are similar to other forms of trauma and have been extensively studied. However, the etiology and mechanisms of the primary injury are not well characterized, and therefore, the main focus of this research. Primary blast injury is the result of a blast-wave impacting bodily tissue and is considered a unique injury modality separate from the other categories. The exposure of bodily tissue, such as the brain, to a shock wave results in a complex series of events. The impact force caused by the shock wave is particularly detrimental to the central nervous system (CNS).

The pathology of blast injury can be through direct contact of the pressure wave to the cranium and/or indirectly to the thoracic region [28-31]. Tissue disruptions in the brain can damage the walls of blood vessels and glial end-feet, which can lead to the disruption of the blood brain barrier (BBB) resulting in increased vascular permeability and subsequent pro-inflammatory response [32, 33]. It is still unclear whether this inflammatory response is beneficial or deleterious, however the over stimulation of this bio-signaling cascade and pro-inflammatory responses following injury can induce neurodegeneration in the neighboring neurons and can result in scar formation [33, 34]. From these events, it is apparent that BINT compromises the brain's defense systems in the BBB in some manner. Neurons require a stable environment to thrive and signal

properly and, as such, the post-BINT brain microenvironment can induce large amounts of stress on neuronal tissue.

Additionally, direct damage to the neuronal membranes can lead to an ionic imbalance, and therefore can cause the influx of calcium in the extracellular space. This can trigger an apoptotic-signaling cascade via calcium dependent kinase and protease activation. Activated calcium-dependent proteases such as calpain have been shown to dismantle proteins that anchor myelin to the axonal membrane [35]. Furthermore, damaged neurons can generate reactive oxygen species (ROS), elevate oxidative stress, and lead to further cellular and mitochondrial membrane damage via lipid peroxidation. These damages can lead to the production of reactive aldehydes such as acrolein, which can generate more ROS. This process of self-propagation can easily overcome the endogenous antioxidant defense system of the neurons and thus can lead to neurodegeneration.

It is clear that the biochemical events (secondary injury) following BINT play a critical role in the pathological progression of the injury. Of particular interest is the production of ROS after a blast injury, which can lead to the production of reactive aldehydes such as acrolein, and can perpetuate to generate more ROS. Acrolein, a highly reactive product of lipid peroxidation that produces toxic intermediates, highly reacts with glutathione, the most abundant antioxidant found in cells [36, 37]. A self-propagating cycle, local acrolein-induced depletion of glutathione may play a significant role in the pathogenesis of blast injury and perhaps its long-term consequences. Currently, there is no effective treatment to halt secondary injury processes partly due to the lack of understanding of the pathological pathways of these injuries following a traumatic brain injury [38], particularly, BINT. This study proposes that oxidative stress, particularly that perpetuated by acrolein, plays a major role in the pathology of blast injuries and can contribute to long-term consequences such as Parkinson's disease.

1.3 Oxidative Stress, Lipid Peroxidation, Acrolein Production and Scavengers

Oxidative stress is a collective term used to describe free radical overproduction due to cellular processes. Accumulation of reactive oxidative species (ROS) leads to the activation of several pathways, and is thought to be a major cause of various diseases such as of Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) [39]. The elevation of ROS induces lipid peroxidation, an oxidative degradation of lipids [40]. In the central nervous system, the majority of oxidative stress is manifested by lipid peroxidation, and this leads to the generation of reactive aldehydes such as acrolein and 4-hydroxy-2-nonenal (4HNE) [41-43]. These aldehydes have been shown to modify amino acids, proteins, and nucleic acids through covalent interactions to form irreversible adducts, thereby inhibiting their synthesis and interfering with their function [44-47]. Furthermore, these products have been shown to induce cell death in cultured neurons [45, 48].

Acrolein is the most reactive of the α, β-unsaturated aldehydes produced endogenously during lipid peroxidation. Acrolein interacts with the sulfhydryl group of cysteine, the amino group of lysine, and imidazole group of histidine forming covalent adducts [44, 49, 50]. Due to its high reactivity towards biomolecules, the excessive endogenous production of acrolein can inflict significant damage within the CNS. Specifically in spinal cord injury, acrolein can induce chemical or secondary damage following the mechanical trauma and has been shown to be highly elevated [51]. Similarly, microinjection or *in vitro* exposure of acrolein to the spinal cord promotes tissue damages including demyelination and cell death. These damages were associated with the motor and sensory behavioral deficits of spinal cord injury [37, 52-55]. With acrolein's relatively long half-life and its proven neurotoxicity, eliciting damages observed in neuronal trauma and degenerative diseases [56, 57], we speculate similar biochemical changes and damages in the brain neurons as a result of the mechanical stress induced by BINT. Therefore, mechanical trauma resulting from BINT can provide an initial source of acrolein in a lipid-rich environment of the brain, which is highly susceptible to lipid peroxidation due to its high lipid content attributed to abundance of

myelin and axonal membrane. Additionally, it has been reported that overproduction of acrolein significantly elevated oxidative stress through depletion of glutathione [54]. This leads to a self-regeneration concept, where oxidative stress via lipid peroxidation leads to the production of acrolein, and acrolein in itself can perpetuate oxidative stress (Figure 1). Therefore, the removal of acrolein stunts this cycle proving beneficial for therapeutic intervention and ideally prevention of disease pathology.

Due to the highly reactive properties of acrolein and its ability to modify cell macromolecules underlying its toxicity, a trapping agent would be ideal to prevent such damage. The antihypertensive drug, hydralazine is to date the most studied and well characterized of the acrolein scavengers. It has been demonstrated to bind and neutralize acrolein [58-60] and acrolein-protein adducts [61-63]. Additionally, it has also prevented acrolein-mediated cell death and injuries in vitro [64, 65]. The hydrazine group of hydralazine has been identified to react with acrolein, at a 1:1 ratio [60, 63] (Figure 2). However, hydralazine's vasodilatory effect is one of the limitations for therapeutic purposes, mainly because it would be undesirable for a patient that suffered from spinal cord injury or blast, as they may be likely suffering from a neurogenic shock. In addition, the half-life of hydralazine does not exceed one hour, which potentially limits its therapeutic efficacy in suppressing acrolein generation and related chronic oxidative stress processes [66].

Phenelzine (PLZ), an irreversible non-selective monoamine oxidase inhibitor (MAOI), has been primarily utilized for the treatment of depression [67-69], but also in other psychiatric disorders such as panic disorders [70, 71], social anxiety disorders and post-traumatic disorders [72-74]. Phenelzine, like hydralazine has a hydrazine group rendering it a potential acrolein scavenger (Figure 2). PLZ has also been shown to be neuroprotective in a gerbil model of forebrain ischemia by reacting with 3-aminopropanal (3-AP) to form hydrozone, and provides neuroprotection from acrolein induced LDH release *in vitro* [75]. Additionally, PLZ alleviates oxidative stress through acrolein scavenging in a traumatic spinal cord injury rat model [37]. PLZ is not a vasodilator and can be administered safely at higher doses compared to hyrdralazine. A 15 mg/kg dosage was given subcutaneously to gerbils after ischemia-reperfusion brain injury and

effectively neutralized reactive aldehydes such as acrolein, providing neuroprotection [75]. In this study, we used PLZ as an acrolein scavenger following blast injury in efforts to prevent PD-like symptoms in our rat model.

1.4 Parkinson's Disease (PD)

Parkinson's disease (PD) is an age-related neurodegenerative disease affecting about 1-3% of the population over 50 years of age [76, 77]. PD is characterized by relatively selective, progressive degeneration of the dopaminergic (DA) neurons in the substantia nigra and the presence of LB inclusions in the affected brain regions. The lack of dopamine supply to the striatum is the major contributing factor to motor dysfunction phenotypes of PD, including shaking tremors, rigidity and gait disturbances. The current therapeutic interventions include drugs such as dopamine agonists and MAO-B inhibitors to increase dopamine levels and at time deep brain stimulation are currently available. However, these treatments only provide relief rather than a cure or preventive measure for combating disease symptoms and the risk-benefit profiles of current treatment options are still inadequate.

This shortage of effective treatments for PD is partially due to our limited understanding of the mechanisms of dopaminergic neuronal death. Therefore, the current treatments only provide symptomatic relief rather than addressing the major cause of the disease. α-Synuclein, the major component of LB inclusions is thought to play an essential role in the pathogenesis of PD [78]. Modified α-synuclein is found within LB inclusions in a PD brain, and mutations in the α-synuclein gene are associated with familial PD [79, 80]. The formation of LBs has been linked with oxidative stress, which is also consequently elevated in PD patients [81-83]. α-Synuclein also has abundant lysine-rich repeats, making it more vulnerable to oxidative stress and the reaction with acrolein [84]. Therefore, we can speculate that α-synuclein aggregation is a major player in DA cell death seen in PD.

It is well established that oxidative stress and lipid peroxidation play important roles in mediating the death of DA neurons [85, 86]. The challenge in the field of PD is to further understand mechanisms of oxidative stress and to identify novel and more effective targets to prevent DA cell death. As mentioned previously, acrolein, produced by lipid peroxidation, can directly damage nerve cells and generate free radicals. Our lab has also shown that in a cell-free system and in vitro studies, acrolein can induce α-synuclein aggregation and leads to neuronal death (data not shown). These findings have led us to postulate that acrolein plays a critical role in neurodegeneration associated with PD. Consequently, it is also important to consider other contributing factors that lead to PD, such as traumatic brain injury (TBI). Epidemiological studies have demonstrated that TBI is a risk factor for PD [87], however the link between the two remains unclear. Previously, our lab has shown the role of acrolein in a PD rat model and in a blunt-force impact TBI model, but not in a BINT model. We further speculate that the acrolein post-blast injury can contribute to the development of a PD-like pathology. This study investigates the susceptibility to PD following a blast injury and we speculate acrolein plays a major role in linking blast injury and PD.

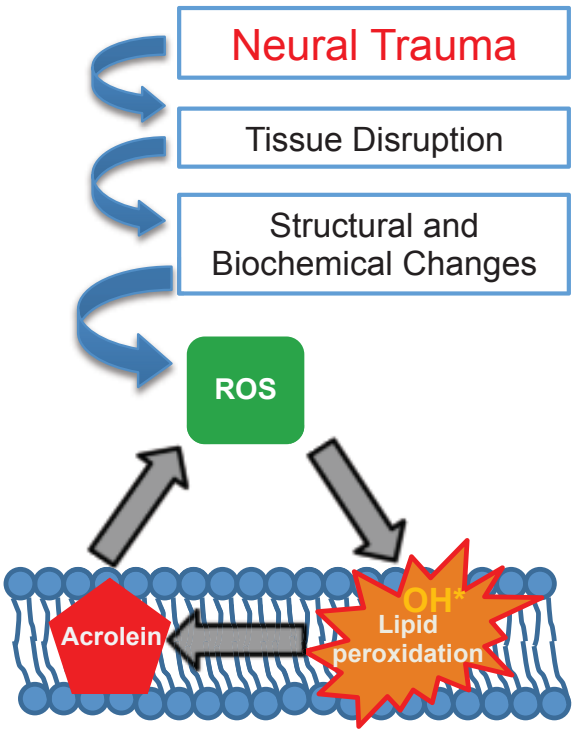

Figure 1. Acrolein regeneration cycle after neural trauma. A diagram showing the cascade of cellular responses after a neural trauma. The tissue disruption and structural damages from a neural trauma leads to a cascade of biochemical changes in the brain. In particular, ROS (such as H_2O_2) from oxidative stress can readily generate hydroxyl radicals (OH*). These radicals induce lipid peroxidation and produce reactive aldehydes such as acrolein, which can directly and indirectly generate more ROS, perpetuate this cycle and generate further insult.

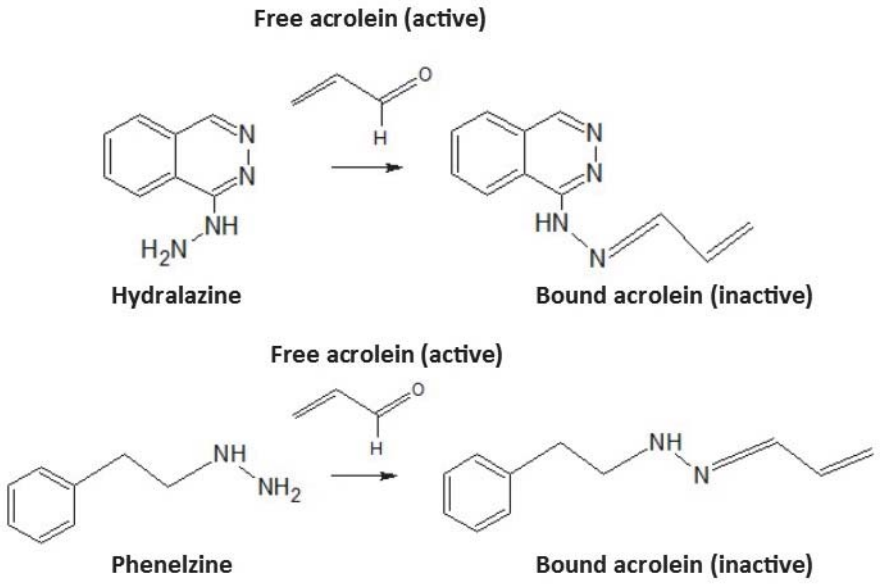

Figure 2. Inactivation of acrolein by acrolein scavengers: hydralazine and phenelzine

CHAPTER 2 – RESEARCH PROPOSAL AND AIMS

2.1 Research Rationale

BINT injuries are usually accompanied by affective disorders and have been linked to an increased risk of developing PD [4, 9, 14-17]. The primary physical trauma sustained from an injury, such as blast injury, is exacerbated by altered physiological conditions including cerebral blood flow, intracranial pressure, inflammatory response activation and phospholipid metabolism, which lead to a delayed phase of chemically-mediated damage termed "secondary injury" [88-93]. Particularly, in human post-mortem brain tissue analysis and animal studies, TBI can induce abnormal α-synuclein accumulations in the axonal swellings, dystrophic neurite formation [94-96], and inflammatory response [97]; similar to what is observed in PD pathology. However, due to inadequate knowledge of underlying mechanisms of both TBI and PD, common pathogenic features remain elusive and therapeutic options are limited. Recently, oxidative stress, the underlying mechanism of secondary injury, has emerged as an important feature of both neural trauma [28, 98] and neurodegenerative diseases, particularly PD [99-102]. However pharmacologically eliminating free radicals offers marginal neuroprotection and has had limited success in attenuating further progression of damage. *Our lab has identified acrolein, a highly reactive aldehyde that persists days to weeks following injury and perpetuates oxidative insult, as a potential therapeutic target to curtail chemically-mediated damage, a common feature of TBI and PD.*

Despite strong interest, the cellular mechanisms of blast-induced brain injury are essentially unknown due to limitations of human studies and insufficient investigation in animal models. We have recently established a novel rat blast-induced brain injury model that displays significant biochemical and behavioral deficits in the absence of

conspicuous acute motor deficits, shown in Figure 1, [103] a phenotype that closely resembles the mild BINT human condition [104]. The development of such model will allow us to quantify the injury sequelae as well as investigate potential treatments to alleviate potential neurodegeneration. In order to provide a holistic model of injury, as it would progress in clinical cases, we must use an approach that allows us to monitor the injury *in vivo*.

Using the blast-model, we have found that acrolein, a well-known key neural toxin and marker of oxidative stress [44, 91], is significantly elevated in brain tissues (using dot blot) and in urine through the measurements of 3-HPMA, an acrolein metabolite, in days post-injury (data not shown). On the other hand, our lab also investigated that in a 6-OHDA-induced PD animal model, acrolein is elevated. Furthermore, lowering of acrolein using a well-known acrolein scavenger, hydralazine, could lead to alleviation of motor deficits implicating a pathological role of acrolein in PD. Consistent with role of acrolein in PD pathology, our lab in collaboration with Dr. Rochet, has shown that acrolein can promote α-synuclein aggregation, one of the hallmarks of PD *in vitro* and *in vivo*. In addition, acrolein is a known pro-inflammatory aldehyde [105, 106] further suggesting its putative role in inflammation, both in blast injury and PD. *In summary, our data strongly suggests that acrolein plays a critical role in the pathogenesis of PD by directly promoting α-synuclein aggregation, and instigating neuroinflammation, which further exacerbates α-synuclein aggregation*.

2.2 Research Proposal and Aims

In light of these findings, we propose that acrolein may be the underlying culprit rendering blast victims vulnerable to PD development. Specifically, we postulate that elevated acrolein levels instigated by blast injury may synergistically work with some other possible factors to trigger or exacerbate PD pathology. With relevance to our animal models, we predict that the known event of post-blast injury acrolein elevation in combination with sub-threshold 6-OHDA (sub clinical dose causing no behavioral

deficits) may produce more severe PD like symptoms than either injury alone. We also predict that lowering acrolein post blast would eliminate or reduce the vulnerability to PD. This would strongly suggest acrolein to be the key mechanistic link between blast-brain injury and PD. **The purpose of this project is to investigate the molecular mechanism underlying the susceptibility of PD in post-blast rats.** *Our hypothesis is that acrolein is the key, linking blast injury and the development of PD in our rat model.*

AIM 1. Characterize behavioral deficits and evaluate oxidative stress-related biochemical changes after mild blast-induced traumatic brain injury, and its possible causal effects to enhance susceptibility to PD.

AIM2. Investigate the behavioral deficits following mild blast-induced traumatic brain injury and sub-threshold 6-OHDA nigral injection.

AIM3. Investigate the neuroprotective role of acrolein scavengers such as phenelzine, to mitigate PD-like behavioral deficits following blast-induced traumatic brain injury.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Animal Models

Mild Blast-traumatic Brain Injury Model

Rats were anesthetized with 80 mg/kg ketamine and 20 mg/kg xylazine and a body shield was placed over the animals for protection during injury allowing for the study of mild BINT (mBINT) without systemic confounders. mBINT was produced by a blast wave generator, which delivered a global blast pressure wave in a laboratory setting. Blast generation was achieved when pressure built up in a reservoir until it exceeded the burst strength of the diaphragm. The blast wave was directed downward at a distance of 50 mm from the nozzle of the blast generator to the head of the animal, with a peak pressure of 150 kPa. Sham animals were anesthetized accordingly and place in the same room of the blast set-up but outside the blast wave range. Our injury model resembles the human condition by successfully correlating blast pressure intensity wave and motor function after mBINT (Figure 3).

The 6-Hydroxydopamine (6-OHDA) Rat Model

The 6-OHDA is one of the well-known PD animal models and it has been established in our lab. In brief, the 6-OHDA at 8μg diluted in sterile saline was administered using a Hamilton syringe with a stainless steel cannula gauge injector to obtain a unilateral nigrostriatal lesion. The rats were anesthetized with 100 mg/kg of ketamine and 10 mg/kg xylazine using intraperitoneal (i.p.) injections. A burr hole was made in the skull using a dermal drill, and after careful piercing of the dura mater; the needle was inserted vertically according to the stereotaxic coordinates using Paxinos as reference. Two microliters of the 6-OHDA solution was infused at a rate of 1 μL/min for

2 minutes. Sham-operated 6-OHDA rats (Group 6) rats received 2 microliters of saline delivered at the same rate. Sub-threshold 6-OHDA injection is induced laterally into the substantia nigra at this following coordinates: -5.4AP, +3.0ML, -8.2DV with a 6-OHDA concentration of 4μg/2μL and a full 6-OHDA with a higher concentration of at 8μg/2μL. The concentration of 6-OHDA has been previously used in our lab, and the brain coordinates are suggested by Bergstrom et al., 2001 [107]. For Group 3, sub-threshold 6-OHDA was induced 3 days after blast. Since symptoms of PD do not present until dopamine loss in the putamen exceeds approximately 80%, the denervation produced by partial lesions as suggested is more suitable for investigating the compensatory adaption in the pre-symptomatic or preclinical phase of the disease [107].

3.2 Behavioral Assessments

Rotarod Test

The rotarod is a performance test and was first developed in 1957 by Dunham and Miya [108], and is commonly used as a screening test for evaluating the neurological effects of drugs [109]. The device consists of a metal frame with a motorized, rotating assembly of rods. The device in the Hamm et al. paper consisted of 18 rungs (1 mm stainless steel rods). Jones & Roberts (1968) showed that gradually increasing the speed of rotation led to greater sensitivity of the test [110]. Animals are allowed first to remain stationary for 10s. The speed then gradually increased by 3 rpm per 10s until 30 rpm is reached (rotational speed at which naïve, uninjured rat will not fall of during 2 min test interval). The animal must remain on the apparatus for the remainder of the 2 min test interval at this 30 rpm speed. The trial will end if the rat completely falls off the rungs, or grips the device and spins around twice without actually walking on the rungs. The purpose of the rotarod test is to assess the rat's sensory motor coordination. The test is sensitive to damage in the basal ganglia and cerebellum and to drugs that affect motor function. The speed of rotation is gradually increased and the rat's ability to remain on the rotating rod is recorded. Baseline behavior for each animal is recorded before surgery.

Open Field Activity Detector

Animals were placed in a Plexiglas activity box (100 cm×100 cm×20 cm) in a darkened room. A red light and a camera are placed on top of the box to record the activity of the animal. Each rat is placed on the box and activity is recorded for 5-15 minutes. The area covered and distance travelled per animals will be evaluated using an automated video tracking system (ANY-maze) to evaluate these behavioral parameters.

3.3 Statistical Analysis

Unless otherwise specified, a one-way analysis of variance (ANOVA) comparing group means for control and experimental groups for each of the experiments was performed to determine the differences of protein expression in the biochemical studies and motor deficits on the behavioral tests. The null hypothesis was rejected if $P<0.05$. The Tukey's Test was used for post-hoc comparisons to control, and the null hypothesis was rejected if $P<0.05$. SEM was used for the standard error bars.

3.4 Experimental Approach For Each Aims

Aim 1

Our blast injury model has been recently modified to provide consistent pressure wave and mimic a blast-injury observed in human blast injuries (Figure 3). Male Sprague Dawley rats (weight 350-450 grams) were used in this study. The experiments were performed in strict accordance with PACUC guidelines. Upon arrival to animal facility, animals were housed individually in a temperature- controlled room at 25^{o}C and on a 12 h light/12 h darkness cycle with free access to food and water for at least a week before testing. Animals were handled daily 3 days prior any procedure. Rats were in the following groups: mBINT (n=4) and sham (n=4). We characterize short-term motor and behavioral deficits using rotarod and open-box activity seven days post-mBINT.

Aim 2

Male Sprague Dawley rats (weight 350-450 grams) were used in this study. The rats were kept on a 12 h light/12 h darkness cycle and free access to water and food, and at controlled temperature of 25 ^{o}C. The experiments were performed under the strict accordance with PACUC guidelines. mBINT was performed as described in Figure 3. Our time line for the mBINT and sub-6-OHDA injection is shown in Figure 4. The groups for this study:

Group 1: Sham-Blast (n=4)

Group 2: Blast only/mBINT (n=5)

Group 3: Blast/mBINT + Sub-threshold 6-OHDA (n=5)

Group 4: Sub-threshold 6-OHDA only (n=5)

Group 5: 6-OHDA (n=5)

Group 6: Sham- 6-OHDA (n=4)

Aim 3

Male Sprague Dawley rats (weight 350-450 grams) were used in this study. The rats were kept on a 12 h light/12 h darkness cycle and free access to water and food, and at controlled temperature of 25 ^{o}C. The experiments were performed under the strict accordance with PACUC guidelines. Blast injury was performed as described in Aim 1 and sub-threshold 6-OHDA in Aim 2. A dose of 15 mg/kg of phenelzine was administered to each animal post-mBINT. Figure 5 shows a time line of the experiment for this aim.

3.5 Biochemical Assessments

Detection of Acrolein

The quantification of acrolein and its metabolites in urine has recently been established using liquid chromatography-mass spectroscopy (LC/MS) providing a non-invasive detection to monitor acrolein and investigate its pathological role in brain injuries and other diseases [45, 58, 111-115]. Specifically, measurement of acrolein metabolites known as mercapturic acids in blood, urine and feces using LC/MS have

shown promise as reliable biomarkers to detect acrolein [116, 117]. N-acetyl-S- (3-hydroxypropyl) cysteine (3-HPMA), the most common mercapturic acid derived from reduction of acrolein by aldehyde reductase, is a stable and non-toxic compound with feasibility of detection [118, 119]. Our lab has optimized a consistent method of detecting 3-HPMA through LC/MS/MS providing a systemic measurements on acrolein dynamics as described in Lingxing et al., 2013 [120].

Western blotting of post-mortem brain tissue

We evaluated protein markers that are relevant to oxidative stress (acrolein Lys-adducts) and PD following blast-injury in rats. In brief, rats were induced with blast as described above and sacrificed after two days and seven days post-mBINT. Whole brain tissues were frozen and pulverized. Total protein lysates were isolated from each group: Control (uninjured rats, n=4), two-days after injury (n=4), and seven-days after injury (n=3). Protein concentrations were measured using the Bicinochoninic Acid protein assay kit (Pierce, Rockford, IL, USA) and SPECTRAmax (Molecular Devices, Sunnyvale, CA). Fifty micrograms of protein with 20% SDS, β-mercaptoethanol, and 2x Laemmli buffer were loaded to a 12% Tris-HCL gels and electrophoresed at 100 volts for 1.5 hours at 4 ^{o}C. Proteins were then transferred to a nitrocellulose membrane by electro blotting in 50 volts for 2 hours at 4 ^{o}C in a 1x transfer buffer (Tris-Glycine buffer from BioRad, Hercules, CA and methanol). Membranes were blocked in 5% milk and washed with Tris-Buffered Saline with 0.1% Tween-20 accordingly. The blots were subjected to the primary antibody of interests: (Anti-Acrolein (Abacm #ab37110, Anti-TH (Cell Signaling #2792S), Anti-pTHSer40 (Novus Biologicals #NB300-173), Anti-α-Synuclein (BD Transduction Labs #610786), Anti-CRF (Abcam #ab8901), and Anti-Actin (Imgenex #IMG-5142A). An HRP-tagged secondary antibody was used and an enhanced chemiluminescence (ECL) system was used for detection. Protein bands were visualized and quantified using the AlphaView software system (Protein Simple, San Jose, CA). Data are normalized with actin and expressed as percent control.

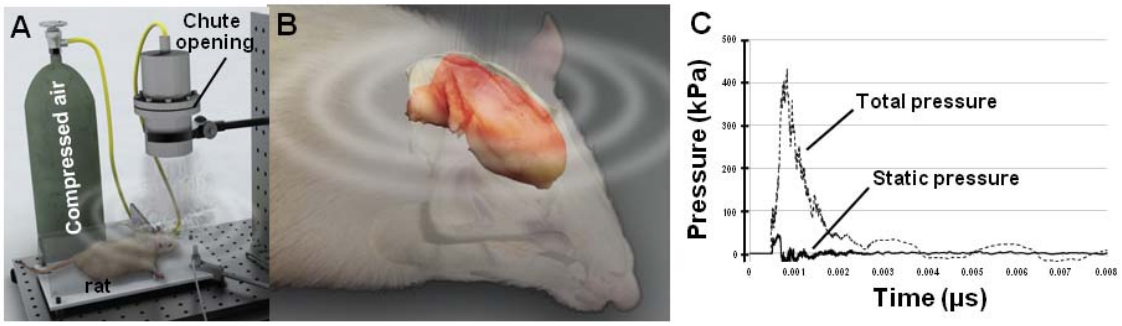

Figure 3. **Rat blast-induced brain injury model.** **(A)** Illustration of rat blast experimental set-up. **(B)** Enlarged area view of the surface of the rat brain where the blast was aimed. **(C)** Average blast pressure waveforms. Total pressure represents pressure generated by blast while static pressure represents control situations–no blast.

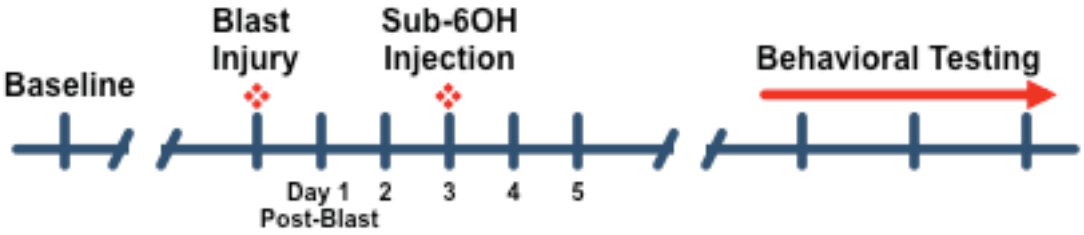

Figure 4. Aim 2 Experimental Design and Timeline. Rats were trained on the rotarod and acclimated within the open box before collecting baseline. A blast injury was performed on the experimental group and three days after blast a sub-threshold dose of 6-OHDA (4 μg/2 μl) was stereotaxically injected into the substantia nigra. Behavioral tests were performed for a certain period of time and animals were sacrificed according to PACUC guidelines after the study.

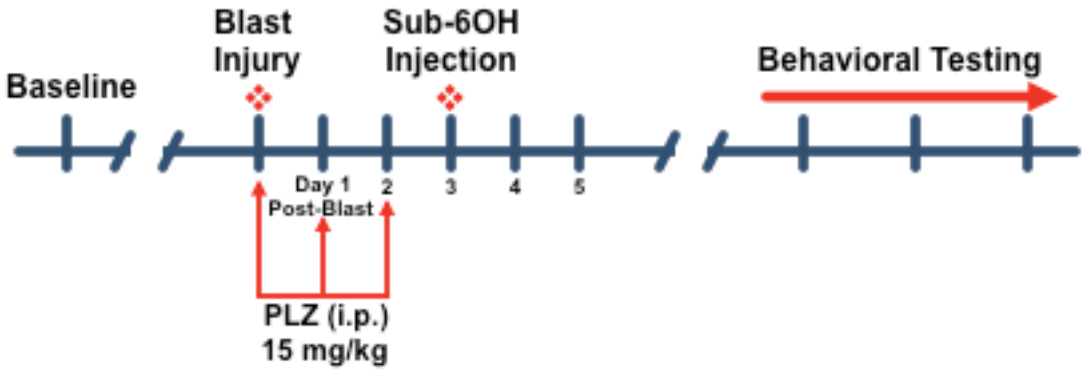

Figure 5. Aim 3 Experimental Design and Timeline. Rats were trained on the rotarod and acclimated within the open box before collecting baseline. A blast injury was performed on the experimental group and 5 minutes after blast an injection of phenelzine (PLZ) at 15 mg/kg was administered intraperotineally (i.p.). Phenelzine was administered again 24 and 48 hours post-blast. On Day 3, a sub-threshold dose of 6-OHDA (4 µg/2 µL 6-OHDA) was stereotaxically injected into the substantia nigra. Behavioral tests were performed for a certain period of time and animals were sacrificed according to PACUC guidelines after the study.

CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Characterization of behavioral deficits and evaluation of oxidative stress-related biochemical changes following a mild-blast induced traumatic brain injury, and the possible causal effects for enhancing susceptibility to PD

mBINT Injury and Behavioral Test

Despite the aforementioned short and long term consequences of mild blast injuries, the underlying mechanisms governing the associated functional loss are poorly investigated. This lack of understanding is due in part to limitations of human studies and insufficient investigation in animal models. As such, we have successfully established a rodent model of mild Blast Induced Neurotrauma (mBINT), which resembles the human condition. By developing this model of mBINT, we can elucidate underlying pathophysiological cellular responses and better identify targets for therapeutic intervention. Our model utilizes a consistent pressure intensity wave to induce mBINT (data not shown). Assessment of motor function, using a rotarod task, showed no statistically significant difference between the sham and mBINT animals suggesting that our mBINT model does not elicit motor deficits (Figure 6).

Biochemical assessment of post-mortem tissue following mBINT

Acrolein: Role in Oxidative Stress and Inflammation

Oxidative stress markers have been shown to substantially increase in disease states such as PD and TBI. Acrolein, a byproduct of oxidative stress, is a highly reactive aldehyde product of lipid peroxidation that has been shown to react with DNA, lipids, and proteins. Acrolein also stimulates the production of free radicals and therefore

perpetuates oxidative stress. Our immunoblot results show a (19.2 ± 7.2 %) increase of acrolein-modified protein levels two days post-mBINT which continues to increase until seven days post-mBINT (31.5 ± 2.1 %) (Figure 7A). Each lane was quantified by summing the density of all bands, and was subsequently normalized to beta-actin protein expression (see Methods for software parameters). Data are expressed as mean (±SEM) of the percent control values for normalized relative quantities. Statistical tests showed a significant increase in acrolein-lysine adduct proteins levels two days (19.2 ± 7.2 %, $P<0.05$) and seven days (31.5 ± 2.5 %, $P<0.001$) post-mBINT compared to the control group. These results further support previous data from our lab, which demonstrates that acrolein levels are increased following blast injury (data not shown). Here, we further validate that our blast model mBINT induces extensive biochemical changes. This also supports the notion that oxidative stress is elevated in blast injuries [121-123]. We speculate that the biochemical changes post-mBINT can trigger and perpetuate additional long-term damage (Figure 1). Additionally, acrolein possesses pro-inflammatory capabilities and can partially contribute to the inflammatory response following mBINT thereby exacerbating the secondary injury biochemical cascades post-blast injury [105, 106]. We suspect that the elevated acrolein levels we observed post-mBINT are responsible in part for increasing susceptibility to PD.

Tyrosine Hydroxylase (TH)

TH catalyzes the amino acid tyrosine to Levodopa (L-dopa), which is the first rate-limiting step in synthesizing the catecholamine dopamine [124, 125]. In PD patients, TH activity is decreased in the nigro-striatal area, which results in the reduction of dopamine levels [126]. TH is essential in the synthesis of dopamine and it is an essential biomarker implicated in the neurodegeneration process of PD. Our data show an overall decreased of TH activity by measuring the protein expression of TH phosphorylation at serine 40 (pSer40) two (49.4 ± 6.4 %) and seven days (55 ± 12.6 %) post-mBINT relative to the control group (Figure 8). The bar graph shows the average means for each group (± SEM) of the percent control values normalized relative quantities to unphosphorylated TH (Figure 8B). Statistical analysis showed a significant decrease of pSer40 TH activity

two days (49.4 ± 6.4 %, P<0.01) and seven days (55 ± 12.6 %, P<0.01) post-mBINT. This suggests that the "secondary injuries," particularly acrolein, post-mBINT injury can contribute to the biochemical pathology observed in PD. As evident by the elevated acrolein levels we observed post-mBINT (Figure 7), BINT increases ROS such as superoxide radicals [121, 123], which thereby enhances lipid peroxidation. We believe that acrolein plays a significant role in TH regulation post-mBINT.

Due to the myelin content of brain tissue, lipid peroxidation (LPO) is a major process of neurodegeneration-related oxidative stress. Among the end products of LPO is acrolein, a far more reactive molecule than other aldehydes products (Figure 1). Although acrolein has been shown to modify proteins *in vitro* [47, 112] and acrolein-modified proteins have been observed in PD brains [127], the mechanisms by which it interacts with TH remain unclear. It is possible that acrolein can modulate the function of TH through direct or indirect signaling that is thereby affecting dopamine synthesis. TH is an enzyme in which its activity is regulated by kinases and phosphatases, activators and deactivators respectively. The main kinases that regulate TH phosphorylation at Ser40 are the cAMP-dependent protein kinase A (PKA) and the mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) [128-130]. In addition, the adapter protein, 14-3-3 enhances both kinases phosphorylation of TH at Ser40 and TH function [131]. On the other hand, protein kinase Cδ (PKCδ) has been shown to decrease TH activity and DA synthesis. PKCδ is also highly expressed in the nigral dopaminergic neurons and co-localizes with TH [128, 132]. The potential of oxidative stress, specifically acrolein, to modulate any one of these proteins may be critical for PD progression.

Possibly the most important regulatory protein of TH is α-synuclein since it is directly implicated in PD pathology. The function of α-synuclein, the major protein found in the in Lewy body inclusions of PD, is not fully understood. However, the abnormal expression of α-synuclein has been demonstrated to inhibit TH gene expression and its activity in both *in vitro* and *in vivo* studies [128]. TH function is hindered in both transgenic mice overexpressing α-synuclein and in dopaminergic cells transfected with α-synuclein [133, 134]. Additionally, transfection of α-synuclein in MES 23.5

dopaminergic cell line induces cell injury and decreases TH gene expression and protein levels [135]. Furthermore, α-synuclein has similar protein homology as 14-3-3 and is thought to function as a chaperone protein [136-140]. The 14-3-3 chaperone protein enhances the kinase's function to activate TH at pSer40 thereby increasing DA synthesis [141-143]. Interestingly, α-synuclein is found to activate phosphatases, particularly phosphatase 2A (PP2A), leading to the inhibition of TH activity [144] and could explain the decrease in TH activity and gene expression in the aforementioned *in vitro* and *in vivo* studies. Furthermore, phosphorylation of α-synuclein at Ser129 attenuates PP2A activity and increases TH activity. This suggests that overexpression of α-synuclein can independently trigger PP2A activation and TH inhibition leading to the interaction of both enzymes or possibly TH inhibition is an additional consequence and it can activate PP2A [144]. Further investigation of the exact mechanism underlying α-synuclein effects on TH and PP2A, particularly delineating its soluble forms, is need not only for PD targeted therapeutics but also to potentially regress or prevent the onset of PD following blast injury.

The mechanisms by which acrolein affects biological systems are not yet fully understood; however, many of its effects are attributed to activation of such kinases mentioned above, particularly (MAPKs) and c-Jun N-terminal kinase (JNK) [145, 146], as well as chemokine production and the induction of apoptosis [146, 147]. The reactive properties of acrolein allow it to readily target these proteins by Michael addition to the cysteine, lysine and histidine residues, and thereby can dysregulate their function and affect downstream signaling pathways, particulary the dopaminergic synthesis and neurotransmission. In relation to the interplay between the kinases and phosphatases mentioned above, TH and α-synuclein in the presences of acrolein needs to be further validated to fully understand the pathology of blast injury and how it can potentiate PD pathology.

Additionally PD pathology is marked by increased inflammation and levels of pro-inflammatory cytokines, such as TNF-α [148]. This event can trigger proteosomal degradation of TH and contribute to reduced TH levels [149]. This implicates the role of inflammation in the regulation of TH, and with acrolein's pro-inflammatory role,

increased acrolein levels post-mBINT may directly affect TH activity as observed in our blots. Our results present further investigation on the role of acrolein post-mBINT and on the mechanisms and pathways of TH activation and deactivation to further elucidate its contribution to PD pathology. Also, investigation of the multi-site phosphorylation of TH is warranted.

α-Synuclein

α-Synuclein is the main protein present in the Lewy Bodies (LB) found in the surviving dopaminergic neurons of PD patients. It is a small soluble protein (14 kDa) that is highly localized in the presynaptic terminals [128, 150], and can be essential in normal brain function. However, the exact function and neurotoxic level of α-synuclein remains unclear. α-Synuclein is very sensitive to environmental factors and genetic modifications which trigger its misfolding and an eventual loss of normal function. Point mutations in the α-synuclein gene can enhance its aggregation and thus contributes to the development of the familial and rare forms of PD. Currently, little is known about the mechanism leading to the aggregation of α-synuclein, however one study suggests that the dimerization of the protein can be a rate-limiting step in its aggregation leading to the formation of LBs [151]. The ubiquitin proteasome system (UPS) and oxidative stress are two mechanisms, which can promote α-synuclein aggregation. UPS is the main biochemical pathway for degrading both normal and abnormal (mutated, misfolded) intracellular proteins [152, 153], such as α-synuclein. Malfunction of this system leads to protein accumulation and cell death [154, 155]. Several studies have shown that in PD this ubiquitin-dependent protein degradation is impaired [153]. In vivo post-translational modifications such as phosphorylation and ubiquitination can interfere with the function and degradation of α-synuclein and alter its native state in a way that enables aggregation. Tofaris et al. (2003) used a two-dimensional gel electrophoresis approach to characterize the pathogenic species of α-synuclein in LBs isolated from the post-mortem brain tissue of PD individuals [142]. They found a highly modified species of α-synuclein at 22-24 kDa that was ubiquitinated. This species was conjugated with at least 1-3 ubiquitins. Furthermore, this 22-24 kDa α-synuclein species was

hyperphosphorylated on its serine residues. The phosphorylation of α-synuclein at Ser-129 has been highly associated with LB α-synucleins and accelerates neurodegeration in a rat model of PD [156]. In addition, as mentioned previously, this Ser129 site can also regulate TH activity and further implicates these post-translational modifications of α-synuclein in the pathogenesis of PD.

Our Western blot shows two distinct bands of α-synuclein, 17 kDa and 25 kDa (Figure 9A). We performed a control experiment to confirm these bands by blocking the α-synuclein antibody with purified α-synuclein proteins before incubating the blot. Our results show convincing evidence that these two bands observed are α-synuclein (Figure 16). We speculate that the band observed at 17 kDa is the monomeric form of α-synuclein which is the "normal" unmodified form, and the 25 kDa is the modified form of α-synuclein, possibly by post-translational modifications. We expressed our data as means (±SEM) of the percent control values for normalized relative quantities and all bands of interests were first normalized with beta-actin. Our analysis of the unmodified 17 kDa α-synuclein bands show a significant decrease two days (51.5 ± 4.0%, $P<0.05$) and seven days (82.8 ± 3.5%, $P<0.05$) post-mBINT (Figure 9B). On the other hand, our analysis of the modified 25 kDa α-synuclein band shows an opposite trend, where a statistically significant increase was observed two days (43.6 ± 5.5% $P<0.05$) and seven days (65 ± 10.2%, $P<0.01$) post-mBINT (Figure 9C). Our data suggests both two and seven days post-mBINT can induce post-translational modifications of α-synuclein.

It is essential to conduct further experiments to validate the 25 kDa α-synuclein bands on our results and the specific α-synuclein post-translational modification mechanism. We suspect that perhaps, acrolein can contribute to modification of α-synuclein since we also show that in an *in vitro* study of acrolein can promote α-synuclein aggregation in dopaminergic neuronal cell lines (Data not shown). We speculate that increased levels of modified of α-synuclein suggests a disruption of the physiological integrity of the cells and thus may be contributing to the decrease in TH activity observed in Figure 8. Similarly *in vitro* studies have demonstrated that overexpression of α-synuclein reduces the levels of TH mRNA and protein [157, 158]

and thereby can regulates TH expression and affects dopamine synthesis in the brain, further exacerbating PD pathology.

4.2 The susceptibility of PD-like motor deficits following a blast injury and the role of acrolein

Loss of motor function is the predominant symptom of PD which is observed in the most common 6-hydroxydopamine (6OHDA) rat model of the disease. Our lab has established the 6-OHDA rat model by stereotaxically injecting 8 μg/2 μl of 6-OHDA into the substantia nigra (SN) to induce PD-like motor deficits. 6-OHDA promotes neurodegeneration of dopaminergic neurons in the substantia nigra further mimicking the pathology of PD. For our sub-threshold 6-OHDA model we injected at the same site (see methods for coordinates) but at a lower dosage of 6-OHDA (4 μg/2 μl). This is the first time we employed this sub-threshold dosage and expect to not show motor deficits. Sham-6OHDA, injection of saline into the SN, is our negative control for this model. As seen in Figure 3, our mBINT rat model was assessed using sham-mBINT as the control, as well as the combination of mBINT and sub-threshold 6OHDA (see Figure 4). We trained each animal on the rotarod prior to collecting baseline measurements, which included three testing times per day and 210 seconds on the rotarod was considered a perfect performance test. We averaged the motor performance for each animal and within groups which was used to plot our data. We followed these animal groups' motor performance for approximately two months and collected data at different time points.

The overall trend of motor performance on the rotarod for each of the six groups of animals is shown in Figure 10. Our motor performance test on the rotarod indicates that the combination of mBINT and sub-threshold 6-OHDA (172.2 ± 10.4 s) resulted in motor deficits similar to a full 6-OHDA PD model (180.1 ± 3.8 s) and is consistent throughout the study. However, the mBINT only (204 ± 2.3 s) and sub-threshold 6-OHDA (206 ± 1.8 s) groups did not show motor deficits similar to the control groups: sham-mBINT (209.2 ± 0.32 s) and sham-6-OHDA (208.5 ± 1.37 s). At the end of the

study the motor performance test for each group was averaged and plotted on a bar graph (Figure 11). ANOVA shows a significant group effect [$F(5,22)$, $p<0.001$]. Post-hoc comparison between mBINT and mBINT+sub-6-OHDA ($P<0.05$), sub-6-OHDA and mBINT+sub-6-OHDA ($P<0.05$), and sub-6-OHDA and 6-OHDA ($P<0.01$) all show statistically significant differences. The error bars represent SEM values. These results show that mBINT can heighten the susceptibility to PD-like motor deficits. Motor deficits are absent in our mBINT only and sub-6-OHDA only groups, but the combination of these produce a PD-like motor deficits further supporting our hypothesis that mBINT can promote PD onset (Figures10 and 11). These data further validates our mBINT model, mimicking phenotypes typically observed in human mild-blast injury cases, and we have established an effective sub-threshold dosage of 6-OHDA.

Urine levels of the acrolein metabolite, 3-HMPA, were measured from these animal samples. In Figure 12 we show that 3-HPMA is elevated in the 6-OHDA group compared to the sub-6-OHDA and the control, sham-6-OHDA groups. The data are expressed as mean (±SEM) of the percent control values for normalized relative quantities. We demonstrate that at five days post surgery there is no statistically significant group effect. However fifteen days post-surgery there is a statistically significant group effect, ANOVA [$F(2,10)=7.01$, $P<0.01$]: sub-6-OHDA group (17 ± 14%) decrease and 6-OHDA (72 %± 37%) increase from the sham-6-OHDA control group (100 ± 8.5%. Post-hoc comparison between sham-6OHDA vs 6-OHDA ($P<0.05$) and sub-6-OHDA vs 6-OHDA ($P<0.01$) show significant increased levels of 3-HPMA in the 6OHDA group. This supports previous studies that oxidative stress, particularly acrolein, is increased in PD and perhaps is a major contributor of ROS production and thereby contributes to neurodegeneration. Additionally, these 3-HMPA measurements can be correlated to the motor deficits behavior tests (Figure 11), thus demonstrating that the increased levels of 3-HPMA induces motor deficits in the 6-OHDA PD rat model.

Furthermore, we also measured the 3-HMPA levels in the mBINT and mBINT+sub-6-OHDA group. Our data are expressed as mean (±SEM) of the percent control valueis for normalized relative quantities (Figure 13). We show that two days post-mBINT injury there is an increased levels of 3-HPMA in the mBINT group (12.6 ±

16.1 %) and in mBINT+sub6OHDA (7 ± 31.5 %) relative to control, but not statistically significant. Subsequently, fifteen days after mBINT injury and twelve days after sub-threshold 6OHDA injection, the levels of 3-HPMA continued to increase in the mBINT group (75.47 ± 30.1%) and mBINT+sub-6-OHDA (168 ± 92 %) relative to the control, sham-blast; however this increase is not statistically significant. Here, we show that the combination of mBINT and sub-threshold 6-OHDA can potentiate the increase of 3-HPMA levels in the urine as compared to the mBINT only and relatively to the sub-6-OHDA group, indicating that the combination of these two injuries increases oxidative stress similar to the 6-OHDA PD model. These levels of 3-HMPA can also be correlated to the motor performance test in Figure 11.

4.3 The investigation of the neuroprotective role of phenelzine, an acrolein scavenger, to mitigate PD-like behavioral deficits post-blast-induced TBI

Previous experiments from our lab demonstrate that acrolein scavengers, such as phenelzine, can mitigate motor deficits in our PD model, and we also show that the injection of acrolein to the brain show PD-like motor deficits. We hypothesized that elevated acrolein following mBINT can exacerbate the biochemical insults post injury and can potentiate further damage which eventually contributes to the pathology of PD. In Figure 14A we shows that the treatment of phenelzine post-mBINT, before induction of sub-6-OHDA (Blast/PLZ/Sub6OH group; 208 ± 4.8 s) shows an improvement of motor performance compared to the non-phenelzine treated group (Blast+sub-6-OHDA; 172 ± 10.4 s). The average motor performance was calculated at the end of the study and represented on each bar (Figure 14B). ANOVA analysis shows significant group effects [$F(2,9)=5.88$, $P<0.05$] and a post-hoc comparison between blast/PLZ/sub-6-OHDA (203 ± 4.8 s) and Blast+sub-6-OHDA (172 ± 10.4 s) shows a statistically significant improvement in motor performance in the PLZ treated group ($P<0.05$). Therefore, we show that phenelzine treatment post-blast can be effective for alleviating these deficits.

These data further implicate the role of acrolein post-mBINT and perhaps with other factors can contribute to the susceptibility to PD-like motor deficits.

Furthermore, we measured acrolein indirectly through urine 3-HPMA detection from each sample group. Figure 15A shows the time line of our treatment and experimental testing. Figure 15B shows bar graphs of the normalized levels of 3-HPMA in the urine two days post mBINT: mBINT+sub-6-OHDA group (3.3 ± 1.3 a.u.) versus mBINT/PLZ/sub-6-OHDA (0.83 ± 0.34 a.u.); one day post-sub-6-OHDA injection: mBINT+sub-6-OHDA group (4.3 ± 1.0 a.u.) versus mBINT/PLZ/sub-6-OHDA (1.6 a.u. ±0.54) and fifteen days post-mBINT. mBINT+sub-6-OHDA group (3.9 ± 1.3a.u.) versus mBINT/PLZ/sub-6-OHDA (0.95 ± 0.12 a.u.). Using at one-tailed t-test to compare between groups on each time point, we show a significant decrease of 3-HPMA levels in the phenelzine treated group compared to the non-phenelzine treated group one day post sub-6-OHDA injection ($P<0.05$) which continually decreases even at fifteen days post surgery ($P<0.05$). Error bars represent SEM. 3-HPMA urine measurement results demonstrate that phenelzine can mitigate acrolein levels. Given that these data can be correlated with the behavior motor tests in Figure 14, and the reduction of acrolein through phenelzine can mitigate these deficits, it suggests that acrolein post-mBINT may have a key role in promoting the susceptibility of PD-like motor deficits.

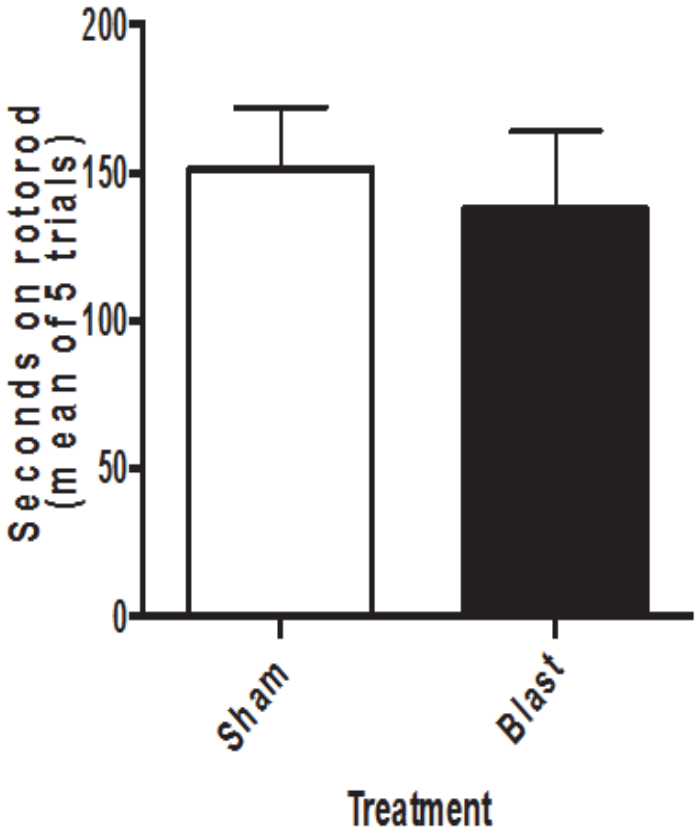

Figure 6. Motor Performance on the rotarod for each animal group after injury. Length of time on rotarod test does not differ between mbTBI and sham rats (unpaired t-test, n=9).

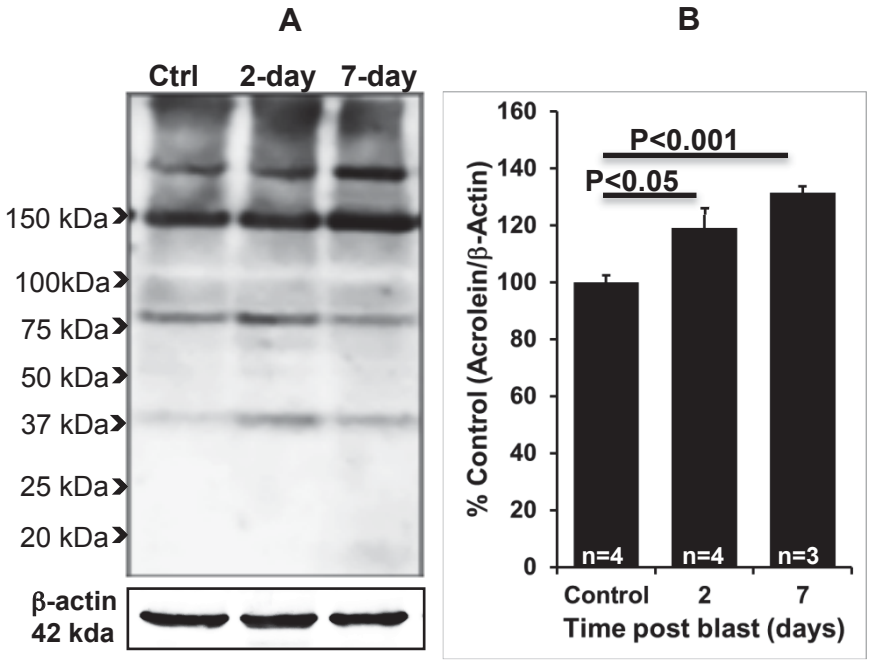

Figure 7. Levels of acrolein modified proteins increase post-mBINT. **(A)** Western Blot image of acrolein modified proteins (FDP-Lys) and β-actin for each group. **(B)** Data are expressed as mean (±SEM. of the percent control values for normalized relative quantities. ANOVA shows a significant group effect [$F(2,8)=13.19$, $P<0.01$]. A post-hoc comparison using Tukey's Test was used to compare control vs. 2 days ($P<0.05$) and control vs. 7 days ($P<0.001$) groups and show significant differences between groups. Error bars represent SEM.

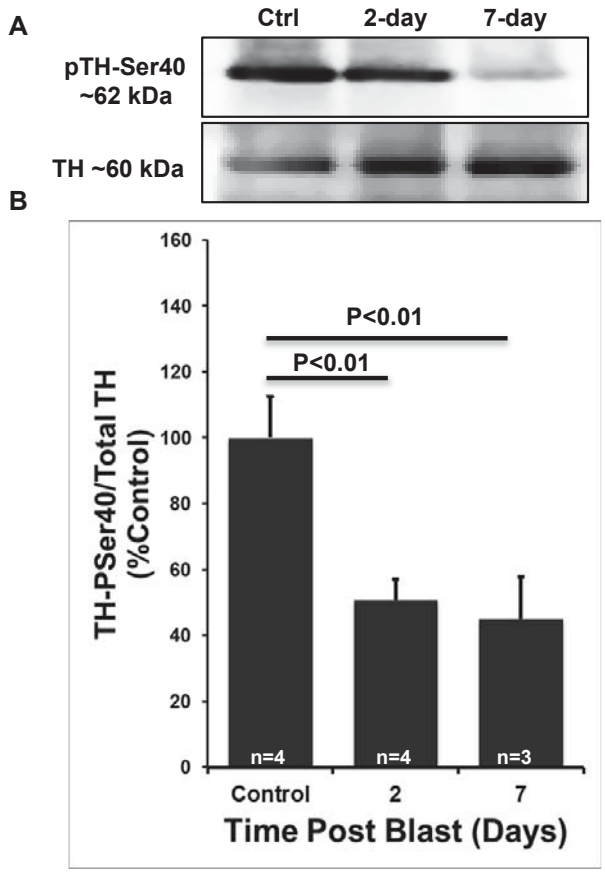

Figure 8. Tyrosine hydroxylase (TH) activity is decreased post-mBINT. **(A)** Western Blot image of tyrosine hydroxylase phosphorylated at Serine 40 (pTH-Ser40) and unphosphorylated TH **(B)** Data are expressed as mean (±SEM) of the percent control values for normalized relative quantities. ANOVA shows a significant group effect [$F(2,8)=11.19$, $P<0.005$]. A post-hoc comparison using Tukey's Test was used to compare control vs. 2 days ($P<0.01$) and control vs. 7 days ($P<0.01$) groups and shows significant differences between groups. Error bars represent SEM.

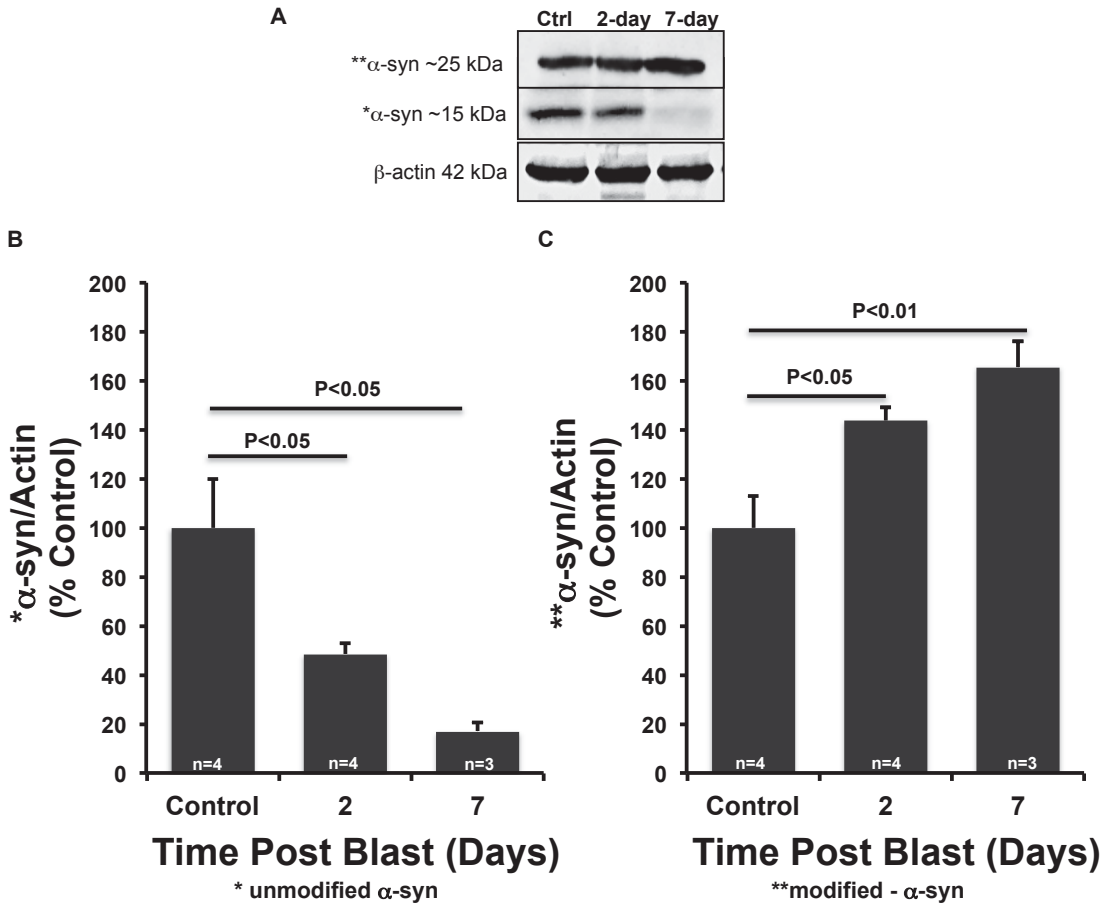

Figure 9. The levels of 25 kDa modified α-Synuclein (**) is increased post-mBINT. **(A)** Western Blot image of modified α-syn at 25kDA, unmodified α-syn at 17kDA and β-actin. **(B)** Data are expressed as mean (±SEM) of the percent control values for normalized relative quantities. ANOVA shows a significant group effect [$F(2,8)=12.86$, $P<0.005$]. A post-hoc comparison using Tukey's Test was used to compare control vs. 2 days ($P<0.05$) and control vs. 7 days ($P<0.05$) groups and show significant differences between groups for the unmodified (*) α-syn observed at 14 kDa. **(C)** ANOVA shows a significant group effect [$F(2,8)=14.39$, $P<0.005$]. A post-hoc comparison using Tukey's Test was used to compare control vs. 2 days ($P<0.05$) and control vs. 7 days ($P<0.01$) groups and show significant differences between groups for the modified (**) α-syn observed at 25 kDa. Error bars represent SEM.

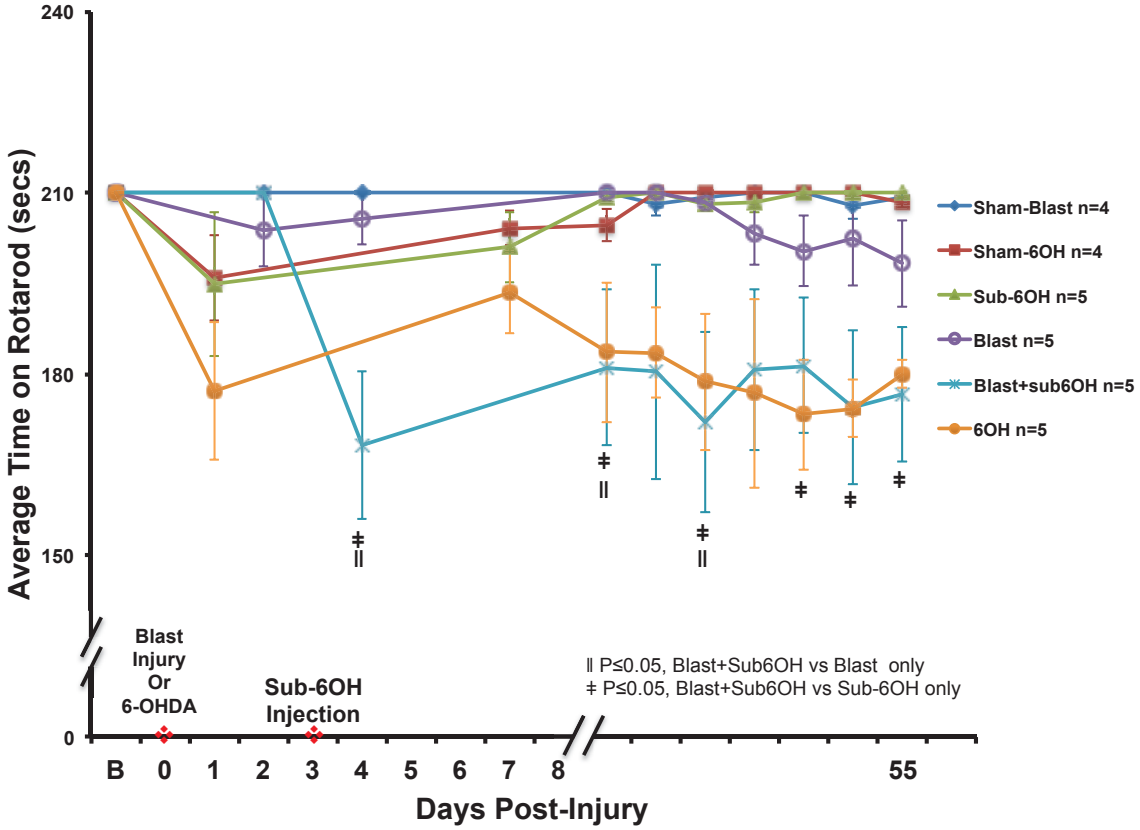

Figure 10. The combination of mBINT and sub-threshold 6-OHDA nigral injection induces a PD-like motor deficits. The Sub-6OH group and Blast only group show no motor deficits similar to the control groups (Sham-Blast and Sham-6OH). However, when blast injury is combined with a sub-6OH injection to the substantia nigra (Blast+sub6OH) it produces motor deficits similar to the 6-OH group. A comparison between Blast+Sub6OH vs. Blast-only and Blast+Sub6OH vs. Sub6OH only show significant differences as indicated by the symbols. Error bars represent SEM.

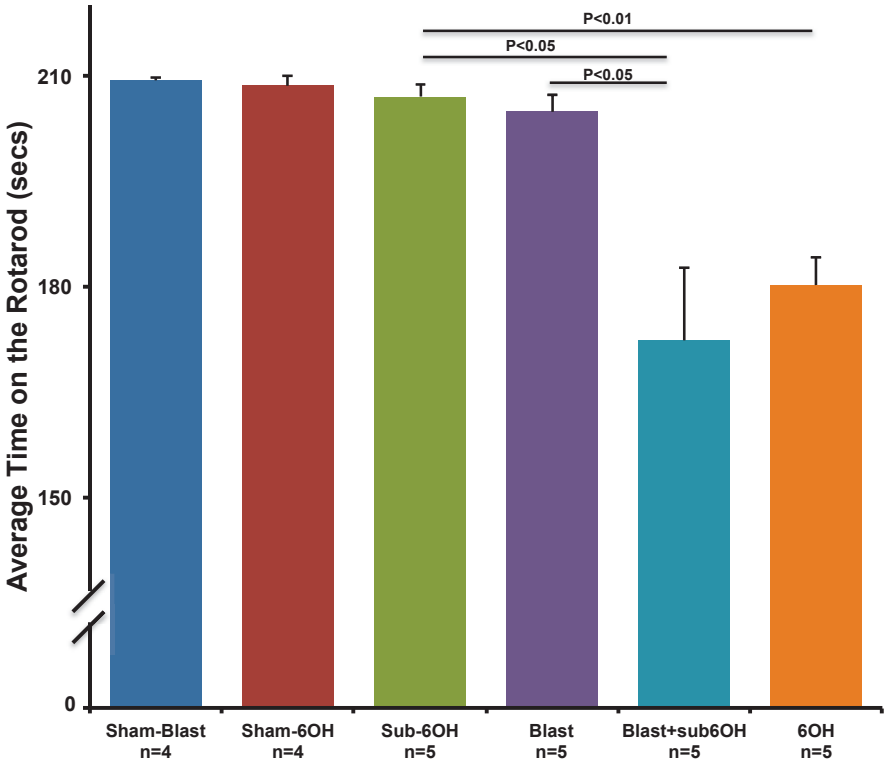

Figure 11. The combination of mBINT and sub-threshold 6-OHDA nigral injection induces a PD-like motor deficits. At the end of the study, the average motor activity test for each rat within each group was calculated. ANOVA shows a significant group effect [$F(5,22)=6.8$, $P<0.001$]. A post-hoc comparison using Tukey's Test was used to compare Blast and Blast+sub6OH and shows a statistically significant difference ($P<0.05$). The comparison between Sub-6OH and Blast+sub6OH show a statistically significant difference ($P<0.05$). In addition, the comparison between Sub-6OH and 6-OH (PD rat model) shows statistically significant difference ($P<0.01$). Error bars represent SEM.

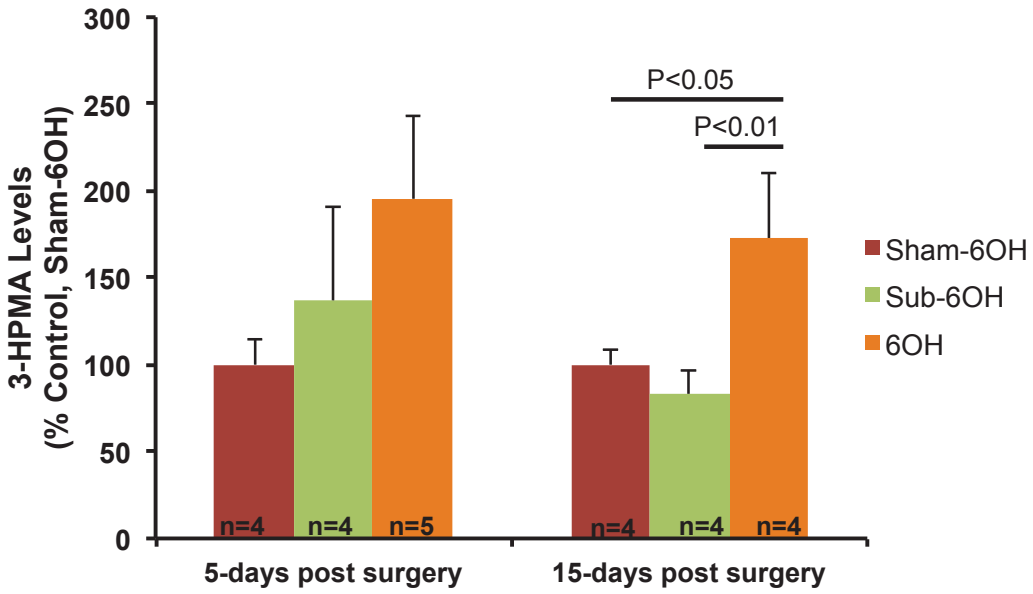

Figure 12. 3-HMPA, an acrolein metabolite, is increased in urine of the Parkinson's Disease rat model (6-OHDA). Data are expressed as mean (±SEM) of the percent control values for normalized relative quantities. Group treatment summary: Sham-6OH is the control group (injection of saline in the SN), Sub-6OH (4 µg/2 µl) injection of 6-OHDA into the SN, and 6OH (8 µg/2 µl) injection of 6OHDA into SN. At 5 days post surgery, ANOVA shows no significant group effect. The ANOVA test 15 days post-surgery show a significant group effect [$F=(2,10)7.01$, $P<0.05$]. A post-hoc comparison using Tukey's Test was used to compare Sham-6OH vs. 6OH ($P<0.05$) and Sub-6OH vs. 6OH ($P<0.01$) groups and showing significant differences between groups. Error bars represent SEM.

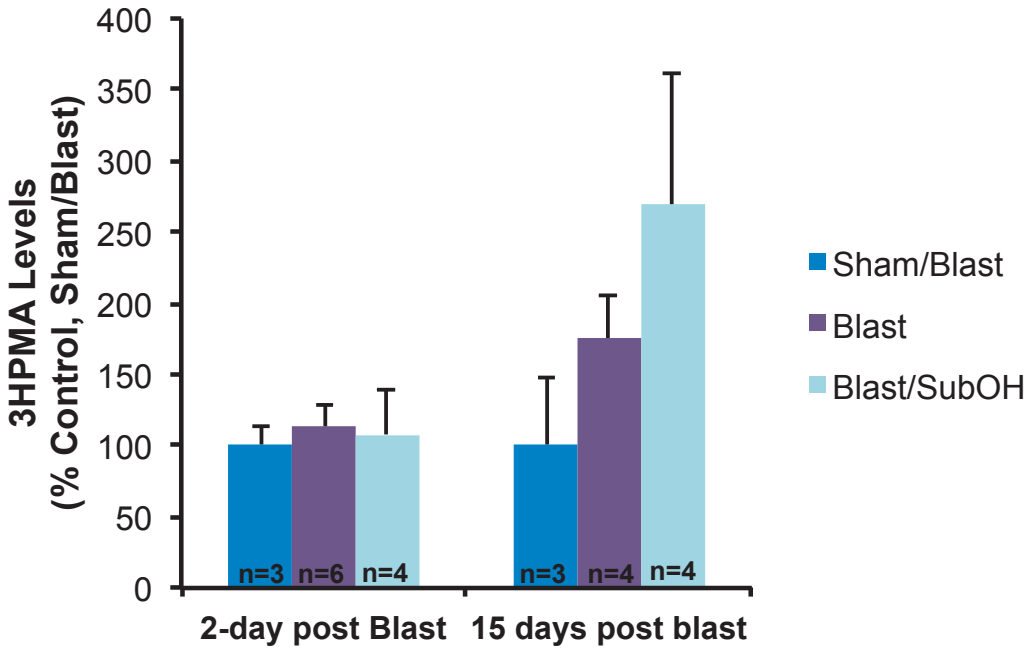

Figure 13. The combination of mBINT and sub-threshold 6OHDA nigral injection heightens the 3-HMPA, an acrolein metabolite, compared to only mBINT animals. Data are expressed as mean (±SEM) of the percent control values for normalized relative quantities. Group treatment summary: Sham-Blast is the control group (anesthetized and placed in the room where the blast occurs), Blast (as described on the Methods section), Blast/SubOH (Blast and 3 days after a 4 µg/2 µl 6OHDA was injected into the SN). 2 days post-mBINT, the animals show an increase of 3-HPMA, but not significant. Fifteen days later, and the injection of sub-6OH 3 days after blast injury (Blast/SubOH group), the 3HPMA levels are heightened compared to the blast only group, however ANOVA show no significant differences amongst groups. Error bars represent SEM.

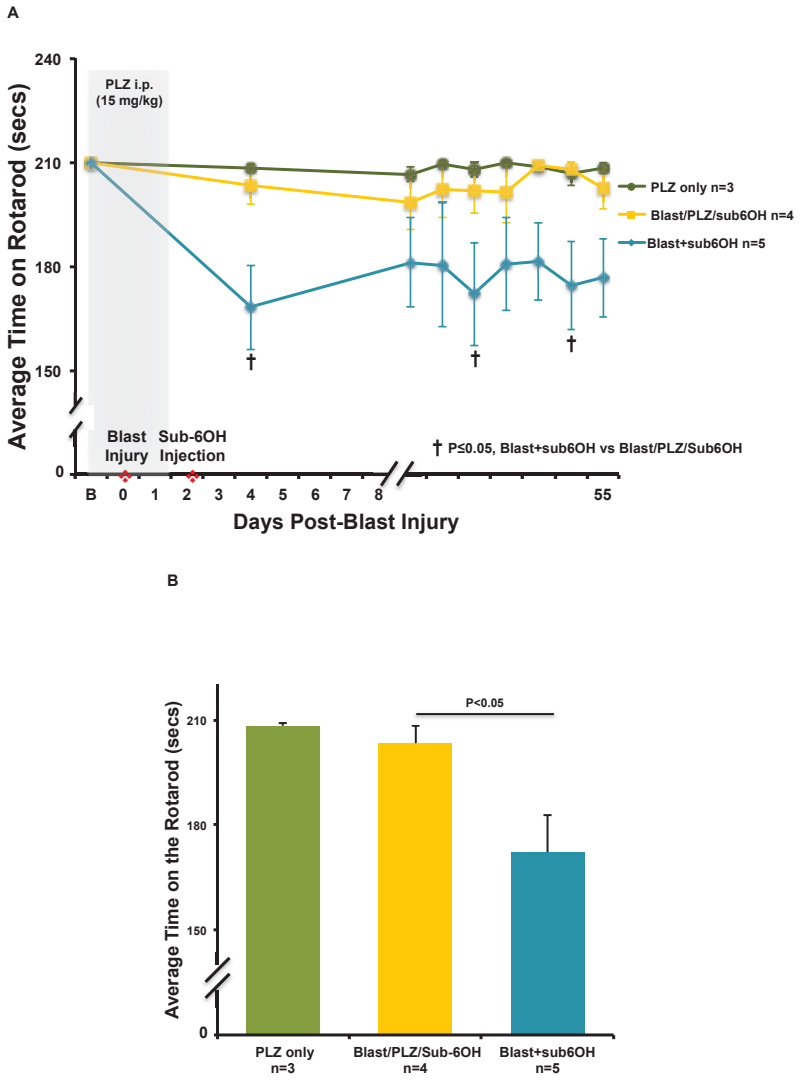

Figure 14. The treatment of phenelzine post-blast improves motor performance on the rotarod. **(A)** Shows that phenelzine treatment (15 mg/kg, i.p.) post-mBINT shows improvement of motor performance (Blast/PLZ/Sub-6OH group) compared to the non-phenelzine treated group (Blast+Sub-6OH). **(B)** The average motor performance of each group were calculated and represented on by bar. ANOVA shows significant group effects [$F(2,9)=5.88$, $P<0.05$). A post-hoc comparison using Tukey's Test was used to compare Blast/PLZ/Sub-6OH vs. Blast+Sub6OH ($P<0.05$) show significant differences in motor activity. Error bars represent SEM.

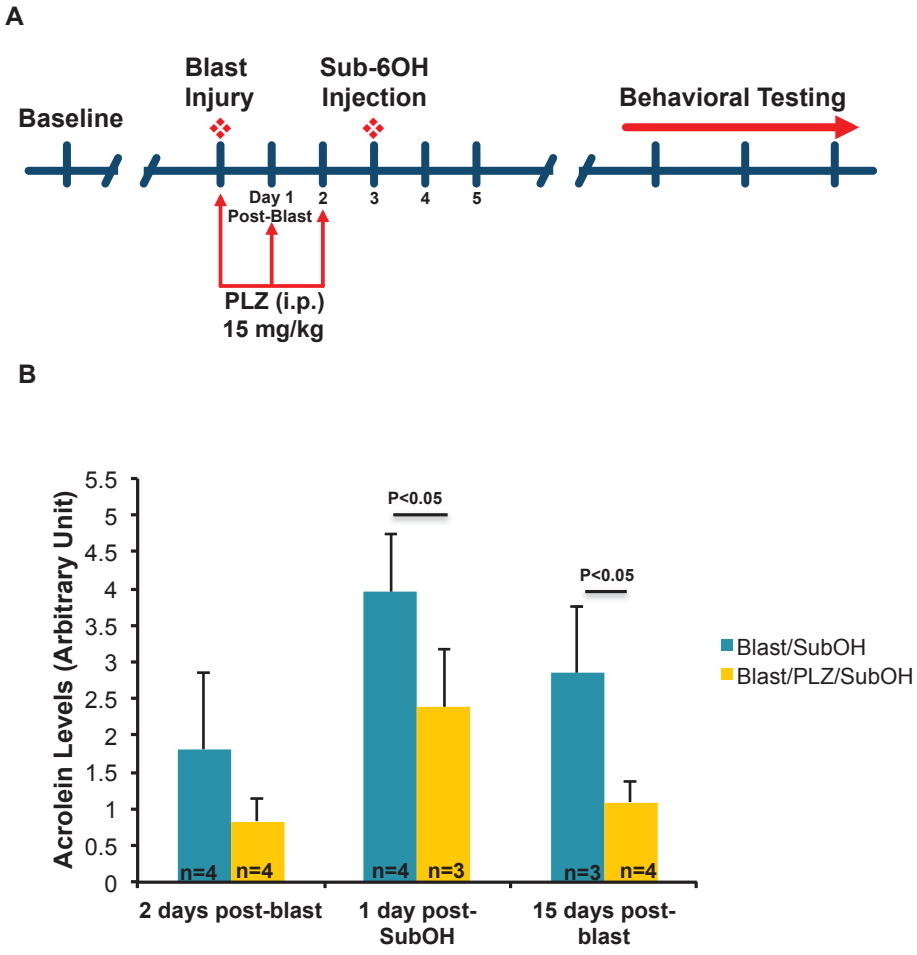

Figure 15. Treatment of phenelzine post-mBINT injury reduces the levels of 3-HPMA, an acrolein metabolite, in the urine. (A) Timeline of the injury and treatment. (B) Bar graphs are normalized levels of 3-HPMA in the urine 2 days post-mBINT, 1 day post sub-6OH nigral injection and 15 days post-mBINT comparison between the phenelzine treated group (Blast/PLZ/6OH) and the non-treated group. A comparison between the Blast/Sub6OH and Blast/PLZ/Sub6OH using a two-sample t-test assuming equal variance show significant differences 1 day post-SubOH ($P<0.05$) and 15 days post-mBINT ($P<0.05$). Error bars represent SEM.

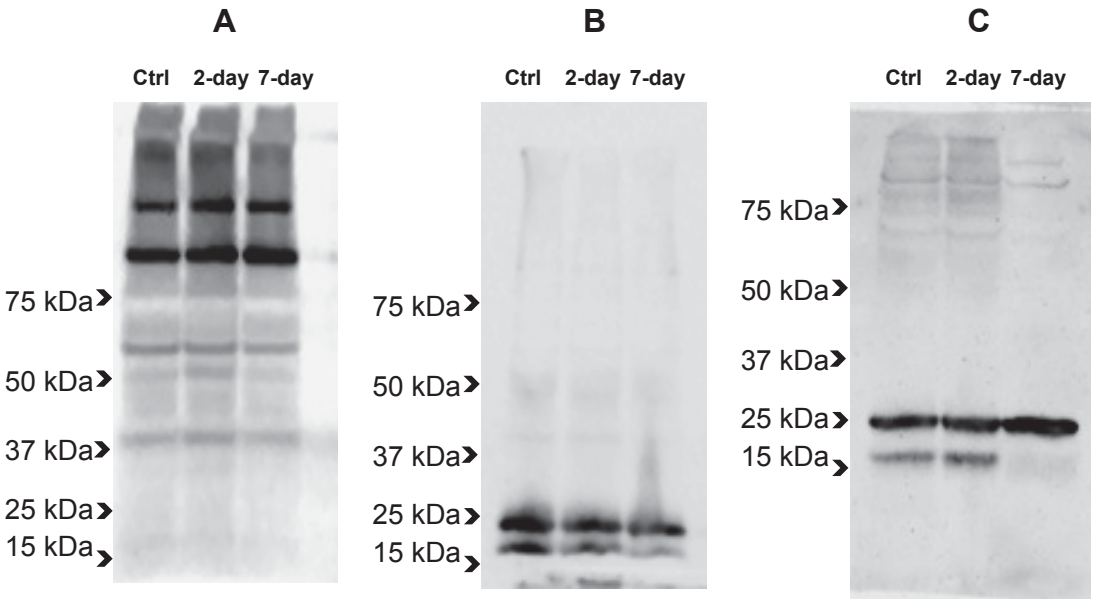

Figure 16. Western Blot Confirming the α-Synuclein bands observed in Figure 9. Each group is represented on each blot: Ctrl (Control), 2 days and 7 days post-mBINTgroups. **(A)** Western blot image of blocked α-syn antibody at 25 and 17 kDA. For this experiment, α-syn antibody was pre-incubated with a purified α-syn protein before adding to the blot. **(B)** Western blot image of non-blocked α-syn where α-syn antibody was not pre-incubated before addition to the blot. This was run simultaneously with blot (A). **(C)** Western blot image of non-blocked α-syn, same conditions as (B); but electrophoresis was run at slower rate and shorter time span. This blot was used for analysis as shown in Figure 9.

CHAPTER 5 – CONCLUSIONS AND FUTURE DIRECTIONS

The lack of understanding of mBINT pathophysiological mechanisms as well as the limited number of effective PD therapies motivates us to find alternative avenues for therapeutic targets, particularly to treat in the window of time between post-BINT and pre-PD symptoms. We hypothesize that oxidative stress, specifically acrolein, plays a critical role in linking mBINT and PD. Our results from this study demonstrate that acrolein scavenging may provide a more effective method for neuroprotection from such neuronal insults. We demonstrate that our mBINT rat model does not show motor deficits similar to those observed clinically. Because we are limited with the variability of these injuries in human studies and lack of animal models, a consistent and reliable model is essential to further understand the pathology of mBINT. Our mBINT model shows no motor deficits on our rotarod performance test, while post-mortem tissue analysis shows extensive biochemical changes (Figure 6, 7, 8, 9). From these results, we can speculate that secondary injuries such as acrolein are elevated post-mBINT and can be a critical factor in regulating PD biomarkers such as TH and α-synuclein.

PD is a complex disease and the mechanisms of dopaminergic neuronal death are still largely uncharacterized. Senescence and genetic mutations are the most common factors for increased susceptibility, but other factors such as traumatic brain injury have been gaining attention [87]. As one of the most frequent injuries suffered from increased military conflicts, the main focus of this research is mBINT. Individuals that are deployed to war conflicts have a higher exposure to blast injuries and most of them suffer from long term consequences such as post-traumatic stress disorder, depression, and PD, our main research focus. Our biochemical results show that mBINT can modulate PD markers such as increased levels of modified α-synuclein alpha (Figure 9) and decreased TH activity (Figure 8), hence supporting that mBINT increases susceptibility to PD.

Since acrolein has been identified as an essential mediator of secondary injury in spinal cord injury and its high reactivity to biomolecules [36, 44, 47, 147, 159], points toward the idea that the elevated acrolein levels post-mBINT (Figure 7) are contributing in part to the dysregulation of PD biomarkers, particularly tyrosine hydroxylase and α-synuclein (Figures 8, 9). Due to acrolein's longer half-life, on the order of hours to days [36], it provides a longer time period compared to the transient free radicals (ROS), which can further impose injury to the CNS. Therefore, acrolein scavenging may provide a more effective method for neuroprotection from such neuronal insults.

However, further investigation is warranted to understand precisely how these PD markers are affected by acrolein. Specifically, future studies need to focus on how acrolein induces post- translational modifications of α-synuclein, particulary ubiquitination, since we observed a species of suspected to be ubiquitinated α-synuclein (25 kDa, Figure 9C) post post-mBINT. Future *in vitro* experiments are needed to test if acrolein directly induces ubiquitination. Measuring ubiquitinated α-synuclein in post-mortem tissue of blast-injured animals would potentially validate those results. In addition, we can further investigate on how ubiquitinated α-synuclein species can regulate the activity of TH at pSer 40 and also further investigate the other TH phosphorylation sites. Future experiments are warranted to investigate both the regulation of TH through PKA and PKCδ and how acrolein affects these kinases to further understand the observed post-mBINT decrease in TH activity. Thorough investigation of acrolein effects the molecules such as kinases and phosphatases which regulate the key players in PD pathology, specifically TH and α-synuclein, will help us understand the pathogenesis of mBINT and how it can potentiate the development of PD. This will further our knowledge on how to treat and find avenues for therapeutic intervention and/or potentially prevent the long-term consequences of mBINT, particularly PD.

To further resolve the relationship between mBINT and PD, we established a sub-threshold 6OHDA model, a lower dosage of 6-OHDA from the full 6OHDA PD model, which shows no motor deficits, and combine it with mBINT. Our results show that a combination of mBINT and a sub-threshold injection of 6-OHDA produce PD-like motor

deficits, similar to the full 6-OHDA PD model (Figure 10, 11). These motor deficits can be correlated with acrolein measurements obtained by utilizing 3-HPMA detection in the urine (Figure 13), and provide further evidence on the role of acrolein post-mBINT. We also show that treatment with phenelzine, an effective acrolein scavenger, can mitigate these motor deficits and that it also correlated with our acrolein measurements (Figure 15). These results support that acrolein's role post-mBINT in potentiating may susceptibility to PD.

In summary, these studies support the hypothesis that acrolein post-mBINT contributes significantly to the secondary injury mechanisms similar to what is observed in SCI, and can promote development of PD pathology. Our results further demonstrate that acrolein plays a key role in both mBINT and PD, providing additional evidence regarding cellular and biochemical mechanism of acrolein-mediated insults for both pathologies. These studies can serve to expand our knowledge of the role of acrolein in both mBINT and PD; in order to further investigate mechanisms to effectively attenuate oxidative stress and improve recovery after the injury, and collectively promote neuroprotection. Thus, acrolein scavenging may be a novel therapeutic avenue to attenuate oxidative stress following neuronal trauma and can prevent further long- term consequences such as PD.

Our future studies will further assess the ability of acrolein scavenging to mitigate the risk of PD in post-mBINT rats and also the effect of comorbidities such as smoking and alcohol to intensify the susceptibility of PD in post-mBINT. Another desirable future study would be the investigation of multiple mBINTs and how it can potentiate in the development of PD. Nonetheless, the results from our project will advance our understanding of the long-term consequences of mBINT and development of PD. Our findings show potential for therapeutic application to improve the lifestyle of our military personnel and patients with PD as well as to alleviate the emotional and financial costs associated with these diseases. Once established, acrolein-targeting therapeutics could be extended to diseases such as multiple sclerosis, Alzheimer's disease and even cancer. In summary, future studies include:

- Conformation of our biochemical results on the modified alpha-synuclein observed post-blast injury and the relationship between acrolein and tyrosine hydroxylase regulation.
- Investigation of the molecular mechanisms of acrolein-mediated α-synuclein aggregation in both our mBINT and PD rat models.
- Further analysis of the behavioral data from the open activity box to provide a more quantitative and qualitative test of motor behavior.
- Investigation of the correlation of the level of acrolein and the degree of susceptibility of PD post-mBINT.
- Evaluation of the window of treatment to administer anti-acrolein therapy to mitigate the susceptibility of PD post-mBINT.

LIST OF REFERENCES

LIST OF REFERENCES

1. Chen, Y.C., D.H. Smith, and D.F. Meaney, *In-vitro approaches for studying blast-induced traumatic brain injury.* J Neurotrauma, 2009. **26**(6): p. 861-76.
2. DePalma, R.G., et al., *Blast injuries.* N Engl J Med, 2005. **352**(13): p. 1335-42.
3. Long, J.B., et al., *Blast overpressure in rats: recreating a battlefield injury in the laboratory.* J Neurotrauma, 2009. **26**(6): p. 827-40.
4. Bell, R.S., et al., *Military traumatic brain and spinal column injury: a 5-year study of the impact blast and other military grade weaponry on the central nervous system.* J Trauma, 2009. **66**(4 Suppl): p. S104-11.
5. Elder, G.A. and A. Cristian, *Blast-related mild traumatic brain injury: mechanisms of injury and impact on clinical care.* Mt Sinai J Med, 2009. **76**(2): p. 111-8.
6. Taber, K.H., D.L. Warden, and R.A. Hurley, *Blast-related traumatic brain injury: what is known?* J Neuropsychiatry Clin Neurosci, 2006. **18**(2): p. 141-5.
7. Coupland, R.M. and D.R. Meddings, *Mortality associated with use of weapons in armed conflicts, wartime atrocities, and civilian mass shootings: literature review.* BMJ, 1999. **319**(7207): p. 407-10.
8. Ling, G., et al., *Explosive blast neurotrauma.* J Neurotrauma, 2009. **26**(6): p. 815-25.
9. Moochhala, S.M., et al., *Neuroprotective role of aminoguanidine in behavioral changes after blast injury.* J Trauma, 2004. **56**(2): p. 393-403.
10. Defense, D.o., *2008 DoD Survey of Health Related Behaviors Among Active Duty Personnel*, 2009, Department of Defense: Washington DC.
11. Deforest, M.J., *Principles of Improvised Explosive Devices*. 1984, Boulder, CO: Paladin Press.
12. Sciences and Technology Division on Earth and Life Studies., N.R.C.U.S.C.o.D.I.E.D.B.R.t.i.t.I.D.C.N.R.C.U.S.B.o.C., *National Research Council (U.S.) Naval Studies Board Division on Engineering and Physical Sciences. Countering the threat of improvised explosive devices: Basic Research opportunities, abbreviated version*, 2007, National Academies Press: Washington, DC.
13. Turner, S., *Terrorist Explosive Sourcebook: Countering Terrorist use of improvised explosive devices*. 1994, Boulder, CO: Paladin Press.
14. Cernak, I., et al., *Blast injury from explosive munitions.* J Trauma, 1999. **47**(1): p. 96-103; discussion 103-4.

15. Kaur, C., et al., *The response of neurons and microglia to blast injury in the rat brain.* Neuropathol Appl Neurobiol, 1995. **21**(5): p. 369-77.
16. Kaur, C., et al., *Ultrastructural changes of macroglial cells in the rat brain following an exposure to a non-penetrative blast.* Ann Acad Med Singapore, 1997. **26**(1): p. 27-9.
17. Scott, S.G., et al., *Mechanism-of-injury approach to evaluating patients with blast-related polytrauma.* J Am Osteopath Assoc, 2006. **106**(5): p. 265-70.
18. Hoge, C.W., et al., *Mild traumatic brain injury in U.S. Soldiers returning from Iraq.* N Engl J Med, 2008. **358**(5): p. 453-63.
19. Terrio, H., et al., *Traumatic brain injury screening: preliminary findings in a US Army Brigade Combat Team.* J Head Trauma Rehabil, 2009. **24**(1): p. 14-23.
20. Bombardier, C.H., et al., *Rates of major depressive disorder and clinical outcomes following traumatic brain injury.* JAMA, 2010. **303**(19): p. 1938-45.
21. Spikman, J.M., et al., *Social cognition impairments in relation to general cognitive deficits, injury severity, and prefrontal lesions in traumatic brain injury patients.* J Neurotrauma, 2012. **29**(1): p. 101-11.
22. Veitch, D.P., K.E. Friedl, and M.W. Weiner, *Military risk factors for cognitive decline, dementia and Alzheimer's disease.* Curr Alzheimer Res, 2013. **10**(9): p. 907-30.
23. Weiner, M.W., et al., *Military risk factors for Alzheimer's disease.* Alzheimers Dement, 2013. **9**(4): p. 445-51.
24. Braak, H., et al., *Stanley Fahn Lecture 2005: The staging procedure for the inclusion body pathology associated with sporadic Parkinson's disease reconsidered.* Mov Disord, 2006. **21**(12): p. 2042-51.
25. Braak, H., et al., *Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages).* J Neurol, 2002. **249 Suppl 3**: p. III/1-5.
26. Goldman, S.M., et al., *Head injury and Parkinson's disease risk in twins.* Ann Neurol, 2006. **60**(1): p. 65-72.
27. Clemedson, C.J., *Blast injury.* Physiol Rev, 1956. **36**(3): p. 336-54.
28. Cernak, I., et al., *The pathobiology of blast injuries and blast-induced neurotrauma as identified using a new experimental model of injury in mice.* Neurobiol Dis, 2011. **41**(2): p. 538-51.
29. Hemphill, M.A., et al., *A possible role for integrin signaling in diffuse axonal injury.* PLoS One, 2011. **6**(7): p. e22899.
30. Park, E., et al., *A model of low-level primary blast brain trauma results in cytoskeletal proteolysis and chronic functional impairment in the absence of lung barotrauma.* J Neurotrauma, 2011. **28**(3): p. 343-57.
31. Ravin, R., et al., *Shear forces during blast, not abrupt changes in pressure alone, generate calcium activity in human brain cells.* PLoS One, 2012. **7**(6): p. e39421.
32. Cardona, S.M., J.A. Garcia, and A.E. Cardona, *The fine balance of chemokines during disease: trafficking, inflammation, and homeostasis.* Methods Mol Biol, 2013. **1013**: p. 1-16.
33. Werner, C. and K. Engelhard, *Pathophysiology of traumatic brain injury.* Br J Anaesth, 2007. **99**(1): p. 4-9.

34. Giulian, D., *Reactive glia as rivals in regulating neuronal survival.* Glia, 1993. **7**(1): p. 102-10.
35. Banik, N.L., *Pathogenesis of myelin breakdown in demyelinating diseases: role of proteolytic enzymes.* Crit Rev Neurobiol, 1992. **6**(4): p. 257-71.
36. Ghilarducci, D.P. and R.S. Tjeerdema, *Fate and effects of acrolein.* Rev Environ Contam Toxicol, 1995. **144**: p. 95-146.
37. Hamann, K. and R. Shi, *Acrolein scavenging: a potential novel mechanism of attenuating oxidative stress following spinal cord injury.* J Neurochem, 2009. **111**(6): p. 1348-56.
38. Park, E., J.D. Bell, and A.J. Baker, *Traumatic brain injury: can the consequences be stopped?* CMAJ, 2008. **178**(9): p. 1163-70.
39. Syburra, C. and S. Passi, *Oxidative stress in patients with multiple sclerosis.* Ukr Biokhim Zh, 1999. **71**(3): p. 112-5.
40. Yin, H., L. Xu, and N.A. Porter, *Free radical lipid peroxidation: mechanisms and analysis.* Chem Rev, 2011. **111**(10): p. 5944-72.
41. Butterfield, D.A., et al., *Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death.* Neurobiol Aging, 2002. **23**(5): p. 655-64.
42. Pugazhenthi, S., et al., *Differential regulation of c-jun and CREB by acrolein and 4-hydroxynonenal.* Free Radic Biol Med, 2006. **40**(1): p. 21-34.
43. Uchida, K., *4-Hydroxy-2-nonenal: a product and mediator of oxidative stress.* Prog Lipid Res, 2003. **42**(4): p. 318-43.
44. Esterbauer, H., R.J. Schaur, and H. Zollner, *Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes.* Free Radic Biol Med, 1991. **11**(1): p. 81-128.
45. Lovell, M.A., C. Xie, and W.R. Markesbery, *Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures.* Neurobiol Aging, 2001. **22**(2): p. 187-94.
46. Tanaka, N., et al., *Immunohistochemical detection of lipid peroxidation products, protein-bound acrolein and 4-hydroxynonenal protein adducts, in actinic elastosis of photodamaged skin.* Arch Dermatol Res, 2001. **293**(7): p. 363-7.
47. Uchida, K., et al., *Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins.* J Biol Chem, 1998. **273**(26): p. 16058-66.
48. Kruman, I., et al., *Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis.* J Neurosci, 1997. **17**(13): p. 5089-100.
49. Butterfield, D.A., et al., *Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide.* Trends Mol Med, 2001. **7**(12): p. 548-54.
50. Uchida, K., et al., *Protein-bound acrolein: potential markers for oxidative stress.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 4882-7.
51. Luo, J., K. Uchida, and R. Shi, *Accumulation of acrolein-protein adducts after traumatic spinal cord injury.* Neurochem Res, 2005. **30**(3): p. 291-5.
52. Hamann, K., et al., *Critical role of acrolein in secondary injury following ex vivo spinal cord trauma.* J Neurochem, 2008. **107**(3): p. 712-21.

53. Hamann, K., et al., *Hydralazine inhibits compression and acrolein-mediated injuries in ex vivo spinal cord.* J Neurochem, 2008. **104**(3): p. 708-18.
54. Luo, J. and R. Shi, *Acrolein induces axolemmal disruption, oxidative stress, and mitochondrial impairment in spinal cord tissue.* Neurochem Int, 2004. **44**(7): p. 475-86.
55. Shi, Y., et al., *Acrolein induces myelin damage in mammalian spinal cord.* J Neurochem, 2011. **117**(3): p. 554-64.
56. Leung, G., et al., *Anti-acrolein treatment improves behavioral outcome and alleviates myelin damage in experimental autoimmune encephalomyelitis mouse.* Neuroscience, 2011. **173**: p. 150-5.
57. Luo, J. and R. Shi, *Acrolein induces oxidative stress in brain mitochondria.* Neurochem Int, 2005. **46**(3): p. 243-52.
58. Burcham, P.C., et al., *Aldehyde-sequestering drugs: tools for studying protein damage by lipid peroxidation products.* Toxicology, 2002. **181-182**: p. 229-36.
59. Burcham, P.C., P.G. Kerr, and F. Fontaine, *The antihypertensive hydralazine is an efficient scavenger of acrolein.* Redox Rep, 2000. **5**(1): p. 47-9.
60. Kaminskas, L.M., S.M. Pyke, and P.C. Burcham, *Reactivity of hydrazinophthalazine drugs with the lipid peroxidation products acrolein and crotonaldehyde.* Org Biomol Chem, 2004. **2**(18): p. 2578-84.
61. Burcham, P.C., et al., *Protein adduct-trapping by hydrazinophthalazine drugs: mechanisms of cytoprotection against acrolein-mediated toxicity.* Mol Pharmacol, 2004. **65**(3): p. 655-64.
62. Burcham, P.C. and S.M. Pyke, *Hydralazine inhibits rapid acrolein-induced protein oligomerization: role of aldehyde scavenging and adduct trapping in cross-link blocking and cytoprotection.* Mol Pharmacol, 2006. **69**(3): p. 1056-65.
63. Kaminskas, L.M., S.M. Pyke, and P.C. Burcham, *Strong protein adduct trapping accompanies abolition of acrolein-mediated hepatotoxicity by hydralazine in mice.* J Pharmacol Exp Ther, 2004. **310**(3): p. 1003-10.
64. Liu-Snyder, P., R.B. Borgens, and R. Shi, *Hydralazine rescues PC12 cells from acrolein-mediated death.* J Neurosci Res, 2006. **84**(1): p. 219-27.
65. Liu-Snyder, P., et al., *Acrolein-mediated mechanisms of neuronal death.* J Neurosci Res, 2006. **84**(1): p. 209-18.
66. Reece, P.A., *Hydralazine and related compounds: chemistry, metabolism, and mode of action.* Med Res Rev, 1981. **1**(1): p. 73-96.
67. McGrath, P.J., et al., *Phenelzine treatment of melancholia.* J Clin Psychiatry, 1986. **47**(8): p. 420-2.
68. Paykel, E.S., et al., *Response to phenelzine and amitriptyline in subtypes of outpatient depression.* Arch Gen Psychiatry, 1982. **39**(9): p. 1041-9.
69. Paykel, E.S., et al., *Influence of acetylator phenotype on antidepressant effects of phenelzine.* Br J Psychiatry, 1982. **141**: p. 243-8.
70. Buigues, J. and J. Vallejo, *Therapeutic response to phenelzine in patients with panic disorder and agoraphobia with panic attacks.* J Clin Psychiatry, 1987. **48**(2): p. 55-9.

71. Sheehan, D.V., J. Ballenger, and G. Jacobsen, *Treatment of endogenous anxiety with phobic, hysterical, and hypochondriacal symptoms.* Arch Gen Psychiatry, 1980. **37**(1): p. 51-9.
72. Frank, J.B., et al., *A randomized clinical trial of phenelzine and imipramine for posttraumatic stress disorder.* Am J Psychiatry, 1988. **145**(10): p. 1289-91.
73. Liebowitz, M.R., et al., *Pharmacotherapy of social phobia: an interim report of a placebo-controlled comparison of phenelzine and atenolol.* J Clin Psychiatry, 1988. **49**(7): p. 252-7.
74. Mackenzie, J.E. and L.W. Frank, *Influence of pretreatment with a monoamine oxidase inhibitor (phenelzine) on the effects of buprenorphine and pethidine in the conscious rabbit.* Br J Anaesth, 1988. **60**(2): p. 216-21.
75. Wood, P.L., et al., *Aldehyde load in ischemia-reperfusion brain injury: neuroprotection by neutralization of reactive aldehydes with phenelzine.* Brain Res, 2006. **1122**(1): p. 184-90.
76. Khandhar, S.M. and W.J. Marks, *Epidemiology of Parkinson's disease.* Dis Mon, 2007. **53**(4): p. 200-5.
77. Starkstein, S.E., et al., *Anxiety Has Specific Syndromal Profiles in Parkinson Disease: A Data-Driven Approach.* Am J Geriatr Psychiatry, 2013.
78. Stefanis, L., *alpha-Synuclein in Parkinson's disease.* Cold Spring Harb Perspect Med, 2012. **2**(2): p. a009399.
79. Lo Bianco, C., et al., *alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10813-8.
80. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies.* Nature, 1997. **388**(6645): p. 839-40.
81. Castellani, R., et al., *Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease.* Brain Res, 1996. **737**(1-2): p. 195-200.
82. Owen, A.D., et al., *Indices of oxidative stress in Parkinson's disease, Alzheimer's disease and dementia with Lewy bodies.* J Neural Transm Suppl, 1997. **51**: p. 167-73.
83. Yan, M.H., X. Wang, and X. Zhu, *Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease.* Free Radic Biol Med, 2013. **62**: p. 90-101.
84. Shamoto-Nagai, M., et al., *In parkinsonian substantia nigra, alpha-synuclein is modified by acrolein, a lipid-peroxidation product, and accumulates in the dopamine neurons with inhibition of proteasome activity.* J Neural Transm, 2007. **114**(12): p. 1559-67.
85. Coyle, J.T. and P. Puttfarcken, *Oxidative stress, glutamate, and neurodegenerative disorders.* Science, 1993. **262**(5134): p. 689-95.
86. Xu, S., et al., *Oxidative stress induces nuclear translocation of C-terminus of alpha-synuclein in dopaminergic cells.* Biochem Biophys Res Commun, 2006. **342**(1): p. 330-5.
87. Chade, A.R., M. Kasten, and C.M. Tanner, *Nongenetic causes of Parkinson's disease.* J Neural Transm Suppl, 2006(70): p. 147-51.

88. Elsayed, N.M., et al., *Antioxidant depletion, lipid peroxidation, and impairment of calcium transport induced by air-blast overpressure in rat lungs.* Exp Lung Res, 1996. **22**(2): p. 179-200.
89. Saljo, A., et al., *Exposure to short-lasting impulse noise causes neuronal c-Jun expression and induction of apoptosis in the adult rat brain.* J Neurotrauma, 2002. **19**(8): p. 985-91.
90. Saljo, A., et al., *Expression of c-Fos and c-Myc and deposition of beta-APP in neurons in the adult rat brain as a result of exposure to short-lasting impulse noise.* J Neurotrauma, 2002. **19**(3): p. 379-85.
91. Shi, R., T. Rickett, and W. Sun, *Acrolein-mediated injury in nervous system trauma and diseases.* Mol Nutr Food Res, 2011. **55**(9): p. 1320-31.
92. Shi, R.Y., et al., *Calcium antagonists fail to protect mammalian spinal neurons after physical injury.* J Neurotrauma, 1989. **6**(4): p. 261-76; discussion 277-8.
93. Whalen, M.J., et al., *Acute plasmalemma permeability and protracted clearance of injured cells after controlled cortical impact in mice.* J Cereb Blood Flow Metab, 2008. **28**(3): p. 490-505.
94. Newell, K.L., et al., *Alpha-synuclein immunoreactivity is present in axonal swellings in neuroaxonal dystrophy and acute traumatic brain injury.* J Neuropathol Exp Neurol, 1999. **58**(12): p. 1263-8.
95. Schmidt, M.L., et al., *Tau isoform profile and phosphorylation state in dementia pugilistica recapitulate Alzheimer's disease.* Acta Neuropathol, 2001. **101**(5): p. 518-24.
96. Smith, D.H., et al., *Protein accumulation in traumatic brain injury.* Neuromolecular Med, 2003. **4**(1-2): p. 59-72.
97. Morganti-Kossmann, M.C., et al., *Modulation of immune response by head injury.* Injury, 2007. **38**(12): p. 1392-400.
98. Bhattacharjee, Y., *Neuroscience. Shell shock revisited: solving the puzzle of blast trauma.* Science, 2008. **319**(5862): p. 406-8.
99. Graham, D.G., *Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones.* Mol Pharmacol, 1978. **14**(4): p. 633-43.
100. Jenner, P., *Oxidative stress in Parkinson's disease.* Ann Neurol, 2003. **53 Suppl 3**: p. S26-36; discussion S36-8.
101. Jenner, P. and C.W. Olanow, *Understanding cell death in Parkinson's disease.* Ann Neurol, 1998. **44**(3 Suppl 1): p. S72-84.
102. Jenner, P. and C.W. Olanow, *The pathogenesis of cell death in Parkinson's disease.* Neurology, 2006. **66**(10 Suppl 4): p. S24-36.
103. Connell, S., H. Ouyang, and R. Shi, *Modeling blast induced neurotrauma in isolated spinal cord white matter.* J Med Syst, 2011. **35**(5): p. 765-70.
104. Bryan, C.J., et al., *Loss of consciousness, depression, posttraumatic stress disorder, and suicide risk among deployed military personnel with mild traumatic brain injury.* J Head Trauma Rehabil, 2013. **28**(1): p. 13-20.
105. Andre, E., et al., *Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents.* J Clin Invest, 2008. **118**(7): p. 2574-82.

106. Facchinetti, F., et al., *Alpha,beta-unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages.* Am J Respir Cell Mol Biol, 2007. **37**(5): p. 617-23.
107. Bergstrom, B.P., et al., *Partial, graded losses of dopamine terminals in the rat caudate-putamen: an animal model for the study of compensatory adaptation in preclinical parkinsonism.* J Neurosci Methods, 2001. **106**(1): p. 15-28.
108. Dunham, N.W. and T.S. Miya, *A note on a simple apparatus for detecting neurological deficit in rats and mice.* J Am Pharm Assoc Am Pharm Assoc (Baltim), 1957. **46**(3): p. 208-9.
109. Hamm, R.J., et al., *The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury.* J Neurotrauma, 1994. **11**(2): p. 187-96.
110. Jones, B.J. and D.J. Roberts, *The quantiative measurement of motor inco-ordination in naive mice using an acelerating rotarod.* J Pharm Pharmacol, 1968. **20**(4): p. 302-4.
111. Ando, Y., et al., *Histochemical detection of 4-hydroxynonenal protein in Alzheimer amyloid.* J Neurol Sci, 1998. **156**(2): p. 172-6.
112. Calingasan, N.Y., K. Uchida, and G.E. Gibson, *Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease.* J Neurochem, 1999. **72**(2): p. 751-6.
113. Chen, J., et al., *Formation of malondialdehyde adducts in livers of rats exposed to ethanol: role in ethanol-mediated inhibition of cytochrome c oxidase.* Alcohol Clin Exp Res, 2000. **24**(4): p. 544-52.
114. Comporti, M., *Lipid peroxidation and biogenic aldehydes: from the identification of 4-hydroxynonenal to further achievements in biopathology.* Free Radic Res, 1998. **28**(6): p. 623-35.
115. Uchida, K., *Role of reactive aldehyde in cardiovascular diseases.* Free Radic Biol Med, 2000. **28**(12): p. 1685-96.
116. Perbellini, L., N. Veronese, and A. Princivalle, *Mercapturic acids in the biological monitoring of occupational exposure to chemicals.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **781**(1-2): p. 269-90.
117. Schettgen, T., A. Musiol, and T. Kraus, *Simultaneous determination of mercapturic acids derived from ethylene oxide (HEMA), propylene oxide (2-HPMA), acrolein (3-HPMA), acrylamide (AAMA) and N,N-dimethylformamide (AMCC) in human urine using liquid chromatography/tandem mass spectrometry.* Rapid Commun Mass Spectrom, 2008. **22**(17): p. 2629-38.
118. Parent, R.A., et al., *Metabolism and distribution of [2,3-14C]acrolein in Sprague-Dawley rats. II. Identification of urinary and fecal metabolites.* Toxicol Sci, 1998. **43**(2): p. 110-20.
119. Stevens, J.F. and C.S. Maier, *Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease.* Mol Nutr Food Res, 2008. **52**(1): p. 7-25.
120. Zheng, L., et al., *Determination of urine 3-HPMA, a stable acrolein metabolite in a rat model of spinal cord injury.* J Neurotrauma, 2013. **30**(15): p. 1334-41.

121. DeWitt, D.S. and D.S. Prough, *Blast-induced brain injury and posttraumatic hypotension and hypoxemia.* J Neurotrauma, 2009. **26**(6): p. 877-87.
122. Tumer, N., et al., *Overpressure blast-wave induced brain injury elevates oxidative stress in the hypothalamus and catecholamine biosynthesis in the rat adrenal medulla.* Neurosci Lett, 2013. **544**: p. 62-7.
123. Vuceljic, M., et al., *Relation between both oxidative and metabolic-osmotic cell damages and initial injury severity in bombing casualties.* Vojnosanit Pregl, 2006. **63**(6): p. 545-51.
124. Nagatsu, T., M. Levitt, and S. Udenfriend, *Tyrosine Hydroxylase. The Initial Step in Norepinephrine Biosynthesis.* J Biol Chem, 1964. **239**: p. 2910-7.
125. Zhu, Y., J. Zhang, and Y. Zeng, *Overview of tyrosine hydroxylase in Parkinson's disease.* CNS Neurol Disord Drug Targets, 2012. **11**(4): p. 350-8.
126. Bademci, G., et al., *A rare novel deletion of the tyrosine hydroxylase gene in Parkinson disease.* Hum Mutat, 2010. **31**(10): p. E1767-71.
127. Yoritaka, A., et al., *Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease.* Proc Natl Acad Sci U S A, 1996. **93**(7): p. 2696-701.
128. Khan, W., et al., *A brief overview of tyrosine hydroxylase and alpha-synuclein in the Parkinsonian brain.* CNS Neurol Disord Drug Targets, 2012. **11**(4): p. 456-62.
129. Sutherland, C., et al., *Phosphorylation and activation of human tyrosine hydroxylase in vitro by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2.* Eur J Biochem, 1993. **217**(2): p. 715-22.
130. Thomas, G., J. Haavik, and P. Cohen, *Participation of a stress-activated protein kinase cascade in the activation of tyrosine hydroxylase in chromaffin cells.* Eur J Biochem, 1997. **247**(3): p. 1180-9.
131. Salvatore, M.F., et al., *Stoichiometry of tyrosine hydroxylase phosphorylation in the nigrostriatal and mesolimbic systems in vivo: effects of acute haloperidol and related compounds.* J Neurochem, 2000. **75**(1): p. 225-32.
132. Polanski, W., H. Reichmann, and G. Gille, *Stimulation, protection and regeneration of dopaminergic neurons by 9-methyl-beta-carboline: a new anti-Parkinson drug?* Expert Rev Neurother, 2011. **11**(6): p. 845-60.
133. Beal, M.F., *Mitochondria, oxidative damage, and inflammation in Parkinson's disease.* Ann N Y Acad Sci, 2003. **991**: p. 120-31.
134. Zhang, Y., et al., *Intravenous nonviral gene therapy causes normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism.* Hum Gene Ther, 2003. **14**(1): p. 1-12.
135. Daadi, M.M., et al., *Distribution of AAV2-hAADC-transduced cells after 3 years in Parkinsonian monkeys.* Neuroreport, 2006. **17**(2): p. 201-4.
136. Fujita, M., et al., *Chaperone and anti-chaperone: two-faced synuclein as stimulator of synaptic evolution.* Neuropathology, 2006. **26**(5): p. 383-92.
137. Kim, T.D., S.R. Paik, and C.H. Yang, *Structural and functional implications of C-terminal regions of alpha-synuclein.* Biochemistry, 2002. **41**(46): p. 13782-90.
138. Kim, T.D., et al., *Structural changes in alpha-synuclein affect its chaperone-like activity in vitro.* Protein Sci, 2000. **9**(12): p. 2489-96.

139. Ostrerova, N., et al., *alpha-Synuclein shares physical and functional homology with 14-3-3 proteins.* J Neurosci, 1999. **19**(14): p. 5782-91.
140. Souza, J.M., et al., *Chaperone-like activity of synucleins.* FEBS Lett, 2000. **474**(1): p. 116-9.
141. Itagaki, C., et al., *Stimulus-coupled interaction of tyrosine hydroxylase with 14-3-3 proteins.* Biochemistry, 1999. **38**(47): p. 15673-80.
142. Tofaris, G.K., et al., *Ubiquitination of alpha-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function.* J Biol Chem, 2003. **278**(45): p. 44405-11.
143. Toska, K., et al., *Regulation of tyrosine hydroxylase by stress-activated protein kinases.* J Neurochem, 2002. **83**(4): p. 775-83.
144. Lou, H., et al., *Serine 129 phosphorylation reduces the ability of alpha-synuclein to regulate tyrosine hydroxylase and protein phosphatase 2A in vitro and in vivo.* J Biol Chem, 2010. **285**(23): p. 17648-61.
145. Ranganna, K., et al., *Acrolein activates mitogen-activated protein kinase signal transduction pathways in rat vascular smooth muscle cells.* Mol Cell Biochem, 2002. **240**(1-2): p. 83-98.
146. Tanel, A. and D.A. Averill-Bates, *P38 and ERK mitogen-activated protein kinases mediate acrolein-induced apoptosis in Chinese hamster ovary cells.* Cell Signal, 2007. **19**(5): p. 968-77.
147. Kehrer, J.P. and S.S. Biswal, *The molecular effects of acrolein.* Toxicol Sci, 2000. **57**(1): p. 6-15.
148. Nakashima, A., et al., *A possible pathophysiological role of tyrosine hydroxylase in Parkinson's disease suggested by postmortem brain biochemistry: a contribution for the special 70th birthday symposium in honor of Prof. Peter Riederer.* J Neural Transm, 2013. **120**(1): p. 49-54.
149. Shi, X. and B.A. Habecker, *gp130 cytokines stimulate proteasomal degradation of tyrosine hydroxylase via extracellular signal regulated kinases 1 and 2.* J Neurochem, 2012. **120**(2): p. 239-47.
150. Maroteaux, L., J.T. Campanelli, and R.H. Scheller, *Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal.* J Neurosci, 1988. **8**(8): p. 2804-15.
151. Pivato, M., et al., *Covalent alpha-synuclein dimers: chemico-physical and aggregation properties.* PLoS One, 2012. **7**(12): p. e50027.
152. Ciechanover, A., *Linking ubiquitin, parkin and synphilin-1.* Nat Med, 2001. **7**(10): p. 1108-9.
153. Recchia, A., et al., *Alpha-synuclein and Parkinson's disease.* FASEB J, 2004. **18**(6): p. 617-26.
154. McNaught, K.S., et al., *Altered proteasomal function in sporadic Parkinson's disease.* Exp Neurol, 2003. **179**(1): p. 38-46.
155. McNaught, K.S. and C.W. Olanow, *Proteolytic stress: a unifying concept for the etiopathogenesis of Parkinson's disease.* Ann Neurol, 2003. **53 Suppl 3**: p. S73-84; discussion S84-6.

156. Sato, H., et al., *Authentically phosphorylated alpha-synuclein at Ser129 accelerates neurodegeneration in a rat model of familial Parkinson's disease.* J Neurosci, 2011. **31**(46): p. 16884-94.
157. Baptista, M.J., et al., *Co-ordinate transcriptional regulation of dopamine synthesis genes by alpha-synuclein in human neuroblastoma cell lines.* J Neurochem, 2003. **85**(4): p. 957-68.
158. Gao, N., et al., *Effect of alpha-synuclein on the promoter activity of tyrosine hydroxylase gene.* Neurosci Bull, 2007. **23**(1): p. 53-7.
159. Witz, G., *Biological interactions of alpha,beta-unsaturated aldehydes.* Free Radic Biol Med, 1989. **7**(3): p. 333-49.