TRICHLOROETHYLENE EXPOSURE AND TRAUMATIC BRAIN INJURY INTERACT AND PRODUCE DUAL INJURY BASED PATHOLOGY AND PIOGLITAZONE CAN ATTENUATE DEFICITS FOLLOWING TRAUMATIC BRAIN INJURY

Andrew David Sauerbeck
University of Kentucky, sauerbecka@gmail.com

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation
Sauerbeck, Andrew David, "TRICHLOROETHYLENE EXPOSURE AND TRAUMATIC BRAIN INJURY INTERACT AND PRODUCE DUAL INJURY BASED PATHOLOGY AND PIOGLITAZONE CAN ATTENUATE DEFICITS FOLLOWING TRAUMATIC BRAIN INJURY" (2011). University of Kentucky Doctoral Dissertations. 133.
https://uknowledge.uky.edu/gradschool_diss/133

This Dissertation is brought to you for free and open access by the Graduate School at UKnowledge. It has been accepted for inclusion in University of Kentucky Doctoral Dissertations by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
Abstract of Dissertation

Andrew D. Sauerbeck

The Graduate School
University of Kentucky
2011
TRICHLOROETHYLENE EXPOSURE AND TRAUMATIC BRAIN INJURY INTERACT AND PRODUCE DUAL INJURY BASED PATHOLOGY AND PIOGLITAZONE CAN ATTENUATE DEFICITS FOLLOWING TRAUMATIC BRAIN INJURY

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Andrew David Sauerbeck
Lexington, Kentucky
Director: Dr. Patrick G. Sullivan,
Professor of Anatomy and Neurobiology
Lexington, Kentucky

2011

Copyright © Andrew David Sauerbeck 2011
ABSTRACT OF DISSERTATION

TRICHLOROETHYLENE EXPOSURE AND TRAUMATIC BRAIN INJURY INTERACT AND PRODUCE DUAL INJURY BASED PATHOLOGY AND PIOGLITAZONE CAN ATTENUATE DEFICITS FOLLOWING TRAUMATIC BRAIN INJURY

The development of Parkinson's disease (PD) in humans has been linked to genetic and environmental factors for many years. However, finding common single insults which can produce pathology in humans has proved difficult. Exposure to trichloroethylene (TCE) or traumatic brain injury (TBI) has been shown to be linked to PD and it has also been proposed that multiple insults may be needed for disease development.

The present studies show that exposure to TCE prior to a TBI can result in pathology similar to early PD and that the interaction of both insults is required for impairment in behavioral function, and cell loss. Following exposure to TCE for 2 weeks there is a 75% impairment in mitochondrial function but it has yet to be shown if the addition of a TBI can make this worse. If the exposure to TCE is reduced to 1 week and combined with TBI a 50% reduction in mitochondrial function is observed following the dual injury which requires both insults. These studies provide further support for the hypothesis that PD may result from a multifactorial mechanism.

It had been established that regional differences exist in mitochondrial function across brain regions. The present studies indicate that previous findings are not likely to be the result of differences in individual mitochondria isolated from the cortex, striatum, and hippocampus. Further analysis of the effect of mitochondrial inhibitors on enzyme activity and oxygen consumption reveal that the different regions of the brain are similarly affected by the inhibitors. These results suggest that findings from previous studies indicating regionally specific deficits following systemic toxin exposure, such as with TCE, are not the result of regional differences in the individual mitochondria.

Given that TBI results in significant dysfunction, finding effective therapeutics for TBI will provide substantial benefits to individuals suffering an insult. Treatment with Pioglitazone following TBI reduced mitochondrial dysfunction, cognitive impairment, cortical tissue loss, and
inflammation. These findings provide initial evidence that treatment with Pioglitazone may be an effective intervention for TBI.

KEYWORDS: Trichloroethylene, Traumatic brain injury, Parkinson's disease, Mitochondrial dysfunction, Pioglitazone
TRICHLOROETHYLENE EXPOSURE AND TRAUMATIC BRAIN INJURY INTERACT AND PRODUCE DUAL INJURY BASED PATHOLOGY AND PIOGLITAZONE CAN ATTENUATE DEFICITS FOLLOWING TRAUMATIC BRAIN INJURY

By

Andrew David Sauerbeck

__________________________

Patrick G. Sullivan Ph.D.
Director of Dissertation

Wanye A. Cass Ph.D.
Director of Graduate Studies
RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

Name

Date
TRICHLOROETHYLENE EXPOSURE AND TRAUMATIC BRAIN INJURY INTERACT AND PRODUCE DUAL INJURY BASED PATHOLOGY AND PIOGLITAZONE CAN ATTENUATE DEFICITS FOLLOWING TRAUMATIC BRAIN INJURY

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky
By
Andrew David Sauerbeck
Lexington, Kentucky
Director: Dr. Patrick G. Sullivan,
Professor of Anatomy and Neurobiology
Lexington, Kentucky

2011

Copyright © Andrew David Sauerbeck 2011
Acknowledgements

My work as a graduate student would not have been possible if not for the help and support of numerous individuals. I would especially like to thank my mentor Dr. Patrick Sullivan for guiding me through my graduate career and allowing me to grow as an independent researcher. I would also like to thank my committee members Dr. Don Gash, Dr. James Pauly, Dr. Edward Hall, and Dr. Gouying Bing for providing thoughtful guidance to me over the years. If not for the guidance and support I have received from these individuals, since the start of my time in the Department of Anatomy and Neurobiology, I would not have been able to complete the work I have done.

Many thanks are also deserved by the numerous individuals who have helped me develop my technical skills over the years. I would like to especially thank Randy Hunter, Jignesh Pandya, and Yi Ai for helping me to develop crucial skills needed for the completion of this work. I would also like to thank the members of the Sullivan lab and the Spinal Cord and Brain Injury Research Center that have provided continued assistance over the years.

Finally, I would also like to thank my family and friends who have supported me throughout my graduate career. Their constant help and support will never be forgotten. I am indebted to many individuals, both those mentioned and not, and this work would never have been completed if not for the involvement of many people.

Thank you all.
# Chapter 1: Introduction

## Mitochondria

- Normal physiological function
- Regional differences in basal mitochondrial function
- Synaptic vs. non-synaptic mitochondrial function
- Regional mitochondrial differences following systemic toxin exposure

## Pathological mitochondrial function

- Traumatic brain injury
  - Epidemiology
  - Primary injury mechanisms
  - Secondary injury mechanisms
  - Mitochondrial dysfunction following traumatic brain injury
  - Mitochondrial treatments for traumatic brain injury
  - Excitotoxicity following traumatic brain injury
  - Mitochondrial permeability transition pore
  - Traumatic brain injury induced changes in the dopaminergic system

- Parkinson's disease
  - Epidemiology
  - Anatomical circuitry of the basal ganglia
  - Etiology
  - Trichloroethylene
  - Multifactorial pathogenesis Parkinson's disease

## Peroxisome proliferator activator receptors

- PPAR's in normal cellular function
- PPAR's in CNS disorders

## Summary

---

# Chapter 2: Traumatic brain injury and trichloroethylene exposure interact

and produce functional, histological, and mitochondrial deficits

## Introduction

---

## Methods

- Trichloroethylene treatment and controlled cortical impact brain injury
- Behavioral analysis
- Rotarod testing
- Cylinder testing
- Histological analysis
Western blot analysis.................................................................................................................. 46
Mitochondrial isolation and oxygen consumption analysis..................................................... 47
Seahorse protocol for isolated mitochondria............................................................................. 48
Statistical analysis....................................................................................................................... 49
Results........................................................................................................................................ 50
  Motor impairment occurs following exposure to TCE and a moderate TBI.............................. 50
  Exposure to TCE and a moderate TBI leads to a loss of tyrosine hydroxylase positive neurons in the substantia nigra............................................................... 52
  Exposure to TCE and TBI does not affect striatal tyrosine hydroxylase fiber density of levels of the dopamine transporter and dopamine receptor...................................................... 54
  Exposure to TCE does not affect cortical tissue loss following TBI........................................ 54
  Two week TCE exposure impairs mitochondrial bioenergetic function in the striatum.......... 56
  One week exposure to TCE followed by TBI results in synergistic mitochondrial dysfunction in the striatum......................................................... 58
Discussion..................................................................................................................................... 60

Chapter 3: Regional mitochondrial function and toxin susceptibility........................................ 71
  Introduction................................................................................................................................... 71
  Methods........................................................................................................................................ 74
  Mitochondrial isolation............................................................................................................... 74
  Preparation of mitochondrial substrates and inhibitors.............................................................. 75
  Preparation and calibration of seahorse sensor cartridge sample plate................................ 76
  Seahorse protocol for isolated mitochondria............................................................................. 77
  Analysis of data generated by seahorse bioscience XF24.......................................................... 79
  Mitochondrial Complex I assay.................................................................................................. 79
  Mitochondrial Complex II assay................................................................................................. 80
  Statistical analysis....................................................................................................................... 80
Results............................................................................................................................................ 81
  Basal mitochondrial bioenergetic function across brain regions.............................................. 81
  Effect of mitochondrial Complex I and II inhibitors on enzyme activity.................................. 83
  Effect of mitochondrial Complex I and II inhibitors on oxygen consumption...................... 83
  Time dependent inhibition of Complex I by rotenone............................................................... 85
Discussion....................................................................................................................................... 87
Chapter 4: Pioglitazone attenuates mitochondrial dysfunction cognitive impairment, cortical tissue loss, and inflammation following traumatic brain injury

Introduction.................................................................................................. 93
Methods....................................................................................................... 96
  Controlled cortical impact brain injury.............................................. 96
  Pioglitazone treatment...................................................................... 97
  Morris water maze behavioral assessment................................. 97
  Histological analysis....................................................................... 98
  Mitochondrial isolation and bioenergetic analysis....................... 99
  Statistical analysis......................................................................... 100
Results........................................................................................................ 101
  Pioglitazone protects mitochondria from injury-induced............ 101
  mitochondrial dysfunction
  Pioglitazone treatment improves morris water maze performance.. 103
  following traumatic brain injury
  Pioglitazone treatment reduces cortical damage following .......... 105
  traumatic brain injury
  Pioglitazone attenuates the neuroinflammatory response after...... 107
  traumatic brain injury
Discussion.................................................................................................... 109

Chapter 5: Summary and conclusions.......................................................... 117

References............................................................................................................... 124
Vita.......................................................................................................................... 140
LIST OF FIGURES

Figure 1.1: Diagram of electron transport chain....................................................... 2
Figure 1.2: Diagram of decrease in electron energy level....................................... 2
Figure 1.3: Causes of traumatic brain injury.......................................................... 11
Figure 1.4: Excitotoxic calcium induce mitochondrial death..................................... 19
Figure 1.5: Basal ganglia circuitry.......................................................................... 26
Figure 1.6: Trichloroethylene structure and environmental contamination............ 30
Figure 2.1: Exposure to TCE and a moderate TBI synergistically interact................. 51
resulting in functional impairment
Figure 2.2: Exposure to TCE and TBI interact and result in TH-positive neuron.. 53
loss in the substantia nigra
Figure 2.3: Exposure to TCE, TBI, or the dual injury does not lead to changes.... 55
in TH-fibers, DAT, and D2R
Figure 2.4: Exposure to TCE prior to a moderate TBI does not increase cortical.. 56
tissue loss
Figure 2.5: Two week exposure to TCE results in decreased bioenergetics............. 57
in the striatum
Figure 2.6: One week exposure to TCE and TBI results in synergistic............... 59
mitochondrial deficits
Figure 3.1: Regional differences exist in brain mitochondrial function................. 82
Figure 3.2: Mitochondrial Complex I and II inhibitors affect enzyme activity........ 84
equally across brain regions
Figure 3.3: Regional differences exist in the susceptibility to rotenone but not....... 85
malonate
Figure 3.4: Exposure to 10pM rotenone produces a progressive decrease in..... 86
mitochondrial oxygen consumption
Figure 4.1: Pioglitazone treatment attenuates mitochondrial dysfunction after..... 102
traumatic brain injury
Figure 4.2: Pioglitazone treatment reduces cognitive impairment following........ 104
traumatic brain injury
Figure 4.3: Pioglitazone treatment reduces cortical tissue loss.......................... 106
Figure 4.4: Pioglitazone treatment reduces post-injury microglial activation....... 108
following traumatic brain injury
Chapter 1

Introduction

Mitochondria

Normal physiological function:

The mitochondrion is a cellular organelle composed of two lipid bilayers, the inner and outer mitochondrial membranes, and complex protein machinery necessary to produce large amounts of adenosine-triphosphate (ATP), a required substrate for numerous cellular functions. Historically, mitochondria were independent bacteria which were incorporated into mammalian cells, leading to the ability for complex organisms to develop (Lane, 2006). Prior to becoming part of the mammalian cell, the mitochondria had a large genome containing thousands of genes (Timmis et al., 2004). Through many years of evolution, mammalian mitochondria now only contain 37 genes and require genes encoded in the nucleus for 99% of all mitochondrial proteins (Lane, 2006). The production of ATP by the mitochondria requires electrons to be moved sequentially through a series of proteins resulting in the generation of a proton gradient (Figure 1.1)(Nicholls and Ferguson, 2002). The inter-membrane space (IMS) lies between the inner and outer mitochondrial membranes and protons are “pumped” here during mitochondrial respiration. The process of moving protons from the matrix, the area inside of the inner mitochondrial membrane, into the IMS utilizes energy from the flow of electrons through the electron transport chain (ETC). As an electron moves through the ETC, the energy level of the electron decreases as protons are pumped into the IMS (Figure 1.2). In order for the mitochondria to obtain a high energy electron, food from the diet is first broken down and metabolized into smaller molecules, such as glucose, which can be further processed by the cell. The process of glycolysis converts
The mitochondrial electron transport chain (ETC) utilizes high energy electrons to move protons from the mitochondrial matrix into the intermembrane space. Electrons enter the ETC through Complex I and Complex II as the result of the oxidation of NADH and FADH$_2$. Electrons move from Complexes I and II via Ubiquinone (CoQ10) to Complex III and from Complex III to Complex IV via Cytochrome C. As electrons are transferred through the ETC, protons are moved through Complexes I, III, and IV generating a proton concentration gradient. The mitochondrial membrane potential, generated by the concentration of protons in the intermembrane space, is utilized by Complex V to generate ATP.

The proteins of the mitochondrial electron transport chain (ETC) have different redox potentials which range from $\sim-300$ mV at Complex I to $\sim600$ mV at Complex V. The sequential change in redox potential facilitates the directional flow of electrons through the ETC. As the electrons are moved through the ETC, the energy state of the electron is reduced significantly as a result of the usage of energy to translocate protons from the mitochondrial matrix to the intermembrane space.
glucose into pyruvate through a process of ten enzymatic steps. Pyruvate can be further processed in the mitochondrial matrix by the tricarboxylic acid cycle (TCA cycle) in order to produce NADH and FADH$_2$. Complex I (NADH-Ubiquinone Oxidoreductase) can oxidize NADH to NAD$^+$ by transferring an electron from NADH to an iron-sulphur (Fe-S) center which resides in Complex I. This first electron transfer results in the movement of protons into the IMS. Complex II (Succinate Dehydrogenase) can oxidize the FADH$_2$ produced by the TCA cycle into FAD in a similar manner to the oxidation of NADH by Complex I. Even though Complex II does receive an electron from FADH$_2$ no proton translocation to the IMS occurs during this step. Both Complexes I and II can then transfer the electron obtained from either NADH or FADH$_2$ to Complex III via ubiquinone (Coenzyme Q10), which resides in the IMS. Complex III can subsequently transfer the electron to Cytochrome C and move additional protons into the IMS. Cytochrome C then transfers the electron to Complex IV (Cytochrome C Oxidase), which is able to utilize energy from the electron to move an additional four protons into the IMS while consuming oxygen in the form of O$_2$. The consumption of oxygen by Complex IV results in the formation of two molecules of water and the elimination of four electrons. The process by which electrons move through the ETC from complex to complex while “pumping” protons into the IMS results in the formation of a mitochondrial membrane potential ($\Delta \Psi$). As a result of the generated $\Delta \Psi$, Complex V (ATP Synthase) is able harness the energy in the proton gradient by allowing protons to flow down the proton concentration gradient and through Complex V, resulting in the production of ATP. The mitochondrial respiratory process is vital to normal cellular function and survival and even transient inhibition of the process can lead to a loss of ATP and cell death.
Regional differences in basal mitochondrial function:

Given the diversity of the nervous system and the regional differences observed in mitochondrial function in different experimental models, it is currently understood that regional differences play an important role in mitochondrial function (for review see (Dubinsky, 2009)). As with many other aspects of cellular function, calcium plays an important role in mitochondrial function. Regional differences in the levels and regulation of calcium can directly affect the function of the mitochondria. In response to increases in intracellular calcium levels, the aspartate-glutamate carrier becomes activated and imports NADH (Pardo et al., 2006). The increased intracellular concentration of NADH is thought to underlie the observed increases in activities of the α-ketoglutarate, Pyruvate, and oxoglutarate dehydrogenases following increases in intracellular calcium (Denton et al., 1988). In addition to calcium induced changes in the activities of some of the dehydrogenases, regional differences have also been observed for Malate dehydrogenase and creatine kinase (Ryder, 1980, Gupta et al., 2000). In addition basal differences, neuronal activity has been shown to transcriptionally regulate mitochondrial subunit expression (Wong-Riley et al., 1997). Reducing neuronal activity with the application of tetrodotoxin has been shown to result in a loss of Cytochrome C oxidase levels (Nie and Wong-Riley, 1996), further indicating a close relationship between levels of neuronal activity and mitochondrial function. An additional difference in mitochondrial function between different regions of the brain can be observed by comparing spare respiratory capacity, which is the ability for mitochondria to increase the respiratory rate. Fern compiled data from many sources in order to compare the amount of spare respiratory capacity across brain regions and across mitochondrial complexes. It was observed that regions of the brain such as the striatum, which are highly metabolically active, have relatively lower levels of spare respiratory capacity (Fern, 2003), indicating
that even though mitochondrial subunit levels are correlated with neuronal activity there may be an upper limit to the amount of mitochondrial function for a given brain region. One of the most striking findings from Fern’s analysis is that the regions with the lowest spare respiratory capacity for a given mitochondrial complex are also the regions of the brain which are the most susceptible to mitochondrial inhibitors which block that complex. The striatum was the next to lowest region of the brain with regards to Complex II spare respiratory capacity (Fern, 2003) and this is also a region of the brain highly susceptible to the Complex II inhibitor 3-Nitropropionic acid (Beal et al., 1993). Further evidence supporting the existence of regional differences in brain mitochondria comes from work looking at the response of mitochondria to ischemic insults. Following a 30 minute ischemic event and six hours of reperfusion the paramedian cortex exhibited no decrease in mitochondrial function while the dorsal-lateral striatum exhibited approximately a 25% decrease in mitochondrial state 3 function (Sims, 1991), further indicating that the striatum is more susceptible to bioenergetic inhibition.

**Synaptic vs. non-synaptic mitochondrial function:**

In addition to different regions of the brain exhibiting differences in mitochondrial function, differences have been observed between synaptic and non-synaptic mitochondria from the same region of the brain (Davey et al., 1997, Brown et al., 2006, Naga et al., 2007, Pathak and Davey, 2008). By using inhibitors specific for Complexes I, III, and IV the susceptibility of synaptic and non-synaptic mitochondria to mitochondrial inhibition has been investigated. Synaptic mitochondria isolated from the whole brain are markedly more susceptible to Complex I inhibition by rotenone than non-synaptic mitochondria (Pathak and Davey, 2008). Non-synaptic mitochondria required a 72% inhibition of Complex I before any changes were observed in mitochondrial oxygen consumption while synaptic mitochondria began showing mitochondrial respiration
deficits after only 25% inhibition of Complex I (Pathak and Davey, 2008). In contrast to the large differences between synaptic and non-synaptic mitochondria with regards to Complex I inhibition and mitochondrial oxygen consumption, no major differences are observed between Complex III and Complex IV inhibition and mitochondria oxygen consumption with tissue isolated from the whole brain (Pathak and Davey, 2008). When looking within sub-regions of the brain, the cortex and CA1 region of the hippocampus exhibit similar effects of Complex I inhibition with both regions showing that synaptic mitochondria show oxygen consumption deficits with only 25% inhibition of Complex I while non-synaptic mitochondria require 60% inhibition (Pathak and Davey, 2008). In addition to being more susceptible to mitochondrial Complex I inhibition, synaptic mitochondria have also been shown to regulate calcium differently than non-synaptic mitochondria (Brown et al., 2006, Naga et al., 2007). Synaptic mitochondria isolated from cortical tissue are not able to buffer as much calcium as non-synaptic mitochondria before undergoing mitochondrial permeability transition pore (mPTP) formation (Brown et al., 2006). Even the addition of Cyclosporine A, which can inhibit the formation of the mPTP, cannot raise the calcium buffering capacity of synaptic mitochondria up to that of basal non-synaptic mitochondria (Brown et al., 2006). Further evidence shows that the major underlying difference between synaptic and non-synaptic mitochondrial calcium buffering capacity resides in the significantly higher levels of Cyclophilin D in synaptic mitochondria (Naga et al., 2007). Removing Cyclophilin D from synaptic mitochondria by utilizing knockout mice results in an increase in the calcium buffering capacity of the synaptic mitochondria to that of wild-type non-synaptic mitochondria (Naga et al., 2007). The collection of findings looking at synaptic and non-synaptic mitochondria further defines the significance of regional differences in mitochondrial function since not only are there differences among mitochondria from different areas of the brain but there are
differences in mitochondria between different portions of the cellular architecture of a given brain region, specifically synaptic and non-synaptic regions.

**Regional mitochondrial differences following systemic toxin exposure:**

An important aspect of regionally specific mitochondrial differences can be observed following the systemic administration of mitochondrial toxins, where specific regions of the brain are more affected by a given toxin. The toxins 3-nitropropionic acid (3-NP), cyanide, and trichloroethylene (TCE) have all been shown to inhibit mitochondrial function and produce regionally specific damage following systemic administration (Hicks, 1950, Beal et al., 1993, Gash et al., 2008, Liu et al., 2010). The toxin 3-NP irreversibly inhibits Complex II (succinate dehydrogenase) of the mitochondrial electron transport chain (Alston et al., 1977) and systemic exposure to 3-NP produces an excitotoxic lesion in the striatum but not in other regions of the brain (Beal et al., 1993). Regional differences in how the mitochondria are affected and regulated following exposure to 3-NP may underlie the observed regionally specific susceptibility to 3-NP. Following exposure to 3-NP, striatal neurons but not cortical neurons up regulate Cytochrome-C oxidase (COX) levels which results in increases in ATP levels with the side effect of increased peroxide production, leading to increases in oxidative stress (Singh et al., 2010). Additionally, the striatum has been shown to have one of the lowest spare respiratory capacities for Complex II of any brain region accessed (Fern, 2003) and this may drive a proportionally higher degree of energy impairment in the striatum following exposure to 3-NP. With a lower basal spare respiratory capacity, mitochondrial inhibition by 3-NP likely drives the striatum to up regulate COX in an attempt to maintain ATP production and cell viability. Even though the increase in COX leads to more ATP production, the increased peroxide production would make the cells more vulnerable and play a role in the cell loss observed in the
striatum (Singh et al., 2010). Similar to 3-NP, TCE has been shown to produce regionally specific deficits following systemic administration (Gash et al., 2008, Liu et al., 2010). Following six weeks of TCE exposure in rats, mitochondrial bioenergetic impairment was observed in the substantia nigra but not in the striatum or liver (Gash et al., 2008). At both two and six weeks following exposure to TCE there are increases in markers of oxidative stress in the substantia nigra along with significant impairment in mitochondrial Complex I activity (Liu et al., 2010). In the striatum there is an increase in Complex I activity following six weeks of TCE exposure (Gash et al., 2008) and this may represent a compensatory response similar to the striatal increase in COX following 3-NP exposure (Singh et al., 2010). In addition to predominately affecting mitochondria bioenergetic function in the substantia nigra after six weeks of exposure, TCE also results in death of dopaminergic neurons but not cholinergic or GABAergic neurons (Liu et al., 2010), further expanding the level of complexity in regional mitochondrial deficits to including cell type specific responses. Cyanide, a mitochondrial Complex IV inhibitor, has also been shown to produce regionally specific damage, most notably to white matter (Hicks, 1950), and like 3-NP its toxicity is correlated the amount of spare respiratory capacity for the region it affects (Fern, 2003). Exposure to cyanide does affect mitochondrial functions in other regions, such as the cortex where reductions in COX activity and ATP levels are observed shortly after exposure (MacMillan, 1989), however, the long term damage still remains mainly in the white matter of the corpus callosum following sub lethal exposure (Brierley et al., 1976). The major apparent similarity observed with the different toxin models is that pathology tends to follow basal differences in mitochondrial function and regional requirements for energy sources. Regions which have lower levels of spare mitochondrial respiratory capacity for a given mitochondrial complex are more likely to show damage following exposure to a toxin inhibiting that complex. Additionally, cell populations with high energy demands, such as
dopamine neurons, are more susceptible to inhibition of mitochondrial function than other cell types from the same brain region (Zeevalk et al., 1997). Brain regions which cannot maintain sufficient mitochondrial function and ATP production can become damaged and undergo cell loss while regions which can maintain sufficient energy production survive.

**Pathological mitochondrial function:**

The complexity of the electron transport chain process leaves the system vulnerable to many insults and disturbances. For example, following TBI an ischemic insult can result in a loss of oxygen needed by the mitochondria at Complex IV to remove electrons from the ETC. Following reperfusion of the hypoxic tissue with oxygenated blood there is a rapid increase in the production of reactive oxygen species (ROS) which can overcome endogenous antioxidant systems and lead to further damage and dysfunction (for review see (Sugawara and Chan, 2003)). Given its critical role in normal cellular function by producing ATP, the mitochondria's susceptibility to a wide range of inhibitors and insults, and its ability to initiate cell death cascades, it is not surprising that the mitochondria has been found to play an important role in numerous pathological conditions. Mitochondrial dysfunction has been observed following TBI or exposure to environmental toxins such as TCE as well as in degenerative neurological conditions such as Parkinson's disease (PD). The role the mitochondria plays in these separate yet related pathologies is discussed in more detail in the sections specific to each insult/pathology.
**Traumatic brain injury**

**Epidemiology:**

Every year in the United States there are approximately 1.7 million individuals that obtain medical care at a hospital emergency department (ED) for treatment related to a traumatic brain injury (TBI) (Faul et al., 2010). Of the individuals that go to an ED for care related to a TBI, approximately 52,000 die, 275,000 become hospitalized, and 1.36 million are released (Faul et al., 2010). The level of severity for a TBI can vary significantly between individuals with some people sustaining a mild TBI with short term alterations in cognition and mental status to individuals having a severe TBI which results in chronic long term disability and impairment. Current estimates predict that in the United States there are approximately 5.3 million individuals living with prolonged deficits due to a TBI (Thurman et al., 1999). The prolonged impairments from a TBI can affect multiple aspects of normal daily function including cognition, sensorimotor function, language, and emotion (NINDS, 2002). The four most common causes of TBI are falls (28%), motor vehicle accidents (20%), impact with an object (19%), and assaults (11%) (Figure 1.3) (Langlois et al., 2006). Not only does TBI impart significant hardships on the individual sustaining the injury but it is also estimated that in the United States alone TBIs cost $60 billion annually as a result of medical expenses and lost productivity (Finkelstein et al., 2006). The incidence of TBI can vary widely between gender, ethnic, and age groups, and men are twice as likely as women to sustain a TBI (Langlois et al., 2006). On average people over the age of 75 are more likely to die from a TBI and African Americans have the highest likelihood of death compared to other ethnic backgrounds (Langlois et al., 2006). The primary cause of a TBI can vary widely and plays a direct and important role in the pathology which ensues.
Figure 1.3: Causes of traumatic brain injury

Causes of traumatic brain injury

Traumatic brain injury (TBI) results from various different causes. The most common mechanisms leading to TBI pathology are falls, motor vehicle accidents, impact with an object, and assaults. These four causes are responsible for 78% of all TBIs in the United States.

Primary injury mechanisms:

TBI pathology results from both a primary injury and a secondary injury cascade. The primary injury is due to biomechanical damage which results in the shearing and compression of neuronal, glial, and vascular tissue. TBIs can result from numerous different causes (Figure 1.3), present with a variety of different anatomical pathologies, and present with injury levels ranging from mild to severe. Current research on human TBI has generally utilized neurological injury severity criteria to classify the severity of
the injury (Narayan et al., 2002, Saatman et al., 2008) with the 15-point Glasgow Coma Score (GCS) (Teasdale and Jennett, 1974) being the most common (Narayan et al., 2002). Though the GCS has been utilized extensively for clinical trials seeking to enroll patients with severe TBIs, the GCS does not work well for certain populations such as infants, it does not distinguish less severe injuries, and it cannot distinguish the underlying type of injury (Saatman et al., 2008). Beyond determining the severity of a TBI, it is important to understand the causative mechanism of the injury since different types of injuries require different medical care and have different long term outcomes. TBI can result in various neuroanatomical changes which include epidural hematoma, parenchymal hematoma, diffuse axonal injury (DAI), subdural hematoma, subarachnoid hemorrhage, intraventricular hemorrhage, and diffuse brain swelling (Saatman et al., 2008). Of the various primary injuries, the four main pathologies observed in human TBI are subarachnoid hemorrhage, DAI, contusion, and hematoma (Saatman et al., 2008). Patients can present with any combination of pathoanatomical changes or even just a single injury type. It was recently shown that patients presenting with an initial GCS <8, indicating a severe TBI, can exhibit single injury types which cannot be distinguished by the GCS score (Saatman et al., 2008). The problems associated with utilizing a GCS score for patient treatment decisions indicates that new strategies for rapidly classifying injury type need to be found since the type of injury affects patient outcome (Gennarelli et al., 1982). Given the variety of types of injuries, it is crucial to understand what type of injury a patient has so that appropriate medical care can be given.

**Secondary injury mechanisms:**

The cascade of secondary injury damage, which occurs in the hours and days following the initial insult, is due to activation of pathophysiological cascades, consisting of complex biochemical and cellular pathways that influence progression of the injury.
Secondary injury processes include alterations in excitatory amino acids (Yamamoto et al., 1999, Rose et al., 2002), increased reactive oxygen species (ROS) production (Marklund et al., 2001, Hall et al., 2004, Tavazzi et al., 2005), disruption of calcium homeostasis (Mattson and Scheff, 1994, Xiong et al., 1997, Sullivan et al., 1999c), post-traumatic neuroinflammation (Morganti-Kossmann et al., 2001, Vlodavsky et al., 2006) and mitochondrial dysfunction (Azbill et al., 1997, Xiong et al., 1997, Sullivan et al., 1998b, Sullivan et al., 1999a, Sullivan et al., 1999b). As a result of these secondary injury processes, there are significant reductions in ATP levels (Sullivan et al., 1998a), increases in lipid peroxidation (Sullivan et al., 1998a), release of Cytochrome C (Sullivan et al., 2002) and activation of apoptotic pathways (Sullivan et al., 2002), all of which can lead to the initiation of cell death pathways.

**Mitochondrial dysfunction following traumatic brain injury:**

Mitochondria are a major component of this secondary injury pathway because they function as a highly sensitive regulator of cell death mechanisms and as the primary energy producer for the cell. The mitochondria play a pivotal role in cerebral energy metabolism, intracellular calcium homeostasis, and ROS generation and detoxification. Following TBI, a significant disruption of mitochondrial homeostasis has been documented that results in a decline in cellular bioenergetics, increased mitochondrial ROS production and a decline in synaptic equilibrium (Azbill et al., 1997, Xiong et al., 1997, Sullivan et al., 1998b, Sullivan et al., 1999a, Sullivan et al., 1999b). Immediately after the TBI reductions in the amount of ATP have been observed in the cortex following a cortical impact brain injury and this impairment in mitochondrial function precedes increases in levels of lipid peroxidation which begin to increase by 30 minutes post injury (Sullivan et al., 1998b).
Looking at more detailed mechanisms of the mitochondrial dysfunction which occurs after TBI has revealed that in cortical synaptosomes isolated 30 minutes after the injury there is a loss of mitochondrial membrane potential, increases in intracellular mitochondrial calcium loading, increased susceptibility to calcium induced mitochondrial permeability transition, and increases in the production of ROS (Sullivan et al., 1999c). The mitochondrial deficits which occur after the injury can lead to both mitochondrial and cellular damage and dysfunction. The loss of the mitochondria’s ability to produce ATP can impair the cell’s ability to maintain both normal and post-injury functions and ultimately lead to cell death (Nicholls and Budd, 2000). Additionally, factors such as mitochondrial ROS production and increases in levels of mitochondrial lipid peroxidation can result in mitochondrial bioenergetic impairments resulting in further reductions in mitochondrial dependent ATP production as well as the induction of mitochondrial dependent cell death pathways (Nicholls and Budd, 2000). Considering the multiple mechanisms of mitochondrial dysfunction that occur following TBI, the degree of mitochondrial injury or dysfunction can be an important determinant of cell survival or death (for reviews see (Robertson, 2004, Sullivan et al., 2005, Robertson et al., 2006) and it has been shown that therapeutic treatments designed to protect and stabilize the mitochondria have the ability to reduce injury in preclinical studies (Sullivan et al., 2000a, Pandya et al., 2007).

**Mitochondrial treatments for traumatic brain injury:**

Given the important role which the mitochondria play in both normal and post-TBI pathology, and the desire to find therapeutic interventions which result in neuroprotection, drugs targeting mitochondrial function have been pursued as potential effective interventions. Mitochondrial therapeutics which have been pursued for the treatment of TBI include Cyclosporine A (CsA), mitochondrial uncouplers such as 2,4-
Dinitrophenol (2,4-DNP) and FCCP, as well as alternative energy sources for the mitochondria.

Cyclosporin A is an immunosuppressive drug which is capable of binding to mitochondrial Cyclophilin D thereby inhibiting the formation of mitochondrial permeability transition pore (mPTP) (Broekemeier et al., 1989, Broekemeier and Pfeiffer, 1995). Following TBI there is significant mitochondrial dysfunction which occurs acutely after the injury (Sullivan et al., 1998b, Sullivan et al., 1999c, Sullivan et al., 2004). Treatment with CsA 15 minutes after a TBI has shown the ability to ameliorate multiple aspects of mitochondrial pathology by preventing the formation of the mPTP, preserving membrane potential, reducing mitochondrial calcium accumulation, and reducing the production of mitochondrial ROS (Sullivan et al., 1999c). It has also been observed that treatment with CsA is capable of reducing the amount of cortical tissue loss by up to 50% following a single bolus injection (Sullivan et al., 1999c) and up to 74% following a continuous infusion of CsA (Sullivan et al., 2000c). Further work has indicated that significant mitochondrial improvements can still be obtained even if treatment with CsA is delayed for 24 hours following the injury (Sullivan et al., 2000b), which should provide a large therapeutic window of opportunity for the usage of CsA in humans. Confirming the mitochondrial findings observed with CsA treatment, administration of the non-immunosuppressive CsA analog NIM811 was shown to protect the mitochondria following TBI (Mbye et al., 2008). Treatment with NIM811 resulted in preservation of the mitochondrial respiratory control ratio (RCR), mitochondrial bioenergetic function, and prevented increases in the oxidative damage markers 4-hydroxynonenol (4-HNE) and 3-Nitrotyrosine (3-NT) (Mbye et al., 2008). Given the effectiveness of CsA in preclinical research, the clinical testing of CsA in humans for the treatment of TBI has begun (Hatton et al., 2008, Mazzeo et al., 2009).
A second therapeutic intervention targeting the mitochondria is the use of chemical mitochondrial uncouplers. Mitochondrial uncoupling is a process by which protons are moved from the mitochondrial intermembrane space to the matrix without going through Complex-V of the mitochondria, thereby rapidly reducing mitochondrial membrane potential (for review see (Sullivan et al., 2004, Mattiasson and Sullivan, 2006)). Given that after TBI there is a rapid increase in excitotoxicity which results in intracellular calcium accumulation (Fiskum, 2000) it was proposed that this calcium accumulation would lead to mitochondrial calcium overload (Pandya et al., 2007). The hypothesis was generated that treatment with mitochondrial uncouplers would reduce the mitochondrial calcium overload since the mitochondrial accumulation of calcium is membrane potential dependent (Pandya et al., 2007). A single intraperitoneal injection of either 2,4-DNP or FCCP five minutes after a TBI led to reductions in the amount of cortical tissue loss and hippocampal Fluoro Jade-B positive neurons, and this was correlated with preservations in cognitive function (Pandya et al., 2007). As was hypothesized, animals treated with mitochondrial uncouplers exhibited preserved levels of mitochondrial bioenergetic function, reductions in \textit{in situ} calcium loading, and reductions in mitochondrial ROS production (Pandya et al., 2007). Given that a five minute post-injury treatment time is not feasible for translating to human usage, a delayed time course for administration was tested (Pandya et al., 2009). Delaying the administration of the mitochondrial uncoupler FCCP up to six hours after the injury produced the same beneficial effect as observed following a five minute post-injury injection, indicating a broad therapeutic window of opportunity for the treatment of TBI with a mitochondrial uncoupler (Pandya et al., 2009).

Additional work has pursued the use of alternative energy sources for the mitochondria following TBI to maintain ATP levels. Following either a moderate or
severe TBI, alterations in the brain’s usage of oxygen, glucose, and lactate have been observed in humans which suggests the brain changes how it utilizes energy sources for the production of ATP (Glenn et al., 2003). Given that the brain changes how it generates ATP after a TBI, it was proposed by Prins et al. that administration of the ketone beta-hydroxybutarate (B-HB) after TBI would ameliorate energy deficits observed after the injury (Prins et al., 2004). Following a three hour infusion of B-HB beginning immediately after the injury, treatment prevented the 20% reduction in ATP levels which was observed in vehicle-treated injured animals (Prins et al., 2004). It was also observed that sham animals did not exhibit an increase in cerebral uptake of B-HB, which was observed in the injured animals, further suggesting that the TBI directly alters how the brain responds to mitochondrial energy sources and what fuel sources the brain utilizes after a TBI (Prins et al., 2004).

Supporting the concept that alternative energy sources can lead to neuroprotection after TBI, research has shown that post-injury fasting promotes neuroprotection by reducing cortical tissue loss, reducing mitochondrial calcium loading, maintaining mitochondrial bioenergetic function, and preventing increases in oxidative damage (Davis et al., 2008). Given that fasting increases both insulin and ketone levels in the blood, both were testing following TBI to determine if either component of post-injury fasting was responsible for the neuroprotection. Davis et al. showed that the ketone B-HB but not insulin was capable of increasing tissue sparing after the injury (Davis et al., 2008), providing further evidence and support that following TBI providing the brain with alternative fuels, specifically the ketone B-HB, is capable of providing neuroprotection and reducing post injury deficits.
Excitotoxicity following traumatic brain injury:

The concept of excitotoxicity was developed by Olney after subcutaneous injections of monosodium glutamate were shown to produce neuronal loss in various brain regions (Olney, 1969). The mechanism of cell death resulting from glutamate exposure has been shown to result from the influx of calcium into the cell (Choi, 1987) and that the NDMA receptor but not voltage-sensitive calcium channels are the cytotoxic source of the calcium (Tymianski et al., 1993). It has been shown that the link between NMDA receptor activation and the resulting mitochondrial dysfunction results from their close proximity to each other (Peng and Greenamyre, 1998) and this connection results in the mitochondria being considered the primary cause of excitotoxic cell death (Fiskum, 2000). A major mechanism underlying glutamate induced damage is through the effects of calcium influx on the mitochondria and damage to calcium extrusion pathways, which inhibit mitochondrial bioenergetic function (Ward et al., 2000). Both aspects of mitochondrial impairment can eventually lead to cellular deregulation of calcium and the loss of ATP production (Figure 1.4) (Ward et al., 2000). It has further been shown that the increase in mitochondrial calcium concentrations is mitochondrial membrane potential dependent. Treatment with mitochondrial uncouplers can reduce the accumulation of calcium in the mitochondria following TBI (Pandya et al., 2009).

TBI in humans has been shown to lead to elevations in the levels of excitatory amino acids (EAAs), specifically the neurotransmitter glutamate, in cerebrospinal fluid (CSF) (Bullock et al., 1998, Koura et al., 1998, Yamamoto et al., 1999). Patients with elevated CSF glutamate levels have been shown to have increased post-injury intracranial pressure and a worse outcome six months after the injury (Koura et al., 1998). Animal models of TBI have shown that the increases in glutamate levels are
Figure 1.4: Excitotoxic calcium induced mitochondrial death

Following TBI, the cascade of excitotoxic damage leads to both cell death and damage to neighboring cells. As a result of glutamate activation of the NMDA receptor, calcium enters the cell and accumulates in the mitochondria. The mitochondrial accumulation of calcium can lead to either necrotic or apoptotic cell death. Both cell death pathways can result in the further release of calcium which can perpetuate the process.

linked to injury severity with moderately injured animals showing a 282% increase in glutamate and severely injured animals showing a 940% increase when compared to sham animals (Faden et al., 1989). Even though glutamate levels have been shown to increase after injury and be correlated with a worse outcome, simply showing post-injury increases in EAA levels is not sufficient to show that these changes directly play a role in increased pathology. To determine whether the increased levels of EAAs play a direct
role in outcome and pathology, various NMDA receptor antagonists have been utilized to prevent the activation of the NMDA receptor by glutamate (Faden et al., 1989, Shapira et al., 1990). Following a fluid percussion injury paradigm, administration of NMDA receptor antagonists have shown the ability to improve motor function and bioenergetic status (Faden et al., 1989) and reduce brain damage and edema (Shapira 1990). Even with the success of NMDA antagonists in animals models of TBI, attempts to move compounds into clinical usage has been met with much difficulty (for review see (Ikonomidou and Turski, 2002)). The usage of NMDA antagonists in humans has been linked to possible neurological damage (Davis et al., 2000) and animal models suggest NMDA antagonists may have a short and potentially unusable therapeutic window of efficacy in humans (Meldrum, 1990). Even though the clinical usage NMDA antagonists to block excitotoxicity has not reached success as a treatment for brain injuries in humans, the mechanisms by which glutamate induced excitotoxicity leads to neuronal death is widely accepted.

**Mitochondrial permeability transition pore:**

A downstream outcome from increases in mitochondrial calcium loading, such as from excitotoxicity, is the formation of the mitochondrial permeability transition pore (mPTP) (Sullivan et al., 2000c, Sullivan et al., 2005). The mPTP is a multi-protein complex which consists of the adenine nucleotide translocase (ANT), the voltage-dependent anion channel (VDAC), and Cyclophilin D (Baines, 2009). The proteins which form the mPTP can be found both in the inner and outer mitochondrial membranes which allows for the efflux of substances from the IMS and matrix into the cytosol when the pore opens. Cytochrome C, endonuclease G, and apoptosis inducing factor (AIF) are three important substances released after the opening of the mPTP since their release from the mitochondria can lead to the activation of cell death cascades. Endonuclease
G and AIF play an important role during apoptosis since they can initiate controlled DNA degradation. When Cytochrome C is released into the cytosol it is capable of interacting which the cytosolic protein Apaf-1 and form the apoptosome. The apoptosome recruits procaspase-9 and initiates the activation of caspases, such as Caspase-3, which drive the characteristic protein degradation observed with apoptosis. Therapeutic intervention with Cyclosporin A (CsA) can delay the opening of the mPTP and this intervention has been shown to have a beneficial effect in experimental models of TBI (Scheff and Sullivan, 1999, Sullivan et al., 1999c, Sullivan et al., 2000b, Sullivan et al., 2000c). Similar to excitotoxic mechanisms of cellular injury after TBI, intervening rapidly after a TBI in order to prevent the opening of the mPTP is critical to prevent the initiation the cell death cascades.

**Traumatic brain Injury induced changes in the dopaminergic system:**

The dopaminergic (DA) neurons in the brain influence numerous functions including cognition, movement, and emotion (for reviews see (Jackson and Westlind-Danielsson, 1994, Floresco and Magyar, 2006)). Dopamine neurons which originate in the substantia nigra (SN) send the majority of their projections into the striatum (ST), however, axons also extend into the cortex and hippocampus as well (for review see (Van den Heuvel and Pasterkamp, 2008)). The DA fibers which project from the SN to the ST modulate the function of the basal ganglia (BG) by activating both D1 and D2 dopamine receptors (Graybiel, 2000). The D1 and D2 receptors differ greatly in protein structure (Jackson and Westlind-Danielsson, 1994) and it is currently thought that activation of the D1 receptor serves to excite the direct pathway of the BG while activation of the D2 receptor functions to inhibit the indirect pathway (Graybiel, 2000). The summation of all of the interconnections within the BG culminates at the thalamus and results in a single refined signal being sent to the cortex.
Following TBI, alterations in the DA system have been shown to occur and these changes are thought to underlie many of the deficits observed after TBI (for review see (Bales et al., 2009)). In humans, Donnemiller showed that following a severe TBI, where the average GCS was 5.8±4.2, patients exhibited alterations in both the dopamine transporter (DAT) and the D2 receptor (Donnemiller et al., 2000). Compared to controls, individuals which sustained a severe TBI had a loss of 55% of the DAT binding and 27% loss of D2R binding, however, the overall degree of change in Striatal DAT or D2R was not correlated with the severity of the TBI (Donnemiller et al., 2000). The lack of a direct correlation between TBI severity and the amount of change in the DA system indicates that even though TBI can produce DA deficits, since the GCS does not specifically measure DA function it cannot be used to predict the amount of DA dysfunction. Similar to what has been observed in humans, in a rodent model of severe TBI a 40% reduction in the levels of the DAT have been observed in the striatum 14 days after the injury (Wagner 2005). In these same animals, striatal evoked dopamine release was significantly reduced in injured animals ipsilateral to the injury and the $V_{\text{max}}$ for dopamine clearance was only 52.1% of naïve controls (Wagner et al., 2005).

Further evidence supporting a role for the DA system after TBI comes from the success of using the DA reuptake inhibitor methylphenidate as a treatment for cognitive and motor dysfunction after injury (Kaelin et al., 1996, Plenger et al., 1996, Whyte et al., 1997). Plenger et al. showed in a double-blind placebo controlled study that at 30 days following subacute treatment with methylphenidate patients receiving the drug exhibited improvements in attention and motor function (Plenger et al., 1996). In another clinical trial, Whyte et al. showed that individuals who sustained a severe TBI showed significant improvements in information processing, attentiveness during individual work tasks, and caregiver ratings of attention (Whyte et al., 1997). Both of these reports only provided
indirect evidence of alterations in the DA system after TBI since the clinical trial studies only showed that the drug lead to improvements in function and not that there were deficits in DA system. It wasn’t until studies such as those conducted by Donnemiller et al. which showed changes in the DAT and D2R that evidence existed to show TBI dependent changes in the DA system. In order to elucidate how treatment with methylphenidate might be affecting function after TBI, Wagner et al. utilized a rodent model of severe TBI to look at the effects of methylphenidate on striatal dopamine function. After only a single injection of methylphenidate, injured animals did exhibit an increase in the amount of striatal evoked dopamine, however, the increase was not as high as what was observed in sham animals treated with a single dose of methylphenidate (Wagner et al., 2009), indicating that the injured brain responds differently to DA agonists. When the treatment regimen of methylphenidate was extended for two weeks, animals which received a severe TBI and treatment with methylphenidate exhibited both an increase in the total rate and amount of dopamine release in the striatum and these changes resulted in evoked dopamine levels similar to sham animals (Wagner et al., 2009). The results from the animal studies utilizing methylphenidate treatment after TBI show that injury does result in reductions in dopamine levels and that treatment with a DA agonist can reverse these changes if the drug is given long enough. Results from the animal studies further support what was observed in humans by Plenger et al. where stopping methylphenidate treatment resulted in the loss of a difference between drug and placebo groups (Plenger et al., 1996). When methylphenidate is allowed to wear off the increases in evoked dopamine release return to post-injury levels (Wagner et al., 2009). The human and rodent data suggest that following TBI the DA systems in the brain, especially in the striatum, become compromised and do not function adequately for normal function. Administration of a dopamine agonist, such as methylphenidate, is capable of overcoming some of the
deficits in the DA system and improving function, however, it does not lead to permanent changes.
Parkinson's disease:

Epidemiology:

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by the symptoms: tremor, rigidity, bradykinesia, hypokinesia, akinesia, and postural instability (Marsden, 1994), which result from the loss of dopaminergic (DA) neurons within the substantia nigra. PD generally affects individuals after age 65 and in the United States it is estimated that 60,000 new cases are diagnosed each year leading to a total of 1.5 million people currently suffering with the neurological dysfunction which characterizes the disease. The motor impairment found in patients suffering with PD occurs in a progressive manner and worsens with time. The loss of DA input into the striatum from the substantia nigra is the major neurological cause of the motor dysfunction (Marsden, 1994) and human evidence suggests that loss of the majority of DA neurons in the substantia nigra must occur before motor dysfunction becomes apparent (Riederer and Wuketich, 1976, Morrish et al., 1996). Since Dr. James Parkinson first described the pathology of PD in his essay on “Shaking Palsy” (Parkinson, 2002), no definitive cause has been found which describes the etiology for all cases of sporadic PD.

Anatomical circuitry of the basal ganglia:

The basal ganglia (BG) is a collection of brain nuclei which includes the striatum, globus pallidus, subthalamic nucleus (STN), and substantia nigra. The BG plays important roles in motor, associative, and limbic functions within the brain (Alexander et al., 1986, Middleton and Strick, 2000). DA projections from the substantia nigra innervate D1 and D2 receptors in the striatum and influence the interconnections between the remaining regions of the BG, which are interconnected by inhibitory GABAergic and excitatory glutamatergic fibers (Figure 1.5) (Graybiel, 2000). The effect
of the BG circuitry on motor control is the most understood of its functions (Obeso et al., 2008) and lesions in the STN and substantia nigra result in frequently characterized motor dysfunctions in humans. The arrangement of the neuronal connections within the BG are somatotopically organized (Takada et al., 1998) with the regions controlling motor function residing dorsolaterally (DeLong and Coyle, 1979). The substantia nigra impacts the function of the BG by exciting D1 neurons of the direct pathway and inhibiting D2 neurons from the indirect pathway (Figure 1.5) (Graybiel, 2000). The activities of the direct and indirect pathways result in the regulation of the amount of inhibition on the thalamus, thereby modulating motor control through the thalamo-cortical projections (Graybiel, 2000).

**Figure 1.5: Basal ganglia circuitry**

The normal circuitry of the basal ganglia (BG) involves interconnections between dopaminergic (DA), glutamatergic (Glu), and GABAergic (GABA) fibers. The regions of the BG are interconnected in such a way that allows for the control of thalamic activity and ultimately thalamic modulation of cortical function. In Parkinson’s disease, the DA neurons in the substantia nigra are lost resulting in a loss of nigrastral DA input. The loss of nigral input into the striatum ultimately results in an increase in the inhibitory GABAergic single into the thalamus. The resulting inhibition of the thalamus leads to a decrease in the amount of input sent to the cortex from the thalamus which results in a decrease in motor output from the cortex.
Etiology:

Though much is known about the structure and function of the BG and its role in the development of PD, finding a single cause for the majority of idiopathic PD has remained elusive. Current evidence indicates that both genetic and environmental factors play an important role in the development of PD (for review see (Veldman et al., 1998)). Genetic analysis utilizing twins has shown that for the majority of cases there is not a significant effect of genetics on the development of PD (Duvoisin et al., 1981, Marsden, 1987, Marttila et al., 1988), however, a rare mutation in the gene for α-synuclein has shown that an autosomal dominant form of PD does exist (Golbe et al., 1990, Golbe et al., 1996, Polymeropoulos et al., 1996). With the likelihood of developing PD solely because of a genetic mutation being low, the most likely causes for PD involve an environmental component (Semchuk et al., 1993, Veldman et al., 1998). Exposure to toxins such a paraquat (Liou et al., 1997), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983), or TCE (Gash et al., 2008) have been shown to be linked to the development of PD in humans. Even with links between certain chemicals and the development of PD, a study looking a environmental causes of PD could not establish a link between the duration or level of exposure and disease development (Hertzman et al., 1994). The difficulty in establishing a human link between the level of chemical exposure and the development of PD is likely to be further complicated by the belief that PD arises from a multifactorial process (Carvey et al., 2006). The premise of a multifactorial model for PD is that a single insult is insufficient to drive disease development and that the interaction of multiple insults results in a greater level of pathology. Considering the potential necessity for more than one insult opens the possibility that sub-pathological levels of single insults can interact and either additively or synergistically lead to pathology.
Traumatic brain injury is another factor which has been linked to the development of PD and could interact in a multifactorial mechanism. Bower et al. showed that individuals which experienced either a TBI which resulted in a loss of consciousness or a severe TBI had an increased risk of developing PD later in life (Bower et al., 2003) and TBI was shown in a separate study to be the second most likely cause of PD in humans (Semchuk et al., 1993). TBI has been shown to produce alterations in the striatum which are similar to PD (Donnemiller et al., 2000) and experimental studies have shown that following a severe TBI there are deficits in the striatal DA system (Wagner et al., 2005). The link between TBI and PD likely involves both direct and multifactorial mechanisms. Human and experimental research indicates that, independently of other insults, TBI can damage the BG and produce alterations similar to what has been observed in human PD patients. If an individual who experiences a TBI is also exposed to an environmental toxin it could be expected that the pathological outcome would be more severe.

A common factor between PD, TBI, and toxins linked to PD is the involvement of mitochondrial dysfunction (Ramsay et al., 1986, Vyas et al., 1986, Schapira et al., 1990, Sullivan et al., 1998b, Sullivan et al., 1999c, Sherer et al., 2003, Lifshitz et al., 2004, Bender et al., 2006). The environmental pollutant TCE (Gash et al., 2008, Liu et al., 2010), the pesticide rotenone (Sherer et al., 2003), and the toxin MPTP (Nakamura et al., 2000) have all been shown to produce mitochondrial dysfunction in the brain and are linked to the development of PD. Mitochondrial dysfunction has been further linked to the development of PD since two treatments which act at the mitochondrial level, CoQ10 (Shults et al., 2002) and creatine (Matthews et al., 1999), have shown promise in treating disease symptoms in preclinical studies. The mechanisms by which mitochondrial toxins lead to PD involve impairment of mitochondrial bioenergetic function through the inhibition of mitochondrial respiration. By blocking the function of the
electron transport chain, inhibitors lead to reductions in ATP levels and increases in damaging ROS, both of which can lead to cell death (Chen et al., 2007b). In addition to toxin induced mitochondrial dysfunction, genetic mutations in several genes encoding mitochondrial proteins have also been linked to the development of PD. Mutations in DJ-1 (Bonifati et al., 2003), Pink1 (Valente et al., 2004), and Parkin (Kitada et al., 1998) have all been linked to PD in human studies and the mechanisms of pathology involve mitochondrial components (for review see (Cho et al., 2010).

The link between genetic mutations in mitochondrial proteins further supports the role of a multifactorial mechanism since studies utilizing the toxin 6-hydroxydopamine have shown a requirement for specific mitochondrial proteins in order to develop pathology (Gomez-Lazaro et al., 2008). The evidence from human and experimental studies strongly supports the notion that there are many factors which can play a role in the development of PD. Few cases exist that show a clear single insult mechanism of disease development and that alone supports the concept of multi-insult pathway for developing PD. The interactions between genetics and environmental insults likely drive the loss of the DA neurons in the substantia nigra and lead to the classical histological and motor impairments of PD.

**Trichloroethylene:**

Trichloroethylene (TCE), \( C_2HCl_3 \), is a halogenated hydrocarbon that has been widely used as a solvent in the United States for degreasing, dry cleaning, food processing, paint production, and as an anesthetic (EOHS, Bakke et al., 2007). It is estimated that as of 1997 over 400,000 workers were annually exposed to TCE in the workplace (ATSDR, 1997). TCE has been detected at 861 National Priorities List hazardous waste sites, with 213 of these sites having completed exposure pathways.
Trichloroethylene consists of a carbon-carbon double bond which is attached to three chloride ions. As of 1997, 861 National Priorities List toxic sites were contaminated with TCE and of these, 213 also had complete exposure pathways which resulted in the contamination of ground water systems and the potential exposure of the communities around the site. The number of toxic sites containing TCE can vary widely between states, however, every state which had data available contained at least one location contaminated by TCE. (n.d. = no data provided, figure edited from ATSDR, 1997)

allowing TCE to reach the population in proximity to the site (Figure 1.6) (ATSDR, 1997). Acute exposure to TCE can lead to narcotic symptoms and chronic exposure can lead to encephalopathy, polyneuropathy, trigeminal affection, and hearing loss (EOHS). Following exposure to TCE, deficits including memory impairment, sensorimotor neuropathy, facial muscle twitching, and decreased facial sensation have been shown to persist for years (Feldman et al., 1985). Kilburn conducted an age matched study of people exposed to TCE by inhalation and well water and found that exposed individuals exhibited significant deficits in reaction time, balance, blink responses, cognitive function, and motor speed as compared to regional referents (Kilburn, 2002). Similar to the finds of Kilburn, Gash et al. observed that in factory workers chronically exposed to TCE for many years that exposure was linked to the development movement impairments (Gash et al., 2008). Strikingly, the findings by Gash showed a link between
the level of exposure to TCE and the development of pathology since individuals which were directly working with the TCE developed worse symptoms than individuals which worked more distantly and therefore exposed to lower levels of TCE by inhalation (Gash et al., 2008).

Given the link between mitochondrial dysfunction and PD (Schapira et al., 1989) and the evidence that TCE exposure can lead to PD (Kilburn, 2002, Gash et al., 2008), rodent experiments sought to further elucidate the connection between TCE and PD (Gash et al., 2008, Liu et al., 2010). Rats which were exposed to TCE for six weeks exhibited significant mitochondrial bioenergetic impairment, a loss of tyrosine hydroxylase positive neurons in the SN, and changes in dopamine turnover kinetics in the striatum (Gash et al., 2008). Further analysis of the effects of TCE on the brain revealed that exposure led to increases in mitochondrial oxidative damage, accumulation of α-synuclein both in the SN and the dorsal motor nucleus of the vagus, and motor impairment (Liu et al., 2010). The results from the animal studies support what has been observed in humans exposed to TCE since experimental exposure to TCE resulted in symptoms of PD including motor impairment.

It was hypothesized by Bringmann that the downstream metabolite of TCE, 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo), may be the component of TCE exposure responsible for the deficits. TaClo has been shown in in vitro models to significantly inhibit Complex I of the mitochondrial respiratory chain (Janetzky et al., 1995) and induce apoptosis through the release of mitochondrial Cytochrome C (Akundi et al., 2004). With the availability of both experimental (Grote et al., 1995, Janetzky et al., 1995, Akundi et al., 2004) and clinical reports (Guehl et al., 1999, Kochen et al., 2003) of mitochondrial and dopaminergic toxicity following exposure to either TCE or its metabolite TaClo, evidence has accumulated to implicate TCE (Kochen et al., 2003) and
TaClo (Bringmann et al., 1995b, Riederer et al., 2002) as relevant neurotoxins in the development of PD. It has been shown that exposure to an environmental factor long before the onset of symptoms of PD correlates with the future development of pathology (Veldman et al., 1998, Brown et al., 2005, Landrigan et al., 2005) and this may be the result of either a slow continuous neuronal decline or a predisposition towards increased susceptibility to future insults.

Multifactorial pathogenesis of Parkinson’s disease:

The search for a single causative factor responsible for the development of PD in humans has been a major difficulty in understanding the pathogenesis of the disease. Causative factors such as exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983) or familial genetic mutations in the gene encoding α-synuclein (Golbe et al., 1990, Golbe et al., 1996, Polymeropoulos et al., 1996) have been shown to independently result in pathology, however, these factors are not likely to be the cause for most cases of idiopathic sporadic PD. With the difficulty finding single insults which can independently produce models PD that involve a progressive loss of dopamine neurons, attention has been given to the concept of a multifactorial mechanism (Veldman et al., 1998, Carvey et al., 2006). Age is considered to be a major factor in the development of PD and after age 50 up to 6% of dopamine neurons in the substantia nigra are lost (Gibb and Lees, 1991). Given that the normal loss of nigral cells is thought to be insufficient to independently produce functional impairment (McGeer et al., 1977, Kish et al., 1992), Carvey proposed the idea that the combination of age and a second insult may be required to drive disease development (Carvey et al., 2006). Given that age is linked with the development of PD but the normal loss of nigral neurons is
less than typically needed for functional impairment, a combination of genetics, environmental factors such as herbicides, pesticides, and head trauma may be needed to interact over the course of an individual’s lifetime to lead to pathology (for review see (Veldman et al., 1998)).

Multifactorial experiments have shown that neurons can be made more susceptible to future insults following exposure to a toxin (Arundine et al., 2004, Ling et al., 2004b). Numerous studies show that a variety of dual injuries can produce increased pathology in animal models of PD (Thiruchelvam et al., 2000a, Thiruchelvam et al., 2000b, Arundine et al., 2003, Arundine et al., 2004, Ling et al., 2006, Fei and Ethell, 2008) and these represent potential scenarios for how human PD develops. Exposure the herbicide Paraquat has been linked to an increased risk of PD development (Liou et al., 1997), however, other reports indicate there is not a direct link between Paraquat and PD (Koller, 1986). Exposure to another insult in addition to Paraquat exposure may be the necessary link which allows some studies to find a link to PD development while other studies do not. Thiruchelvam et al. showed that exposure the pesticide Maneb in addition to Paraquat exposure produced even greater deficits to the DA system in animal models (Thiruchelvam et al., 2000a, Thiruchelvam et al., 2000b, Thiruchelvam et al., 2002). Combining age with toxin exposure revealed that older animals are more susceptible to exposure to either Paraquat or Maneb alone and that the combination of age, Paraquat exposure, and Maneb exposure produced the greatest deficits to the dopaminergic system (Thiruchelvam et al., 2003).

In addition to insults which individuals experience as adults, it is also thought that childhood insults may play a role in the development of PD later in life (Martyn and Osmond, 1995). Rural living has been linked to the development of PD (Rajput et al., 1986, Rajput et al., 1987) and decreased numbers of nigral dopamine neurons
(Thiessen et al., 1990) and this may represent early life exposure to environmental factors which caused these differences. In addition to rural living, exposures in utero, such as from maternal bacterial infections, may play a role in disease development. In an experimental prenatal exposure model, it has been shown that prenatal exposure to the bacteriotoxin lipopolysaccharide (LPS) results in a decrease in the number of DA neurons at birth (Ling et al., 2002) and that when the prenatal exposure is combined with postnatal toxin exposure the loss of DA neurons is even more pronounced (Ling et al., 2004a, Ling et al., 2004b). Whether toxin exposure occurs during development or after birth, there is clear evidence that the combination of exposure to multiple toxins can result in greater deficits in the DA system.

**Peroxisome proliferator activator receptors:**

**PPARs in normal cellular function:**

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and regulate gene expression using various ligand-dependent and independent molecular processes. Three isoforms of the PPARs exist: PPARα, PPARγ, and PPARδ, and each isoform is the product of a separate gene (Dreyer et al., 1992, Michalik and Wahli, 1999, Torra et al., 2001). While these isoforms have similar protein sequence and structure, they differ in their ligand-binding domains and have different ligand specificity, tissue distribution, and biological actions (Guan et al., 2002). PPARs regulate the expression of their respective target genes through the interaction with 9-cis retinoic acid receptors and the formation of heterodimers (Kliwer et al., 1992). Defects in PPAR genes do not result in major changes to basal function, however, these changes lead to alterations in the ability to activate the PPAR receptors.
Fatty acids and prostaglandins serve as endogenous activators of the PPAR system under normal conditions (Forman et al., 1997, Kliewer et al., 1997, Berger et al., 2005) and exogenously fibric acids and thiazolidinediones (TZDs) have been utilized as pharmaceutical ligands (Forman et al., 1995, Lehmann et al., 1995, Forman et al., 1997). Under normal physiological conditions PPARα and PPARγ function to control lipid and glucose metabolism respectively (Berger et al., 2005). The pathways interact in various ways such as the ability of PPARγ to initiate the storage of lipids (Debril et al., 2001) and PPARα to control lipid metabolism.

Under injury conditions PPAR activation has been shown to modulate inflammatory responses. The most common method utilized by PPARs to regulate gene expression is through transrepression (Moraes et al., 2006). PPARs have been shown to significantly modulate inflammatory processes through the regulation of genes such as nuclear factor-kappa B (NFκB), activator protein-1 (AP-1), signal transducer and activator of transcription (STAT), and nuclear factor of activated T cells (NFAT) (Ricote et al., 1998, Delerive et al., 2001, Park et al., 2003, Bernardo and Minghetti, 2006). The regulation of these genes allows the PPAR system to affect macrophages, microglia, dendritic cells, endothelial cells, B cells, and T cells (Clark, 2002, Daynes and Jones, 2002, Hunter, 2007)). Inflammation and oxidative stress have been shown to play an important role in cell death after injury (Andersen, 2004) and given the ability for PPARs to regulate proteins such as NFκB, it has been proposed that they will be effective in various neurological disorders (Feinstein, 2003).

**PPARs in CNS disorders:**

The PPARγ agonists Pioglitazone and Rosiglitazone are both FDA approved drugs for diabetes treatment (for review see (Sood et al., 2000) and have been utilized
as therapeutics in animal models of CNS injury (Besson et al., 2005, Kiaei et al., 2005, Schutz et al., 2005, Collino et al., 2006, Chen et al., 2007a, McTigue et al., 2007, Park et al., 2007, Chen et al., 2008, Feng et al., 2008, Hyong et al., 2008, Sun et al., 2008, Yi et al., 2008, Allahtavakoli et al., 2009). Rosiglitazone has been previously shown to have a higher binding affinity for the PPARγ receptor (Young et al., 1998), however, Pioglitazone has been shown to more readily cross the blood brain barrier (BBB) (Berger and Moller, 2002) as well as partially activate the PPARα receptor (Sakamoto et al., 2000). Currently evidence exists showing that activation of either the PPARα (Chen et al., 2007a, Chen et al., 2008) or PPARγ (Yi et al., 2008) pathways are protective in models of TBI. However, given Pioglitazone’s increased brain penetration and activation of two separate PPAR pathways it may yield a greater therapeutic potential for the treatment of TBI (for review see (Kapadia et al., 2008).

Treatment with the PPARγ agonist Pioglitazone following lipopolysaccharide (LPS) induced brain inflammation has shown the ability to prevent both mitochondrial impairment and neuronal cell loss (Hunter et al., 2007). The therapeutic use of various PPARs has shown benefit in multiple CNS injury models including spinal cord injury (SCI) (McTigue et al., 2007, Park et al., 2007), TBI (Besson et al., 2005, Chen et al., 2007a, Chen et al., 2008, Yi et al., 2008), and stroke (Collino et al., 2006, Allahtavakoli et al., 2009). Of the three known PPAR isoforms, PPARα and PPARγ have been the most well studied in CNS injury and have been shown to reduce lesion size both in SCI (McTigue et al., 2007, Park et al., 2007) and TBI (Yi et al., 2008), reduce inflammation (Besson et al., 2005, Chen et al., 2007a, Park et al., 2007, Hyong et al., 2008, Kapadia et al., 2008), minimize oxidative damage (Chen et al., 2007a, Yi et al., 2008), spare neurons (McTigue et al., 2007, Park et al., 2007), and preserve behavioral function (Chen et al., 2007a, McTigue et al., 2007, Park et al., 2007, Chen et al., 2008). In
addition to the anti-inflammatory properties of PPAR ligands, treatment with PPAR agonists have also been shown to protect neuronal vasculature after an ischemic insult (Inoue et al., 2003). The protective effects of PPARs on the vasculature after injury has been shown to involve the prevention of increases in matrix-metalloproteinase-9 along with increasing levels of the protective protein Hemeoxygenease-1 (Pereira et al., 2005). Along with traumatic and acute injuries, PPAR ligands have shown effectiveness in experimental models of PD (Breidert et al., 2002). Following administration of the toxin MPTP, treatment with the PPARγ ligand Pioglitazone resulted in reductions in glial activation and cell loss in the substantia nigra (Breidert et al., 2002) as a result of increases in IkBα and reductions in NFκB (Dehmer et al., 2004). PPAR activation has been shown to be protective in other neurological disorders such as Alzheimers disease (Heneka et al., 2005) and multiple sclerosis (Feinstein et al., 2002, Lovett-Racke et al., 2004) through similar mechanistic processes. It is most likely the neuroprotective effects afforded by treatment with PPAR agonists involve a pleotropic process whereby activation of the PPAR system affects multiple pathways.

Summary:

A common aspect of many of the toxins linked to the development of PD is that they can inhibit mitochondrial bioenergetic function. The maintenance of mitochondrial function is critical to preventing cell death since neuronal ATP stores can be depleted within minutes and lead to the initiation of cell death pathways (Nilsson, 2001). A toxin which is capable of inhibiting the mitochondrial respiratory chain such as MPTP (Ramsay et al., 1986, Vyas et al., 1986), rotenone (Sherer et al., 2003), TCE (Gash et al., 2008, Liu et al., 2010), or TaClo (Janetzky et al., 1995), could produce a chronic decrease in ATP levels making neurons more susceptible to future insults since the cell would already be in a compromised state from energy depletion. Similar to the toxin
exposure models showing increased loss of dopamine neurons after exposure to two different toxins, the combination of mitochondrial inhibition and a second insult have the potential to interact and produce greater pathology.

Head trauma has also been linked to the development of PD (Semchuk et al., 1993, Bower et al., 2003) however it has not been shown to be independently causative for the development of PD (Factor et al., 1988). Though it remains yet to be proven, the ability for trauma to drive PD may involve other factors or insults to be on going (Koller et al., 1989). Currently there is no complete understanding of how idiopathic sporadic PD develops in humans. A likely scenario is that multiple environmental and genetic factors exist which can potentiate disease development but that under most conditions human exposure to only one factor is not sufficient to independently cause PD and therefore a multifactorial insult is more capable of producing PD.

With the ability of TCE to affect mitochondrial function (Gash et al., 2008), it is possible that exposure prior to a TBI may lead to a worse outcome. Additionally, regional differences in mitochondrial function and/or the susceptibility to mitochondrial inhibition may affect to development of pathology following toxin exposure and how the interaction between the toxin and TBI occurs. Given the role TBI may play in a dual injury process, and the impact TBI has as an individual condition, finding effective therapeutic interventions will likely help in ameliorating both of these issues.
Chapter 2

Traumatic brain injury and trichloroethylene exposure interact and produce functional, histological, and mitochondrial deficits.

Introduction:

One of the pivotal, common pathways shared between traumatic brain injury (TBI), toxin induced neurodegeneration, and Parkinson's disease (PD) is the disruption of normal mitochondrial function (Ramsay et al., 1986, Vyas et al., 1986, Schapira et al., 1990, Sullivan et al., 1998a, Sullivan et al., 1999c, Sherer et al., 2003, Lifshitz et al., 2004, Bender et al., 2006). To date, research has shown that these three insults may be able to interact at the mitochondrial level. Given the similar changes in mitochondrial function observed with these pathologies, exposure to a mitochondrial toxin prior to a TBI is hypothesized to produce a multifactorial injury with pathological changes greater than either insult alone.

Exposure to an environmental factor long before the onset of symptoms of PD has been shown to correlate with the future development of pathology (Veldman et al., 1998, Brown et al., 2005, Landrigan et al., 2005) and this may be the result of either a slow continuous neuronal decline or a predisposition towards increased susceptibility to future insults such as TBI. One such factor environmental factor which has been linked to PD is trichloroethylene (TCE), C₂HCl₃. TCE is a halogenated hydrocarbon carbon that has been widely used as a solvent in the United States for degreasing, dry cleaning, food processing, paint production, and as an anesthetic (EOHS, Bakke et al., 2007). It is estimated that as of 1997 over 400,000 workers were annually exposed to TCE in the workplace (ATSDR, 1997). TCE has been detected at 861 National Priorities List
hazardous waste sites, with 213 of these sites having completed exposure pathways allowing TCE to reach the population in proximity to the site (ATSDR, 1997). Exposure to TCE has been shown to cause mitochondrial dysfunction (Gash et al., 2008) and a loss of dopaminergic neurons in the substantia nigra (Liu et al., 2010). The downstream metabolite of TCE, 1-trichloromethyl-1,2,3,4-tetrahyro-β-carboline (TaClo), has been shown to inhibit Complex I of the mitochondrial respiratory chain (Janetzky et al., 1995) and induce apoptosis through the release of mitochondrial Cytochrome C (Akundi et al., 2004). With the availability of both experimental and clinical reports of mitochondrial and dopaminergic toxicity following exposure to either TCE or its metabolite TaClo, evidence has accumulated to implicate TCE (Kochen et al., 2003, Gash et al., 2008, Liu et al., 2010) and TaClo (Bringmann et al., 1995b, Riederer et al., 2002) as relevant neurotoxins in the development of PD.

The development of PD has been linked to TBI both in epidemiological studies (Factor and Weiner, 1991, Stern, 1991, Semchuk et al., 1993, Taylor et al., 1999, Bower et al., 2003) and in clinical evaluations (Nayernouri, 1985, Doder et al., 1999). Patients sustaining a mild head trauma with a loss of consciousness or a severe head trauma have a significant increase in the probability of developing PD later in life (Bower et al., 2003). Although there have been multiple reports linking TBI to PD (Factor and Weiner, 1991, Stern, 1991, Semchuk et al., 1993, Bower et al., 2003), current evidence only shows TBI to be a risk factor and has failed to show a causative link (Factor et al., 1988). The disruption of mitochondrial function and the initiation of cell death mechanisms may play a direct role linking TBI and PD. Mitochondrial dysfunction after TBI can result in reductions in ATP levels (Sullivan et al., 1998a), increases in lipid peroxidation (Sullivan et al., 1998a), release of Cytochrome C and activation of apoptotic pathways (Sullivan et al., 2002), resulting in cell death. The role of the mitochondria is critical after TBI since it
is capable of functioning as a regulator of cell death mechanisms and these changes likely worsen an individual's outcome from an additional insult.

Given the numerous factors linked to PD, including TCE and TBI, the concept that the development of PD involves a multifactorial injury has emerged (Semchuk et al., 1993). Multifactorial experiments have shown that neurons can be made more susceptible to future insults following exposure to a toxin (Arundine et al., 2004, Ling et al., 2004b). A toxin, such as TCE, which is capable of inhibiting the mitochondrial respiratory chain (Gash et al., 2008), could make neurons more susceptible to future insults. The chronic inhibition of Complex I by TCE and a resulting decline in energy stores may be the link allowing TCE and TBI to interact. Based on previous studies showing dual injuries can produce increased pathology (Thiruchelvam et al., 2000a, Thiruchelvam et al., 2000b, Arundine et al., 2003, Arundine et al., 2004, Ling et al., 2006, Fei and Ethell, 2008), we sought to test the hypothesis that TCE exposure and TBI can interact to produce a greater level of disease pathology. This is the first report of these two insults being studied together and provides further support that sporadic PD may be the result of a multifactorial process.
Methods:

All studies were approved by the University of Kentucky Institutional Animal Care and Usage Committee and utilized male Fischer 344 rats 16 weeks old (Harlan Laboratory). Animals were housed in groups of three per cage with food provided *ad libitum* and 12-hour light/dark cycles. For the first set of studies there were six treatment groups which consisted of animals which were exposed to trichloroethylene or vehicle followed by either a sham, mild (1.0mm), or moderate (2.0mm) brain injury. For lesion volume and mitochondrial studies the mild (1.0mm) group was not generated. A total of 156 animals were utilized for the completion of these studies.

Trichloroethylene treatment and controlled cortical impact brain injury:

Animals were orally gavaged 0.6ml of either the olive oil vehicle (Kroger Pure Olive Oil) or 1000mg/kg trichloroethylene (Sigma Aldrich) daily for either 1 week or 2 weeks using a 100mm gavage needle (Fine Science Tools) with a 16 gauge tip sized to prevent insertion of the needle into the trachea. The health of the animals was monitored during the entire duration of the experiments to ensure no animals became morbidly sick. Following treatment with trichloroethylene, animals received either a sham brain injury or a controlled cortical impact brain injury using previously established methods (Sullivan et al., 1998b, Sullivan et al., 1999c, Sullivan et al., 2000c). Animals were anesthetized with 2% isofluorane and placed in a Kopf stereotaxic frame for positioning under a pneumatic impactor (Precision Science Instruments). A 6 mm craniotomy was performed, with a hand trephine, lateral to the central fissure on the left side of the skull centered between lambda and bregma. Animals in injury groups received a unilateral
injury directly to the surface of the brain. The injury parameters consisted of a 1.0mm deep impact for mild injured animals and a 2.0 mm deep impact for moderate injured animals at 3.5 meters/second for 500ms. Sham animals received a craniotomy but did not receive an impact to the brain. Following the injury a piece of Surgicel (Johnson&Johnson) sized to fit into the craniotomy was placed directly on the brain. The skull cap was replaced and secured in place with dental acrylic. Once the acrylic was allowed time to harden, the scalp incision was closed with surgical staples. Animals were removed from isofluorane and placed in a clean cage and body temperature was maintained at 37°C with the use of a heating pad. Five to six animals per group were utilized throughout all of the experiments. Following the brain injuries, animals were housed in their home cages until post-injury behavioral analysis was begun.

**Behavioral analysis:**

**Rotarod testing:** Nine days after the brain injury surgeries Rotarod testing was begun for all treatment groups. Animals were trained on a Rotarod (Med Associates Inc.) for three days at speeds of 8rpm, 12rpm, and then 16rpm. On the fourth day (12 days post TBI) animals were tested on the Rotarod using an accelerating speed starting at 3rpm and holding at 30rpm. The time animals were able to stay on the Rotarod on the fourth day was determined by a timer built into the Rotarod which was stopped when the animal fell off the Rotarod and blocked an infrared light sensor. Animals were tested twice on the fourth day and the times for each trial were averaged for each animal.

**Cylinder testing:** Thirty days after the brain injury, animals underwent cylinder testing to access alterations in paw placement. Animals were placed in a clear plastic cylinder with a diameter of 19cm which was mounted on a plexiglass surface with an angled mirror underneath to allow video recording of the animals for 5min. Video recordings were performed using a digital video camera, and the footage was analyzed
using QuickTime software (Apple Computer Inc.) with playback set at ½ actual speed. The number of contralateral and ipsilateral paw touches was quantified blinded to treatment group. The percentage of contralateral forepaw usage was calculated using the formula \[ \frac{\text{Contralateral touches}}{\left(\frac{\text{Contralateral touches}}{2} + \text{Ipsilateral touches}\right)} \times 100 = \% \text{ Contralateral Touches}. \]

**Histological analysis:**

Following the completion of the cylinder testing, animals were sacrificed for post injury histological analysis. Animals were anesthetized with sodium pentobarbital (Abbot Laboratories) and transcardially perfused with saline followed by 4% paraformaldehyde. The brains were removed and placed in 4% paraformaldehyde and 30% sucrose in PBS for 24hrs. After 24hrs the brains were transferred to a 30% sucrose PBS buffer without paraformaldehyde. Coronal sections 30µm thick were cut with a freezing microtome throughout the rostral caudal extent of the entire brain. Nissl staining was performed to visualize the intact regions of the cortex for cortical tissue sparing analysis. Quantitative assessment of cortical damage employed an unbiased protocol using the Cavalieri method as previously described (Sullivan et al., 2000c, Sullivan et al., 2002). All slides were assessed blindly with respect to treatment group. For immunohistochemistry, sections containing regions of interest were incubated overnight at 4°C with a primary antibody. Slices were stained for tyrosine hydroxylase using a primary monoclonal antibody (Chemicom) followed by a biotinylated anti-mouse secondary antibody (Vector). Following the secondary antibody treatment sections were treated with an Avidin Biotin Peroxidase Complex, Vectastatin ABC Kit (Vector). The tissue was developed with a diaminobenzidine tetrahydrochloride (DAB) solution with nickel intensification to allow stable long term staining and visualization with a light microscope. Bioquant Image Analysis software (Bioquant, Nashville, TN) was used to estimate total cell number in the
region of interest using the optical fractionator method (Mouton, 2002). This method represents an unbiased quantitative technique that is independent of size and shape or any conformational changes of cells. Detailed procedures regarding stereological counting are similar to our previously published studies (Liu et al., 2008). Coronal sections containing the substantia nigra (interaural 2.7-4.2) (Paxinos and Watson, 1998) were used to estimate the total number of tyrosine hydroxylase (TH) positive cells in the SN. A set of 6 systematic sections were sampled in an independent-random manner from a total of 36-39 sections containing the entire reference space. On each stained section the area containing the SN was identified using conventional landmarks (Paxinos, 2004). An unbiased grid was overlayed on each section and a random number system embedded in the software used to identify areas of the grid for counting. On each identified grid position an unbiased counting frame was used for the optical dissector. The counting frame was 150 X 150μm centered on the grid intersection. The height of each dissector was determined with a digital z-axis encoder (Boeckeler Instruments, Tucson, AZ). Cells were identified using a 40X objective with a high numerical aperture. The total number of cells (N) was estimated using the formula \[ N = Q \cdot \frac{1}{hsf} \cdot \frac{1}{asf} \cdot \frac{1}{ssf} \] where Q is the total number of cells actually counted, hsf is the height sampling fraction, asf is the area sampling fraction, and ssf is the section sampling fraction. Image Pro software was used for the quantification of the area of the striatum immunoreactive for TH in a blinded fashion. High resolution images throughout the entire striatum stained for TH were taken using an Olympus AX80 microscope. Using Image Pro, the perimeter of the striatum was outlined and the percentage area which was immunoreactive for TH was calculated. The average amount of immunoreactivity for a single animal was calculated separately for both the ipsilateral and contralateral sides of the brain.
Western blot analysis:

Quantification of dopamine transporter and receptor levels in the striatum was performed using standardized western blot protocols. Animals were asphyxiated with CO₂ and rapidly decapitated. The striatum was rapidly dissected, placed into plastic tubes, and the tubes were placed into a bath of dry ice and ethanol to rapidly freeze the tissue. Samples were frozen at -80°C until homogenized. At the time of homogenization samples were placed into lysis buffer (0.1M NaCl, 0.01M Tris-Cl, 0.001 EDTA, 1ug/ml aprotinin, 100ug/ml phenylmethylsulfonyl fluoride) and homogenized using low intensity sonication for 30 seconds. Samples were centrifuged at 13,800xG for 30 min at 4°C. The protein concentrations were determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560nm with a Biotek Synergy HT plate reader (Winooski, Vermont). Protein separation was performed using a 4% stacking gel followed by a 10% separation gel. Gels were run for one hour at 150 volts and 4°C with constant stirring. Following gel separation, proteins were transferred to nitrocellulose utilizing a wet transfer setup at 100 volts for one hour. Following protein transfer to the nitrocellulose, blots were washed three times with TBS and blocked with 5% milk in TBS for one hour at room temperature. After blocking, blots were washed three times with TTBS at room temperature with constant agitation. Primary antibodies towards the proteins of interest were prepared in 5% milk in TTBS. For quantification of the dopamine transporter, 40ug of protein was loaded per well and the primary antibody (Millipore) was applied at a concentration of 1:1500 and incubated overnight. For quantification of the dopamine receptor, 60ug of protein was loaded to the gel and the primary antibody (Santa Cruz) was applied at a concentration of 1:800 and incubated overnight. Following incubation with the primary antibody, blots were washed with TTBS and incubated with an infrared secondary antibody (Rockland) at a concentration of 1:5000. Band intensities were determined using a Licor Odyssey infrared imager. Levels
of each protein were expressed as a ratio of the protein of interest compared to the amount of actin for the same sample. Six animals per group were utilized for western blot analysis.

**Mitochondrial isolation and oxygen consumption analysis:**

Mitochondria from the striatum and substantia nigra were isolated using differential centrifugation, nitrogen disruption, and a Ficoll gradient. Animals were asphyxiated with CO₂ and rapidly decapitated. Dissected tissue was removed and immediately placed in ice-cold isolation buffer (215mM Mannitol, 75mM Sucrose, 0.1% BSA, 1mM EGTA, and 20mM Hepes at pH 7.2). Due to the small size of the substantia nigra, three animals were combined for each mitochondrial sample, resulting in an n=1 for these three animals. Samples were homogenized and centrifuged at 1300xG for 3 minutes. Following the first spin the supernatant was placed in a fresh tube and the pellet was resuspended in isolation buffer and spun at 1300xG for 3min. The supernatant from the first and second spins were collected in separate tubes and spun at 13,000rcf for 10 minutes. The pellets from both tubes were combined, resuspended in 400ul isolation buffer and placed in a nitrogen bomb at 1,200psi for 10min. The pressure in the nitrogen bomb was rapidly released and the sample was placed as the top layer on a Ficoll separation column which consisted of a 10% Ficoll layer and a 7.5% Ficoll layer. The Ficoll column with sample was centrifuged at 100,000xG for 30min at 4°C using a Beckman SW 55Ti rotor and ultra-centrifuge. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a final concentration of approximately 10mg/ml, and stored immediately on ice. To normalize the results, the protein concentrations were determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560nm with a Biotek Synergy HT plate reader (Winooski, Vermont). Following two week exposure to TCE, mitochondrial
oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments, Norfolk, England) in a continuously stirred, sealed chamber at 37°C as previously described (Sullivan et al., 2003). Isolated mitochondrial protein (100µg) was suspended in respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH2PO4 at pH 7.2) in a final chamber volume of 0.25 mL. Mitochondrial bioenergetic analysis was measured by the sequential administration of substrates: 5mM Pyruvate, 2.5mM Malate, 150nM ADP, 1uM Oligomycin, and 1uM FCCP. Five to six animals per groups were utilized for all of the mitochondrial studies.

**Seahorse protocol for isolated mitochondria:**

Experiments utilizing 1 week exposure to TCE were performed with the Seahorse Biosciences XF24 for analysis of mitochondrial oxygen consumption. The following protocol was utilized for the analysis of bioenergetic function in purified mitochondria using the Seahorse Biosciences XF24 Flux Analyzer. Pyruvate/Malate and ADP, Oligomycin, FCCP, or Rotenone and Succinate were injected sequentially through ports A, B, C, and D, respectively, in the Seahorse Flux Pak cartridges to yield final concentrations of 5 mM (Pyruvate), 2.5 mM (Malate), 1mM (ADP), 1 µg/ml (Oligomycin), 1 µM (FCCP) and 100 nM (Rotenone), 10 mM (Succinate) respectively.
The following sequence was utilized for analysis following 1 week exposure to TCE and TBI:

<table>
<thead>
<tr>
<th>Step</th>
<th>Command</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Calibrate probes</td>
</tr>
<tr>
<td>2</td>
<td>Mix 1 minute</td>
</tr>
<tr>
<td>3</td>
<td>Time delay 1 minute and 30 seconds</td>
</tr>
<tr>
<td>4</td>
<td>Mix 25 Seconds</td>
</tr>
<tr>
<td>5</td>
<td>Measure 2 minutes</td>
</tr>
<tr>
<td>6</td>
<td>Mix 1 minute</td>
</tr>
<tr>
<td>7</td>
<td>inject Port A (Pyruvate/Malate/ADP)</td>
</tr>
<tr>
<td>8</td>
<td>Mix 25 Seconds</td>
</tr>
<tr>
<td>9</td>
<td>Measure 2 minutes</td>
</tr>
<tr>
<td>10</td>
<td>Mix 1 minute</td>
</tr>
<tr>
<td>11</td>
<td>inject Port B (Oligomycin)</td>
</tr>
<tr>
<td>12</td>
<td>Mix 25 Seconds</td>
</tr>
<tr>
<td>13</td>
<td>Measure 2 minutes</td>
</tr>
<tr>
<td>14</td>
<td>Mix 1 minute</td>
</tr>
<tr>
<td>15</td>
<td>inject Port C (FCCP)</td>
</tr>
<tr>
<td>16</td>
<td>Mix 25 Seconds</td>
</tr>
<tr>
<td>17</td>
<td>Measure 2 minutes</td>
</tr>
<tr>
<td>18</td>
<td>Mix 1 minute</td>
</tr>
<tr>
<td>19</td>
<td>inject Port D (Rotenone/Succinate)</td>
</tr>
<tr>
<td>20</td>
<td>Mix 25 Seconds</td>
</tr>
<tr>
<td>21</td>
<td>Measure 2 minutes</td>
</tr>
</tbody>
</table>

Statistical analysis:

All experimental groups contained 3-6 animals/group. All data were evaluated with a two-way analysis of variance (2-way ANOVA) followed by a post-hoc Bonferroni test with significance set at p<0.05.
Results:

Motor impairment occurs following exposure to TCE and a moderate TBI

Motor impairment is the major functional pathology associated with Parkinson’s disease. To investigate the potential of this multifactorial model to produce behavioral impairment, two behavioral tests were utilized: the Rotarod and Cylinder Test. Animals were tested on the Rotarod 12 days post TBI and it was found that following exposure to TCE and a moderate CCI a significant reduction of approximately 50% (Figure 2.1A, p<0.01) in the amount of time spent on the Rotarod was observed, indicating impairment in motor coordination and balance. At 30 days post TBI animals were analyzed using a cylinder test to determine longer-term deficits in forepaw usage. With the cylinder test a significant reduction in the usage of the contralateral forepaw was observed in animals that were exposed to TCE and a moderate CCI, which decreased to 34% (Figure 2.1B, p<0.05) of control animals. With the decrease in forepaw usage only being present following a dual exposure to both TCE and a moderate CCI there is again support for a dual injury mechanism. Both behavioral tests indicate that the dual injury consisting of TCE exposure and a moderate TBI is required for functional deficits to appear and that any of the single insults tested are incapable of producing these functional deficits.
Exposure to both TCE for 2 weeks and a moderate controlled cortical impact TBI result in significant motor impairment. **A)** Twelve days after the TBI animals exhibited significant motor impairment as exhibited by a reduced ability to stay on a rotating rod. Animals exposed to TCE and a moderate TBI have approximately a 50% reduction in the length time able to stay on the Rotarod. **B)** Thirty days after the TBI forepaw usage was assessed using the cylinder test and contralateral forepaw usage decreased to 34%. Both behavioral tests show the necessity for both insults to be present before functional impairment occurs.

#=p<0.01, *=p<0.05; n=6; 2-Way ANOVA with Bonferroni post-test; Mean±SE
Exposure to TCE and a moderate TBI leads to a loss of tyrosine hydroxylase positive neurons in the substantia nigra

The major histological feature of postmortem Parkinson’s disease diagnosis is the loss of dopamine neurons in the substantia nigra (SN). Following exposure to either TCE or TBI alone there was no significant loss of TH positive neurons in the SN, however, following the dual injury with TCE and either a mild or moderate cortical impact there was a 13-17% loss of TH positive neurons in (Figure 2.2A, P<0.05). The presence of a significant loss of TH+ cells only following the dual injury indicates that exposure to both TCE and TBI can lead to a synergistic loss of dopamine neurons in the SN and produce the major histological symptom of PD even with injury levels which are incapable of independently producing pathology. To determine if the loss of TH+ cells was the result of cell death or a loss of the tyrosine hydroxylase protein, Nissl positive cells were also counted. Nissl staining revealed no significant loss of cells indicating that the loss of TH+ neurons was the result of a loss of tyrosine hydroxylase and not cell death.
Exposure to both TCE for 2 weeks and a mild or moderate controlled cortical impact TBI resulted in significant decreases in the number of TH-positive neurons in the substantia nigra at 30 days post TBI. A) The dual injury consisting of TCE exposure and a TBI brain injury resulted in a 13-17% loss of dopamine neurons in the substantia nigra. B) Quantification of Nissl cells in the substantia nigra did not reveal any measurable amount of cell loss indicating the loss of TH-positive neurons is due to a loss of the TH protein and not the loss of cells. C) Representative images of the substantia nigra stained for TH.

*=p<0.05; n=6; 2-Way ANOVA with Bonferroni post-test; Mean±SE
**Exposure to TCE and TBI does not affect striatal tyrosine hydroxylase fiber density or levels of the dopamine transporter and dopamine receptor:**

Following the loss of dopamine neurons in the substantia nigra in human PD there is a resulting loss of both TH+ fibers and the pre-synaptic dopamine transporter (DAT) in the striatum along with an increase in the type-2 post-synaptic dopamine receptor (D2R). Following either exposure to TCE, TBI, or both, no changes were observed in the area of fibers positive for tyrosine hydroxylase in the striatum (Figure 2.3A). Additionally, there were no observable changes in the levels of DAT or D2R following any of the single insults or the dual injury (Figure 2.3C,D).

**Exposure to TCE does not affect cortical tissue loss following TBI**

Following a controlled cortical impact (CCI) injury there is the formation of a cortical cavity which results from the loss of neuronal tissue. Given the ability for TCE to impair mitochondrial function, and the role mitochondrial dysfunction plays in post-TBI cortical tissue loss, the amount of spared cortical tissue was measured in both single and dual injured animals. Following a moderate CCI there is approximately a 30% loss of tissue (Figure 2.4A, *=p<0.0001). Exposure to the mitochondrial toxin TCE prior to the CCI did not lead to an increase in the size of the cortical lesion (Figure 2.4A). Unlike the behavioral results, there was no additive effect in the cortex following exposure to both TCE and TBI.
Figure 2.3 Exposure to TCE, TBI, or the dual injury does not lead to striatal changes in TH-fibers, DAT, and D2R.

Neither exposure to TCE for 2 week, a mild or moderate TBI, nor any combination of the insults resulted in histological deficits in the striatum. **A)** The amount of area in the striatum immunoreactive for TH was quantified and no reduction in TH-positive fibers was measured. **B)** Representative images of the striatum stained for TH. **C,D)** Western blot analysis for levels of the dopamine transporter and type-2 dopamine receptor revealed no changes in either protein. n=6; 2-Way ANOVA; Mean±SE
Animals which were exposed to TCE for 2 weeks prior to a moderate TBI did not exhibit an increase in the amount of cortical tissue loss. **A)** With this model and moderate TBI resulted in approximately a 30% loss of cortical tissue at 30 days post-injury. Exposure to TCE had no effect on the loss of cortical tissue. **B)** Representative images of Nissl stained cortex 30 days post-injury.

\*\*=p<0.0001; n=6; 2-Way ANOVA with Bonferroni post-test; Mean±SE

**Two week TCE exposure impairs mitochondrial bioenergetic function in the striatum**

Mitochondrial dysfunction is a common feature of TBI, PD, and TCE exposure. The ability for both TBI and TCE exposure to independently inhibit mitochondrial function is hypothesized to be a potential mechanism allowing these insults to interact and increase pathology. Mitochondria were isolated six hours after the CCI and total mitochondria from both the striatum and substantia nigra were analyzed to determine mitochondrial bioenergetic function. No reductions in mitochondrial function were
observed in the substantia nigra six hours after the TBI or following the two week exposure to TCE (Figure 2.5A). At six hours post injury exposure to TCE produced significant mitochondrial impairment in the striatum which was observed by reductions in both the basal and maximal rates of Complex I dependent oxygen consumption by approximately 75% (Figure 2.5B, *=p<0.05). Exposure to the TBI alone resulted in a non-significant trend towards reduced Complex I function in by approximately 30% in the striatum. The combination of TCE exposure and a TBI did not result in a measureable change mitochondrial function in the striatum when compared to TCE exposure alone.

Figure 2.5: Two week exposure to TCE results in decreased bioenergetics in the striatum.

Animals were exposed to TCE for 2 weeks and then subjected to a moderate TBI and mitochondrial tissue was isolated from the striatum and substantia nigra six hours post-injury. A) Analysis of mitochondrial bioenergetic function in the substantia nigra revealed no significant difference between any of the treatment groups. B) Exposure to TCE for 2-weeks produced significant reductions of approximately 75% in Complex I dependent basal and maximal rates of mitochondrial oxygen consumption. TBI alone resulted in a trend towards reduced mitochondrial function in the striatum, however, this decrease did not reach significance.

*=p<0.05, n=5-6; 2-Way ANOVA with Bonferroni post-test; Mean±SE
One week exposure to TCE followed by TBI results in synergistic mitochondrial dysfunction in the striatum

Given the 75% reduction in Complex I driven mitochondrial function in the striatum following 2 week exposure to TCE (Figure 2.5), and the difficulty this produces in elucidating whether an interaction between TCE exposure and TBI exists, additional animals were subsequently administered TCE for only 1 week. Following exposure to TCE alone for 1 week there was not a reduction in Complex I driven mitochondrial respiration. Similarly to the previous 2 week exposure studies, a moderate TBI resulted in a non-significant decrease in mitochondrial bioenergetic function of approximately 30%. When the 1 week exposure to TCE was combined with a moderate TBI, mitochondrial bioenergetic function was reduced by approximately 50% (Figure 2.6, *=p<0.05). These studies indicate that TCE and TBI are capable of interacting at the mitochondrial level which leads to significant mitochondrial dysfunction requiring the presence of both insults.
Figure 2.6: One week exposure to TCE followed by a moderate TBI results in mitochondrial dysfunction in the striatum.

Exposure to either TCE for 1 week or a moderate TBI did not result in significant mitochondrial dysfunction in the striatum. When the two separate insults were combined together there was a significant reduction in the Complex I driven rate of mitochondrial oxygen consumption. The dual injury paradigm resulted in significant mitochondrial impairment which was measured as a 50% reduction in mitochondrial respiration.

* = p<0.05, n=3-4; 2-Way ANOVA with Bonferroni post-test; Mean±SE
Discussion:

It has been proposed that in humans, the development of Parkinson’s disease (PD) may involve a multifactorial mechanism (Semchuk et al., 1993). Genetic factors (for review see (Nuytemans et al., 2010)), age (Naoi and Maruyama, 1999), and the toxin MPTP (Langston et al., 1983, Langston and Ballard, 1983) have been shown to independently lead to the development of PD in humans independently of other factors, however, with the vast majority of other insults, such as TBI, or environmental exposures it has been difficult to show causation for disease development (for review see (Veldman et al., 1998)). Previous work from our group has shown that both in humans and rodents chronic exposure to trichloroethylene (TCE) is capable of producing pathologic features of PD such as motor impairment, loss on dopamine neurons in the substantia nigra and striatal deficits in tyrosine hydroxylase (Gash et al., 2008, Liu et al., 2010). Even though chronic high dose exposure is capable of leading to the development of Parkinsonism or complete PD in both humans and animals (Gash et al., 2008, Liu et al., 2010), it is unknown how many individuals would be exposed to high levels of TCE chronically. In the present studies we investigated the potential for a lower exposure to TCE combined with a traumatic brain injury (TBI) to produce a multifactorial model of PD. These studies provide an initial indication that TCE and TBI can interact in a multifactorial mechanism.

It has been established using various combinations of insults and toxin exposures that a multifactorial injury can produce a greater insult both in vitro (Arundine et al., 2003, Arundine et al., 2004, Fei and Ethell, 2008) and in vivo (Thiruchelvam et al., 2000a, Thiruchelvam et al., 2000b, Ling et al., 2006). In neuronal culture, cells which are exposed to a mechanical stretch injury prior to NMDA exposure are made more susceptible to the toxic insult (Arundine et al., 2003, Arundine et al., 2004). In rodents it has been shown that prenatal toxin exposure can make animals more susceptible to
future insults, resulting in a progressive model of PD (Ling et al., 2006), and that multifactorial toxin exposure can produce a greater insult than exposure to a single insult (Thiruchelvam et al., 2000a, Ling et al., 2006). Epidemiological studies have attempted to find a potential link between TBI and PD (Bower et al., 2003). However, a causative link between TBI and PD has been difficult to show and as a result it has been proposed that the link between TBI and PD may involve a multifactorial mechanism (Semchuk et al., 1993).

The major pathological symptoms of PD are progressive impairment in motor control and loss of dopamine neurons in the substantia nigra. To investigate the potential for a dual injury model to recapitulate the motor impairments and cell loss observed in PD, both Rotarod and cylinder testing were utilized to assess motor function and tyrosine hydroxylase (TH) staining was utilized to analyze both dopamine neurons in the substantia nigra and dopamine fibers in the striatum. For our initial studies two different severities for the TBI were utilized since it has been shown in human studies that the severity of the injury significantly affects the link between TBI and PD (Bower et al., 2003). Following a 2 week exposure to TCE and a moderate controlled cortical impact (CCI) TBI, impairments were observed both with the Rotarod (Figure 2.1A) and cylinder test (Figure 2.1B) indicating not only that the dual injury paradigm in these experiments is required for motor impairment but also that a moderate and not a mild TBI is required for the presentation of motor impairment. To determine whether a loss of dopamine neurons in the substantia nigra was linked to the dual injury, TH-positive neurons were quantified and it was observed that, in contrast to the behavioral data which indicated that TCE exposure and a mild TBI did not result in dysfunction, exposure to TCE and either a mild or a moderate TBI is sufficient to produce a loss of TH-positive neurons (Figure 2.2A). Additionally, the loss of TH-positive cells was also shown to be the result
of a loss of the TH protein and not cell loss, since quantification of Nissl cells did not result in any group differences (Figure 2.2B). Though the behavioral analysis and TH-positive cell counts did reach statistical significance in these studies, the deficits were not as substantial as we hypothesized they would be. The results indicate that the individual insults are insufficient to produce any impairment independently which is desirable in this model since the major goal was to show a dual injury effect, however, different injury parameters may have yielded us a more substantial dual injury model. Specifically with regards to the TBI, had the injury been located directly over the striatum or been more severe we may have been able to observe more profound alterations in our endpoints.

Since a moderate TBI was required for motor impairment to occur, further experiments were conducted utilizing this level of injury. Given the loss of TH-positive neurons, the area of TH-positives fibers in the striatum was determined. As was expected for only a 13-17% loss of cells in the substantia nigra, no measurable decrease in TH-fiber density was observed in the striatum (Figure 2.3A). Since the levels of the dopamine transporter have been shown to be reduced in a similar model of TBI which utilizes a severe injury (Wagner et al., 2005), striatal tissue was analyzed both for levels of the dopamine transporter (DAT) and the type-2 dopamine receptor (D2R). Following either single insult or the dual injury, no reductions in the levels of DAT or D2R were observed one month following the injury (Figure 2.3C,D). The lack of a reduction in the DAT is likely the result of a more central and less severe TBI than previous reports (Wagner et al., 2005). Further tissue analysis was conducted looking at cortical tissue loss following the dual injury paradigm. A 30% loss of cortical tissue was observed following the moderate TBI and exposure to TCE did not increase this loss (Figure 2.4A). Since there was not an increase in cortical tissue loss, the data suggests that the main
TCE effects are not in the cortex. The lack of observable changes in the levels of DAT and D2R further indicate that at one month following the dual injury, this paradigm produces a very mild dual injury model.

Mitochondrial dysfunction is a major pathological mechanism involved in TBI (Sullivan et al., 1998a, Sullivan et al., 1999c, Singh et al., 2006) as well as a common factor of many toxins linked to PD (Nakamura et al., 2000, Sherer et al., 2003, Gash et al., 2008, Liu et al., 2010). It has been previously shown that either exposure to TCE (Gash et al., 2008, Liu et al., 2010) or the metabolite TaClo (Bringmann et al., 1995a, Janetzky et al., 1995) are capable of producing significant impairment in mitochondrial bioenergetic function. In order to understand if exposure to TCE is capable of leading to greater mitochondrial dysfunction following TBI, animals were exposed to TCE for two weeks and then subjected to a moderate CCI injury. Tissue was isolated from both the striatum and substantia nigra six hours after the brain injury since mitochondrial dysfunction has been shown to peak early after TBI (Singh et al., 2006). Samples from the substantia nigra revealed no significant impairments in mitochondrial function at six hours after the injury (Figure 2.5A). In contrast to the lack of an effect in the substantia nigra at six hours post-TBI, the striatum exhibited significant mitochondrial dysfunction. In these studies a significant reduction in mitochondrial function was produced as a result of exposure to TCE (Figure 2.5B), which made it difficult to observe a dual injury effect since it appears that mitochondrial bioenergetic function has potentially reached a maximal amount of impairment which cannot be increased. This bottoming out effect likely occurs since any further decrease in bioenergetic function would most likely lead to cell death and the mitochondria would no longer survive for analysis. Exposure to TCE resulted in approximately a 75% reduction in both basal and maximal rates of Complex I dependent mitochondrial bioenergetic function. A trend towards a reduction in
mitochondrial function was observed in the striatum following exposure to TBI alone, however, this effect did not reach significance. As predicted for the other endpoints, had the TBI occurred directly over the striatum it is hypothesized that this decrease would become more pronounced. Given the large reduction in mitochondrial function in the striatum following the 2 week exposure to TCE and the difficulty this presents for measuring increases in this deficit due to a subsequent TBI, further experimentation was conducted using a 1 week exposure to TCE. Following exposure to TCE for 1 week there was no decrease in Complex I driven mitochondrial function in the striatum. As observed in the previous mitochondrial experiments, exposure to the TBI alone resulted in a non-significant trend towards mitochondrial impairment of approximately 30%. When the 1 week exposure to TCE was combined with the TBI, a significant reduction of approximately 50% was observed in the striatum six hours after the injury (Figure 2.6). The experiments utilizing the 1 week exposure of TCE show that the two insults are capable of interacting at the mitochondrial level to produce a greater amount of dysfunction than either insult alone is capable of.

The requirement for a multifactorial injury to produce the pathology shown in this study is in line with what has been seen in humans, where many insults have been determined to be risk factors for the development of human PD, but it has been difficult to show whether these same risk factors are able to independently produce pathology. This dual injury model utilizes toxin and injury levels which are individually insufficient to produce motor impairment or a loss of TH-positive neurons, hallmarks of human PD. However the dual injury produces significant reductions in both outcomes, indicating that sub-acute exposure to multiple insults can interact to produce a significant injury and lead to disease development. Previous studies have shown that insults targeting the striatum can result in the progressive loss of TH-positive neurons in the substantia nigra
(Sauer and Oertel, 1994) and that striatal mitochondrial inhibition results in DA
dysfunction (Zeevalk et al., 1997). Since both TCE exposure and TBI are capable of
producing reductions in striatal mitochondrial function, it is predicted that this
mitochondrial impairment leads to the reductions in TH-positive nigral neurons observed
in these studies in the same way as other models. What is striking about this study is
that motor impairment is present with levels of dopamine neuron loss which are lower
than typically seen from nigral neuron loss alone (Lee et al., 1996). The likely cause for
the motor impairment in this model is that in addition to the observed nigral deficits there
is striatal mitochondrial dysfunction which impairs normal synaptic function and a loss of
cortical tissue. The presence of neuronal death and dysfunction in multiple
interconnected regions of the brain likely leaves the system unable to overcome the
combinatorial insult. Given the lack of a significant decrease in tyrosine hydroxylase
positive fiber density in the striatum it would be unexpected that the typical therapeutic
utilized in humans, levodopa, would be capable of alleviating any of the motor
impairment. Since a percentage of human PD patients are non-responsive to levodopa,
this dual injury paradigm my recapitulate certain aspects of non-levodopa responsinve
PD.

Our initial study sought to utilize insults which would be insufficient to produce
pathology in our endpoints since we wanted to show a dual injury effect. However,
utilizing insults that do not independently produce pathology makes it difficult to know
what levels of the insults are ideal for this dual injury model. The results presented in
these studies do suggest that the dual injury hypothesis is valid; however, the lack of
overwhelming histological changes makes determining the role of these changes in PD
development difficult to prove. The lack of an effect on striatal protein expression at 30
days post-TBI suggests that the injury parameters utilized in the present study are not
sufficient to elicit permanent changes in the striatum. Given the changes in striatal proteins in human PD patients (Donnemiller et al., 2000) and that other animal models recapitulate some of these changes (Wagner et al., 2005), future attempts to combine a toxic insult with TBI should focus on injury parameters which directly target the striatum and are thus more likely to elicit these changes. The mitochondrial results following the 1 week exposure to TCE do show that these two insults can interact at the mitochondrial level to produce significant dysfunction.

These studies are the first to shown an in vivo dual injury model which utilizes TBI, a major risk factor of PD, as a component of the injury paradigm. These studies further implicate the mitochondria as a central component of TCE exposure, TBI, and PD, especially in the striatum. Given the lack of increased cortical tissue loss when TCE is administered prior to the TBI and previous evidence that more chronic exposure to TCE can lead to deficits in the substantia nigra (Gash et al., 2008, Liu et al., 2010), it is likely that the basal ganglia, especially the striatum, is more susceptible to TCE exposure. Previous studies have shown that other mitochondrial toxins do appear to predominately affect the basal ganglia (Koutouzis et al., 1994, Ferrante et al., 1997) and that this effect is likely due to a these regions having lower levels of spare electron transport chain capacity (Fern, 2003). These studies provide further support that TBI can play a role in the development of PD and that a multifactorial injury paradigm can produce greater pathology. Given the observed deficits in this study, future work incorporating TBI in a dual injury PD model should attempt to more directly target the basal ganglia in order to have a greater ability to elicit deficits. The present findings do support the concept of a multifactorial mechanism for the development of PD and further experimental refinement of this model will help in elucidating the complex interactions of these insults across multiple brain regions.
A short coming in this work, which could be addressed with further experimentation, is the limited time points utilized for the various endpoints. The initial goal of the project was to determine if TCE and TBI could interact and produce a dual injury model of PD. Given that PD is a chronic condition which does not improve on its own, acute time points were not utilized for the behavioral analysis. Previous work in rodents has shown that following many experimental models of traumatic injury there is a spontaneous recovery in behavioral function which occurs. Since the individual behavioral tests were only conducted on single days, we are unable to determine in the dysfunction is constant, improves with time, or gets worse with time. Future experimentation with the model would be made stronger if behavioral analysis for the individual tasks were performed over multiple days, both acute and chronic, for each test. In addition to performing more time points for the behavioral analysis, the quantification of dopamine neurons in the substantia nigra would be made stronger by the addition of later time points. In the present experiments cell counts were performed 30 days post injury, only a loss of phenotypic dopamine neurons was observed. Given the progressive nature of PD in humans, determining the number of dopamine neurons in the substantia nigra at more time points would provide at least two benefits. First, it could be further determined in the loss of phenotypic dopamine neurons continued or if the cells went on to die or were able to recover and start producing dopamine again. Secondly, by performing cell counting at later time points it could be concluded if cell loss increases with time in a similar manner as observed in humans. By further expanding the time points for the behavioral and histological analysis the ability to determine if this model results in progressive degeneration, similar to what is observed in humans, and to further understand the interaction of the two insults would be made stronger.
In addition to expanding the number of time points utilized in these studies, incorporation of additional endpoints would increase the understanding of this model. In human PD there is the development of α-synuclein inclusions and exposure to TCE in rats has also been shown to result in the formation these inclusions. Given the link between both TCE induced α-synuclein inclusion formation and the occurrence of these inclusions in human PD, this endpoint would help both by providing and endpoint which one of the factors in this model, TCE, is already known to affect and could be potentially made worse by a second endpoint as well as providing further endpoints shown to be affected in human PD. Additionally, markers of neuronal death such as silver-stain or Flurojade staining could be utilized which would help to assess damage not only in the cortex of basal ganglia as has already been performed but also in other regions of the brain which as of yet have not been utilized for analysis. Furthermore, analysis of the post-synaptic neurons could provide significant understanding about the current model. The present experiments looked mainly at the pre-synaptic dopamine neurons which project from the substantia nigra to the striatum. Recent unpublished data has shown that following traumatic brain injury in rats there is a significant alteration of the phosphorylation status of the post-synaptic protein DARPP-32 (bales poster). Given the role this protein plays in the normal function of dopaminergic signaling in the basal ganglia (for review see (Fisone et al., 2007) it would be expected that changes in the proteins regulation would affect basal ganglia functioning. Given the previous evidence of changes to this protein following TBI, and that many other post synaptic proteins may have been altered in the present studies which result in deficits in motoring functioning, detailed analysis of the post-synaptic neurons would help in determining if TBI, TCE, or the interaction of the two affect the pre-synaptic and post-synaptic neurons differently.
As mentioned previously, significant modification to the method of performing the TBI would be expected to have a profound effect on the endpoints in the current model. Previous experiments utilizing cortical impact to look at changes to the dopaminergic system have positioned the injury directly over the striatum and utilized a more severe impact and this injury paradigm results in more significant changes than were observed in the experiments present here (Wagner et al., 2005, Wagner et al., 2009). Had we utilized different injury parameters we expect that the observed effects of the TBI on the striatum would be more severe. What should also be cautioned against though is producing too severe of an injury which would prevent or hinder the ability to observe a dual injury affect. As was observed with the 2 week TCE mitochondrial analysis, if the mitochondrial dysfunction is too large it can be impossible to determine if the dual injury resulted in more impairment. If the location of the TBI is altered in future experiments some endpoints such as changes to proteins like the DAT or D2R could become significant, however, other endpoints such as mitochondrial dysfunction may increase to the point where a dual injury effect is not able to be observed. In addition to changing the location of the TBI, changing the type of TBI could have a profound effect on the effect of the dual injury and on the understanding of the developed of human PD and the role of TBI in disease development. Recent unpublished work from the University of Kentucky has indicated that moderate severity diffuse brain injury results in significant dysfunction to the substantia nigra (Lifshitz poster). Following this injury paradigm there is a significant increase in microglial activation and reactive astrogliosis along with a loss of neurons in the substantia nigra. The cell loss associated with this TBI paradigm is greater at month following the brain injury alone than was observed with the TCE TBI paradigm present here. Future work looking at the interaction of TCE and TBI should strongly consider model of TBI utilized in the experiments since damage to the substantia nigra and potentially the rest of the basal ganglia could be must greater if a
diffuse brain injury instead of a cortical impact based brain injury is combined with the TCE exposure.

The current studies are limited by endpoints and time points utilized for the determination of the insults both independently and together. Future work on this model should seek to expand the endpoints utilized for histological analysis as well as expand the number of time points utilized for motor function analysis. Changes to both of these aspects of the current project would greatly improve the ability to determine the effect the insults have and whether the model truly does represent of potential mechanism for the development of PD in humans. Additionally, and potentially more significantly than adding and expanding endpoints, careful planning should be performed to determine what model(s) of TBI should be utilized for future studies. It could be of particular interest to independently test both changes to the parameters of the current model as well as changing to a more diffuse injury paradigm since this would give the best ability to determine not only if TBI plays a role in the development of human PD but also what type(s) of TBI produce the most significant damage to the basal ganglia. These studies provide an initial indication that TCE and TBI may be able to interact and produce more pathology than either insult alone and future experiments should be able to help in extending the findings of the current work.
Chapter 3
Regional mitochondrial function and toxin susceptibility

Introduction:

It is understood that regional differences play an important role in mitochondrial function (for review see (Dubinsky, 2009)), however, there is limited evidence profiling basal mitochondrial function or the susceptibility to mitochondrial inhibitors across multiple brain regions. As shown in the previous chapter, the toxin trichloroethylene has been shown to affect mitochondria in the striatum and substantia nigra differently, however, there is no good understanding at this time why the two regions are differentially affected. Many different factors have been shown to play a role the observed regional differences in mitochondrial function such as enzyme activities (Ryder, 1980, Gupta et al., 2000), requirements for oxidative phosphorylation (Hevner et al., 1995), neuronal activity (Nie and Wong-Riley, 1996, Wong-Riley et al., 1997), and spare respiratory capacity (Fern, 2003). Mitochondrial function has been shown to be tightly linked with the cellular environment and events such as neurotransmitter induced increases in intracellular calcium have been shown to directly affect the activity of mitochondrial enzymes (Denton et al., 1988). Aside from rapid induction of changes in mitochondrial function, regions which have high utilization of glucose have been shown to contain more mitochondrial protein as determined by the level of Cytochrome Oxidase (Humphrey and Hendrickson, 1983), indicating that long term changes in the regulation of mitochondrial bionenergetics also exist. Additionally, the opposite effect has been shown by reducing the activity of a particular brain region either by administration of the sodium-channel blocker tetrodotoxin (Nie and Wong-Riley, 1996) or by blocking peripheral sensory input (Tieman, 1985). These reductions in neuronal activity have been shown to result in decreased amounts of mitochondria and mitochondrial proteins.
(Tieman, 1985, Nie and Wong-Riley, 1996). It is generally believed that one of the most important factors controlling regional mitochondrial differences is higher levels of neuronal activity which demand more ATP (Sokoloff et al., 1977). Even though regional differences in oxygen consumption and mitochondrial numbers have been shown to be linked to neuronal activity, this does not directly indicate that the individual mitochondria are actually different. There are mitochondrial enzymes which do show regional differences (Ryder, 1980, Gupta et al., 2000), however, this is not the case for every enzyme. Many components of the TCA cycle have been shown to have similar activity levels across brain regions suggesting that the mitochondria in different brain regions may function similarly to each other (Marzatico et al., 1987, Curti et al., 1989, Dagani et al., 1989, Villa and Gorini, 1991). A major difficulty in understanding whether regional differences in mitochondrial function exist is that most studies typically utilize very few brain regions, whole brain homogenates, or focus on synaptic versus non-synaptic mitochondria. Factors such as the control of oxygen consumption by electron transport chain components are similar in the cortex and hippocampus but differ significantly between synaptic and non-synaptic mitochondria from the same brain region (Pathak and Davey, 2008). The activity of Complex II of the mitochondrial electron transport chain has also been shown to be similar between brain regions even though it differs from non-neuronal tissue such as the heart and the liver (Mirandola et al., 2010). Analysis of currently available experimental data suggests that the individual mitochondria may function similarly across brain regions (Dubinsky, 2009) even though some proteins have shown activity differences. Even though evidence suggests oxygen consumption by isolated mitochondrial may be similar across brain regions, the assumption has yet to be directly tested by looking at mitochondrial consumption of oxygen across brain regions within a single study.
Beyond just basal mitochondrial function, another aspect of regional mitochondrial differences is the susceptibility of different brain regions to inhibition of the mitochondrial electron transport chain (ETC). It has been established that toxins such as 3-nitropropionic acid or trichloroethylene produce regionally specific mitochondrial dysfunction when given systemically (Beal et al., 1993, Gash et al., 2008) even though it would be expected that more than just the affected brain regions are actually exposed to the toxins. Mirandola et al. showed that there are regional differences in the activation of the mitochondrial permeability transition by 3-NP even though inhibition of Complex II enzyme activity is affected proportionally across brain regions (Mirandola et al., 2010). A significant factor which likely plays a direct role in the regionally different susceptibilities to mitochondrial inhibitors is the amount of spare respiratory capacity for a given complex within a specific brain region (Fern, 2003). Interestingly, the regions with the lowest spare respiratory capacities for a given mitochondrial complex appear to be the ones most susceptible to inhibitors blocking that particular complex (Fern, 2003). The current evidence on the effect of mitochondrial inhibitors across brain regions is difficult to fully interpret since many of the studies only take limited factors into account. Davey et al. showed that mitochondrial inhibitors which block different ETC complexes can produce significantly different effects on oxygen consumption, however, the study utilized whole brain mitochondria and so determining if regional differences exist is impossible to determine (Davey et al., 1998). Mirandola et al. investigated the effect of 3-NP on Complex II enzyme activity across brain regions, however, when investigating the effect of 3-NP of oxygen consumption only a minimally dissected brain sample was utilized (Mirandola et al., 2010). Studies investigating systemic toxin exposure of mitochondrial toxins have shown regionally specific histological damage (Beal et al., 1993, Liu et al., 2010) which suggests that mitochondrial function may be differentially affected across brain regions. Since the amount of inhibition of enzyme activity is not
always directly proportional to the amount of inhibition of oxygen consumption (Davey et al., 1998), it is important to investigate both the inhibition of enzyme activity and oxygen consumption in order to determine whether effects differ across brain regions. Given the evidence which shows regionally specific damage following systemic toxin exposure, it was hypothesized that regional differences in basal mitochondrial function or the susceptibility of the mitochondria to Complex I or Complex II inhibitors may exist which underlie the regionally specific histological damage.

**Methods:**

All studies were approved by the University of Kentucky Animal Care and Usage Committee. Throughout all of the experiments, 16 week old male Fischer 344 rats (Harlan) were utilized.

**Mitochondrial isolation:**

Total mitochondria were isolated using differential centrifugation, nitrogen disruption, and a Ficoll gradient as previously reported. Animals were asphyxiated with CO₂ and rapidly decapitated. Following decapitation, the brain was rapidly removed and placed in a beaker of ice-cold isolation buffer (215mM Mannitol, 75mM Sucrose, 0.1% BSA, 1mM EGTA, and 20mM Hepes at pH 7.2) to briefly cool. Anatomical regions of interest were rapidly dissected apart and the tissue placed in ice-cold isolation buffer until homogenization. Samples were homogenized and then centrifuged at 1300xG for 3 minutes at 4°C. Following the first spin the supernatant was placed in a fresh tube and the pellet was resuspended in isolation buffer and spun at 1300xG for 3min at 4°C. The supernatants from the first and second spins were collected in separate tubes and spun
at 13,000xG for 10 minutes at 4°C. The pellets from both tubes were combined, resuspended in 400ul isolation buffer and placed in a nitrogen bomb at 1,200psi for 10min. The pressure in the nitrogen bomb was rapidly released and the sample was placed as the top layer on a Ficoll separation column which consisted of a 10% Ficoll layer and a 7.5% Ficoll layer. The Ficoll column with sample was centrifuged at 100,000xG for 30min at 4°C using a Beckman SW 55Ti rotor and ultra-centrifuge. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a final concentration of approximately 10mg/ml, and stored immediately on ice. Protein concentrations for each sample were determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560nm with a Biotek Synergy HT plate reader (Winooski, Vermont).

**Preparation of mitochondrial substrates and inhibitors:**

Stocks of mitochondrial substrates were prepared as follows and stored at -20°C until further use. The concentrations for mitochondrial substrates were based upon experience with the Clark-type oxygen electrode (Pandya et al., 2007, Pandya et al., 2009) and optimized to obtain mitochondrial bioenergetic profiles which fit with previously observed findings obtained with the oxygen electrode. 500mM Pyruvate/250mM Malate was prepared by combining 550 mg Pyruvate (Sigma P-2256), 335 mg Malate (Sigma M-7397), and 200 µl of 1M HEPES in 10 ml diH₂O and the pH was adjusted to pH 7.2. A 30mM ADP stock was prepared by combining 128.2mg ADP (Sigma A-5285) and 200 µl of 1M HEPES in 10 ml diH₂O and pH was adjusted to pH 7.2. A 1mg/ml Oligomycin-A stock was prepared by combining 1 mg Oligomycin-A (Biomol CM-111) with 1ml methanol. A 1mM FCCP stock was prepared by combining 2.542 mg FCCP (Biomol CM-120) with 10 ml 100% ethanol. A 1M Succinate stock was
prepared by combining 2.36g Succinic Acid (Sigma S-7501) with 400 µl of 1 M HEPES in 20ml diH$_2$O and the pH was adjusted to pH 7.2. Rotenone was prepared by first combining 3.944mg rotenone (Biomol CM-117) with 10ml 100% ethanol for a final concentration of 1mM. Rotenone was further serial diluted with 100% ethanol to obtain final concentrations of 100µM, 10µM, 1µM, 100nM, and 10nM rotenone. Malonate stock was prepared by first combining 1.04g Malonic Acid (Sigma M1296) with 10mL diH$_2$O and the pH was adjusted to 7.2 for a final concentration of 5M. KOH and HCl were utilized for all pH adjustments.

**Preparation and calibration of Seahorse sensor cartridge sample plate:**

A Seahorse Bioscience XF24 extracellular flux analyzer was used to measure mitochondrial function in intact isolated mitochondria. The XF24 creates a transient, 7 ul chamber in specialized microplates that allows for the determination of oxygen and proton concentrations in real time. The day before the planned experiment, 1ml of XF Calibrant solution (Seahorse Bioscience) was added to each well of a 24 well dual-analyte sensor cartridge (Seahorse Bioscience). The sensor cartridge was placed back on the 24 well calibration plate and put in a 37 ºC incubator without CO$_2$ (Seahorse Bioscience) overnight. The day of the experiment, the injection ports on the sensor cartridge were loaded with the appropriate mitochondrial substrates or inhibitors at 10X concentrations. Once the sensor cartridge was loaded with all of the experimental reagents it was placed into the Seahorse XF24 Flux Analyzer for automated calibration. During the sensor calibration, isolated mitochondria were then seeded in 50 ul volume of respiration buffer containing 2.5 µg or 5 µg of protein (BCA method) per well in polyethyleneimine-coated XF24 V7 cell culture microplates. Following the centrifugation
of the plates at 2000 rpm for 4 minutes at 4°C, 575 ul of respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH2PO4 at pH 7.2) at 37°C was gently added to each well for a final volume of 625 ul per well at the beginning to the experiment. Plates were immediately placed into the calibrated Seahorse XF24 flux analyzer for mitochondrial bioenergetic analysis.

**Seahorse protocol for isolated mitochondria:**

The following protocol was utilized for the analysis of bioenergetic function in purified mitochondria using the Seahorse Biosciences XF24 Flux Analyzer. Pyruvate/Malate and ADP, Oligomycin, FCCP, or Rotenone and Succinate were injected sequentially through ports A, B, C, and D, respectively, in the Seahorse Flux Pak cartridges to yield final concentrations of 5 mM (Pyruvate), 2.5 mM (Malate), 1mM (ADP), 1 μg/ml (Oligomycin), 1 μM (FCCP) and 100 nM (Rotenone), 10 mM (Succinate) respectively.
The following sequence was utilized for basal functional analysis:

<table>
<thead>
<tr>
<th>Protocol for standard mitochondrial analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>16</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>19</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>21</td>
</tr>
</tbody>
</table>

The same protocol was utilized for the log dose response experiments except that rotenone or malonate were injected through Ports B, C, and D. For the time dependent effect of rotenone administration a modified Seahorse mitochondrial protocol was utilized for the repeated measure of oxygen consumption from the same well following the injection of various low concentrations of rotenone (10fM, 10pM, 10nM).
**Analysis of data generated by Seahorse biosciences XF24:**

For optimal analysis of mitochondrial bioenergetic function, the data generated by the XF24 was further analyzed beyond the average oxygen consumption ratio generated for a given measure. The methods for analyzing the Seahorse data are based upon similar protocols for analyzing the rate of oxygen consumption using a Clark-type oxygen electrode. During a given measure, the rate of oxygen consumption varies with time as substrates bind with their targets or are consumed by the mitochondria. Using the Excel software package (Microsoft), point-by-point rates are generated using the AKOS algorithm written by Seahorse Bioscience. For Complex I and Complex II function, the highest point-by-point rate is taken since as substrates are consumed the rate begins to decrease. For the Oligomycin-A rate, an average of the last three point-by-point rates is taken since the effect of Oligomycin-A can take at least one minute to reach maximal effect and an average of the last 30 seconds of the measure provides the most consistent result.

**Mitochondrial Complex I assay:**

Mitochondrial Complex I enzyme activity was determined by measuring the decrease in NADH absorption at 360nm in the presence and absence of rotenone, as previously described (Gash 2008, Brown 2004). Frozen purified mitochondrial were thawed and then diluted in 10mM KPO₄ pH 7.4 to a concentration of 1μg/μl. Samples then underwent three cycles of five minute freezing on dry ice followed by five minutes of thawing at 37°C followed by five seconds of sonication. The assay was performed in a BioTek Synergy HT plate reader (BioTek, Winooski, VT) with excitation at 360nm and emission at 460nm. 6μg of mitochondrial protein was added to 25mM KPO₄ pH 7.2, 5mM MgCl₂, 1mM KCN, 1mg/ml bovine serum albumin, and 150μM NADH in a total volume of 69μl. Either 1μl of 1mM rotenone or 1μl of 25mM KPO₄ pH 7.2 was added so
that with and without rotenone samples could be compared. The reaction was started by the addition of 30ul of coenzyme Q1 (final concentration 50uM). Enzyme activity was calculated by subtracting the change in NADH emission in the absence of rotenone from the change in NADH emission in the presence of rotenone.

**Mitochondrial Complex II assay:**

Mitochondrial Complex II enzyme activity was determined by measuring the change in absorbance of DCIP at 600nm using a BioTek Synergy HT plate reader (BioTek, Winooski, VT). Frozen purified mitochondrial were thawed and then diluted in 10mM KPO₄ pH 7.4 to a concentration of 1ug/ul. Samples then underwent three cycles of five minute freezing on dry ice followed by five minutes of thawing at 37°C followed by five seconds of sonication. 3ug of mitochondrial was added to 200mM KPO₄ pH 7.0, 20mM K-Succinate, 10uM EDTA, 0.01% Triton X-100, 0.5ug/50ul coenzyme Q10, ~20% DCIP for a total volume of 50ul. The starting optical density was between 0.6-0.8 at 600nm and the exact concentration of DCIP was made so that this starting density was obtained. The change in absorbance was measured at the beginning of the experiment and two minutes later. Enzyme activity was determined by subtracting the final optical density from the initial optical density.

**Statistical analysis:**

For all experiments significance, was set at p<0.05. Analysis of the mitochondrial protein dependent rate of Complex I oxygen consumption was performed with a two-tailed t-test. Comparisons of basal mitochondrial function were conducted using a one-way analysis of variance followed by post-hoc analysis to determine group differences. The log dose response experiments and the time dependent rotenone inhibitory effect were analyzed using a two-way analysis of variance followed by post-hoc analysis to determine group differences.
Results:

Basal mitochondrial bioenergetic function across brain regions:

Mitochondria isolated from different regions of the brain exhibit differences in mitochondrial bioenergetic function with the cerebellum and the striatum exhibiting significant differences. Compared with the striatum, the cerebellum exhibited a 36% slower rate of ADP dependent Complex I driven respiration. Additionally, analysis of maximal Complex II driven respiration revealed that the striatum was approximately 1.6 times faster than the cerebellum. Both the striatum and the hippocampus exhibited higher rates of residual oxygen consumption in the presence of the Complex V inhibitor Oligomycin-A compared to the cerebellum. The respiratory control ratio (RCR) was calculated to determine the overall health and integrity of the mitochondria and no significant differences were observed between any of the brain regions. Additionally, the RCR determinations revealed that the mitochondria utilized in these experiments were all healthy since the RCRs were greater than 5.
Mitochondrial bioenergetic analysis reveals regionally specific variations in mitochondrial function. A) Compared with the striatum, the cerebellum exhibited a 36\% slower rate of Complex I driven mitochondrial oxygen consumption. B) Similar to the Complex I findings, analysis of Complex II dependent oxygen consumption revealed that the cerebellum had a significantly slower rate of oxygen consumption. C) The striatum and the hippocampus exhibited nearly two-fold higher rates of oxygen consumption in the presence of Oligomycin-A. D) No significant differences exist between brain regions with respect to the mitochondrial respiratory control ratio.

n=6/group. *=p<0.05 with One-way ANOVA and SNK-post test; #=p<0.05 with One-way ANOVA and protected Fischer’s LSD
Effect of mitochondrial Complex I and II inhibitors on enzyme activity:

Measurement of enzymatic activity was performed in order to determine the direct action of the inhibitors on their respective mitochondrial targets. Analysis of the effect on the Complex I specific inhibitor rotenone revealed that the concentration of rotenone required to inhibit 50% of enzyme activity, the IC50, ranged from 163-238pM and that no significant difference in the IC50 existed across brain regions (Figure 3.2). Further analysis of the effect of rotenone revealed that Complex I enzyme activity was equally affected across brain regions as the concentration of the inhibitor was increased. Similar to the effects of rotenone, exposure to the Complex II inhibitor malonate resulted in no significant differences in the calculated IC50 with values ranging from 440-580µM across brain regions (Figure 3.2). As the concentration of the inhibitor was increased, the resulting decline in Complex II enzyme activity was equal across all brain regions tested.

Effect of mitochondrial Complex I and II inhibitors on oxygen consumption:

Mitochondrial enzyme activity does not always change proportionally to mitochondrial oxygen consumption following treatment with a mitochondrial enzyme inhibitor. Direct measurement of enzymatic activity was performed following inhibitor administration to determine the effect of the inhibitors on their respective target enzymes. Baseline values for the two inhibitors were calculated from mitochondrial samples in the absence of inhibitor. Acute exposure to rotenone resulted in an immediate dose dependent reduction in mitochondrial Complex I driven oxygen consumption. Significant differences were observed across brain regions as a result of exposure to rotenone. Following exposure to 10pM rotenone the cerebellum and hippocampus were significantly more inhibited than the striatum or the cortex (Figure
3.3, *=p<0.05). The hippocampus remained more inhibited than the striatum following exposure to 100pM rotenone. All differences between brain regions went away at concentrations of rotenone 1nM and higher. Exposure to malonate resulted in a dose dependent decrease in mitochondrial driven oxygen consumption with no observed differences across brain regions.

Figure 3.2. Mitochondrial Complex I and II inhibitors affect enzyme activity equally across brain regions.

A) No differences were observed in the response of the different brain regions to Complex I enzyme inhibition. As the amount of rotenone was increased, all of the tested brain regions had similar percentage reductions in enzyme activity. B) The response to Complex II inhibition was not significantly different across brain regions. Similar to the effects observed following rotenone administration, as the concentration of malonate was increased the various brain regions had similar decreases in enzyme activity. C) No significant differences were observed in the calculated IC50 values across brain regions and the reduction in enzyme activity in the presence of rotenone was comparable across all brain regions tested. The calculated IC50 values for malonate did not differ significantly between the brain regions tested.

n=3-5/group. Two-way ANOVA
**Figure 3.3:** Regional differences exist in the susceptibility to rotenone but not malonate.

A) At low concentrations of the mitochondrial Complex I inhibitor rotenone the cerebellum and hippocampus are more susceptible to inhibition of oxygen consumption than the striatum or the cortex. As the concentration of rotenone increased, the differences between brain regions eventually went away. B) Exposure to the Complex II inhibitor malonate produced dose dependent reductions in mitochondrial oxygen consumption which did not differ across brain regions for a given concentration of inhibitor.

n=5/group. *=p<0.05 Two-way Anova with Bonferroni post-test

**Time dependent inhibition of Complex I by rotenone:**

Human exposure to a toxin can occur over time frames longer than *in vitro* experiments which typically assess inhibitory values are conducted. Isolated cortical mitochondria treated with 10nM of the Complex I inhibitor rotenone exhibited a significant reduction in mitochondrial oxygen consumption immediately following exposure. Lower doses of rotenone, 10pM and 10fM, did not result in any reduction in oxygen consumption up to eight minutes following exposure. If exposure to rotenone at 10pM was allowed to continue a significant reduction in mitochondrial oxygen consumption became apparent as early as 12 minutes following exposure (Figure 3.4, *=p<0.05). The decrease in oxygen consumption following 10pM rotenone exposure eventually reached a 75% reduction in mitochondrial oxygen consumption.
Figure 3.4: Exposure to 10pM rotenone produces a progressive decrease in mitochondrial oxygen consumption.

Samples which were exposed to 10nM rotenone produced an immediate reduction in oxygen consumption similar to previous finding. Similarly, exposure to 10fM rotenone resulted in no reduction in oxygen. In contrast to acute measurements rotenone dependent reductions in Complex I driven oxygen consumption, exposure to 10pM resulted in a progressive reduction in mitochondrial oxygen consumption. For at least eight minutes following exposure to 10pM rotenone no reduction in oxygen consumption was observed. As early as 12 minutes following exposure to 10pM rotenone a significant reduction in oxygen consumption occurred that continued to decrease with time. A 75% reduction in Complex I driven oxygen consumption was observed at 16 minutes post-exposure and this level of inhibition continued beyond this time point without further reduction.

n=5/group, *=p<0.05 Two-way ANOVA with Bonferroni post-test
Discussion:

Regional differences have been observed in the activity of specific mitochondrial enzymes (Ryder, 1980, Gupta et al., 2000). Neuronal activity has been shown to play a direct role in the expression of mitochondrial proteins (Wong-Riley et al., 1997) and reductions in synaptic activity can lead to reduced Cytochrome-C levels (Nie and Wong-Riley, 1996) and the number of mitochondria (Tieman 1985). Additionally, ischemic injury experiments have shown regionally different mitochondrial susceptibility to the injury (Sims, 1991) which could be the result of mitochondrial or cellular environment differences. Multiple groups have further, established that, in addition to brain region differences in mitochondrial function there is a significant difference between synaptic and non-synaptic mitochondria (Davey et al., 1997, Brown et al., 2006, Naga et al., 2007, Pathak and Davey, 2008). Numerous factors are likely to play role in the differential susceptibility of mitochondria such as which region of the brain the mitochondria are from (Singh et al., 2010) or the cell containing the mitochondria (Zeevalk et al., 1997).

In order to further elucidate the role of the mitochondria in regionally specific toxin susceptibility, analysis of basal mitochondrial function was performed across various regions of the brain. We hypothesized that regions which appear more susceptible to Complex I or Complex II inhibition in vivo may have reduced basal mitochondrial function. At the initial onset of these experiments we attempted to utilize TaClo, a metabolite of TCE which has been shown to produce robust inhibition of mitochondrial Complex I driven oxygen consumption (Janetzky et al., 1995). Though we were able to obtain a sample of TaClo, problems with the solubility of the compound and a limited amount of it precluded its use to the present studies. Given that TaClo is known to inhibit mitochondrial Complex I, the much more usable inhibitor rotenone was utilized.
in these experiments for technical reasons. The results indicate that the greatest
differences in Complex I and Complex II dependent oxygen consumption exist between
the cerebellum and the striatum (Figure 3.1). The finding of reduced basal oxygen
consumption in the cerebellum compared to other brain regions has not been shown
before and therefore the underlying reason for this effect is currently unknown. Previous
work has show a reduced activity of Complex II enzyme activity in the cerebellum
(Fagundes et al., 2007) and this could be an underlying reason for the reduced Complex
II dependent oxygen consumption observed in these studies. It has recently been shown
that resting aerobic glycolysis is lower in the cerebellum than other brain regions
(Vaishnavi et al., 2010) and this may be the result of different ratios of neuronal and non-
neuronal cells in the cerebellum (Azevedo et al., 2009). Interactions between neurons
and astrocytes during *in vivo* oxygen consumption has been shown to occur (Kasischke
et al., 2004) with the two cell types providing different roles during energy metabolism
(for review see (Magistretti, 2006). Given the higher percentage of neurons to astrocytes
in the cerebellum, the regional differences in the mitochondrial respiration observed in
the present studies may be affected by different cell type ratios. Basal Complex I and
Complex II dependent mitochondrial oxygen consumption did not differ between the
cortex, striatum, or hippocampus in these experiments which indicates that mitochondria
from these regions have similar bioenergetic capacities and profiles. Interestingly, the
cerebellum had a significantly slower rate of oxygen consumption in the presence of the
Complex V inhibitor Oligomycin-A compared to the striatum and hippocampus (Figure
3.1B). The difference in oxygen consumption in the presence of Oligomycin-A may be
the result of lower levels of glutathione and superoxide dismutase in the striatum and
hippocampus relative the cerebellum (Sanchez-Iglesias et al., 2009) which could
possibly allow for increased damage to the inner mitochondrial membrane and increased
proton leak across the damaged membrane. Calculation of the mitochondrial Respiratory
Control Ratio revealed high levels of coupling between the pumping of protons across the inner membrane with the formation of ATP by Complex V (Figure 3.1D).

Differences in mitochondrial spare respiratory capacity have been documented which may provide insight into the regional heterogeneity of \textit{in vivo} mitochondrial toxin models. Previous reports utilizing the toxin trichloroethylene (Gash et al., 2008, Liu et al., 2010) or 3-Nitroproionic acid (2-NP) (Beal et al., 1993) have shown that different regions of the brain do appear more susceptible than other regions. Mirandola showed that following \textit{in vitro} systemic exposure to the mitochondrial Complex II inhibitor 3-NP there are regional differences in the susceptibility to mitochondrial permeability transition in mitochondria isolated from the brain. However, in isolated mitochondria \textit{in vitro} treatment with 3-NP affected mitochondria equally across the same brain regions (Mirandola et al., 2010). It has been suggested that one of the major reasons certain brain regions are more susceptible than other to a particular toxin is not the mitochondria itself but the environment the cells are in (Mirandola et al., 2010). Analysis of the susceptibility of isolated mitochondria to Complex I or Complex II inhibition indicates that there are no differences between brain regions with regards to the effect these toxins have on enzyme activity (Figure 3.2). This finding is in agreement with results looking at Complex II activity and the susceptibility to 3-NP where the cortex, striatum, and cerebellum had similar basal enzyme activities and susceptibilities to inhibition (Mirandola et al., 2010). It has previously been shown that the percentage inhibition of mitochondrial Complex I activity is correlated with the percentage reduction in mitochondrial oxygen consumption (Davey et al., 1998). The current results support this previous finding since the dose dependent reduction in mitochondrial oxygen consumption in the presence of the Complex I inhibitor rotenone (Figure 3.3A) is similar to of the reduction in Complex I enzyme activity (Figure 3.2A). The present study further extends this effect to Complex II
dependent oxygen consumption since the effect of the inhibitor malonate had a similar concentration dependent effect on oxygen consumption (Figure 3.2B) as it did on enzyme activity (Figure 3.3B). These results further indicate that at low concentrations of the inhibitor rotenone differences in the percentage reduction of oxygen consumption does differ between brain regions (Figure 3.3A).

Previous unpublished experiments from our group indicated that a time dependent effect of rotenone on Complex I dependent mitochondrial function may exist at low concentrations of the inhibitor. To test if this effect was real, repetitive measurements were taken of mitochondrial samples treated with either 0, 10fM, 10pM, or 10nM rotenone. As shown in our previous experiments (Figure 3.4), the 10nM rotenone produced an immediate significant reduction in oxygen consumption while the 10pM rotenone did not produce an immediate reduction in oxygen consumption (Figure 3.4). Strikingly, continued exposure to the 10pM rotenone produced a clear time dependent reduction in Complex I dependent oxygen consumption which became significant by 12 minutes post injection and reach a 75% reduction in oxygen consumption by 16 minutes (Figure 3.4). This is an important finding to consider when attempting to determine the IC50 for a compound or comparing results from multiple groups which utilize different methodologies.

These results show that there are no significant differences in basal mitochondrial bioenergetic function in samples isolated from the cortex, striatum, or hippocampus. The cerebellum did exhibit significant differences in basal mitochondrial function but the susceptibility to mitochondrial Complex I and Complex II inhibitors remained proportional to the other brain regions. Like the cerebellum, the other regions of the brain were relatively similar to mitochondrial enzyme inhibition except at low concentrations of rotenone where regional differences did become apparent. The effect
of rotenone on Complex I function is further shown to be significantly time dependent at a 10pM concentration.

A major factor which must also be considered when interpreting these results is that the studies are carried out with equal amounts of purified mitochondria. These studies provide insight into the function of isolated mitochondria from different brain regions but do not give evidence for the amount of in vivo mitochondrial oxygen consumption across these regions. Previous reports have established that reductions in neuronal activity can lead to reduced numbers of mitochondria (Tieman, 1985) or amounts of mitochondrial proteins (Nie and Wong-Riley, 1996), which would impact the amount of oxygen consumption per volume of tissue and factors such as these are not addressed with these studies. Future experimentation should utilize techniques such as immunohistochemical or western blot analysis for the amount of mitochondrial proteins or the quantification of the number of mitochondria in order to determine regional differences in the amount mitochondria across brain regions. Furthermore, positron emission tomography has been utilized to assess the consumption of pyruvate within the brain in vivo (Toyoda et al., 1989) and further investigation utilizing this technique or measurement of brain glucose utilization would help to further the understanding of regional mitochondrial differences. Utilizing in vivo techniques for the measurement of mitochondrial function would also allow for the determination of the effect of the various mitochondrial toxins directly on the intact brain regions. An additional level of in vivo differences which these studies do not address is the role of metabolic coupling between neurons and astrocytes. Evidence has accumulated which indicates coupling of energy processing machinery between these two cell populations in such a way that their metabolic processes function as a single unit (Kasischke et al., 2004) (for review see (Magistretti, 2006)). Considering that the mitochondria in the current experiments are no
longer inside individual cells, it is conceivable that the mitochondrial pathways may function differently \textit{in vitro} compared with \textit{in vivo}. By using techniques which do not disturb the brain regions, cells, or mitochondria it is possible that the results of these experiments could be significantly different than what is present presently. The major findings from these studies are that the mitochondrial machinery function similarly across brain regions, with the exception of the cerebellum. Additionally, mitochondrial Complex I and Complex II inhibitors equally affect different regions of the brain except for lower concentrations of rotenone. These studies further suggest that the observed regionally specific histological changes which occur following systemic exposure to a mitochondrial inhibitor (Beal et al., 1993, Liu et al., 2010) are not likely to be the result of regional differences in the individual mitochondria.
Pioglitazone attenuates mitochondrial dysfunction, cognitive impairment, cortical tissue loss, and inflammation following traumatic brain injury.

Introduction:

Traumatic brain injury (TBI) pathology results from both a primary injury and a secondary injury cascade. The primary injury is due to biomechanical damage which results in the shearing and compression of neuronal, glial, and vascular tissue. The cascade of secondary injury damage, which occurs in the hours and days following the initial insult, is due to activation of pathophysiological cascades, consisting of complex biochemical and cellular pathways that influence progression of the injury, such as alterations in excitatory amino acids (Yamamoto et al., 1999, Rose et al., 2002), increased reactive oxygen species (ROS) production (Marklund et al., 2001, Hall et al., 2004, Tavazzi et al., 2005), disruption of calcium homeostasis (Mattson and Scheff, 1994, Xiong et al., 1997, Sullivan et al., 1999c), post-traumatic neuroinflammation (Morganti-Kossmann et al., 2001, Vlodavsky et al., 2006) and mitochondrial dysfunction (Azbill et al., 1997, Xiong et al., 1997, Sullivan et al., 1998b, Sullivan et al., 1999a, Sullivan et al., 1999b). As a result of these secondary injury processes, there are significant reductions in ATP levels (Sullivan et al., 1998a), increases in lipid peroxidation (Sullivan et al., 1998a), release of cytochrome c (Sullivan et al., 2002) and activation of apoptotic pathways (Sullivan et al., 2002), all of which can lead to the initiation of cell death pathways. Mitochondria are a major component of this secondary injury pathway because they function as a highly sensitive gatekeeper of cell death mechanisms and as the primary energy producer for the cell. As such, mitochondria play
a pivotal role in cerebral energy metabolism, intracellular calcium homeostasis, and ROS generation and detoxification.

Following TBI, a significant disruption of mitochondrial homeostasis has been documented that results in a decline in cellular bioenergetics, increased mitochondrial ROS production and a decline in synaptic equilibrium (Azbill et al., 1997, Xiong et al., 1997, Sullivan et al., 1998b, Sullivan et al., 1999a, Sullivan et al., 1999b). Therefore, following TBI, the degree of mitochondrial injury or dysfunction can be an important determinant of cell survival or death (for reviews see Robertson, 2004, Sullivan et al., 2005, Robertson et al., 2006) and therapeutic treatments designed to protect and stabilize the mitochondria have demonstrated the ability to reduce injury in preclinical studies (Sullivan et al., 2000a, Pandya et al., 2007). Although preclinical research has identified neuroprotective agents which target mitochondrial function, inflammation, and oxidative damage, attempts to move therapies into clinical usage have so far been unsuccessful (Schouten, 2007). Given the complexity of the secondary injury, it has been suggested that drugs which target multiple pathological pathways may yield more effective therapeutic approaches for TBI. The PPARγ agonist Pioglitazone has been shown to reduce inflammation (Besson et al., 2005, Chen et al., 2007a, Park et al., 2007, Hyong et al., 2008, Kapadia et al., 2008) and oxidative damage (Chen et al., 2007a, Yi et al., 2008), attenuate mitochondrial dysfunction (Hunter et al., 2007), and reduce cell death (McTigue et al., 2007, Park et al., 2007) following CNS injury. Pioglitazone’s ability to target multiple injury mechanisms may provide it with an advantage over other therapeutics for TBI which target a single secondary injury cascade. Treatment with Pioglitazone following LPS induced brain inflammation has shown the ability to prevent both mitochondrial impairment and neuronal cell loss (Hunter et al., 2007). The use of various PPARs has shown benefit in multiple CNS
injury models including spinal cord injury (SCI) (McTigue et al., 2007, Park et al., 2007), traumatic brain injury (TBI) (Besson et al., 2005, Chen et al., 2007a, Chen et al., 2008, Yi et al., 2008), and stroke (Collino et al., 2006, Allahtavakoli et al., 2009). Of the three known PPAR isoforms, PPARα and PPARγ have been the most well studied in CNS injury and have been shown to reduce lesion size both in SCI (McTigue et al., 2007, Park et al., 2007) and TBI (Yi et al., 2008), reduce inflammation (Besson et al., 2005, Chen et al., 2007a, Park et al., 2007, Hyong et al., 2008, Kapadia et al., 2008), minimize oxidative damage (Chen et al., 2007a, Yi et al., 2008), spare neurons (McTigue et al., 2007, Park et al., 2007), and preserve behavioral function (Chen et al., 2007a, McTigue et al., 2007, Park et al., 2007, Chen et al., 2008).

The PPARγ agonist Pioglitazone is an FDA approved drug for diabetes treatment (for review see (Sood et al., 2000)) and has been utilized as a therapeutic in multiple animal models of CNS injury (Besson et al., 2005, Kiaei et al., 2005, Schutz et al., 2005, Collino et al., 2006, Chen et al., 2007a, McTigue et al., 2007, Park et al., 2007, Chen et al., 2008, Feng et al., 2008, Hyong et al., 2008, Sun et al., 2008, Yi et al., 2008, Allahtavakoli et al., 2009). Pioglitazone has been shown to more readily cross the blood brain barrier (BBB) than the similar drug Rosiglitazone (Berger and Moller, 2002) as well as partially activate the PPARα receptor (Sakamoto et al., 2000). Pioglitazone’s increased brain penetration and activation of two separate PPAR pathways may yield a greater therapeutic potential for the treatment of TBI (for review see (Kapadia et al., 2008)). Currently, evidence exists showing that activation of either the PPARα (Chen et al., 2007a, Chen et al., 2008) or PPARγ (Yi et al., 2008) pathways are protective in models of TBI, however, no studies currently exist showing the effect of Pioglitazone following TBI. Because of the success of PPAR agonists in multiple models of CNS injury and their offer of a broad range of potentially protective properties, it is
hypothesized that PPAR activation by Pioglitazone will be beneficial in an animal model of controlled cortical impact (CCI) that has hallmarks of human TBI. The current project addresses the hypothesis that Pioglitazone will offer significant neuroprotection leading to maintenance of mitochondrial function, sparing of cortical tissue, attenuation of inflammation, and preservation of cognitive function following TBI.

Methods:

All studies were approved by the University of Kentucky Institutional Animal Care and Usage Committee. Male Sprague Dawley rats weighing 250 grams (Harlan Laboratory, IN) were utilized throughout all experiments.

Controlled cortical impact brain injury:

Animals were anesthetized with 2% isofluorane and placed in a Kopf stereotaxic frame for positioning under a pneumatic impactor (Precision Science Instruments). A 6 mm craniotomy was performed, with a hand trephine, lateral to the central fissure on the left side of the skull centered between lambda and bregma. Animals in injury groups received a unilateral injury directly to the surface of the brain. The injury parameters consisted of a 1.5mm deep contusion at 3.5 meters/second for 500ms. Sham animals received a craniotomy but did not receive an impact to the brain. Following the injury a piece of Surgicel (Johnson&Johnson) sized to fit into the craniotomy was placed directly on the brain. The skull cap was replaced and secured in place with dental acrylic. Once the acrylic was allowed time to harden, the scalp incision was closed with surgical staples. Animals were removed from isofluorane and placed in a clean cage and temperature was maintained at 37°C with the use of a heating pad.
Pioglitazone treatment:

Following the completion of the brain injuries, animals were randomly assigned to drug treatment groups. Pioglitazone and the antagonist T0070907 were prepared by adding 1mg of the compound to 25µL of 100% Ethanol, 1µL 38% HCl, and 25µL of 0.9% saline. This mixture was vortexed briefly and then 200µL of 0.9% saline was added. Each day, the drugs were made fresh for each set of injections. Pioglitazone or the PPARγ antagonist T0070907 were administered at 10mg/kg/injection. The vehicle administration group was administered the same solution used to dissolve the Pioglitazone and the antagonist. All injections were administered intraperitoneally. For mitochondrial analysis experiments treatments were started 15 minutes following the injury and depending upon the group a second injection was administered 24 hours later. For the behavioral and histological experiments treatment was begun 15 minutes after the injury and continued with subsequent injections at 24h, 48h, 72h and 96h after the first injection.

Morris water maze behavioral assessment:

Morris water maze testing was begun 10 days post-surgery and consisted of four trials per day for five consecutive days. The maze consisted of a dark black circular pool (170 cm diameter, 56 cm high) filled with water (27°C) to a depth of 30 cm. A clear circular Plexiglas platform 13 cm in diameter was placed 2 cm below the surface and served as the goal platform. A video camera placed directly above the center of the pool recorded swimming performance. Each video record was processed by a video motion analyzer (Videomex V; Columbus Instruments, Columbus, OH). The swim speed was measured for each animal to ensure that observed changes were not secondary to impairments in motor function. For each animal, goal latency and distance traversed to the platform were measured for each of the four daily trials and averaged.
**Histological analysis:**

After the completion of the behavioral testing, animals were sacrificed for histological analysis. Animals were anesthetized with sodium pentobarbital (Abbot Laboratories) and transcardially perfused with saline followed by 4% paraformaldehyde. The brain were removed and placed in 4% paraformaldehyde and 30% sucrose in PBS for 24hrs. After 24hrs the brains were transferred to a 30% sucrose PBS buffer without paraformaldehyde. Coronal sections 35µm thick were cut with a freezing microtome throughout the rostral caudal extent of the damaged cortex. Sections were stained with neutral red and subjected to image analysis for lesion volume assessment. Quantitative assessment of cortical damage employed an unbiased stereological protocol using the Cavalieri method as previously described (Sullivan et al., 2000c, Sullivan et al., 2002). All slides were assessed blindly with respect to treatment group. For immunohistochemistry, sections containing regions of interest were incubated overnight at 4°C with a primary antibody. The primary antibody for OX-42 (monoclonal, 1:2000, PharMingen, San Diego, CA) was used to detect microglia. After washes and incubation with an appropriate secondary antibody (Vector Laboratories, Burlingame, CA), immunoreactive cells were visualized by the avidin-biotin immunoperoxidase method (ABC kits, Vector Laboratories) with chromogen 3,3'-diaminobenzidine tetrahydrochloride (Sigma). For each animal, sections at an interval of 770µm throughout the injured cortex were utilized for microglia. Five sections were selected for each animal and five non-overlapping adjacent fields of each section were selected which surrounded the edge of the cortical lesion. A Nikon Eclipse 80i light microscope with an attached Nikon microscope camera was used to obtain data images using a 200X objective. The images were captured and imported into Simple PCI image processing software (Compix Inc.) for activated microglias counting.
Mitochondrial isolation and bioenergetic analysis:

Cortical mitochondria were isolated using differential centrifugation, nitrogen disruption, and a Ficoll gradient. Animals were asphyxiated with CO₂ and rapidly decapitated. A cortical punch encompassing the injury site was removed and immediately placed in ice-cold isolation buffer (215mM Mannitol, 75mM Sucrose, 0.1% BSA, 1mM EGTA, and 20mM HEPES at pH 7.2). Samples were homogenized and centrifuged at 1300xG for 3 minutes. Following the first spin the supernatant was placed in a fresh tube and the pellet was resuspended in isolation buffer and spun at 1300xG for 3 min. The supernatant from the first and second spins were collected in separate tubes and spun at 13,000xG for 10 minutes. The pellets from both tubes was combined, resuspended in 400ul isolation buffer and placed in a nitrogen bomb at 1,200psi for 10min. The pressure in the nitrogen bomb was rapidly released and the sample was placed as the top layer on a Ficoll separation column which consisted of a 10% Ficoll layer and a 7.5% Ficoll layer. The Ficoll column with sample was centrifuged at 100,000xG for 30min at 4°C using a Beckman SW 55Ti rotor and ultra-centrifuge. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a final concentration of approximately 10mg/ml, and stored immediately on ice. To normalize the results, the protein concentrations were determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560nm with a Biotek Synergy HT plate reader (Winooski, Vermont). Mitochondrial oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments, Norfolk, England) in a continuously stirred, sealed chamber at 37°C as previously described (Sullivan et al., 2003). Isolated mitochondrial protein (100µg) was suspended in respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl₂, 2.5 mM KH₂PO₄ at pH 7.2) in a final chamber volume of 0.25 mL.
Mitochondrial bioenergetic analysis was measured by the sequential administration of substrates: 5mM Pyruvate, 2.5mM Malate, 150nM ADP, 1uM Oligomycin, and 1uM FCCP.

**Statistical analysis:**

All experiments were analyzed with a one-way ANOVA with post-hoc statistical analysis to determine group differences. Significance for all experiments was set at p<0.05.
Results:

Pioglitazone protects mitochondria from injury-induced mitochondrial dysfunction.

Following TBI there is significant damage to the mitochondria resulting in impaired bioenergetic function. In order to elucidate the effect of Pioglitazone on mitochondrial bioenergetic function, two different treatment paradigms for Pioglitazone administration were utilized. In the first set of animals, Pioglitazone was administered 15min after the injury (10mg/kg) and animals were sacrificed 25hrs after the injury. In the second set of animals Pioglitazone was administered at 15min and 24hrs after injury (10mg/kg/injection) and animals were sacrificed at 25hrs after the injury. Following injury in both sets of animals, reductions in Pyruvate/Malate (PM), ADP, and FCCP (uncoupled) respiration rates were seen in vehicle treated animals (Figure 4.1, *=p<0.01). With only a single 15min injection there was a slight but non-significant increase in respiration rates following Pioglitazone treatment (Figure 4.1A); however, when Pioglitazone was given at both 15min and 24hrs after the injury a significant increase in mitochondrial function was observed (*=p<0.01, Figure 4.1B), indicating that under these conditions Pioglitazone treatment leads to preservation of the mitochondria’s ability to produce ATP.
In order to assess the ability of Pioglitazone to prevent the mitochondrial dysfunction which occurs with this model of TBI, mitochondrial bioenergetic function was analyzed 1 day following a controlled cortical impact TBI. **A.** Following a single injection of Pioglitazone (10mg/kg) 15 minutes after the injury, no improvements in mitochondrial function were observed. **B.** When Pioglitazone was administered at both at 15 minutes and 24 hours after the injury (10mg/kg/injection) a significant increase in mitochondrial bioenergetics was observed. These studies indicate that treatment with Pioglitazone at 15 minutes and 24 hours is capable of preventing mitochondrial dysfunction following TBI.

(* p<0.01 by one-way ANOVA with SNK post-test).
Pioglitazone treatment improves Morris water maze performance following traumatic brain injury.

Cognitive impairment is a significant pathological outcome with both human and rodent cortical impact TBI. In order to assess the ability of Pioglitazone to reduce cognitive impairment following injury, animals were administered vehicle, Pioglitazone, or Pioglitazone plus the PPARγ antagonist T0070907 with an initial injection at 15 min post-injury and subsequent injections at 24h, 48h, 72h and 96h after the first injection. Pioglitazone and T0070907 were administered at 10 mg/kg at every dose. Morris water maze (MWM) assessments were performed 10 days post-injury and consisted of 4 days of trials. Following repeated measures analysis of the MWM results, a significant effect of day (p<0.0001) and a trend towards effect of treatment (latency p=0.0693; distance p=0.0648) were observed. To further elucidate the effect of Pioglitazone on MWM behavior and increase statistical power, groups were collapsed across days. When averaged across all testing days, the latency and distance to the platform showed significant effects. Injured vehicle-treated animals showed impairments in both distance (Figure 4.2A, *=p<0.05) and latency (Figure 4.2B, *=p<0.05) to the platform and treatment with Pioglitazone significantly improved behavior. Treatment with the PPARγ antagonist T0070907 and Pioglitazone together prevented the beneficial effects of Pioglitazone following injury (Figure 4.2 p<0.05 Sham vs. Pio+Ant) since animals given the antagonist with Pioglitazone performed similarly to injured vehicle-treated animals, indicating direct involvement of the PPARγ receptor. No significant differences in swim speed were observed among the treatment groups, suggesting that the differences in MWM performance were not related to motor deficits.
Figure 4.2. Pioglitazone treatment reduces cognitive impairment following traumatic brain injury.

With this model of TBI there are impairments in Morris Water Maze (MWM) performance following the injury. Pioglitazone was administered for 5 days following the injury in order to determine if treatment with Pioglitazone is capable of preventing cognitive impairment with the MWM test. Treatment with Pioglitazone produced a significant reduction in both the distance and latency to find the platform following TBI. Additionally, animals which were also treated with the PPARγ antagonist T0070907 exhibited no preservation of function indicating that PPARγ receptor activation is required for Pioglitazone’s ability to preserve cognitive functioning.

(* p<0.05 by one-way ANOVA).
Pioglitazone treatment reduces cortical damage following traumatic brain injury.

Quantification of the cortical lesion was utilized to assess the efficacy of Pioglitazone to reduce the cortical tissue loss which occurs following CCI brain injury. Administration of Pioglitazone and the antagonist T0070907 was conducted in the same manner as for the behavioral analysis, with injections of 10mg/kg beginning 15 minutes after the injury and continuing at 24h, 48h, 72h and 96h after the first injection. A moderate CCI injury (1.5 mm) resulted in a significant loss of cortical tissue (Figure 4.3). At 16 days post-injury, the cortical contusion volume in the vehicle-treated control group was measured to be 5.09±0.73mm$^3$ (Figure 4.3B). Treatment with Pioglitazone resulted in reduction of the lesion size by 55% to 2.27±0.27mm$^3$ which was significantly smaller compared to the vehicle treated TBI group (Figure 4.3B, *=p<0.05). Co-treatment of Pioglitazone with the PPARγ antagonist T0070907 abolished the effect seen with Pioglitazone treatment alone. Animals that were treated with Pioglitazone and the antagonist had an average lesion size of 4.32±0.60 mm$^3$ which was larger than the lesion size in the Pioglitazone group (Figure 4.3B, *=p<0.05 Pio vs. Pio+Ant), suggesting that the PPARγ receptor activation is involved in Pioglitazone’s ability to spare cortical tissue. Administration of the PPARγ antagonist alone following the injury did not affect the size of the cortical lesion.
Following a cortical impact brain injury there is significant loss of cortical tissue which occurs in the days and weeks following the injury. Pioglitazone was administered for 5 days following a unilateral cortical contusion in order to access the ability of Pioglitazone to reduce cortical damage. In these studies Pioglitazone treatment reduced the size of the lesion which occurs in this CCI rat model of TBI. These studies also indicate that activation of the PPARγ receptor is required for this aspect of Pioglitazone’s neuroprotective function since treatment with the PPARγ antagonist blocked the therapeutic effect. A. Representative coronal sections stained with neutral red for animals treated with vehicle, Pioglitazone, or T0070907 and Pioglitazone at 16 days post-injury. B. Lesion volume quantification showed a significantly reduced lesion volume in Pioglitazone-treated CCI animals when compared with the vehicle group and antagonist treatment prevented the effect (*=p<0.05, one-way ANOVA with SNK post-test).
Pioglitazone attenuates the neuroinflammatory response after traumatic brain injury.

Following TBI there is a significant increase in microglial activation and inflammation and Pioglitazone was administered in attempts to reduce post-injury inflammation. At 16 days after TBI, Pioglitazone-treated rats showed significantly fewer OX-42 positive cells in the injured cortex adjacent to the injury site, suggesting a reduction in both microglial activation and macrophage infiltration. The effect of Pioglitazone on inhibiting the inflammatory process was assessed by stereological quantification of the activated microglia. OX-42 immunoreactive cells exhibited two morphologies: resting microglia with small cell body and multiple thin processes and activated microglia with enlarged amoeboid cell body and pyknic processes. Following injury there was a significant increase in the number of activated microglia (p<0.05 Figure 4.4B). In the injured cortex, the total number of activated microglia was reduced in the Pioglitazone-treated group compared vehicle treatment (Figure 4.4B). Compared with animals which received a sham injury, animals treated with Pioglitazone did not show a significant increase in the number OX-42 positive cells. Treatment with the PPARγ antagonist T0070907 did not prevent the effect of Pioglitazone on preventing the increase in inflammation, indicating that the effect was not dependent upon the PPARγ receptor.
Figure 4.4. Pioglitazone reduces post-injury microglial activation following traumatic brain injury.

With this cortical contusion model of TBI there is significant inflammation which occurs following the injury. Pioglitazone was administered for 5 days following the injury in order to assess its ability to reduce inflammation following TBI. In this model, Pioglitazone attenuated the neuroinflammatory response in the injured animals. These studies also show that Pioglitazone does not require the PPARγ receptor in order to reduce inflammation following injury since treatment with the PPARγ inhibitor T0070907 did not block Pioglitazone’s neuroprotective effects. A. Immunohistochemistry for OX-42 was shown in cerebral cortex adjacent to the cortical lesion. The bottom images are high magnification views of the boxed regions. Scale bar = 100µm. B. Total number of activated microglia was counted in the cortex and a reduction in the number of activated microglia was found in Pioglitazone treated animals compared to vehicle treated animals. Compared with sham animals, Pioglitazone treated animals exhibited no significant increase in activated microglia.

* p<0.05 by one-way ANOVA with SNK post-test.
Discussion:

Previous reports from our group have shown that following TBI there is significant impairment of cortical mitochondria which begins acutely and substantially damages the mitochondria over the course of the first day following the injury (Pandya et al., 2007, Gilmer et al., 2009, Pandya et al., 2009). Interventions that are capable of protecting mitochondrial function following TBI have been demonstrated to reduce cortical damage and behavioral impairment (Scheff and Sullivan, 1999, Sullivan et al., 2000c, Pandya et al., 2007, Pandya et al., 2009). In the present studies, mitochondrial function was analyzed one day following the injury. We chose this time point based on previous experiments which have shown that at this time post-injury there is significant mitochondrial dysfunction in our model of cortical impact injury (Pandya et al., 2009). We found that with Pioglitazone treatment there are improvements in mitochondrial function which are observed in the Complex-I dependant, Pyruvate/Malate-driven, rates of oxygen consumption as well as the State III (ADP-present) and maximal (uncoupled) respiration rates, a measure of electron transport capacity/reserve (Figure 4.1B). From the mitochondrial experiments it can be concluded that animals treated with Pioglitazone maintain more functional mitochondria which are capable of producing more ATP and therefore providing the brain with the much needed energy source required for normal function as well as post-injury protective and reparative mechanisms. The mitochondrial data from these experiments also suggests that at least part of Pioglitazone’s ability to improve mitochondrial function involves a direct effect on the mitochondria since a single 15 minute post injury injection only produced a non-significant trend towards improved mitochondrial bioenergetics (Figure 4.1A) while a subsequent injection of Pioglitazone one hour prior to analyzing the tissue resulted in a dramatic increase in mitochondrial bioenergetic function (Figure 4.1B). Since the improvement in mitochondrial function was
only observed after giving the second injection, the mitochondrial effect is most likely not
due to only an up regulation of protein expression. Previously published in vitro
experiments have shown that treatment with Pioglitazone can up regulate mitochondrial
electron transport proteins, however, these changes take at least two days to produce
significant increases in protein levels (Miglio et al., 2009). Up regulation of mitochondrial
protein expression is not likely the cause for the improvements in mitochondrial function
seen in these experiments after the first 24 hours of Pioglitazone treatment, especially
considering the rapid change in mitochondrial function observed one hour following the
second Pioglitazone treatment. In order to help in determining whether Pioglitazone has
a direct effect on mitochondrial function, future experiments should incorporate
modifications to the current treatment and testing paradigm. Similar to the testing
following the second injection of Pioglitazone at 24 hours post injury, further
experimentation should include analysis of mitochondrial function one hour following the
first 15 minute post injury injection. Since the majority of the Pioglitazone should be
metabolized from the body 25 hours after a single injection, the lack of a therapeutic
effect on mitochondrial function following the single injection paradigm could simply be
that the Pioglitazone is no longer in the system. Treatment with Pioglitazone will likely
produce even greater improvements in mitochondrial function at time points beyond 24
hours when significant increases in mitochondrial proteins are likely occur. The rapid
increase in mitochondrial function seen after the second injection of Pioglitazone may
involve the mitochondrial outer membrane bound protein MitoNEET. Previously it has
been shown that Pioglitazone binds to MitoNEET and stabilizes its conformational
structure (Paddock et al., 2007) and that knocking-out the MitoNEET protein results in
reductions in mitochondrial electron transport chain function (Wiley et al., 2007). In
addition to Pioglitazone’s ability to rapidly increase mitochondrial function as seen in
these studies, considering that PPAR agonist treatment leads to reductions in oxidative
damage (Chen et al., 2007a, Yi et al., 2008) and inflammation (Besson et al., 2005, Chen et al., 2007a, Park et al., 2007, Hyong et al., 2008, Kapadia et al., 2008) following injury, Pioglitazone treatment will promote a less hostile cellular environment which will lead to reductions in secondary injury cascades that cause further mitochondrial dysfunction and propagate bioenergetic impairments.

To further understand the effects of Pioglitazone following TBI, we wanted to determine if Pioglitazone was capable of reducing lesion volume and improving cognition. PPARγ agonists, in particular Pioglitazone, have been examined for protective properties in several models of CNS injury and disease (Sundararajan et al., 2006), including spinal cord injury. Following spinal cord injury, Pioglitazone offers protection against the induction of inflammatory genes, astrogliosis, and microgliosis even if treatment is delayed for up to two hours after the injury (Park et al., 2007). The neuroprotective actions of Pioglitazone may be a general characteristic of PPARγ activation, as this phenomenon has been noted in other models of CNS injury. For instance, Pioglitazone treatment in murine models of Parkinson’s disease promoted neuronal sparing within the substantia nigra (Breidert et al., 2002, Dehmer et al., 2004). Potent neuroprotection of motor neurons was also induced by Pioglitazone in transgenic mouse models of amyotrophic lateral sclerosis (Kiaei, 2008). Additionally, several studies have detected enhanced neuroprotection and decreased lesion sizes in animal models of stroke and intracerebral hemorrhage (Ou et al., 2006, Victor et al., 2006). To determine if any measurable effect on cognition or lesion volume was the result of activation of the PPARγ pathway, the selective PPARγ antagonist T0070907 was co-administered with Pioglitazone. It has previously been shown that following TBI the injury causes cortical damage which correlates well with cognitive dysfunction as measured using the MWM (Marklund et al., 2001). From these studies it was determined that
Pioglitazone is capable of preserving cognitive function, as determined by MWM assessment. It was observed at 15 days post injury that animals treated with Pioglitazone exhibited a decreased latency time and distance to the platform compared to animals treated with either vehicle or animals treated with Pioglitazone and the PPARγ antagonist T0070907 together (Figure 4.2). Since the PPARγ antagonist was able to block the protective effects of Pioglitazone on MWM function, activation of the PPARγ receptor is further implicated as a beneficial therapeutic strategy for TBI. Additionally, treatment with Pioglitazone resulted in a reduction of the size of the cortical lesion at 16 days post injury with the size of the cortical cavity being reduced from 5.09±0.73mm³ (vehicle treated animals) to 2.27±0.27mm³ (Pioglitazone treated animals) (Figure 4.3). Similar to the MWM testing, administration of the PPARγ antagonist T0070907 almost completely blocked the neuroprotective benefit of Pioglitazone on sparing cortical tissue, indicating that activation of the PPARγ pathway is critical to attenuating cortical tissue loss as well as cognitive deficits. As has been observed in other CNS injury models, these studies show that following TBI Pioglitazone is capable of reducing neuronal cell loss and improving behavioral outcomes.

It has been established that TBI is accompanied by a dramatic inflammatory response, which escalates over the first week post-injury and is thought to contribute to the secondary pathology of TBI. Multiple studies link agonism of PPARγ to the attenuation of inflammation (Jiang et al., 1998, Ricote et al., 1998, Drew et al., 2006), such that PPARγ activation influences the development and intensity of the inflammatory response. With the realization that inflammation plays a role in several neurodegenerative diseases, researchers have searched for a role of PPARγ in neurodegeneration. PPARγ activation regulates inflammation by decreasing the expression of a variety of pro-inflammatory genes such as COX-2, iNOS, and several
cytokines (Jiang et al., 1998, Ricote et al., 1998, Kitamura et al., 1999, Bernardo et al., 2000) that have all been associated with inflammation-induced neurodegeneration (Banati et al., 1993, Przedborski et al., 1996, Minghetti and Levi, 1998, Liberatore et al., 1999, Heneka et al., 2000, Arimoto and Bing, 2003, Vijitruth, 2006). Previously it has been shown that Pioglitazone protects against intrastriatal lipopolysaccharide (LPS)-induced neurodegeneration by suppressing the inflammatory response, reducing oxidative damage and preventing mitochondrial dysfunction (Hunter et al., 2008, Xing et al., 2008). Since evidence shows that PPARγ is expressed throughout the brain (Moreno et al., 2004) in neurons (Braissant et al., 1996) and glia (Cullingford et al., 1998, Heneka et al., 1999, Bernardo et al., 2000, Cristiano et al., 2001), it is possible that PPARγ agonism will inhibit neuroinflammation and neurodegeneration in multiple injury and disease states. Because of its wide range of potential therapeutic efficacy, several clinical trials using synthetic PPAR agonists have begun for the treatment of diseases involving aberrant or chronic immune/inflammatory responses (Pershadsingh et al., 2004, Risner et al., 2006).

To further elucidate the benefits of Pioglitazone following TBI, tissue was analyzed for assessment of inflammation following injury. As has been previously shown for other PPARα (Chen et al., 2007a, Chen et al., 2008) and PPARγ (Yi et al., 2008) agonists, treatment with Pioglitazone resulted in a significant reduction in inflammation following TBI in the present study. Following treatment with Pioglitazone, reductions in the number of activated microglia were observed in the area of the cortex adjacent to the site of the cortical lesion (Figure 4.4). In contrast to our data indicating that the effect of Pioglitazone on the cortical lesion and cognitive function is dependent on the PPARγ receptor, treatment with the PPARγ antagonist along with Pioglitazone did not prevent the reduction in activated microglia in these experiments. These studies indicate that
Pioglitazone’s ability to reduce inflammation following TBI is not dependent upon PPARγ receptor activation. Even though Pioglitazone is predominately a PPARγ agonist, Pioglitazone has been shown to also activate the PPARα receptor (Sakamoto et al., 2000). Considering that activation of the PPARα receptor has previously been shown to reduce microglial activation (Xu et al., 2005) it is possible that Pioglitazone’s ability to reduce inflammation following TBI can be through activation of the PPARα pathway. Additionally, since mitochondrial dysfunction can lead to cell death and cause increases in inflammation, it is expected that the protective effects of pioglitazone on mitochondrial function (Figure 3.1B) are likely to lead to further reductions in post-injury increases in inflammation. Regardless of the exact mechanism(s) by which Pioglitazone leads to reductions in inflammation, the evidence from this study and studies in SCI (Park et al., 2007) indicate that Pioglitazone treatment leads to significant reductions in inflammation following injury. In order to further the understanding of how Pioglitazone leads to a reduced inflammatory response following TBI, future experiments should seek to elucidate the role of other PPAR receptors in pioglitazone’s neuroprotective effect. As previously mentioned, since Pioglitazone can activate PPARα, further working utilizing selective PPARα inhibitors or knock-out animals are needed to determine the role of the pathway in pioglitazone’s ability to reduce inflammation. Since at this time only the PPARγ pathways can be excluded from playing a role in the anti-inflammatory actions of Pioglitazone it is unknown whether the effect is mediated by a different PPAR pathway or a completely different mechanism.

These experiments are the first to show a therapeutic effect of Pioglitazone following TBI and they fit well with what has been previous shown regarding the use of PPAR agonists in CNS injury. As has been observed in SCI (McTigue et al., 2007), Pioglitazone is capable of reducing the cortical lesion and improving behavioral outcome.
following injury. These experiments also produce similar findings to studies that have utilized either PPARα or PPARγ agonists following TBI that produce reductions in inflammation (Chen et al., 2007a, Yi et al., 2008), reduction in lesion volume (Yi et al., 2008), and behavioral improvement (Chen et al., 2007a, Chen et al., 2008). This is the first report to our knowledge of any PPAR agonist showing protection of mitochondrial function following TBI. These experiments show that following TBI Pioglitazone is capable of protecting mitochondria, reducing inflammation, minimizing the cortical lesion, and improving cognitive function. Given the results of these experiments and considering that Pioglitazone has been shown to have better BBB permeability than the other PPARγ agonist Rosiglitazone (Berger and Moller, 2002), as well as partially activate the PPARα pathway (Sakamoto et al., 2000), which has been shown to be efficacious following TBI (Chen et al., 2007a, Chen et al., 2008), we feel that Pioglitazone will be a more effective treatment for TBI than other PPAR agonists previously utilized for the treatment of TBI.

Even with the success of the current studies, further work is needed before Pioglitazone should be considered for human clinical usage. Of significant important, the current studies only utilized a single dose of Pioglitazone and began administration 15 minutes after the injury. Both a dose response study and a delayed administration study are needed in order to properly plan a human trial and determine if administration can be delayed longer than 15 minutes, which would be needed for nearly any human treatment window. Additionally, given the heterogeneity of human TBI it would be beneficial if Pioglitazone was tested in multiple different experimental TBI models. Though evidence from other models and similar PPAR agonists suggest that Pioglitazone would be effective for different types of TBIs without empirical evidence directly testing Pioglitazone it is unknown whether it would have any effect or even be potentially
detrimental to specific pathological subtypes. Without testing Pioglitazone in humans it will never be known whether or not it will be beneficial at treating human TBI. However, in order to provide any future clinical trials with the best possible chance of success much more research is needed regarding dosing and differences in efficacy with different types of TBI pathology.
Chapter 5
Summary and conclusions

Further elucidating of the role of both toxins and treatments in traumatic brain injury will help in understanding the pathology which occurs following neurological insults and how to potentially intervene in order to minimize damage. Beyond just the direct and acute deficits which have been shown to occur after traumatic brain injury (Sullivan et al., 1999c, Sullivan et al., 2000a, Sullivan et al., 2000c, Sullivan et al., 2002, Sullivan et al., 2003), a strong link has been shown between history of a brain injury and the development of Parkinson’s disease later in life (Nayernouri, 1985, Bower et al., 2003). Though TBI is linked to PD, it is currently unknown if traumatic brain injury alone is capable of leading to Parkinson’s and evidence from both humans (Semchuk et al., 1993, Carvey et al., 2006) and animals (Thiruchelvam et al., 2000a, Thiruchelvam et al., 2000b, Thiruchelvam et al., 2002, Thiruchelvam et al., 2003, Ling et al., 2004a, Ling et al., 2004b, Fei and Ethell, 2008) suggests that multifactorial paradigms may play a role in disease development.

Like many of the environmental toxins linked to the development of PD in humans, trichloroethylene has been shown to produce neuropathology in animal studies (Gash et al., 2008, Liu et al., 2010) and human investigation has indicated a link between chronic TCE exposure and the development of PD (Gash et al., 2008). Studies utilizing dual injury paradigms should help in further understanding the development of PD in humans by attempting to mimic the complex history of exposures and insults which a person would be exposed to during his/her life. Even with evidence from dual injury models, much work is still needed to fully understand the complexities of disease.
development in humans since many factors have been shown to play a role in disease development.

The studies presented here, utilizing trichloroethylene exposure followed by traumatic brain injury, provide an initial indication that the two insults may in fact be capable producing a dual injury phenotype. However, the model does not result in changes which mimic severe human PD. Behavioral analysis revealed that only following exposure to TCE and a moderate TBI was impairment with both the rotarod and cylinder test was present (Figure 2.1). Though behavioral dysfunction was observed it still remains unknown what the exact cause of the behavioral impairment is since various CNS and non-CNS factors can play a role in these measurements. It was hypothesized that following the dual injury there would be a much greater loss of dopamine neurons in the substantia nigra and dopaminergic impairment in the striatum than was observed in these studies. One month following the dual injury there was a loss of tyrosine hydroxylase positive neurons in the substantia nigra but the cell loss was only about 13-17% (Figure 2.2). Given evidence from humans (Riederer and Wuketich, 1976, Morrish et al., 1996), this level of cell loss should not be capable of producing behavioral impairment independently which suggests a role for the brain injury, the dual injury, or both in the behavioral impairment. Additionally, no observable changes were observed in the striatal dopaminergic system one month following the dual injury (Figure 2.3) which indicates that excessive loss of dopaminergic fibers and specific synaptic proteins has not occurred in this model. As expected for a moderate traumatic brain injury, there was a persistent loss of cortical tissue following the injury which was not affected by exposure to TCE (Figure 2.4). Mitochondrial impairment was observed in the striatum following two week exposure to TCE, however, the level of impairment was great enough with TCE alone that observing a dual injury affect was made nearly
impossible (Figure 2.5). Further studies utilizing a 1 week exposure to TCE showed no measurable decrease in mitochondrial function following either insult alone. However, exposure to TCE for 1 week followed by a TBI resulted in a 50% reduction in Complex I driven mitochondrial oxygen consumption. From the experiment utilizing the 1 week exposure to TCE it is further concluded that TCE and TBI can interact in a dual injury model and that the mitochondria play a pivotal role in the interaction.

Though the behavioral testing does not target a specific single pathway which the neurological dysfunction can be said to originate from, the data still suggests that a dual injury plays a role in the dysfunction. Neither exposure to TCE or TBI alone resulted in any measurable functional impairment but when TCE and a moderate TBI were combined together impairment did occur. Further experimentation is required to elucidate the pathological mechanisms which lead to the functional impairment. Two scenarios exist which could underlie the neurological changes which resulted in functional changes. First, the two insults could affect the same brain region(s) and this synergistic targeting could be responsible for the dysfunction. Second, the two insults could affect different regions of the brain individually and the presence of neurological dysfunction in these different brain regions may be the cause of the functional impairment. The cortex, striatum, and substantia nigra have all been shown to have some deficits in this model, some single insult based and some dual insult based, and given that all three of these regions play a role in dopaminergic function (Figure 1.5) it remains plausible that multiple brain regions may need to be affected in order for functional impairment to occur in this model.

In addition to indicating the possibility of a dual injury mechanism, the mitochondrial studies further support the notion that systemic exposure to TCE can result in mitochondrial impairment in the brain. Even though previous work (Gash et al.,
2008) resulted in a different regional profile for the mitochondrial impairment than the present experiments, these studies utilized total purified mitochondria while previous work utilized synaptosomes for the mitochondrial analysis. It has been previously established that significant differences exist between synaptic and non-synaptic mitochondria in the brain (Davey et al., 1997, Brown et al., 2006, Naga et al., 2007) and this difference may underlie the seemingly conflicting results. Regardless, the analysis of the striatal mitochondria revealed a very significant impairment of mitochondrial function which was present in both groups exposed to TCE for 2 weeks (Figure 2.5).

In order to further understand the ability for TCE to produce regionally different mitochondrial impairment, it is important to understand regional differences in mitochondrial function and susceptibility to toxins. Previous animal studies (Ryder, 1980, Sims, 1991, Davey et al., 1997), and work utilizing animals exposed to trichloroethylene (Gash et al., 2008, Liu et al., 2010) or other mitochondrial toxins (Beal et al., 1993, Mirandola et al., 2010) have shown that regional differences in mitochondrial function and toxin susceptibility do exist. However, the reasons for the regional differences in mitochondrial dysfunction remain elusive. The present studies with naïve mitochondria suggest that with the exception of the cerebellum no major differences exist in mitochondria throughout the cortex, hippocampus, and striatum with respect to Complex I and Complex II dependent function (Figure 3.1). It has been established in vivo that different brain regions have different metabolic demands (Kennedy et al., 1976, Wong-Riley, 1979) and neuronal activity affects the amount of mitochondria and mitochondrial proteins (Tieman, 1985, Nie and Wong-Riley, 1996) and these differences likely have little to do with the function of individual mitochondria. When the amount of mitochondria is controlled for by normalizing to protein content, regional differences in mitochondrial function are not strikingly different. This finding extends to mitochondrial Complex I and
Complex II inhibitors were no differences are seen in the inhibition of enzyme activity (Figure 3.2) and only small differences in oxygen consumption are seen at low concentrations, 10pM and 100pM, of rotenone (Figure 3.3). These findings suggest that the regional differences in mitochondrial dysfunction shown following exposure to TCE may not be the result of regionally different individual mitochondria. Mirandola et al. suggested that the regional differences in 3-NP induced changes in the susceptibility to mitochondrial permeability transition may be the result of regional differences in the environment which the mitochondria exist in (Mirandola et al., 2010). Interestingly, Mirandola found the striatum to be the region most susceptible to 3-NP inhibition (Mirandola et al., 2010) and this is the same region most affected by TCE in these studies. Given the lack of overt regional differences in basal function and susceptibility to inhibitors in purified naïve mitochondria from the cortex, hippocampus, and striatum the most likely conclusion is that the mitochondria are in fact functionally very similar across brain regions. Given that regional differences in mitochondrial function following exposure to TCE do exist, but the affected and non-affected mitochondria are likely the same, interventions which target the pathways responsible for making one region more susceptible than another region may be effective at ameliorating regionally specific deficits.

In addition to the possibility to target pathways which may make a specific brain region more susceptible to a systemic toxin, therapeutically targeting one part of a dual injury insult may provide the ability avoid or minimize the occurrence of functional impairment. Given that traumatic brain injury plays a role in our dual injury model, developing effective therapeutics to treat the brain injury is likely to provide two benefits. First, if an effective TBI therapeutic is developed TBI patients should receive benefit regardless of the presence of an additional insult. Second, if the patient already has an
initial insult or sustains a second insult later in life, effectively treating the TBI may prevent the development of dual insult based pathology.

The studies utilizing Pioglitazone reveal that not only is the drug capable of minimizing some of the mitochondrial dysfunction, the effect is likely to involve a non-PPARγ mediated pathway (Figure 3.1). Additionally, cognitive testing showed that animals treated with Pioglitazone were not significantly different than sham animals while vehicle treated animals were significantly different (Figure 3.2). The behavioral effects suggest that Pioglitazone does provide protection against cognitive impairment and finding an optimal dosing strategy should provide more robust cognitive benefits. Analysis of cortical tissue loss and increases in the number of activated microglia show that Pioglitazone is able to reduce both of these endpoints following TBI even though tissue loss was PPARγ dependent while microglial activation was not. This initial report of the therapeutic benefit of Pioglitazone will hopefully lead to further work which can help human patients who sustain a brain injury. Further work should be done to better understand the optimal dosing of the drug but the potential for a therapeutic benefit has now been shown with these studies.

The findings of the three separate projects come together to suggest a role for toxin, treatments, and brain regions following traumatic brain injury and dual injury models. The overall conclusion is that TCE and TBI are able to interact and produce behavioral dysfunction and a loss of TH-positive neurons in the substantia nigra, however, more work needs to be done refining the experimental model to understand the impact these findings have on Parkinson's disease. The mitochondrial impairment in the striatum following TCE exposure is likely the result of regional differences in the cellular environment within the striatum which these mitochondria exist. The conclusions about the regional TCE effect are made possible because exposure to the complex I
inhibitor rotenone resulted in only small regional differences in mitochondrial oxygen consumption across the brain, specifically at low concentrations of 10pM and 100pM. Furthermore, it is hypothesized that treating a single insult in a dual injury paradigm will prevent the dual injury phenotype, and so effective therapeutics for one of the single insults are necessary to test this hypothesis. Given that Pioglitazone can attenuate pathology following TBI, and the role TBI plays in this dual injury model, future work may show if Pioglitazone is effective at attenuating deficits observed with this dual injury. The studies presented here should assist future research in understanding how toxins and treatments may affect traumatic brain injury and the role or lack thereof in regional mitochondrial function in affecting outcome.
References:


Alston TA, Mela L, Bright HJ (1977) 3-Nitropropionate, the toxic substance of Indigofera, is a suicide inactivator of succinate dehydrogenase. Proc Natl Acad Sci U S A 74:3767-3771.


Chen XR, Besson VC, Beziaud T, Plotkine M, Marchand-Leroux C (2008) Combination therapy with fenofibrate, a peroxisome proliferator-activated receptor alpha agonist, and


Hicks SP (1950) Brain metabolism in vivo; the distribution of lesions caused by cyanide poisoning, insulin hypoglycemia, asphyxia in nitrogen and fluoroacetate poisoning in rats. AMA Arch Pathol 49:111-137, illust.


ANDREW SAUERBECK, B.S., Ph.D.

436 Biomedical & Biological Sciences Research Building (BBSRB)
741 South Limestone Street
Spinal Cord & Brain Injury Research Center (SCoBIRC) and
The Department of Anatomy & Neurobiology
University of Kentucky Chandler Medical Center
Lexington, KY  40536-0509
(859)323-4682 FAX (859)257-5737
Adsaue2@uky.edu

Date of Birth:  July 15, 1982
Born:   Whitesburg, Kentucky
Nationality:  U.S. Citizen

EDUCATION

2005   B.S., University of Kentucky
       (Biology)
2010   Clinical and Translational Sciences training certificate
2011   Ph.D., University of Kentucky
       (Anatomy and Neurobiology)

PROFESSIONAL EXPERIENCE

2005-Present   Graduate Student (Ph.D.); Department of Anatomy & Neurobiology, University of Kentucky.
2004-2005  Undergraduate Researcher; College of Pharmacy, University of Kentucky
2004  Undergraduate Researcher; College of Pharmacy, University of Kentucky, Summer Undergraduate Research Program

AWARDS AND HONORS

2009-2011  Kentucky Opportunities Fellowship
2006-2009  Neurobiology of Aging Training Grant (T32 AG00242)
2007   Poster Award for: A Multifactorial Model for Parkinson’s Disease: Traumatic Brain Injury and Trichloroethylene Exposure
2005-2010  Daniel R. Reedy Quality Achievement Fellowship
2005   Otis A. Singletary Fellowship for Graduate or Professional Study
2005   University of Kentucky Undergraduate Research Fellowship
2005   Graduated Magna Cum Laude (University of Kentucky, Biology B.S.)
2001-2005  Kentucky Educational Enrichment Scholarship
2001-2005  Kentucky Academic Excellence Scholarship
2001-2002  Kentucky Mining Engineering Scholarship
2001-2002  Joy Mining Machinery Scholarship
2001-2004  University of Kentucky Dean’s List

GRANTS

2008  University of Kentucky, Clinical Translational Sciences Seed Grant
Primary Author: Andrew Sauerbeck, PI: Patrick Sullivan (Mentor)

RESEARCH INTERESTES

• The interactions between traumatic brain injury and trichloroethylene exposure which can predispose an individual to develop symptoms of Parkinson’s disease and a worse outcome following traumatic brain injury.
• Role of mitochondrial dysfunction in synergistic interaction of trichloroethylene and traumatic brain injury
• Regional differences in mitochondrial function and susceptibility to mitochondrial toxins.
• Usage of PPARγ agonist Pioglitazone as a therapeutic for traumatic brain injury.
• Therapeutic interventions for CNS Injury targeting the secondary injury cascade.
• Being able to move therapeutics into clinical testing.

PROFESSIONAL SYMPOSIA AND WORKSHOPS ATTENDED

2010  National Neurotrauma Conference, Las Vegas, NV
2009  Neuroscience Day, Bluegrass Chapter of the Society for Neuroscience, Lexington, KY
<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>15th Annual Kentucky Spinal Cord and Head Injury Research Symposium,</td>
</tr>
<tr>
<td></td>
<td>University of Louisville, Louisville, KY</td>
</tr>
<tr>
<td>2009</td>
<td>Center for Clinical and Translational Sciences Conference, University of Kentucky, Lexington, KY</td>
</tr>
<tr>
<td>2009</td>
<td>National Neurotrauma Society Conference, Santa Barbara, CA</td>
</tr>
<tr>
<td>2008</td>
<td>14th Annual Kentucky Spinal Cord and Head Injury Research Symposium, University of Kentucky, Lexington, KY</td>
</tr>
<tr>
<td>2008</td>
<td>Society for Neuroscience Conference, Washington DC</td>
</tr>
<tr>
<td>2007</td>
<td>Society for Neuroscience Conference, San Diego, CA</td>
</tr>
<tr>
<td>2007</td>
<td>National Neurotrauma Society Conference, Kansas City, MO.</td>
</tr>
<tr>
<td>2007</td>
<td>13th Annual Kentucky Spinal Cord and Head Injury Research Symposium, University of Louisville, Louisville, KY</td>
</tr>
<tr>
<td>2007</td>
<td>Neuroscience Day, Bluegrass Chapter of the Society for Neuroscience, Lexington, KY</td>
</tr>
<tr>
<td>2006</td>
<td>12th Annual Kentucky Spinal Cord and Head Injury Research Symposium, University of Kentucky, Lexington, KY</td>
</tr>
<tr>
<td>2005</td>
<td>Mitochondrial Research Symposium, University of Kentucky, Lexington, KY</td>
</tr>
</tbody>
</table>

**PUBLICATIONS**

**Peer-reviewed journal articles:**

A.D. Sauerbeck, J. Gao, R. Readnower, Liu M, J. Pauly, G. Bing, P.G. Sullivan

 Pioglitazone attenuates mitochondrial dysfunction, cognitive impairment, cortical tissue loss, and inflammation following traumatic brain injury. Exp Neurol. 2010 Oct 20

**Published abstracts:**

A.D. Sauerbeck, R. Readnower, J. Gao, J. Pauly, G. Bing, P.G. Sullivan


A.D. Sauerbeck, R. Hunter, G. Bing, D.M. Gash, P.G. Sullivan


A.D. Sauerbeck, R. Hunter, G. Bing, D.M. Gash, P.G. Sullivan


A.D. Sauerbeck, R. Hunter, G. Bing, D.M. Gash, P.G. Sullivan

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>National Neurotrauma Society Conference, Las Vegas, NV</td>
<td>Title: Therapeutic efficacy of Pioglitazone following traumatic brain injury</td>
</tr>
<tr>
<td>2009</td>
<td>National Neurotrauma Society Conference, Santa Barbara, CA</td>
<td>Title: A Dual Injury Synergistic Model of Brain Injury Which Leads to Parkinsonism: Trichloroethylene and Traumatic Brain Injury</td>
</tr>
<tr>
<td>2009</td>
<td>Kentucky Spinal Cord and Head Injury Research Symposium, Lexington, KY.</td>
<td>Title: A Dual Injury Synergistic Model of Brain Injury Which Leads to Parkinsonism: Trichloroethylene and Traumatic Brain Injury</td>
</tr>
<tr>
<td>2009</td>
<td>Clinical and Translational Sciences Conference, Lexington, KY</td>
<td>Title: The Synergistic Interaction of Trichloroethylene and Traumatic Brain Injury Results in Parkinsonism</td>
</tr>
<tr>
<td>2009</td>
<td>Neuroscience Day, Bluegrass Chapter of the Society for Neuroscience, Lexington, Ky</td>
<td>Title: The Synergistic Interaction of Trichloroethylene and Traumatic Brain Injury Results in Parkinsonism</td>
</tr>
<tr>
<td>2008</td>
<td>Kentucky Spinal Cord and Head Injury Research Symposium, Lexington, KY.</td>
<td>Title: A New Dual Injury Model of Parkinson’s Disease, Trichloroethylene Exposure And Traumatic Brain Injury: More is Not Always Better</td>
</tr>
<tr>
<td>2007</td>
<td>Society for Neuroscience Conference, San Diego, CA.</td>
<td>Title: Synergy Between Traumatic Brain Injury and Trichloroethylene Exposure: a Model for Parkinson’s Disease</td>
</tr>
<tr>
<td>2007</td>
<td>National Neurotrauma Society Conference, Kansas City, MO.</td>
<td>Title: A Multifactorial Model for Parkinson’s Disease: Traumatic Brain Injury and Trichloroethylene Exposure</td>
</tr>
<tr>
<td>2007</td>
<td>Neuroscience Day, Bluegrass Chapter of the Society for Neuroscience, Lexington, Ky</td>
<td>Title: A Multifactorial Model for Parkinson’s Disease: Traumatic Brain Injury and Trichloroethylene Exposure</td>
</tr>
<tr>
<td>2004</td>
<td>College of Pharmacy Undergraduate Research Symposium</td>
<td>Title: Assay Human P450 Reductase Mutants for Activity Utilizing the Ames Test</td>
</tr>
</tbody>
</table>