THE ROLE OF APOLIPOPROTEIN E IN RECOVERY FROM TRAUMATIC BRAIN INJURY AND DEVELOPMENT OF CHIMERA: A NOVEL CLOSED-HEAD IMPACT MODEL OF ENGINEERED ROTATIONAL ACCELERATION

by

Dhananjay Rajaram Namjoshi

M.Sc., The University of British Columbia, 2008
M.Pharm., The University of Mumbai, 2003
B.Pharm., The University of Mumbai, 1999

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies
(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

February 2015

© Dhananjay Rajaram Namjoshi, 2015
Abstract

Traumatic brain injury (TBI) is a “silent epidemic” that currently lacks any effective treatment. While a major health care problem in itself, TBI also increases Alzheimer’s disease (AD) risk and leads to the deposition of neurofibrillary tangles and amyloid deposits similar to those found in AD. Agonists of Liver X receptors (LXRs), which regulate the expression of many genes involved in lipid homeostasis and inflammation, improve cognition and reduce neuropathology in AD mice. One pathway by which LXR agonists exert their beneficial effects is through ATP-binding cassette transporter A1-mediated lipid transport onto apolipoprotein E (apoE). In the first part of this thesis, I show that a short-term treatment with synthetic LXR agonist GW3965 improves post-injury outcomes in mice subjected to closed-head, mild, repetitive weight drop TBI (mrTBI). My results suggest that both apoE-dependent and apoE-independent pathways contribute to the ability of GW3965 to promote recovery from mrTBI. While many drugs have shown promising outcomes in preclinical TBI models, clinical drug trials for TBI so far have failed, suggesting that the translational potential of TBI models may require further improvement. As most human TBIs result from impact to an intact skull, closed head injury (CHI) rodent models are highly relevant. Traditional CHI models like weight drop however suffer from large experimental variability that may be due to poor control over biomechanical inputs. To address this caveat we developed a novel CHI model called CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) that fully integrates biomechanical, behavioral, and neuropathological analyses. CHIMERA is distinct from existing neurotrauma model systems in that it uses a completely non-surgical procedure to precisely deliver impacts
of prescribed dynamic characteristics to a closed skull while enabling kinematic analysis of unconstrained head movement. Here I show that repeated TBI in mice using CHIMERA mimics many features of the human TBI including neurological, motor, and cognitive deficits along with persistent neuroinflammation and diffuse axonal injury, and increased endogenous tau phosphorylation up to 14 days with a reliable biomechanical response of the head. This makes CHIMERA well suited to investigate the pathophysiology of TBI and for drug development programs.
Preface

Chapter 1.

Portions of the introductory text are reproduced from the published review article:


Contributions:

Conception and outline of the article were designed by Dhananjay R. Namjoshi and Cheryl L. Wellington. All authors contributed towards the preparation of the manuscript with major contribution from Dhananjay R. Namjoshi.

Portions of the introductory text are reproduced from the published review article:


Contributions:

Conception and outline of the article were designed by Dhananjay R. Namjoshi, Sophie S. Stukas and Cheryl L. Wellington. Manuscript was prepared by Dhananjay R. Namjoshi, Sophie Stukas and Cheryl L. Wellington with equal contribution from Dhananjay Namjoshi and Sophie Stukas.
Chapter 2.

A version is published in:


All the animal experiments were carried out at UBC. All the biochemical and histology experiments, except the cytokine assays described in this chapter were carried out in the Wellington Laboratory at UBC. Cytokine assays were carried out by Kelli Wuerth in the Hancock Laboratory at UBC.

Contributions:

All the experiments were designed by Dhananjay R Namjoshi and Cheryl L. Wellington. James Donkin contributed towards the initial stages of experimental design. The majority of experiments and data analyses were performed by Dhananjay R. Namjoshi. Support for histological experiments and analysis was provided by Georgina Martin, Michael Carr, and Sepediah Tabarestani and that for biochemical assays was provided by Anna Wilkinson, Sophie Stukas, and Jianjia Fan. Manuscript was written by Dhananjay R. Namjoshi and Cheryl L. Wellington.
Chapter 3.

A version is published in:


CHIMERA impactor was constructed by Kurt McInnes in the Cripton Laboratory at UBC. All the animal experiments described in this Chapter were carried out at UBC. All the biochemical and histology experiments described in this Chapter were carried out in the Wellington Laboratory at UBC.

Contributions:

The CHIMERA impactor design was conceived by Dhananjay R. Namjoshi, Wai Hang Cheng, Kurt McInnes, Peter A. Cripton, and Cheryl L. Wellington. Dhananjay R. Namjoshi and Wai Hang Cheng designed and conducted experiments and analyzed data. Support for head injury procedure was provided by Michael Carr and Kris M. Martens. Behavioral assays were conducted by Dhananjay R. Namjoshi, Wai Hang Cheng and Kris M. Martens. Head kinematics analysis was performed by Wai Hang Cheng. Kurt McInnes and Peter A. Cripton provided expert advice on kinematic analysis. Biochemical assays were carried out by Anna Wilkinson and Jianjia Fan. Histology experiments were conducted by Dhananjay R. Namjoshi and Wai Hang Cheng.
Cheng. Support for histology was provided by Michael Carr, Jerome Roberts and Arooj Hayat. Manuscript was prepared by Dhananjay R. Namjoshi, Wai Hang Cheng, and Cheryl L. Wellington.

**Study Approval by UBC Ethics Boards:**

All the animal experiments described in this thesis were conducted according to the protocols approved by the UBC Animal Care Committee as follows:

Protocol # A07-0706: ABCA1 and apoE function in traumatic brain injury.


The candidate's animal care and training certification record:

3937-09: Online animal care training program

RBH-661-09: Rodent biology and husbandry

RA-343-09: Rodent anesthesia

RSHX-268-09: Rodent surgery
# Table of Contents

Abstract ........................................................................................................................................ii
Preface ........................................................................................................................................iv
Table of Contents ............................................................................................................................viii
List of Tables ....................................................................................................................................xv
List of Figures ....................................................................................................................................xvi
List of Abbreviations ...................................................................................................................xix
Acknowledgements .....................................................................................................................xxiii
Dedication ........................................................................................................................................xxv

**Chapter 1: Introduction** ............................................................................................................. 1

1.1 Traumatic Brain Injury (TBI): Definition ............................................................................. 1
1.2 TBI: Epidemiology .................................................................................................................... 4
1.3 TBI: Classification ..................................................................................................................... 7

1.3.1 TBI Classification Based on Clinical Severity ................................................................. 7
1.3.2 TBI Classification Based on Pathophysiology ................................................................. 10
1.3.3 TBI Classification Based on Physical Mechanism ........................................................ 11

1.4 TBI: Biomechanical Principles ............................................................................................... 12

1.4.1 Head Motion during Impact TBI ...................................................................................... 15
1.4.2 Brain Motion during TBI .................................................................................................... 17
1.4.3 The Clinical Picture: Human Tolerance and Related Biomechanical Studies of Human TBI .................................................................................................................. 19

1.5 TBI: Pathophysiology ............................................................................................................. 22

1.5.1 Primary Brain Injury ......................................................................................................... 23
1.5.1.1 Focal Brain Injury ................................................................. 24
  1.5.1.1.1 Cerebral Contusion and Laceration ............................ 25
  1.5.1.1.2 Intracranial Hemorrhage ........................................ 27
1.5.1.2 Diffuse Brain Injury ............................................................. 30
  1.5.1.2.1 Diffuse Axonal Injury ................................................ 30
  1.5.1.2.2 Diffuse Vascular Injury ....................................... 34
1.5.2 Secondary Brain Injury .......................................................... 34
  1.5.2.1 Cerebrovascular Response to TBI ................................. 35
  1.5.2.2 Neurometabolic Changes Following TBI ...................... 36
  1.5.2.3 Neuroinflammation Following TBI ............................. 37
1.6 TBI, Alzheimer Disease, and Apolipoprotein E ............................ 38
  1.6.1 TBI and Alzheimer Disease ............................................... 38
  1.6.2 Alzheimer’s Disease: Pathology ........................................ 39
    1.6.2.1 Amyloid Beta Formation in the CNS ...................... 40
    1.6.2.2 Tauopathy in Alzheimer Disease ......................... 42
  1.6.3 TBI and Alzheimer’s Pathology: Similarities and Differences ... 45
    1.6.3.1 Amyloid Pathology after Moderate to Severe Single TBI .. 45
    1.6.3.2 Consequences of Repetitive TBI: Chronic Traumatic
      Encephalopathy ................................................................. 50
  1.6.4 Apolipoprotein E (ApoE) at the Nexus of TBI and AD Pathology 55
    1.6.4.1 ApoE Synthesis in the CNS .................................... 56
    1.6.4.2 Liver X Receptors (LXR) .......................................... 58
    1.6.4.3 LXR: Molecular Mechanism of Action .................... 59
Chapter 2: The Liver X Receptor Agonist GW3965 Improves Recovery from Mild Repetitive Traumatic Brain Injury in Mice Partly Through Apolipoprotein E .............. 110

2.1 Summary ........................................................................................................ 110

2.2 Introduction ................................................................................................... 111

2.3 Materials and Methods .................................................................................. 115

2.3.1 Animals ................................................................................................ 115

2.3.2 Mild Repetitive Traumatic Brain Injury (mrTBI) .................................... 115

2.3.3 GW3965 Treatment ............................................................................. 119

2.3.4 Cognitive Assessment by Novel Object Recognition ........................... 120

2.3.5 Motor Function Assessment by Accelerating Rotarod ............................. 122

2.3.6 Biochemical Analyses ............................................................................. 122

2.3.6.1 Tissue Collection ..................................................................... 123

2.3.6.2 Protein Extraction .................................................................... 123

2.3.6.3 Western Blot Analyses ............................................................ 124

2.3.6.4 Quantitative Assessment of Endogenous Aβ by ELISA ............... 125

2.3.6.5 Quantitative Assessment of IL-6, TNFα, and MCP-1 by ELISA .......... 125

2.3.7 Histology .............................................................................................. 126

2.3.7.1 Assessment of Axonal Injury by Silver Staining ....................... 126

2.3.7.2 Assessment of Microglial Activation by Iba1 Immunohistochemistry ........................................................................................................ 127

2.3.8 Statistical Analyses .............................................................................. 128

2.4 Results .......................................................................................................... 129
2.4.1 ApoE is Required for GW3965 to Improve Cognitive Function After mrTBI ................................................................. 129
2.4.2 Spontaneous Recovery of Motor Dysfunction After mrTBI is Unaffected by GW3965 ................................................................................................................. 133
2.4.3 GW3965 Prevents mrTBI-Induced Elevation of Endogenous Aβ Levels ......................................................................................... 135
2.4.4 GW3965 Enhances ABCA1 Induction After mrTBI: ..................... 139
2.4.5 ApoE is Required for GW3965-Mediated Suppression of Axonal Damage After mrTBI .................................................................................................................. 142
2.4.6 Weight Drop TBI Model Produces Negligible Neuroinflammation ............................. 148
2.4.7 Retrospective Power Analysis .......................................................................................................................... 152
2.5 Discussion ................................................................................................................................. 154

Chapter 3: Merging Pathology and Biomechanics Using CHIMERA: A Novel, Surgery-Free, Closed-Head Impact Model of Engineered Rotational Acceleration ................. 161
3.1 Summary ................................................................................................................................. 161
3.2 Introduction ............................................................................................................................ 162
3.3 Materials and Methods .......................................................................................................... 165
  3.3.1 CHIMERA Impactor ........................................................................................................ 165
    3.3.1.1 Animal Holding Platform ................................................................. 165
    3.3.1.2 Pneumatic Impactor System .......................................................... 166
    3.3.1.3 Impact Piston and Barrel System ................................................. 166
    3.3.1.4 CHIMERA Calibration .................................................................. 168
  3.3.2 CHIMERA Repetitive TBI (rTBI) Procedure .................................................. 170
3.3.3 High-Speed Videography and Kinematic Analyses ........................................ 172
3.3.4 Behavioral Analyses ................................................................................... 174
3.3.5 Tissue Collection and Processing ............................................................. 178
3.3.6 Iba1 Immunohistochemistry and Silver Staining ..................................... 179
3.3.7 Biochemical Analyses ............................................................................. 180
  3.3.7.1 Tissue Processing .............................................................................. 180
  3.3.7.2 Assessment of TNF-α and IL-1β by ELISA ....................................... 180
  3.3.7.3 Quantitative Assessment of Phosphorylated and Total Tau by Simple Western Analysis ................................................................. 180
3.3.8 Statistical Analyses .................................................................................. 182
3.4 Results ......................................................................................................... 183
  3.4.1 Head Kinematics Following CHIMERA rTBI ........................................ 183
  3.4.2 CHIMERA rTBI Induces Behavioral Deficits ......................................... 189
  3.4.3 CHIMERA rTBI Induces Widespread Diffuse Axonal Injury ............... 193
  3.4.4 CHIMERA rTBI Induces Widespread Microglial Activation ............... 196
  3.4.5 CHIMERA rTBI Increases Proinflammatory Cytokine Levels ............. 202
  3.4.6 CHIMERA rTBI Increases Endogenous Tau Phosphorylation ............ 203
3.5 Discussion ................................................................................................... 205

Chapter 4: Conclusions and Future Directions ..................................................... 213
  4.1 Chapter 2: Conclusions ............................................................................ 214
  4.2 Chapter 2: Study Limitations and Future Directions ................................ 219
  4.3 Chapter 3: Conclusions ............................................................................ 223
  4.4 Chapter 3: Study Limitations and Future Directions ............................... 227
List of Tables

Table 1.1 TBI incidence (in %) by cause across the globe ................................................. 6
Table 1.2: Glasgow coma scale. .......................................................................................... 8
Table 1.3: North American classification of TBI according to severity of clinical signs.... 8
Table 1.4: TBI classification according to European Federation of Neurological Societies guidelines. .................................................................................................................. 10
Table 1.5: Differences in the pathological features of CTE and AD. ......................... 53
Table 1.6: Pathological and clinical stages of CTE. ......................................................... 54
Table 1.7: Summary of physical and physiological properties of apoE alleles. .......... 75
Table 2.1: Details of primary antibodies used for Western blotting. ......................... 124
Table 2.2: Details of primary and secondary antibodies used for cytokine ELISA ...... 126
Table 2.3: Summary of retrospective power calculations ............................................. 154
Table 3.1: Neurological severity score (NSS) tasks. ..................................................... 178
Table 3.2: Details of monoclonal antibodies for probing p-tau and total tau. .......... 181
Table 3.3: Summary of peak values of head kinematic parameters. ......................... 187
Table 3.4: Comparison of head kinematic parameters between rodent TBI models and human TBI ...................................................................................................................... 188
Table 4.1: Comparison of weight drop-rTBI and CHIMERA-rTBI models ............... 226
List of Figures

Figure 1.1: Annual incidence of TBI in people under 40 years of age ......................... 5
Figure 1.2: Biomechanics of head impact and brain tissue deformation ..................... 14
Figure 1.3: Wayne State Tolerance Curve (WSTC). .................................................. 20
Figure 1.4: Primary TBI. ........................................................................................... 23
Figure 1.5: Proposed molecular mechanism of diffuse axonal injury and generation of
Aβ. ................................................................................................................................. 33
Figure 1.6: Amyloidogenic and anti-amyloidogenic processing of APP ....................... 42
Figure 1.7: Regulation of ApoE Lipidation by LXR. .................................................. 62
Figure 1.8: ApoE structure, polymorphism and domain interaction. ............................ 74
Figure 1.9: Animal models of TBI. ............................................................................. 89
Figure 1.10: Fluid percussion injury model. ............................................................... 91
Figure 1.11: Controlled cortical impact model. ......................................................... 92
Figure 1.12: Blast Wave TBI Model. ......................................................................... 97
Figure 1.13: Variability in input parameters and outcomes in weight-drop TBI studies
reported in the literature. ......................................................................................... 100
Figure 2.1: Weight drop CHI model and impact location. .......................................... 118
Figure 2.2: Average animal body weight and drop height across all study groups..... 119
Figure 2.3: Novel object recognition phases. ............................................................ 122
Figure 2.4: Summary of mrTBI, Gw3965 treatment and post-injury outcomes.......... 128
Figure 2.5: ApoE is required for GW3965 to improve NOR performance after mrTBI. 131
Figure 2.6: NOR performance was not affected by motor impairment. .................... 132
Figure 2.7: mrTBI-induced motor impairment recovers spontaneously independent of GW3965 and apoE................................................................. 134

Figure 2.8: GW3965 prevents mrTBI-induced accumulation of endogenous Aβ in WT and apoE-/- mice................................................................. 137

Figure 2.9: APP and APP-CTFα levels remain unchanged following mrTBI. ............... 138

Figure 2.10: GW3965 augments ABCA1 levels in WT and apoE-/- mice following mrTBI. ......................................................................................................................... 140

Figure 2.11: ApoE and LDLR levels are unaffected by mrTBI or GW3965.................... 141

Figure 2.12: Loss of apoE exacerbates axonal injury after mrTBI................................. 143

Figure 2.13: mrTBI leads to mild axonal damage in WT mice...................................... 144

Figure 2.14: Loss of apoE exacerbates axonal damage after mrTBI. ......................... 145

Figure 2.15: ApoE is required for GW3965 to suppress axonal damage after mrTBI.. 147

Figure 2.16: mrTBI does not induce microglial activation in the cortex. .................... 149

Figure 2.17: mrTBI induces negligible hippocampal microglial activation............... 150

Figure 2.18: Pronounced microglial activation is localized only around contused areas. ......................................................................................................................... 151

Figure 2.19: Pronounced microglial activation is localized only around contused areas. ......................................................................................................................... 152

Figure 3.1: CHIMERA impactor and impact pistons. ................................................. 168

Figure 3.2: Piston energy-air pressure calibration curve. ........................................ 169

Figure 3.3: Mouse head and impact position............................................................ 171

Figure 3.4: External markers used for mouse head tracking.................................... 174

Figure 3.5: Open field thigmotaxis............................................................................ 177
Figure 3.6: CHIMERA-rTBI procedure and post-rTBI endpoints ........................................ 182
Figure 3.7: Head kinematics following CHIMERA rTBI ......................................................... 185
Figure 3.8: CHIMERA allows unrestricted head motion during TBI .................................... 186
Figure 3.9: CHIMERA rTBI induces behavioral deficits ......................................................... 192
Figure 3.10: CHIMERA rTBI does not significantly affect general mobility ....................... 193
Figure 3.11: CHIMERA rTBI induces diffuse axonal injury ................................................... 195
Figure 3.12: CHIMERA rTBI induces sustained axonal injury ............................................ 196
Figure 3.13: CHIMERA rTBI induces widespread microglial activation ......................... 199
Figure 3.14: Quantitative analysis of microglial response to rTBI ....................................... 201
Figure 3.15: CHIMERA rTBI increases proinflammatory cytokine levels .......................... 202
Figure 3.16: CHIMERA rTBI increases endogenous tau phosphorylation .......................... 204
List of Abbreviations

ABCA1: Adenosine triphosphate binding cassette transporter A1
Aβ: Amyloid beta peptide
ACRM: American Congress of Rehabilitation Medicine
AICD: Amyloid intracellular domain
ApoE/apoE: Apolipoprotein E
ApoA-I/apoA-I: Apolipoprotein A-I
APP: Amyloid precursor protein
AD: Alzheimer disease
ANOVA: Analysis of variance
BASE-1: Beta site APP cleaving enzyme-1
BBB: Blood-brain barrier
CAA: Cerebral amyloid angiopathy
CCI: Controlled cortical impact
CDC: Centers for disease control and prevention
CG: Center of gravity
CHI: Closed-head injury
CHIMERA: Closed-head impact model of engineered rotational acceleration
CNS: Central nervous system
CSF: Cerebrospinal fluid
CT: Computed tomography
CTE: Chronic traumatic encephalopathy
CTF: APP C-terminal fragment
DAI: Diffuse axonal injury
DI: Discrimination index
DMSO: Dimethyl sulfoxide
DTI: Diffuse tensor imaging
DVI: Diffuse vascular injury
EDH: Epidural hemorrhage/hematoma
EFNS: European federation of neurological societies
FP: Fluid percussion
GCS: Glasgow coma scale
HDL: High-density lipoprotein
HIC: Head injury criterion
ICH: Intracranial hemorrhage/hematoma
ICP: Intracranial pressure
IDE: Insulin degrading enzyme
IL-1β: Interleukin 1 beta
IL-6: Interleukin 6
i.p.: Intraperitoneal
ISF: Interstitial fluid
LDL: Low-density lipoprotein
LDLR: LDL receptor
LRP1: LDLR related protein 1
LOC: Loss of consciousness
LRR: Loss of righting reflex
LTP: Long-term potentiation
LXR: Liver X receptor
LXRE: LXR response element
MAPT: Microtubule associated protein tau
MCP-1: Monocyte chemotactic protein-1
MRI: Magnetic resonance imaging
mTBI: Mild traumatic brain injury
mrTBI: Mild, repetitive traumatic brain injury
MTBR: Microtubule binding repeat
MVA: Motor vehicle accident
NEP: Neprilysin
NFL: National football league
NFT: Neurofibrillary tangles
NMDA: N-methyl-d-aspartate
NOR: Novel object recognition
NSS: Neurological severity score
OHI: Open head injury
PBS: Phosphate-buffered saline
PS-1/2: Presenilin 1/2
PTA: Post-traumatic amnesia
RCT: Reverse cholesterol transport
RIPA: Radioimmunoprecipitation assay
rTBI: Repetitive traumatic brain injury
RXR: Retinoid X receptor
TBI: Traumatic brain injury
SDS: Sodium dodecyl sulfate
s.c.: Subcutaneous
SCI: Spinal cord injury
SDH: Subdural hemorrhage/hematoma
TNF-α: Tumor necrosis factor alpha
VLDL: Very low-density lipoprotein
WHO: World health organization
WSTC: Wayne state tolerance curve
WT: Wild-type (C57Bl/6) mice
Acknowledgements

As I come to this milestone in the pursuit of knowledge, I cannot help but reflect back and acknowledge all those who have contributed towards completion of this work in one or the other way.

First of all, I offer my deepest gratitude to Almighty GOD for showering ITS infinite bounties, graces and mercies on me and giving me strength in the most difficult times. Without ITS wishes and blessings, this work could have remained a dream only.

I would like to dedicate this work to my parents for their unconditional love, constant encouragement, to my wife Archana for her immense love, patience, and unstinting support, who stood with me in every good and bad moment and to my parents-in-law for their constant motivation and support.

I express my deepest gratitude for my mentor Dr. Cheryl Wellington. Thank you Cheryl for your guidance, support, constant encouragement, constructive criticism, and valuable suggestions throughout my work. It has been an enriching experience and privilege working in your laboratory.

I sincerely thank my supervisory committee members, Drs. Brian MacVicar, Wolfram Tetzlaff, and Gordon Francis for providing critical guidance throughout this work.
I thank all the current and past Wellington Lab members, especially, Anna Wilkinson, Jeniffer Chan, Dr. James Donkin, Dr. Veronica Hirsh-Reinshagen, Dr. Kris Martens, Dr. Jianjia Fan, Dr. Sophie Stukas, Tom Cheng, Mike Carr, Georgina Martin, and Arooj Hayat for all the help and support throughout this work. Special thanks to Tom for all the intellectual talks and exchange of ideas and who has a lion’s share in the development of CHIMERA.

Special thanks to Dr. Peter Cripton and Kurt McInnes for helping me understand the biomechanics of traumatic brain injury as well as for the wonderful collaboration that resulted in the development of CHIMERA.

I express my gratitude towards Alzheimer Society of Canada and its patrons for supporting me through Alzheimer Society Research Program doctoral award. This work was also supported by the grants from Canadian Institutes of Health Research to Dr. Cheryl Wellington.

I am grateful to Child and Family Research Institute, Centre for Disease Modeling, as well as Djavad Mowafaghian Centre for Brain Health for allowing me to use their core facilities and space to conduct this research work.

Last but not the least I would like to acknowledge the unsung heroes of this work, the laboratory mice without whom this work would not be possible.
To my parents and parents-in-law.

To my wife, Archana
1.1 Traumatic Brain Injury (TBI): Definition

Traumatic brain injury (TBI) occurs when an external mechanical force traumatically injures the brain resulting in altered mental state. TBI is a type of acquired brain injury that is caused by events after birth as opposed to genetic or congenital defects. As discussed further in this Chapter, TBI pathology is heterogeneous and each TBI is unique and hence defining TBI has been challenging. The definition as well as the classification of TBI has evolved through several attempts by different groups. As discussed further about 75% of the human TBI are mild and therefore in most of the attempts of defining TBI have considered only mild TBI (mTBI). The most commonly used outcomes defining mTBI are Glasgow Coma Scale (GCS) score, loss of consciousness (LOC) and post-traumatic amnesia (PTA). For example, the American Congress of Rehabilitation Medicine (ACRM) defined mTBI as “traumatically induced physiological disruption of brain function as manifested by at least one of the following: 1) any period of LOC, 2) any loss of memory events immediately before or after the accident, 3) any alteration in mental state (e.g., dizziness, disorientation, or confusion) at the time of the accident, and 4) focal neurological deficits which may or may not be transient; but where the severity of injury does not exceed the following: LOC ≤ 30 min, GCS score of 13-15 after 30 min, and PTA < 24 h” (Kay et al., 1993). The U.S. Centers for Disease Control and Prevention (CDC) proposed a general definition of mTBI as “an injury to the head as a result of blunt trauma or acceleration forces that result in: 1) transient confusion, disorientation, or impaired consciousness or 2) dysfunction of memory around the time of injury or 3) LOC < 30 min or 4) neurological or
neuropsychological dysfunction such as seizures following injury (Gerberding and Binder, 2003). The CDC also recommends developing case-specific definition of mTBI based on this general definition. Later, the World Health Organization (WHO) Collaborating Centre for Neurotrauma Task Force on Mild Traumatic Brain Injury published another definition for mTBI as “an acute brain injury resulting from mechanical energy to the head from external physical forces” manifesting into confusion or disorientation, LOC for 30 min or PTA for < 24 h and GCS score of 13-15 after 30 min (Carroll et al., 2004). The WHO definition further emphasizes that mTBI manifestations must not be due to drugs, alcohol, or injuries caused by other mechanisms, e.g., systemic injuries, facial injuries or penetrating craniocerebral injury.

Thus, the ACRM, CDC, and WHO definitions address only the mild spectrum of TBI and not moderate and severe TBIs, including penetrating brain injuries. Moreover, these definitions consider only neurological signs while ignoring any evidence of the resulting brain pathology.

Most recently, the Demographics and Clinical Assessment Working Group of the International and Interagency Initiative toward Common Data Elements for Research on Traumatic Brain Injury and Psychological Health proposed a broader definition of TBI as “an alteration in brain function, or other evidence of brain pathology caused by an external force” (Menon et al., 2010).
The alteration of brain function is assessed by clinical examination and includes any one of the following: 1) any period of LOC, 2) any loss of memory immediately before (retrograde amnesia) or after (PTA) TBI, 3) neurological deficits including weakness, loss of balance, sensory loss, aphasia (problem with speaking, listening, reading and writing), and paralysis, and 4) any alteration in mental state (confusion, disorientation) at the time of injury (Menon et al., 2010). With the advances in modern imaging techniques such as Magnetic Resonance Imaging (MRI), Diffusion Tensor Imaging (DTI) and Computed Tomography (CT), it is now possible to visualize brain damage in some cases. The neurological examination of TBI can therefore be supplemented with these techniques (in addition to potential biomarkers of great interest for future use) to obtain evidence of brain pathology. Lastly, TBI is caused by an external mechanical force, which distinguishes TBI from other types of acquired brain injuries such as cerebrovascular accident (stroke), brain tumors, brain infection, and brain injury caused by substance abuse. The external mechanical force can be an impact (e.g., a moving object striking stationary head such as during an assault or a moving head striking the stationary object such as during a fall) or non-impact (e.g., rapid head movement during whiplash or head exposed to blast waves) or a penetrating force (e.g., a bullet penetrating the skull).

The new definition also encourages using the more appropriate term “traumatic brain injury” rather than “head injury”, which may be limited to injury to the face and the scalp and may not result in neurological deficits.
1.2 TBI: Epidemiology

TBI is a leading cause of death and disability throughout the world. The global annual incidence of TBI is estimated to be ~200 per 100,000 people (Reilly, 2007). The annual incidence of TBI in the North America is greater than the combined incidence of breast cancer, HIV/AIDS, multiple sclerosis, and spinal cord injuries (Figure 1.1A). In the United States, the overall incidence of TBI is estimated to be 538 per 100,000 population, which represents at least 1.7 million new cases per year since 2003 (Gerberding and Binder, 2003; Langlois et al., 2006; Faul et al., 2010). Annually approximately 50,000 Canadians sustain a TBI with more than 11,000 deaths occurring each year (Brain Injury Society of Toronto, 2014). The rate of TBI is reportedly lower in Europe (235 per 100,000) and in Australia (322 per 100,000) (Cassidy et al., 2004; Tagliaferri et al., 2006) although the emerging evidence indicates the otherwise (see below).

Estimating the true burden of TBI is challenging due to several factors. Most of the epidemiological studies report data based on hospitalization and government records that lack systematic epidemiological monitoring. Compounding this is the growing awareness that more than 75% of TBI are mild (mTBI, a term synonymous to concussion) that do not necessarily need hospitalization and therefore are not always reported. As will be noted below, underdiagnosis of mTBI may nonetheless pose challenges to interpretation of potential long-term consequences. Finally, most of the epidemiological studies are retrospective, include small patient number and suffer from recall bias. These limitations have been highlighted by a recent study in which a
substantially higher TBI incidence (749 per 100,000) was observed than previously appreciated (Feigin et al., 2013). In this study, the authors collected data in an urban and rural New Zealand population by both prospective and retrospective surveillance systems. This study suggested that TBI incidence may be far greater than reported in early epidemiological studies that mostly used retrospective data.

Furthermore, although most of the epidemiological data on TBI comes from the developed countries, it is estimated that the incidence of TBI in developing countries is rising and posing a significant health problem (Figure 1.1B). With the increasing worldwide incidence rate as well as high number undiagnosed/underdiagnosed cases, it is not surprising that TBI is often called a “silent epidemic”.

![Figure 1.1: Annual incidence of TBI in people under 40 years of age](image)

(A) TBI has higher incidence rate compared to the combined incidence of other major health issues including multiple sclerosis (MS), spinal cord injury (SCI), HIV/AIDS and breast cancer combined. (B) Estimated annual incidence of TBI across the globe. For data sources, please refer Table 1.1.
Falls and motor vehicle accidents (MVA) are the two most common causes of severe TBI, with assaults/violence often being the third most-common cause (Table 1.1) (Cassidy et al., 2004; Hyder et al., 2007; Faul et al., 2010). TBI resulting from high-contact/collision sports such as boxing, American football, ice hockey, soccer, and rugby account for almost 21% of all head injuries among children and adolescents (Centers for Disease Control and Prevention 2007; American Association of Neurological Surgeons, 2011). TBI is also considered a “signature injury” in modern warfare, as approximately 20% of veterans from the Iraq or Afghanistan wars have experienced a TBI, 80% of which involve blast injury (Taber et al., 2006; Hoge et al., 2008; Elder and Cristian, 2009).

<table>
<thead>
<tr>
<th>Country/Region</th>
<th>Falls</th>
<th>MVA</th>
<th>War</th>
<th>Assault/Violence</th>
<th>Other/Undefined</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>45</td>
<td>23</td>
<td>-</td>
<td>9</td>
<td>10</td>
<td>Canadian Institute for Health Canadian Institute for Health Information (2006)</td>
</tr>
<tr>
<td>USA</td>
<td>35</td>
<td>17</td>
<td>-</td>
<td>10</td>
<td>21</td>
<td>Faul et al. (2010)</td>
</tr>
<tr>
<td>Europe</td>
<td>39</td>
<td>39</td>
<td>-</td>
<td>7.2</td>
<td>19</td>
<td>Tagliaferri et al. (2006)</td>
</tr>
<tr>
<td>Australia</td>
<td>21</td>
<td>40</td>
<td>-</td>
<td>8</td>
<td>25</td>
<td>Reilly (2007)</td>
</tr>
<tr>
<td>New Zealand</td>
<td>38</td>
<td>20</td>
<td>-</td>
<td>17</td>
<td>21</td>
<td>Feigin et al. (2013)</td>
</tr>
<tr>
<td>China</td>
<td>17.5</td>
<td>46.3</td>
<td>-</td>
<td>23.8</td>
<td>21</td>
<td>Zhao and Wang (2001); Wu et al. (2008)</td>
</tr>
<tr>
<td>India</td>
<td>50</td>
<td>22</td>
<td>-</td>
<td>10</td>
<td>22</td>
<td>Gururaj (2002)</td>
</tr>
<tr>
<td>Latin America</td>
<td>24</td>
<td>41</td>
<td>4</td>
<td>25</td>
<td>8</td>
<td>Puvanachandra and Hyder (2008)</td>
</tr>
<tr>
<td>Africa</td>
<td>5</td>
<td>13</td>
<td>53</td>
<td>33</td>
<td>16</td>
<td>Hyder et al. (2007)</td>
</tr>
<tr>
<td>Global</td>
<td>8</td>
<td>62</td>
<td>2</td>
<td>24</td>
<td>4</td>
<td>Hyder et al. (2007)</td>
</tr>
</tbody>
</table>

*Table 1.1 TBI incidence (in %) by cause across the globe.*
MVA: motor vehicle accident.

1.3 TBI: Classification

Traditionally TBI is classified based on three systems: 1. clinical severity, 2. pathophysiology and 3. physical mechanism (Saatman et al., 2008).

1.3.1 TBI Classification Based on Clinical Severity

Conventional clinical TBI taxonomy divides severity of injury into three categories; mild, moderate and severe. The most commonly accepted method to determine injury severity is the GCS score (Table 1.2), which measures the patient’s level of consciousness based on verbal, motor, and eye opening responses after injury. A patient with a GCS score of 3-8 (out of 15) is considered to have sustained a severe traumatic brain injury, 9-12 is moderate, and >12 mild (Table 1.3) (Teasdale and Jennett, 1974). While the GCS is useful for the clinical management of TBI, it does not provide specific information about the neuropathological mechanisms involved. The GCS is also less useful in pediatric TBI. The prognostic ability of this system thus is limited and as such other descriptors have been added. In the most recent iteration of the widely adopted Departments of Defense and Veteran Affairs classification of severity of brain injury, mTBI is further denoted by PTA lasting < 24 h, and a LOC of < 30 min. Similarly, to meet moderate TBI criteria, GCS must be between 9-12, PTA must not exceed one week, and LOC must not last longer than 24 h (U.S Departments of Defense and Veterans United Sates Departments of Defense and Veterans Affairs, 2008) (Table 1.3).
<table>
<thead>
<tr>
<th>Score</th>
<th>Eye Opening Response</th>
<th>Verbal Response</th>
<th>Motor Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>N/A</td>
<td>N/A</td>
<td>Obeys commands for movement</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>Oriented</td>
<td>Purposeful movements to painful stimulus</td>
</tr>
<tr>
<td>4</td>
<td>Spontaneous</td>
<td>Confused, but able to answer questions</td>
<td>Withdraws in response to pain</td>
</tr>
<tr>
<td>3</td>
<td>To verbal stimuli</td>
<td>Inappropriate words</td>
<td>Flexion in response to pain (decorticate posturing)</td>
</tr>
<tr>
<td>2</td>
<td>To pain only (not applied to face)</td>
<td>Incomprehensible speech</td>
<td>Extension in response to pain (decerebrate posturing)</td>
</tr>
<tr>
<td>1</td>
<td>No response</td>
<td>No response</td>
<td>No response</td>
</tr>
</tbody>
</table>

**Table 1.2: Glasgow coma scale.**

Glasgow coma scale determines a patient’s neurological state based on eye opening, verbal and motor response. Adapted from (Teasdale and Jennett, 1974).

<table>
<thead>
<tr>
<th>Injury Severity</th>
<th>GCS Score (Out of 15)*</th>
<th>LOC Duration</th>
<th>PTA Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>&gt; 12</td>
<td>&lt; 30 min</td>
<td>&lt; 24 h</td>
</tr>
<tr>
<td>Moderate</td>
<td>9-12</td>
<td>30 min to 24 h</td>
<td>1 to 7 days</td>
</tr>
<tr>
<td>Severe</td>
<td>3-8</td>
<td>&gt; 24 h</td>
<td>&gt; 7 days</td>
</tr>
</tbody>
</table>

**Table 1.3: North American classification of TBI according to severity of clinical signs.**

* Best available score within 24 h of injury. GCS: Glasgow Coma Score, LOC: Loss of Consciousness, PTA: Posttraumatic Amnesia.

The European Federation of Neurological Societies (EFNS) guidelines have taken a slightly different approach and categorized TBI into four levels of severity: mild, moderate, severe, and critical (Table 1.4) (Vos et al., 2002). The heterogeneity inherent
to mTBI has been addressed with various refinements to this category. For example, the EFNS guidelines further classify mTBI into four categories, 0, 1, 2, and 3. In addition to the parameters used by the North American guidelines (i.e., GCS score, duration of LOC and PTA), the EFNS guidelines also include risk factors for intracranial complications in the grading of mTBI severity. The risk factors for intracranial complications include unclear or ambiguous accident history, continued PTA (GCS verbal score of 4), retrograde amnesia for > 30 min, trauma above clavicles including clinical signs of skull fracture, severe headache, vomiting, focal neurological deficits, seizure, age of < 2 or > 60 years, coagulation disorders, and alcohol/drug intoxication (Vos et al., 2002). In this system, the subcategories of mTBI dictate post-TBI management. Thus, a person with mTBI category 0 is considered to have no TBI and is immediately discharged, mTBI category 1 recommends CT scanning while CT scanning is mandatory for persons with mTBI categories 2 and 3 (Vos et al., 2002). The finer distinction of mTBI may be of questionable value since in a recent major revision of evidence-based guidelines for evaluation and management of sports-related concussions, endorsed by multiple sporting bodies and physician groups, these finer distinctions have now been dropped due to a lack of prognostic utility (Giza et al., 2013).
Table 1.4: TBI classification according to European Federation of Neurological Societies guidelines.
GCS: Glasgow Coma Score, LOC: Loss of Consciousness, PTA: Posttraumatic Amnesia. Adapted from (Vos et al., 2002).

1.3.2 TBI Classification Based on Pathophysiology

The action of external forces on the head leads to a cascade of pathological processes. These processes are broadly classified into primary and secondary brain injuries (discussed in section 1.5). Primary brain injury occurs at the time of mechanical loading on the head leading to structural damage to the brain parenchyma and cerebrovasculature. Primary injury results in laceration or contusion of the cortical surface, diffuse axonal injury (DAI), and hematoma. Damage caused by primary injury is considered to be irreversible and may not respond to any pharmacological intervention. Primary brain injury initiates a plethora of secondary processes that result in complex cellular, inflammatory, neurochemical, and metabolic alterations (McIntosh et al., 1996; Blumbergs, 1997; Davis, 2000; Giza and Hovda, 2001; Werner and Engelhard, 2007;
McAllister, 2011). The secondary injury pathways may be more amenable to pharmacological treatment.

### 1.3.3 TBI Classification Based on Physical Mechanism

TBI is caused by external mechanical forces, which can be static or dynamic. Static stress (e.g., a head caught between elevator doors) does not cause head acceleration and does not lead to brain injury unless the crushing forces exceed the threshold required for skull fracture causing substantial tissue damage (Gennarelli, 1993). The majority of TBIs result from dynamic forces including contact (impact) or non-contact (inertial) forces. Dynamic stresses induce rapid acceleration and/or deceleration of the head followed by distribution of inertial forces to the underlying brain tissue causing tissue damage. Contact injuries are induced by a moving object striking a stationary head or a moving head striking a stationary object and usually involve intense mechanical loading of short duration (Davis, 2000). The basis of classifying TBI on the basis of physical mechanism lies in the observed relation between the type of mechanical force and the resulting neuropathological characteristics. Thus, brain injuries as a result of impact forces are thought to induce localized (focal) damage including contusions, skull fracture, and epidural hematoma while inertial forces tend to cause more widespread (diffuse) brain damage such as diffuse axonal injury and subdural hematoma (McLean and Anderson, 1997; King, 2000; Saatman et al., 2008).
1.4 TBI: Biomechanical Principles

Biomechanics involves the study of the motion (such as head acceleration) of a person, animal or anthropomorphic test device (crash test device) and mechanical loads sustained or applied (such as head impact forces). The work of Gurdjian and Lissner (Gurdjian and Lissner, 1945, 1946, 1947; Gurdjian et al., 1947; Gurdjian and Webster, 1947) and Holbourn (Holbourn, 1943, 1945) pioneered the biomechanical evaluation of TBI to understand the relationship between mechanical loading (force/stress) and the physical response of the head and brain (deformation/strain) and resulting pathology. Besides understanding the mechanism of brain injury, biomechanical studies also help to predict head injury tolerance levels, which are used for developing and standardizing vehicle safety parameters as well as in helmet design to provide improved protection for the head during impact (Versace; Henn, 1998; Eppinger et al., 2000). Although, the mechanisms by which mechanical forces induce brain injury are a subject of considerable debate (Hardy et al., 1994; Drew and Drew, 2004), brain deformation or strain resulting from external mechanical loading (i.e., force/stress) is the currently accepted biomechanical theory of TBI (McLean and Anderson, 1997; King et al., 2003; King et al., 2004; LaPlaca et al., 2007). Brain deformation increases with increasing stress in a non-linear fashion (LaPlaca et al., 2007). In a head impact, dynamic mechanical forces act on the skull and brain to cause both linear and rotational movement of the head and skull. This in turn leads to deformation and structural damage of brain and cerebrovascular tissues and triggers many secondary injury pathways.
Human impact TBI can occur under many conditions. One example involves the slowly moving or stationary head being impacted by a rapidly-moving object, such as when a vehicle strikes a pedestrian’s head. Another example involves a head moving at a high rate of speed impacting a stationary object, such as when a hockey player slides head first into the boards. These impacts cause intense mechanical loading that lasts for only a fraction of a second (< 50 ms) (Gurdjian et al., 1966; Ono et al., 1980; Pellman et al., 2003b) and causes pressure gradients and mechanical strain (i.e., local areas of stretching or compression of the brain tissue) to be induced within the brain tissue as shown in Figure 1.2 (Meaney and Smith, 2011). Head impact can result in pure linear (straight line) motion of the head or combined linear and rotational motion in response to impact. Combined linear and rotational motion is more common than pure linear motion because the head is coupled to the body by the neck and so almost any impact to the head results in a combination of linear and rotational motion of the head due to restraint forces on the head applied by the neck (Greaves et al., 2009). In pure linear motion of the head, the pressure gradients and tissue strains described above will occur. In combined rotational and translational motion of the head the pressure gradients and tissue strains described above will have much larger tissue strains, which arise from the rotation of the head, superimposed on the pressure gradient-related strains (Meaney and Smith, 2011).
Figure 1.2: Biomechanics of head impact and brain tissue deformation.

(A) Impact to the back of the skull with the skull moving downwards (red arrow) causes momentary skull deformation (red outline). (B) The skull stops suddenly (in ~ 50 ms) and the momentum of the brain keeps it moving causing relative motion of the brain with respect to the skull (orange outline). (C) This motion of the brain sets up positive pressure at the impact site (i.e., coup) and negative pressure opposite the coup site at the contrecoup site. Reproduced with permission from Namjoshi et al. (2013a)

Impact is by far the most common cause of TBI in civilian populations. In the military, however, improvised explosive devices (IEDs) can lead to blast forces capable of causing TBI. In contrast to impact TBI, pure blast TBI is non-contact and involves dynamic forces with very short durations on the order of a few microseconds (µs) (Goldstein et al., 2012). Other non-contact TBI mechanisms are hypothesized to occur as a result of “inertial” loading of the head where torso motion, for example when a football player's torso is impacted by another player during a tackle or during “shaken baby” syndrome, causes the head to move even when no direct impact forces are subjected to it. This mechanism of TBI is a matter of some controversy and many investigators have concluded that this does not occur in real world human TBI (Lau et al., 1989; McLean, 1995; Yoganandan et al., 2009; Meaney and Smith, 2011; Wright et
al., 2013). The duration of inertial head loading in these situations varies from 50 to 200 ms (Gennarelli, 1993). Most epidemiological studies also conclude that these injuries cannot feasibly occur in adult humans (Lau et al., 1989; McLean, 1995).

TBI can also result from static or near static loads that essentially crush the brain and skull resulting in direct compression of the brain or contusion injury via bone fragments (Denny-Brown and Russell, 1940; Lopez-Guerrero et al., 2012; Mattei et al., 2012). In these crushing or nutcracker injuries, the head is generally not subjected to rapid linear or rotational movements that occur during impact injuries.

1.4.1 Head Motion during Impact TBI

Head/skull acceleration that occurs during a head impact can be described using three-dimensional linear (translational) and rotational (angular) accelerations. Linear acceleration is defined as the change in velocity over a given time through translational coordinates of the head’s center of gravity (CG) e.g., x-y-z or its resultant, and is usually expressed in units of “g” (one g is the acceleration due to gravity on earth) or m/s².

Rotational acceleration is the change in rotational velocity of the head over a given time and is expressed in units of radians/s² (rad/s²) or degrees/s² (°/s²). One revolution is equal to 360° or 2π rad. Acceleration can be measured using devices called accelerometers.

The relative amounts of linear and rotational head acceleration that result from a particular head impact depend on several factors including the type of impact force, the
direction of the force, the location of force on the skull, and the material properties of the skull and brain. An impulsive contact force applied to the head is a vector with magnitude and direction. A force that passes through the CG of the head (i.e., aligned with the maxilla) will initiate primarily linear motion of the head during the impact. A force that does not pass through the CG (e.g., an impact to the high forehead) will produce an impulsive moment (conceptually a “twisting force”) about the CG and will initiate both linear and rotational acceleration.

There is considerable debate about whether linear (Gurdjian et al., 1955; Haddad et al., 1955; Gurdjian et al., 1961) or rotational (Holbourn, 1943; Gennarelli et al., 1981; Gennarelli and Thibault, 1982; Gennarelli et al., 1982) acceleration is a better predictor of brain injury. Advocates of rotational acceleration argue that pure linear impact is rare in the clinical setting and angular acceleration is the principle mechanism underlying brain injury (Holbourn, 1943; Hardy et al., 1994). Notably, the Head Injury Criterion (HIC), which is a currently incorporated in vehicle safety standards around the world, takes only linear acceleration into account. The HIC is calculated as a function of the acceleration magnitude and duration of acceleration so that high accelerations acting for long time intervals result in high HIC while lower acceleration values or shorter exposure times result in lower HIC. In the context of automotive safety testing, the HIC has been credited with considerably reducing the incidence of MVA-related head injuries for over three decades (King et al., 2004). Moreover, recent studies by King and colleagues contend that helmets significantly reduce linear acceleration without changing rotational acceleration (King et al., 2003), leading these authors to propose
that the response of the brain itself (i.e., deformation of the structures of the brain) to mechanical loading may be a better predictor of brain injury than linear or rotational acceleration of the skull (Hardy et al., 2001; King et al., 2003; King et al., 2004; King et al., 2011). Many authors have proposed metrics that are a combination of both linear and rotational acceleration (and other factors such as HIC and impact force location on the skull) (Gurdjian, 1975; Ono et al., 1980; Pellman et al., 2003b; Greenwald et al., 2008; Rowson and Duma, 2013) as the most predictive mechanisms of brain injury.

1.4.2 Brain Motion during TBI

Like most soft tissue, the brain has viscoelastic properties with non-linear mechanical stress-strain responses (LaPlaca et al., 2007). Importantly, it is shear strain, rather than tissue compression or pressure gradients, which is believed to be the major mechanism underlying most concussion pathology (Meaney and Smith, 2011). Brain motion inside the skull during impact has been studied using a variety of techniques. In the earlier studies, brain motion during impact was directly recorded through a cranial window created by surgically replacing the skull cap of Macaque monkeys with a cap made up of acrylic (called the “Lucite Calvarium”) (Shelden et al., 1944; Pudenz and Shelden, 1946) or polycarbonate (the “Lexan Calvarium”) (Ommaya et al., 1969; Gosch et al., 1970). Brain motion following sub-concussive impacts in the frontal, temporal, and parietal regions was recorded using high-speed film. In these studies, it was found that regardless the direction of impact, the principal brain displacement was in parieto-occipital region with minimum displacement in the frontal region, which is likely due to the anterior cranial fossa that restricts movement of frontal lobes. This study showed
relative movements of brain and the skull, concluding that the relative displacement of the brain depended on the degree of skull movement. Thus, an immobile skull following impact caused little to no brain displacement while a freely moving skull resulted in considerable brain movement. With the advances in high-speed X-ray videography in 1960’s it was possible to record brain movement with minimal surgical manipulation. Using intravascular contrast media and lead targets, brain motion was captured after impact with high speed X-ray videography in dogs (Hodgson et al., 1966) and primates (Shatsky et al., 1974). Hardy and colleagues improved on this technique by using neutral density particles to record brain displacement in human cadavers (Hardy et al., 2001; Hardy et al., 2007). The studies in human cadavers reported brain displacement of ± 5 mm relative to the skull (King et al., 2011). These studies also reported a lag in the brain displacement relative to the motion of the skull. Moreover, these studies also show that the radio-opaque particles return to their original position following displacement suggesting elastic properties of the brain tissue, which may be important for studying brain displacement over repeat impacts. The effects of post-mortem changes in the brain tissue on the brain displacement however are not known. Recently brain motion was studied in human volunteers using tagged MRI. Bayly and colleagues conducted a series of studies in which the head of human volunteers was subjected to deceleration with occipital impact of 2-3 g (Bayly et al., 2005) or angular acceleration of 250-300 rad/s² (Sabet et al., 2008) and the resulting strains were assessed using tagged MRI (tagged MRI enables tracking the motions of tissue by tagging specific tissue points with a sequence of radio-frequency pulses before imaging) although without isolating relative movements of brain and the skull. By tracking the points along
tag lines imposed on MRI images, the authors reported that mechanical inputs that were 10-15% of those experienced by soccer players resulted in strains in the range of 0.02-0.06 (a 0.05 strain corresponds to a 5% change in the dimension of local tissue).

1.4.3 The Clinical Picture: Human Tolerance and Related Biomechanical Studies of Human TBI

The traumatic injury threshold for humans has been investigated using animal models, physical brain surrogate models, analytical models and finite element models and studies in athletes (football and hockey) who experience frequent head impacts and concussion.

Using animal models in the 1950s, researchers at the Wayne State University demonstrated that the duration of intracranial pressure was an important exposure variable in injury tolerance; higher exposure generated a more severe injury in a shorter amount of time (Lissner et al., 1960). Increasingly sophisticated instrumentation allowed head acceleration to be measured at the occiput (posterior part of the skull) along with changes in intracranial pressure in response to forehead impacts for whole and partial cadavers on automotive instrument panels, windshields, and non-yielding surfaces (Evans et al., 1958; Lissner et al., 1960). These experiments led to the preliminary Wayne State Tolerance Curve (WSTC) for head injury. The initial tolerance curve predicted whether head injury would occur as a function of the head impact duration and the average linear acceleration measured at the occiput. This initial curve was further refined through additional cadaver testing and in human volunteers (Eiband,
The revised WSTC, as shown in Figure 1.3, assumed that the underlying experimental impacts that caused a linear skull fracture also caused a moderate to severe concussion (Gurdjian et al., 1966). The first experimental and biomechanics-based, quantitative human brain injury criterion, was based on linear head acceleration. Rotational based human injury criteria would not come until later (Newman, 2002). The WSTC data provides the basis for several widely used injury metrics currently in use such as the Gadd Severity Index (GSI) (Gadd, 1966), and the HIC (Versace, 1971).

![Wayne State Tolerance Curve](image)

**Figure 1.3: Wayne State Tolerance Curve (WSTC).**

The WSTC describes the relationship between linear head acceleration, duration of acceleration, and onset of concussion. The WSTC suggests that the head can withstand very high acceleration for a very short duration. Conversely, any increase in
the duration of impact for the same intensity of acceleration is likely to cause head injury. Reproduced with permission from Namjoshi et al. (2013a).

Several groups have generated head injury risk curves using logistic regression models based on linear acceleration as a measure of exposure (Prasad and Mertz, 1985; Hertz, 1993; Kuppa, 2004). Prasad and Mertz suggest that a HIC15 (measurement of impact over 15 ms) value of 700 represents a less than 5% risk of life-threatening brain injury. More recently, Zhang et al. (2004) used a validated finite element human head model and predicted the maximum resultant linear acceleration at the CG of the head to be 66, 82, and 106 g for a 25%, 50%, and 80% probability of mTBI, respectively, whereas the maximum resultant rotational accelerations for a 25%, 50%, and 80% probability of sustaining a mTBI are estimated at 4600, 5900, and 7900 rad/s², respectively. Funk et al. (2007) estimated a 10% risk of mTBI at 165 g, a HIC of 400, and an angular head acceleration of 9000 rad/s².

To put injury threshold experiments into context with mTBI, many groups have directly measured or reconstructed concussive impacts during sporting events. Using helmets fitted with triaxial accelerometers, Pellman et al. (2003b) reconstructed impacts involving NFL players where players sustained concussions or significant head impacts and determined that peak head accelerations in concussed players averaged 98 g and in uninjured players averaged 60 g. The lowest measured acceleration where a player sustained a concussion was 48 g. The peak angular acceleration in concussed and uninjured players averaged 6432 rad/s² and 4235 rad/s², respectively. The lowest angular acceleration where a player sustained a concussion was 2615 rad/s². These
data are consistent with the predictions made from finite element modeling (FEM) studies.

Researchers at Virginia Tech used instrumented helmets (helmets designed with embedded accelerometers) to record tens of thousands of head impacts in football players including 57 diagnosed concussions. Linear acceleration of 171 g, 192 g, and 214 g were identified to result in a 25%, 50%, and 75% risk of mTBI (Rowson and Duma, 2011). Rotational accelerations of 5821 rad/s², 6383 rad/s², and 6945 rad/s² were associated with a 25%, 50%, and 75% chance of mTBI (Rowson et al., 2012). The most recent study investigated mTBI injury risk as a function of linear acceleration alone, rotational acceleration alone and a combination of both linear and rotational acceleration using logistic regression (Rowson and Duma, 2013). All three models were found to be good predictors of mTBI outcome. Although the combined model is preferred, it was statistically equivalent to the model using linear acceleration alone.

1.5 TBI: Pathophysiology

The TBI event can be divided into four stages: 1) mechanical forces (usually dynamic), act on the skull and brain to cause 2) head motion (rotation and/or translation of the head and skull) and 3) brain motion/deformation causing structural damage of brain and vascular tissues (primary injury), which leads to 4) a delayed biological response of the brain (i.e., deleterious biochemical and cellular processes that cause many TBI patients to gradually deteriorate in the hours and days after injury) referred to as secondary injury.
1.5.1 Primary Brain Injury

The primary brain injury is caused by mechanical forces resulting into structural damage to the brain and the cerebrovasculature. The primary brain injury can be focal or diffuse (Figure 1.4). Most clinical TBIs result in both focal and diffuse brain damage.

*Figure 1.4: Primary TBI.*

Primary TBI results from structural damage caused to the brain parenchyma and cerebrovasculature from mechanical forces. Focal injury results in contusion, laceration and/or vascular injury.
1.5.1.1 Focal Brain Injury

Focal brain injury is caused with or without skull fracture by impact forces such as a moving object striking against a stationary head (e.g., during physical assault) or a moving head striking against a stationary object (e.g., during fall). Focal injuries are thought to account for about two-thirds of TBI-related deaths (Davis, 2000). Whether or not the head is stationary may result in characteristic focal damage. Thus, a moving object striking a stationary head initiates head *acceleration* resulting in focal injury at the side of the impact called “coup injury”. On the other hand, a moving head striking stationary object results in sudden head *deceleration* and increases the probability of focal damage to the side opposite to the impact site called “contrecoup injury” (Dawson et al., 1980). Counterintuitively, the contrecoup injuries tend to be more severe than coup injuries as reported in several clinical observations (Dawson et al., 1980). The mechanisms of coup-contrecoup injuries are not well understood and are subject to controversies and as such several theories are proposed. According to the *positive pressure theory*, as the skull moves before impact, the brain lags and is pressed against the lagging surface of the skull (i.e., side opposite to the forthcoming impact site) (Lindenberg and Freytag, 1960; Drew and Drew, 2004). Also, as the brain lags, the cerebrospinal fluid (CSF) is displaced in the space in the coup site created by the lagging brain. The impact generates pressure waves that further increase compression of the brain against the lagging surface of the skull. Accumulation of CSF near the impact site further cushions against the coup injury causing more contrecoup damage.

The *negative pressure theory* (cavitation theory) argues that upon impact the head suddenly stops while the brain still continues to move towards the impact site (Russel,
This creates negative pressure (cavitation) at the contrecoup site pulling brain towards it. Dawson et al proposed the **angular acceleration theory** which is similar to positive pressure theory (Dawson et al., 1980). This theory is based on the principle of rotation of a tethered object around its center; the more distant the object is from the center of rotation or more massive the object is than to which it is tethered, slower will it move. By applying this principle, when the accelerating head strikes a stationary object, the brain lags resulting injury at the lagging side (i.e., contrecoup side). Using the principles of Newtonian mechanics and based on the difference between the relative densities of the brain (0.96 g/L) and the CSF (1 g/L) Drew and Drew recently proposed an alternate theory. According to this theory, when the moving head suddenly stops by impact, the CSF being denser than the brain, continues to move in the direction of the head movement and accumulates at the site of the impact pushing the brain towards the contrecoup side (Drew and Drew, 2004). This initial displacement of the brain results in more severe contrecoup injury compared to the coup injury.

### 1.5.1.1.1 Cerebral Contusion and Laceration

The most common result of coup-contrecoup injury is **cerebral contusion**, which is observed in about 20-30% of severe TBI (Khoshyomn and Tranmer, 2004). Cerebral contusion usually involves the cortex and is associated with damage to the gyri and small blood vessels often resulting in microhemorrhages (Yokobori and Bullock, 2013). The pia mater remains intact in contusion differentiating it from laceration in which the pia mater is torn (see below). The most common areas susceptible to contusion include
the orbitofrontal cortex, anterior temporal lobes, and posterior portion of superior
temporal gyrus and are thought to be due to the irregular surface of the bony floor
formed by the frontal and middle cranial fossa on which these structures lie
(Khoshyomn and Tranmer, 2004). The acute phase of contusion is characterized by
inflammatory cascades including vascular migration of polymorphonuclear leukocytes
seen within 24 h of injury, microglial activation seen over 3-5 days leading to release of
proinflammaratory cytokines such as tumor necrosis factor–α (TNF-α) and interleukin-1β
(IL-1β) (Holmin et al., 1998; Holmin and Hojeberg, 2004; Clausen et al., 2007),
apoptosis and necrosis (Raghupathi, 2004). This contributes to damage of the blood
brain barrier (BBB) and vasogenic edema resulting in swelling of the areas adjacent to
the contusion site, which is seen in about 30% of all contusions (Khoshyomn and
Tranmer, 2004). Extensive contusions may lead to subdural hematoma and together
they are called a “burst lobe” (Gennarelli and Graham, 2005). In addition to cortical
contusions, shearing forces (often in the sagittal plane) within the white matter can lead
to **gliding contusions** that occur in the subcortical regions causing damage at the gray-
white matter junction. Gliding contusions tend to be bilateral and are often associated
with DAI caused by differential motion of subcortical structures and acute subdural
hematomas caused by tearing of parasagittal veins (Adams et al., 1986; Sganzerla et
al., 1989).

**Laceration** is the most severe form of primary TBI resulting in disruption of the brain
parenchyma and involves torn pia-arachnoid membranes. Laceration requires greater
mechanical force compared to cerebral contusions. Penetrating brain injury or
depressing skull fractures lead to direct laceration, while parenchymal disruption secondary to tissue deformation caused by mechanical stress cause indirect lacerations (Blumbergs, 1997).

1.5.1.1.2 Intracranial Hemorrhage

Intracranial bleeding is a life-threatening complication of TBI and is the most common cause of TBI fatalities. Intracranial bleeding starts at the time of trauma and can expand to hematoma in 30-60% of patients with post-TBI coma (Gennarelli and Graham, 2005). Intracranial bleeding is categorized into four types: epidural hematoma, subdural hematoma, subarachnoid hemorrhage and intracerebral hemorrhage.

**Epidural hematoma/hemorrhage (EDH)** is accumulation of blood in the epidural space (i.e., between the skull and dura) caused by rupture of interposed meningeal vessels due to shearing stress. Usually EDH occurs due to coup injury and results in separation of the dura from periosteum. About two-thirds of EDHs are associated with skull fracture (McKissock et al., 1960). EDH most commonly occurs in the temporoparietal region and usually involves damage to the middle meningeal artery (Blumbergs, 1997). The incidence of EDH is about 2-4% of overall TBI and 15% of severe TBI (Bullock et al., 2006). Compared to adults, EDH is less common in pediatric TBI as the skull is more pliable reducing occurrence of skull fracture, the dura is firmly attached to the skull and the meningeal vessels are not yet embedded in the developing skull of infants (Yokobori and Bullock, 2013). In many cases, patients with EDH have a brief LOC, followed by a lucid interval and may deteriorate if the symptoms are not readily recognized/treated.
('talk and die' phenomenon). The common symptoms of EDH include headache, nausea/vomiting, seizures, and focal neurological deficits including aphagia, weakness, and numbness. EDH treated with neurosurgical intervention have better prognosis.

**Subdural hematoma/hemorrhage (SDH)** is bleeding between the dura and arachnoid membrane resulting from the rupture of bridging veins that cross the subdural space connecting the cerebral surface with the superior sagittal sinus. SDH occurs in about 5% of TBI and is associated with 65% of cases with prolonged LOC. While EDH are localized, SDH tend to spread as the fluid can freely move within the subdural space (Yokobori and Bullock, 2013). SDH is one of the classical triads of shaken baby syndrome, the other two being retinal hemorrhage and cerebral edema. SDH are classified into acute, subacute, and chronic depending on the duration of onset and composition (clotted blood, fluid blood or mixture of clotted and fluid blood) (Blumbergs, 1997; Gennarelli and Graham, 2005). **Acute SDH** is composed of clotted blood and forms within first 48 h of trauma and usually is caused by intense mechanical forces. Acute SDH can be associated with cerebral contusion/laceration and intracerebral hemorrhage together forming ‘burst lobes’ that cause delayed neurological deterioration (Blumbergs, 1997). Acute SDH is life-threatening with 30 to 90% mortality rate unless the patients are operated within the ‘golden hours’ (usually within 4 h) of injury (Seelig et al., 1981). The poor neurological outcome following acute SDH is associated with increased intracranial pressure (ICP) that decreases cerebral perfusion pressure (CPP), which in turn leads to cerebral ischemia (Graham et al., 1978). **Subacute SDH** is composed of both clotted and fluid blood that develops over 3-21 days while **chronic**
**SDH** is composed of fluid blood and develops for > 21 days. Chronic SDH is common in elderly and alcoholic patients who are more prone to cerebral atrophy and/or coagulopathy (Blumbergs, 1997). Subacute and chronic SDH have better prognosis than acute SDH.

**Subarachnoid hemorrhage (SAH)** is bleeding between the arachnoid membrane and pia mater and is the most common intracranial hemorrhage. SAH is observed in 40-65% of moderate-severe TBI and is an independent predictor of poor post-TBI prognosis (Eisenberg et al., 1990; Kakarieka et al., 1994; Taneda et al., 1996; Servadei et al., 2002). In most cases, SAH is mild but the severity of SAH increases when associated with cerebral contusions/lacerations (Blumbergs, 1997).

**Intracerebral hemorrhage (ICH)** is a hematoma 2 cm or greater in size, not in contact with the surface of brain, that is caused by rupture of intraparenchymal blood vessels at the time of impact. ICH is found in 15-20% of autopsy cases of severe TBI (Blumbergs, 1997; Yokobori and Bullock, 2013). Rupture of multiple vessels result in multiple ICH of which about 28% are associated with SDH and 10% with EDH (Soloniuk et al., 1986). Small ICH can occur at multiple sites including corpus callosum, the walls of third ventricle, and basal ganglia. Intraventricular hemorrhage is thought to be caused by deformation and rupture of subependymal veins due to sudden dilation of ventricular system during mechanical impact (Zuccarello et al., 1981) or extension of hematomas in the adjacent regions (Fujitsu et al., 1988).
1.5.1.2 Diffuse Brain Injury

1.5.1.2.1 Diffuse Axonal Injury

Diffuse axonal injury (DAI) is the widespread damage to the white matter and is the most common, most important, and yet the most difficult-to-diagnose TBI pathology. DAI is the major cause of TBI-induced unconsciousness/coma and vegetative state and associated with long-term disabilities (Gusmao and Pittella, 2003; Fork et al., 2005). Although the diagnosis of DAI with traditional imaging techniques such as CT and MRI has been challenging, recent advances in MRI techniques, such as DTI and susceptibility weighed imaging may enable the clinicians to capture this ‘stealth pathology’ even in mild cases (Hunter et al., 2012). DAI was first described by Strich as “diffuse degeneration of the white matter” observed in post-TBI human brain autopsies (Strich, 1956).

The principal biomechanical mechanism of DAI is thought to be rapid inertial loading (e.g., car crash) that induces dynamic shear, tensile and compressive stain in the brain tissue (Gennarelli et al., 1982; Smith and Meaney, 2000; Smith et al., 2003a). The axonal damage during TBI is affected by the unique material properties, size, and mass of the brain tissue, the presence of intracranial folds of dura mater (e.g., falx cerebri) and the rate and magnitude of strain (Smith and Meaney, 2000; Smith et al., 2003a). Several studies have shown that nervous tissue is a nonlinear, viscoelastic material (Donnelly and Medige, 1997; Darvish and Crandall, 2001; Takhounts et al., 2003). In addition, brain is highly inhomogeneous and anisotropic, thus showing regional differences in the biomechanical response to the mechanical loading. Thus, the gray
matter is isotropic while highly-organized white matter shows anisotropy with the greatest anisotropy in the corpus callosum (Prange et al., 2000; Prange and Margulies, 2002). When axons are subjected to slowly applied external load (e.g., during normal head rotation), they behave like a compliant and ductile material and return to their original geometry after removal of the stress (Franze et al., 2009; van Dommelen et al., 2009). On the other hand, under rapid and severe external loading (e.g., during car crash), axons behave like a stiffer and brittle material resulting structural failure. This property of axons along with their high anisotropic nature is thought to make white matter more vulnerable to the mechanical trauma (Geddes et al., 1997; Geddes et al., 2000). The direction of inertial loading and presence of intracranial dural folds my also affect DAI characteristics. For example, mechanical loading in the temporal region causes head rotation in the coronal plane. During such rotation, the falx cerebri (the dural fold that descends vertically between the two cerebral hemispheres) impedes motion of the following cerebral hemisphere while the leading cerebral hemisphere continues moving along the direction of motion. This creates excessive shearing forces along the midline between the two hemispheres. This, along with highly anisotropic nature of the corpus callosum, may lead to greater axonal damage in this region (Smith and Meaney, 2000; Smith et al., 2003a).

In extreme but rare TBI cases mechanical forces can cause direct axonal breakage, called 'primary axotomy'. In most cases, however axonal pathology develops over time following mechanical insult (Buki and Povlishock, 2006). Smith and colleagues conducted a series of elegant studies to understand the mechanism of axonal injury at
the cellular level. Using an *in vitro* axonal stretch injury model, Smith and colleagues showed that immediately following mechanical loading the axons become temporarily undulated at multiple sites, which is attributed to partial breakage of microtubules leading to cytoskeletal damage (Smith et al., 1999a; Tang-Schomer et al., 2010; Tang-Schomer et al., 2012). The initial axonal stretch results in massive Na\(^+\) influx through mechanosensitive voltage-gated Na\(^+\) channels causing membrane depolarization. Increased intracellular Na\(^+\) levels trigger Ca\(^{2+}\) influx, principally via opening of voltage-gated calcium channels and to a modest level, by reversal of Na\(^+\)-Ca\(^{2+}\) exchanger (Wolf et al., 2001; Iwata et al., 2004). The abnormal increase in intracellular Ca\(^{2+}\) leads to calpain-mediated structural proteolysis causing delayed damage to the axonal cytoskeleton and ion channels that ensues over days to weeks (Buki et al., 1999a; Buki et al., 1999b; Iwata et al., 2004; Huh et al., 2006; McGinn et al., 2009). Structural damage to the axonal cytoskeleton along multiple sites leads to disruption of axonal transport and subsequent accumulation of axonal proteins causing characteristic axonal swellings (axonal varicosities or beads) (Tang-Schomer et al., 2012). Two important proteins that accumulate at the site of axonal swellings are amyloid precursor protein (APP) and neurofilament (NF), which are transported by fast and slow transport mechanisms, respectively, and as such are the most commonly-used diagnostic markers of DAI (Gentleman et al., 1993; Grady et al., 1993; Sherriff et al., 1994a; McKenzie et al., 1996; Pierce et al., 1996). Accumulation of APP in axonal varicosities is thought to act as a nidus for the enhanced amyloid beta (A\(\beta\)) production, which has an important implication in the increased risk of Alzheimer disease in TBI survivors (discussed further). The proposed molecular mechanism of DAI and subsequent A\(\beta\)
accumulation are depicted in Figure 1.5. The progressive disruption of the cytoskeleton and subsequent protein accumulation ultimately lead to axonal disconnection, called 'secondary axotomy' characterized by formation of axonal bulbs at the terminal end of axon (Smith and Meaney, 2000; Smith et al., 2003a; Johnson et al., 2013).

Figure 1.5: Proposed molecular mechanism of diffuse axonal injury and generation of Aβ. Following rapid mechanical stretch (A) the axon becomes temporarily undulated (B) and relaxes back (C). This causes partial microtubule breakage at multiple sites (B & C). Axonal stretch also activates mechanosensitive Na\(^+\) channels causing massive Na\(^+\) influx, which in turn activate voltage gated Ca\(^{2+}\) channels. The excessive Ca\(^{2+}\) influx is
thought to exacerbate calpain-mediated disruption of the cytoskeleton (red arrows in E). Microtubule interrupts protein transport along the axon causing accumulation of proteins, including APP along with components of APP-cleaving enzymes, BACE1 and PS1 resulting in axonal varicosities (F). The progressive disruption of cytoskeleton results in axonal disconnection with formation of axonal bulb (G). The cleavage of APP accumulated in the axonal varicosities/bulbs by BACE1 and PS1 is thought to lead to a burst of Aβ production (H). APP: Amyloid Precursor Protein, BACE1: Beta Site APP Cleaving Enzyme 1, PS1: Presenilin-1, VGCC: Voltage-Gated Ca²⁺ Channel, VGNC: Voltage-Gated Na⁺ Channel. Adapted from Tang-Schomer et al. (2012) and Johnson et al. (2010)

1.5.1.2.2 Diffuse Vascular Injury

Diffuse vascular injury (DVI) is characterized by multiple macroscopic and/or microscopic hemorrhagic brain lesions (Tomlinson, 1970; Adams, 1980). Diffuse vascular injury is almost always fatal with short survival duration (< 24 h) post-trauma (Pittella and Gusmao, 2003). DVI and DAI are suggested to be associated with each other and share the same biomechanical mechanism (Pittella and Gusmao, 2003). One major difference between the two pathologies however, is that while DAI can occur at low acceleration, DVI is almost exclusively seen in motor vehicle accidents that involve high-rate inertial forces (Gennarelli and Thibault, 1982; Pittella and Gusmao, 2003).

1.5.2 Secondary Brain Injury

The primary TBI triggers downstream cellular and biochemical cascades that ensue for days to months, called secondary brain injury, which lead to long-term disabilities. In most cases the damage caused by the primary brain injury is irreparable however secondary brain injury is amenable to therapeutic and supportive interventions (Park et
The consequences of secondary injury include disruption of cerebrovasculature including damage to the BBB, alteration in the cerebral blood flow (CBF), edema, neurometabolic changes including disruption of brain energy metabolism, inflammation, free radical formation, necrosis and apoptosis. While a complete discussion of secondary injury pathways is beyond the scope of this thesis, key secondary injury mechanisms are briefly discussed below.

### 1.5.2.1 Cerebrovascular Response to TBI

Brain injury causes changes in cerebrovascular response through alteration in BBB function and CBF that lead to edema, increased intracranial pressure (ICP), cerebral ischemia and hypoxia. The BBB consists of specialized endothelial cells that form tight junctions with each other and are surrounded by the basal lamina matrix, pericytes and astrocyte end feet. This gliovascular unit tightly regulates transport of metabolites, nutrients and xenobiotics between the brain and the periphery (Abbott et al., 2006). TBI is often accompanied by disruption of BBB via a variety of mechanisms including, increased BBB permeability and proinflammatory cytokine activity, loss and/or redistribution of tight junction proteins and changes in the activity of BBB transporters (Zink et al., 2010). Changes in BBB function in response to injury are thought to influence the evolution of injury and response to therapeutic interventions. A compromised BBB allows entry of plasma-derived, osmotically-active molecules such as albumin and fibrinogen into the brain interstitial space leading to development of vasogenic edema (Chodobski et al., 2011).
The brain receives about 20% of the cardiac output. CBF is tightly controlled through constant modulation of vascular resistance and BBB (Zink et al., 2010). TBI decreases CBF as shown by CT and PET scanning (Bouma et al., 1992; Scalfani et al., 2012). The reduction in CBF is thought to occur due to dysfunction of both macro- and microvasculature leading to focal or in severe cases global ischemia (Bouzat et al., 2013).

1.5.2.2 Neurometabolic Changes Following TBI

TBI leads to perturbations of cell membrane leading to redistribution of ions and neurotransmitters. During the acute phase of injury, stretching of axonal membrane opens voltage-dependent K⁺ channels leading to massive K⁺ efflux. This has been shown with increased extracellular K⁺ concentration measured by in vivo microdialysis in various animal models of TBI (Takahashi et al., 1981; Hubschmann and Kornhauser, 1983; Katayama et al., 1990). The duration of K⁺ increase is shown to be dependent on the injury dose with shorter duration following mTBI whereas moderate-severe TBI cause prolonged K⁺ increase (Katayama et al., 1990). The former is associated with increased neuronal firing while the latter coincides with indiscriminate glutamate release (Katayama et al., 1990). In addition to K⁺, excessive glutamate also leads to uncontrolled shifts in Na⁺ and Ca²⁺ homeostasis (Bullock, 1994; Bullock et al., 1998). To compensate for these ionic alterations, the ion pumps, especially Na⁺/K⁺-ATPase go into the overdrive mode further increasing metabolic demand setting a vicious cycle of CBF-metabolism uncoupling to the cell (Werner and Engelhard, 2007).
1.5.2.3 Neuroinflammation Following TBI

Neuroinflammation is an important secondary injury mechanism that contributes to ongoing impairment following TBI. Microglia, the resident macrophages in the central nervous system (CNS) act as the “first responders” mediating the immune response to the brain injury. Microglial activation has been demonstrated as early as 72 hours after human TBI and can persist for years after injury (Engel et al., 2000; Beschorner et al., 2002; Gentleman et al., 2004; Ramlackhansingh et al., 2011). This activation profile is also mirrored in animal models of TBI (Csuka et al., 2000; Koshinaga et al., 2000; Maeda et al., 2007). Studies have shown that under physiological and pathological conditions microglia assume different activation states that have distinct morphological characteristics (Boche et al., 2013). At resting state, microglia show ramified morphology characterized by short, fine processes and are thought to constantly monitor their local environment. In response to an acute insult, such as trauma, resting microglia are activated with dramatic changes in morphology and behavior. The cells retract their processes and assume amoeboid morphology resembling macrophages that proliferate and migrate towards the site of injury (Loane and Byrnes, 2010; Boche et al., 2013). In certain chronic infections, microglia can assume rod morphology characterized by markedly elongated nuclei, scanty cytoplasm and few processes. Recently, rod microglia were observed in the brain regions that have an associated sensory sensitivity following diffuse brain injury in rats (Ziebell et al., 2012) although their significance in TBI pathology is currently unknown. Classically activated microglia, also called M1 microglia release proinflammatory mediators, including cytokines, chemokines, nitric oxide and superoxide free radicals that contribute to neuronal
dysfunction and cell death in response to injury (Loane and Byrnes, 2010). Both human and animal studies have shown rapid elevation of proinflammatory cytokine IL-1β within hours following TBI (Woodroffe et al., 1991; Fan et al., 1995; Winter et al., 2002), which activates proinflammatory pathways such as TNF-α (Rothwell, 2003) through interleukin 1 receptor type 1 expressed in microglia and neurons (Pinteaux et al., 2002; Lu et al., 2005). Recent studies have identified an alternatively activated state of microglia, called M2 microglia (Colton, 2009; Kigerl et al., 2009) that are considered to have anti-inflammatory actions and secrete anti-inflammatory cytokines including IL-10 and neurotrophic factors such as nerve growth factor and transforming growth factor β following injury (Csuka et al., 1999).

1.6 TBI, Alzheimer Disease, and Apolipoprotein E

1.6.1 TBI and Alzheimer Disease

Research over last 30 years indicates an increased risk of dementia, particularly of Alzheimer's disease (AD) in TBI survivors (Van Den Heuvel et al., 2007; May et al., 2011). Moreover, besides age, antecedent TBI is now considered as one of the most important environmental risk factors for dementia. Several epidemiological studies have indicated increased dementia risk in TBI victims (French et al., 1985; Mortimer et al., 1985; Mortimer et al., 1991; van Duijn et al., 1992; Rasmusson et al., 1995; Guo et al., 2000; Plassman et al., 2000; Fleminger et al., 2003; Barnes et al., 2014), although this association is not always observed (Katzman et al., 1989; Li et al., 1992; Fratiglioni et al., 1993). One potential reason for the disagreement between the epidemiological studies is that the data collection in most of these studies was retrospective, based on
low sample number and thus may have suffered from recall bias (May et al., 2011). Nonetheless, a high-powered EURODEM meta-analysis pooled the findings of 11 case-control studies and reported a significant relative risk of 1.82 (95% CI: 1.26-2.67) for head trauma associated with AD (Mortimer et al., 1991). Based on the available epidemiological data, the Committee on Gulf War and Health: Brain Injury in Veterans and Long-Term Health Outcomes recently concluded that “there is sufficient evidence of association between moderate to severe TBI and AD, limited/suggestive evidence of association between mild TBI with LOC and AD and inadequate evidence of association between mild TBI without LOC and AD” (Rutherford et al., 2008). TBI and AD show striking similarities in their pathologies, which may shed light into their association. In the following sub-sections I have discussed similarities and differences between TBI and AD pathologies.

1.6.2 Alzheimer's Disease: Pathology
AD is defined by two neuropathological hallmarks: 1) extraneuronal amyloid plaques mainly composed by aggregated amyloid beta (Aβ) peptides and 2) intraneuronal neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein. Central to the AD pathology is the widely accepted, yet controversial amyloid cascade hypothesis according to which the deposition of Aβ in the brain parenchyma is a crucial step that triggers formation of intraneuronal tau aggregates and ultimately leads to AD (Hardy and Higgins, 1992; Selkoe, 1994; Hardy and Selkoe, 2002; Karran et al., 2011). The amyloid cascade hypothesis was developed on the basis of the discovery of autosomal dominant mutations in APP, the parent protein of Aβ peptides and Presenilin-1 (PS1)
and Presinilin-2 (PS2), the components of γ-secretase (an enzyme involved in cleavage of APP that leads to Aβ generation) that cause Familial/Early Onset AD (EOAD) (Chartier-Harlin et al., 1991; Goate et al., 1991; Citron et al., 1992; Levy-Lahad et al., 1995; Rogaev et al., 1995; Scheuner et al., 1996). On the other hand, apolipoprotein E (apoE), specifically the apoE ε4 allele is an established genetic risk factor for the sporadic or Late Onset AD (LOAD) and is thought to affect several aspects of AD pathology, including Aβ aggregation and clearance, tau phosphorylation, inflammation, neuronal repair and synaptic plasticity (discussed further).

1.6.2.1 Amyloid Beta Formation in the CNS

Aβ peptides are physiologically produced by the sequential enzymatic processing of the highly-conserved type I transmembrane glycoprotein, APP. APP is a single transmembrane domain protein that consists of a large extracellular domain, a hydrophobic transmembrane domain and a short intracellular C terminal tail (Suh and Checler, 2002). The juxtamembrane region of APP contains the Aβ sequence composed of 43 amino acids (amino acids 597-639 of APP). APP is highly expressed in neurons (Slunt et al., 1994; Lorent et al., 1995), undergoes fast anterograde axonal transport (Koo et al., 1990; Buxbaum et al., 1998) and is processed via two mutually-exclusive pathways: anti-amyloidogenic and amyloidogenic (Figure 1.6). The majority of APP is processed through the anti-amyloidogenic pathway that involves enzymatic cleavage, first by α-secretase followed by γ-secretase (Suh and Checler, 2002). Alpha-secretase, a membrane bound, zinc metalloproteinase of the ADAM family, first cleaves the APP within the Aβ sequence between residues Lys16 and Leu17 releasing a large
secreted α APP (sAPPα) domain and a truncated, 83-residue carboxyl terminal fragment (CTFα or C83) that lacks the N-terminal portion of Aβ (Naslund et al., 1994). In the amyloidogenic pathway instead of α-secretase APP is cleaved by β-secretase, which is the rate-limiting step in the formation of Aβ species (Vassar, 2004). The neuronal form of β-secretase, also known as β-Site APP Cleaving Enzyme 1 (BASE1), a membrane-bound aspartyl protease cleaves the extracellular domain of APP generating sAPPβ and a 99-residue CTFβ or C99 that contains the N-terminal domain of Aβ. Both CTFα and CTFβ are substrates of γ-secretase, which is a complex of four subunits: PS-1, PS-2, nicastrin, APH-1 and PEN-2 (Iwatsubo, 2004). Gamma-secretase cleaves CTFα in the transmembrane domain releasing a 3 kDa, N-terminal-truncated Aβ peptide, called P3 in the extracellular fluid and a cytosolic amyloid intracellular domain (AICD). Cleavage of CTFβ by γ-secretase results in the release of 4 kDa Aβ in the extracellular fluid and cytosolic AICD. Gamma-secretase can cut CTFβ at multiple sites; first in the ε site generating 48 or 49-residue Aβ and subsequently every 3-4 amino acid residues generating 39-43 residue Aβ species (Kakuda et al., 2006; Selkoe and Wolfe, 2007). The longer residue Aβ species tend to aggregate more readily and are toxic. Under physiological conditions, γ-secretase cleavage generates less-toxic Aβ40 and more-toxic and fibrillogenic Aβ42 species that account for 90% and <10% of secreted Aβ species, respectively (Thinakaran and Koo, 2008). While Aβ42 is the major component of amyloid plaques, Aβ40 accounts for most of the amyloid deposited in cerebral vessels, which is called cerebral amyloid angiopathy (CAA) and is observed in 80% of AD patients (Biffi and Greenberg, 2011; Masters and Selkoe, 2012).
Figure 1.6: Amyloidogenic and anti-amyloidogenic processing of APP.

The anti-amyloidogenic processing of APP involves sequential cleavage of APP by first \( \alpha \)-secretase that generates sAPP\( \alpha \) and CTF\( \alpha \) followed by cleavage of CTF\( \alpha \) by \( \gamma \)-secretase generating AICD and truncated A\( \beta \) fragment, P3. The amyloidogenic pathway involves cleavage of APP by first \( \beta \)-secretase that generates CTF\( \beta \) and sAPP\( \beta \) followed by cleavage of CTF\( \beta \) generating A\( \beta \) and AICD.

1.6.2.2 Tauopathy in Alzheimer Disease

The second pathological hallmark of AD is intraneuronal neurofibrillary tangles (NFT), which are composed of abnormally hyperphosphorylated tau (p-tau) protein that forms paired helical filaments (PHF) (Iqbal et al., 2010). According to the amyloid hypothesis, while amyloid plaque formation is the initiating event of AD pathology, formation of NFT is a downstream event and executes neuronal death (Hardy and Selkoe, 2002; Karran
et al., 2011). Tau is the major microtubule associated protein (MAP), expressed almost exclusively in neurons and is mostly localized in axons (Binder et al., 1985).

Microtubules are non-covalent cytoskeletal polymers composed of α- and β-tubulins that play an important role in trafficking of organelles and other substances along axons (Conde and Caceres, 2009). Humans express six tau isoforms including three of each of three-repeat (0N3R, 1N3R, and 2N3R) and four-repeat (0N4R, 1N4R, and 2N4R) tau, which are generated by alternative splicing of the MAP tau (MAPT) gene on chromosome 17q21.31 (Neve et al., 1986; Goedert et al., 1989; Andreadis et al., 1992). In contrast to humans, the adult mouse brain expresses only four-repeat isoforms (0N4R, 1N4R, and 2N4R) (Gotz et al., 2010). The two important known functions of tau are 1) facilitation of microtubule assembly and 2) microtubule stabilization by reducing microtubule dynamics through its interaction with tubulin (Weingarten et al., 1975; Bré and Karsenti, 1990). The interaction of tau with tubulin occurs at the C-terminus of tau protein that contains microtubule binding repeat (MTBR) domain composed of three or four tandem repeat motifs (Lee et al., 1989). The binding of tau to tubulin is regulated and is inversely correlated to the degree of tau phosphorylation, especially at Ser262 and Thr231 residues within and flanking the MTBR domain (Wang et al., 2007). Thus, phosphorylation of Ser262 within the flanking region of MTBR significantly reduces binding of tau with microtubules (Biernat et al., 1993). Phosphorylated tau under physiological conditions contains 2-3 moles of phosphate per tau molecule, while hyperphosphorylated tau associated with AD is defined to contain > 6 moles of phosphate per tau molecule (Iqbal et al., 2010). Abnormally p-tau is incapable of binding to tubulin, thus remains free and polymerizes into PHF, which negatively affects
microtubule assembly and stabilization (Alonso et al., 2006). While tau can be phosphorylated by a variety of kinases (e.g., glycogen synthase kinase, cdk5, MAP kinase, JNK, and cdc2), glycogen synthase kinase 3β (GSK3β) plays the most important role in regulating tau phosphorylation under physiological and pathological conditions (Avila et al., 2004). The principle enzyme that controls tau dephosphorylation is protein phosphatase 2A (PP2A). The phosphorylation state of tau is regulated by the balance between the activities of protein kinases and phosphatases. For example, PP2A levels in AD brains are significantly downregulated (Sontag et al., 2004a; Sontag et al., 2004b). On the other hand, Pei et al found approximately 50% increase although without any increase in the activity of GSK3β levels in AD brains (Pei et al., 1997).

While Aβ plaque pathology is seen exclusively in AD, tauopathy besides AD is also associated with other neurodegenerative disorders such as Frontotemporal Dementia and Parkinson’s Disease linked to chromosome 17, Pick’s Disease, Corticobasal Degeneration, Progressive Supranuclear Palsy, Argyrophilic Grain Disease, Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia Complex (Spillantini and Goedert, 2013) and Chronic Traumatic Encephalopathy (CTE) (McKee et al., 2009). A distinct feature of tauopathy seen in AD and CTE compared to other tauopathy-related neurodegenerative disorders is that the NFT in AD and CTE are composed of all six abnormally p-tau isoforms (Goedert et al., 1992; Schmidt et al., 2001). On the other hand, the tauopathy in other neurodegenerative disorders involves abnormal hyperphosphorylation of either four-repeat (e.g., Progressive Supranuclear Palsy, Corticobasal Degeneration and Argyrophilic Grain Disease) or three-repeat (e.g., Pick’s
Disease) tau (Flament et al., 1991; Ksiezak-Reding et al., 1994; Delacourte et al., 1996; Spillantini et al., 1997; Tolnay et al., 2002).

1.6.3 TBI and Alzheimer’s Pathology: Similarities and Differences

1.6.3.1 Amyloid Pathology after Moderate to Severe Single TBI

One of the first studies linking TBI to dementia was by Rudelli et al who reported classical AD pathology seen in the brain autopsy of a 38-year old man who died 16 years following recovery after a single severe head trauma (Rudelli et al., 1982). In subsequent brain autopsy studies it was reported that about 30% of fatal TBI cases had cortical Aβ deposition, strikingly similar to AD plaques (Roberts et al., 1991; Roberts et al., 1994; Gentleman et al., 1997). Moreover, the Aβ deposits seen in severe TBI cases were predominantly composed of insoluble Aβ42 (Gentleman et al., 1997; DeKosky et al., 2007). There are however, important differences in the plaque pathology found in the severe head trauma cases and AD. First, Aβ deposition in these acute TBI cases was observed irrespective of the patient’s age, i.e., even in children, which is in contrast to AD in which the plaque pathology developed in advanced age. Second, severe head trauma triggered rapid Aβ deposition, seen as early as 2 h of TBI, as opposed to AD pathology that develops over years (Ikonomovic et al., 2004). Third, TBI-associated Aβ deposits are not restricted to the gray matter but have also been reported in the white matter (Smith et al., 2003b). Finally, the plaques formed following TBI tend to be diffuse compared to solid, dense-core plaques seen in AD brains. In contrast to the above studies, Adle-Biasette et al found no evidence of Aβ pathology in 23 cases of age
between 17 and 63 years and 17 cases of age between 69 and 79 years who died between 0-76 days after head trauma (Adle-Biassette et al., 1996).

The mechanisms of rapid Aβ deposition following TBI are unclear. A potential source of Aβ is postulated to be the axonal swellings (varicosities) formed by DAI (Johnson et al., 2010). In vitro studies have shown that axonal stretch at high shear rate and intensity induces microtubule breakage at multiple sites along the length of the axon resulting in interruption of axonal transport of various proteins, including APP (as discussed in section 1.5.1.2.1). Intra-axonal accumulation of APP following TBI has been reported in humans (Gentleman et al., 1993; Sherriff et al., 1994a; Gorrie et al., 2002) and animal models (Lewen et al., 1995; Pierce et al., 1996; Smith et al., 1999b). Interestingly, the components of APP cleaving enzymes, BASE1 and PS1 have also been shown to co-accumulate along with APP in the damaged axons in humans (Chen et al., 2004; Uryu et al., 2007) and animals (Chen et al., 2004). The axonal transport of these proteins is shown to be mediated by the axonal transport protein, kinesin-I (Kamal et al., 2000) and APP is thought to act as kinesin-I receptor facilitating transport of BACE-1 and PS1 (Kamal et al., 2001). Based on these observations it is proposed that the co-accumulation of APP, BASE-1 and PS1 within the axonal varicosities form a nidus for Aβ production (Johnson et al., 2010). Supporting this hypothesis, intra-axonal accumulation of Aβ has been reported following TBI (Chen et al., 2004; Uryu et al., 2007; Chen et al., 2009). Thus, DAI seems to create a unique environment bringing together all the machinery necessary for Aβ production (i.e., APP, BACE-1 and PS1), which is thought to lead to a burst of Aβ production that can deposit as amyloid plaques.
(Johnson et al., 2010). To counteract the increased Aβ production, the traumatized brain may also upregulate Aβ clearance mechanisms. For example, Chen et al reported increased intra-axonial accumulation of nepriysin (NEP), a major Aβ degrading enzyme, following TBI (Chen et al., 2009). In support of this observation, Johnson et al reported that a genetic polymorphism in NEP that resulted into longer GT repeats in the enzyme was associated with increased risk of Aβ deposition after TBI while shorter GT repeats NEP decreased the incidence of Aβ deposition (Johnson et al., 2009). Thus, a balance between Aβ production and clearance after TBI may be crucial in preventing Aβ-related pathologies in TBI. In this regard, apoE may also play an important role as it is shown to modulate Aβ clearance mechanisms (discussed further).

While the majority of clinical studies indicate rapid plaque pathology in the acute phase of TBI, the Aβ plaque dynamics in the long-term TBI survivors seems controversial. Chen and colleagues studied 23 cases with post-TBI survival up to 3 years (mean survival 245 days) and found widespread axonal pathology along with intra-axonal accumulation of APP, BASE1, PS1 and Aβ over the long term. The authors also reported NEP immunoreactivity in axons for up to 8 month post-injury. Surprisingly, the authors did not find any evidence of Aβ plaques in the same cases (Chen et al., 2009). The authors suggested that while Aβ plaques are seen in short-term post-TBI survivors, plaques may undergo NEP-mediated regression over months-to-years post-injury. In contrast to the above study, in a recent study by the same group, widespread tau and amyloid pathology was found in 39 single moderate to severe TBI cases with survival up to 47 years (Johnson et al., 2012). It should be noted however that Chen et al have not
reported on any tauopathy as reported by Johnson et al in the absence of Aβ deposition. This may be important for two reasons. First, while amyloid hypothesis is widely accepted as the basis of AD pathology, it is not without controversies. For example, few studies have found no correlation between amyloid plaque load and the degree of cognitive impairment, i.e., some subjects with substantial amyloid plaques do not exhibit clinical signs of AD (Armstrong, 1994; Giannakopoulos et al., 2003). Second, in most of the studies mentioned above, Aβ plaque deposits were found only in one-third of the cases. Thus, as suggested for AD pathogenesis (Hardy and Selkoe, 2002; Karran et al., 2011), while Aβ plaque pathology may be a triggering event, tauopathy may be the executioner of the synaptic and neuronal loss and long-term cognitive deficits in TBI survivors. This is underlined by tauopathy which is the principal pathology of CTE, a progressive neurodegenerative disorder thought to be caused by repeated head trauma (see the next section). Moreover, in a recent study it was shown that inhibition of APP processing by treating 3XTg-AD mice with a γ-secretase inhibitor blocked Aβ accumulation without affecting tauopathy following controlled cortical impact injury suggesting that Aβ accumulation and tauopathy in TBI may be independent pathways (Tran et al., 2011a).

While the data from post-mortem brain analyses indicate increased Aβ production following TBI, Aβ and tau levels have also been measured in CSF and recently in brain interstitial fluid (ISF) of TBI patients and have revealed intriguing changes in Aβ dynamics. For example, two studies reported an initial increase the CSF Aβ42 levels peaking at ~ 5 days after severe TBI (GCS <8) followed by decline over several weeks
(Raby et al., 1998; Olsson et al., 2004). In both these studies, CSF tau levels were not measured. In contrast to these findings, Franz et al found a significant decrease in CSF Aβ42 measured over 1-30 days in severe TBI cases (Franz et al., 2003). Tau levels in the same patients were significantly increased peaking between 5-15 days followed by a decline over 30 days (Franz et al., 2003). Marklund et al assessed total tau and Aβ42 levels in brain ISF collected by real-time in situ microdialysis from eight patients with either focal/mixed TBI or DAI over 8 days following TBI and found a non-significant increase in Aβ42 levels in patients with DAI compared to patients with focal/mixed TBI. Brody et al measured Aβ levels in ISF sample obtained from 18 TBI patients from 12-48 h and observed some very intriguing trends in Aβ dynamics (Brody et al., 2008). The authors found that the ISF Aβ levels correlated positively with the patient’s neurological status as determined by GCS such that ISF Aβ levels declined in patients with worse neurological status, increased as the patient’s neurological status improved and remained stable in clinically stable patients (Brody et al., 2008). In their follow-up study, Brody and colleagues found > 3-fold increase in ISF tau levels at 1-12 h followed by a decline over 61-72 h following TBI (Magnoni et al., 2012). The authors further found that the high initial tau levels were correlated with worse neurological outcomes and inversely correlated with initial low ISF Aβ levels. Based on these surprising findings, the authors hypothesized that low Aβ and high tau levels during the acute post-TBI phase may be associated with reduced synaptic activity (Magnoni and Brody, 2010) as ISF Aβ levels are directly regulated by synaptic activity (Cirrito et al., 2005). Brain ISF Aβ dynamics was also replicated in a controlled cortical impact (CCI) model in AD as well as wild-type mice wherein both human as well as murine ISF Aβ levels were
significantly reduced following TBI (Schwetye et al., 2010). The authors further found that in concordance with ISF, Aβ levels in phosphate buffered saline (PBS)-soluble hippocampal and cortical lysates were also reduced. The pathophysiological implications of differential Aβ dynamics following brain trauma in the short-term vs long-term effects of TBI are unclear and require further investigation.

### 1.6.3.2 Consequences of Repetitive TBI: Chronic Traumatic Encephalopathy

While the data on long-term consequences of moderate to severe single TBI is limited, it is long known that mild, repetitive head trauma can increase the risk of late-life dementia. In 1928, Harrison Martland first described “Punch Drunk” syndrome among boxers who sustained multiple head blows with early symptoms characterized by gait deficits with slow muscular movements, hand tremors and speech hesitancy (Martland, 1928). In the later stages, the fighters with severe head blows suffered from Parkinsonian-like facial tremors, and vertigo ultimately resulting in mental deterioration. Millspaugh coined the term ‘dementia pugilistica’ (pugilist: a professional boxer) to the symptoms described by Martland in boxers (Millspaugh, 1937). The first detailed pathological features of dementia pugilistica were described by Brandenburg and Hallervorden (Brandenburg and Hallervorden, 1954) and later by Corsellis et al (Corsellis et al., 1973). While the earlier studies on the aftermath of repetitive head impacts were focused on boxing, studies in the last decade showed that the clinical symptoms and neuropathology described as dementia pugilistica were also seen in other situations that involved increased risk of repetitive head impacts including contact sports such as American football, wrestling, hockey and association football (soccer).
(Cantu, 2007; McKee et al., 2009) and in military veterans exposed to blast injuries (Omalu et al., 2011; Goldstein et al., 2012). Thus the currently-accepted term for dementia pugilistica is chronic traumatic encephalopathy (CTE).

Presently no biomarkers are available for the diagnosis of CTE. The current neuropathological data comes from the post-mortem analysis of athlete brains, while the clinical features of CTE are mostly based on the interviews of friends and family members. The clinical manifestation of CTE occurs several years after exposure to repetitive TBI and involves cognitive impairments, neuropsychiatric alteration, and motor deficits that are thought to occur over three stages (Chin et al., 2011). Stage 1 is characterized by affective disturbances and psychotic symptoms (paranoia, agitation) and changes in mood and behavior (depression, aggression or violence, irritability, suicidal tendency). Stage 2 involves social instability and memory deficits, with initial symptoms of Parkinsonism. In the last stage the patient manifests general cognitive dysfunction and progression of dementia, speech or gait deficits or full-blown Parkinsonism.

The macroscopic neuropathology of CTE is characterized by marked atrophy of cerebral regions (frontal and temporal regions) (Neubuerger et al., 1959; Corsellis et al., 1973; McKee et al., 2009) and general atrophy of the cerebellum (Williams and Tannenberg, 1996). Another gross neuropathological feature of CTE is cavum septum pellucidum (separation of leaflets of septum pellucidum) with a fenestrated septum (Omalu et al., 2006; McKee et al., 2009; Stern et al., 2011; McKee et al., 2013).
Although many of the clinical features of CTE overlap with those of AD, the microscopic pathology of CTE shows some features that distinguish CTE from AD (Table 1.5). Thus, the primary histological feature of CTE is extensive deposition of p-tau as NFT such that CTE is now often considered as a tauopathy (McKee et al., 2009; McKee et al., 2013). A second distinctive histopathologic feature of CTE is presence of TAR DNA-binding protein 43 (TDP-43) with TDP-43 positive neurites in the cortex, brain stem, and temporal lobe seen in early stages and in frontal subcortical white matter and fornix, brainstem and medial temporal lobes in later stages (McKee et al., 2010; McKee et al., 2013). TDP-43 pathology is seen in about 80% of CTE cases (Baugh et al., 2014) as opposed to a lower incidence (25-30%) in AD (Wilson et al., 2011). Lastly, while amyloid deposition is the neuropathological signature of AD, the presence of Aβ deposition is an inconsistent feature of CTE and is reported in only 25-50% of CTE cases (McKee et al., 2009; McKee et al., 2013).
<table>
<thead>
<tr>
<th>Chronic Traumatic Encephalopathy</th>
<th>Alzheimer Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prominent presence of tau tangles in the astrocytes</td>
<td>Astrocyte tau tangles absent</td>
</tr>
<tr>
<td>Perivascular deposition of NFT</td>
<td>Perivascular NFT absent</td>
</tr>
<tr>
<td>Prominent NFT deposition at depths of cerebral sulci</td>
<td>No NFT deposition at depths of cerebral sulci</td>
</tr>
<tr>
<td>Prominent subpial astrocytic tangles</td>
<td>Absence of subpial astrocytic tangles</td>
</tr>
<tr>
<td>Periventricular astrocytic tangles</td>
<td>Absence of periventricular astrocytic tangles</td>
</tr>
<tr>
<td>Focal NFT seen in the frontal lobe cerebral cortex in early stages</td>
<td>NFT seen in entorhinal cortex, amygdala and hippocampus</td>
</tr>
<tr>
<td>Patchy and irregular NFT distribution in advanced stages</td>
<td>Uniform NFT distribution in advanced stages</td>
</tr>
<tr>
<td>Tau pathology seen in white matter</td>
<td>Virtual absence of tau pathology in white matter</td>
</tr>
<tr>
<td>Amyloid deposits seen only in 25-50% of CTE cases</td>
<td>Amyloid deposits is the signature pathology</td>
</tr>
<tr>
<td>High incidence (~ 80%) of TDP-43 pathology</td>
<td>TDP-43 pathology seen in 25-30% of cases</td>
</tr>
</tbody>
</table>

**Table 1.5: Differences in the pathological features of CTE and AD.**

Adapted from (McKee et al., 2013).

Recently McKee and colleagues proposed four stages of CTE neuropathology based on the severity of pathological and clinical features recorded from 68 cases with confirmed CTE (McKee et al., 2013). The four stages of CTE are briefly described in the Table 1.6.
<table>
<thead>
<tr>
<th>CTE Stage</th>
<th>Neuropathological Features</th>
<th>Associated Clinical Features</th>
</tr>
</thead>
</table>
| I         | • Mild lateral ventricle enlargement seen in some cases  
             • Focal perivascular NFT at depth of cortical sulci | • Headache  
             • Loss of attention and concentration |
| II        | • Mild enlargement of frontal horn of lateral and 3rd ventricles  
             • Small cavum septum pellucidum  
             • NFT in superficial cortical layers adjacent to focal epicenters and in nucleus basalis of Meynert and locus ceruleus | • Depression and mood swings  
             • Explosivity  
             • Headache  
             • Loss of attention and concentration  
             • Short-term memory loss |
| III       | • Mild cerebral atrophy, ventricular dilation, septal abnormalities, a sharply concave contour of 3rd ventricle, depigmentation of locus ceruleus and substantia nigra  
             • Dense p-tau pathology in hippocampus, entorhinal cortex, amygdala and widespread in cortex, diencephalon, brainstem and spinal cord | • Cognitive impairment  
             • Memory loss  
             • Executive dysfunction  
             • Depression  
             • Explosivity  
             • Loss of attention and concentration  
             • Visuospatial abnormalities |
| IV        | • Atrophy of medial temporal lobe, cerebrum, hypothalamus, thalamus, mammillary body, septal abnormalities, ventricular dilation and pallor of locus ceruleus and substantial nigra  
             • Widespread p-tau pathology including white matter  
             • Prominent neuronal loss and gliosis in cortex  
             • Hippocampal sclerosis | • Dementia with profound short-term memory loss  
             • Executive dysfunction  
             • Loss of attention and concentration  
             • Explosivity and aggression  
             • Paranoia, depression, impulsivity  
             • Visuospatial abnormalities |

*Table 1.6: Pathological and clinical stages of CTE.*  
Adapted from McKee et al. (2013).
1.6.4 Apolipoprotein E (ApoE) at the Nexus of TBI and AD Pathology

Familial or early onset AD is caused by mutations in APP, PS1 and PS2 genes and represents less than 10% of total AD cases. The majority of the AD cases has no known causative genetic mutations and is thus called sporadic or late onset AD (LOAD). While the exact cause of LOAD is currently unknown it is believed that the development and progression of AD is affected by multiple environmental and genetic risk factors. For example, advanced aging is considered the greatest environmental risk factor. On the other hand, apolipoprotein E (apoE), particularly the ε4 (apoE4) allele is the most established genetic risk factor for LOAD (Corder et al., 1993; Saunders et al., 1993). Interestingly, apoE4 is also thought to affect recovery from TBI (discussed further).

The CNS is the most lipid-rich tissue in the body, containing over 25% of total body cholesterol in only 2% of total body weight (Dietschy and Turley, 2001; Vance et al., 2005). Almost all of the CNS cholesterol is in the unesterified form and about 70% of this form is present in oligodendrocytes that make myelin sheaths, which accounts for ~50% of the white matter (Bjorkhem and Meaney, 2004). Being insoluble in water, cholesterol is transported on lipoprotein particles that consist of apolipoproteins that surround, stabilize, and solubilize their fatty cargo. The CNS lipid metabolism has some unique features that separate it from the peripheral lipid metabolism. For example, because of the presence of the BBB, there is virtually no exchange of cholesterol and lipoproteins between the CNS and periphery and virtually all cholesterol is synthesized in situ (Dietschy and Turley, 2001). This observation is supported by many studies that showed virtual absence of radioactivity in the CNS following peripheral administration of
radiolabeled cholesterol (Chobanian and Hollander, 1962; Plotz et al., 1968; Meaney et al., 2001). All brain cells synthesize cholesterol during embryonic development, but mature neurons do not produce sufficient cholesterol to meet their lifelong ongoing requirements for membrane synthesis and repair (Dietschy and Turley, 2004). Adult neurons therefore depend upon lipids transported on glial-derived lipoproteins for optimal survival and maintenance. The CNS lipoprotein metabolism is based entirely upon particles that resemble plasma high-density lipoproteins (HDL) in density, size, and composition (LaDu et al., 2000b). A major difference between brain and plasma HDL is that apoE is the major apoprotein in brain HDL whereas apoA-I is the primary apoprotein of plasma HDL (LaDu et al., 2000b).

1.6.4.1 ApoE Synthesis in the CNS

Under physiological conditions, apoE in the CNS is synthesized by primarily by astrocytes (DeMattos et al., 2001) and to a lesser extent by microglia (Gong et al., 2002). ApoE mRNA expression has been reported in cortical and hippocampal neurons but not in the cerebellar neurons (Xu et al., 1999). Some studies suggest that neurons can express apoE under certain conditions like excitotoxic stress (Xu et al., 2006) and is controlled by astrocytes through Erk kinase pathway (Harris et al., 2004). Compared to the peripheral lipoprotein synthesis and metabolism, little is known about CNS lipoprotein metabolism. In the periphery, liver secretes lipid-poor apoA-I containing lipoprotein particles that acquire free cholesterol and phospholipid from the peripheral tissue (Yu et al., 2011). This initial transfer of free cholesterol and phospholipid is mediated by the membrane-bound cholesterol transporter, Adenosine Triphosphate
Binding Cassette A1 (ABCA1) (Oram and Vaughan, 2000). ABCA1-mediated transport of cholesterol is the critical and rate-limiting step in the biogenesis of HDL (Bodzioch et al., 1999; Brooks-Wilson et al., 1999). The partially-lipidated apoA-I particles further acquire more lipids from another ABC transporter, ABCG1 forming nascent HDL₃ (Nakamura et al., 2004; Kennedy et al., 2005). The HDL₃ particles are matured by further acquiring phospholipids, free cholesterol, and triglycerides via lecithin-cholesterol acyltransferase (LCAT), an enzyme that esterifies free cholesterol resulting in spherical HDL₂.

Based on the pathway of peripheral HDL biosynthesis, it is proposed that glia secrete apoE-containing lipoproteins as lipid-poor nascent discoid particles that are composed of phospholipids and free cholesterol but lack a cholesterol ester/triglyceride core (LaDu et al., 1998; Fagan et al., 1999). The nascent apoE particles are remodeled into mature lipoproteins in the brain parenchyma by acquiring cholesterol and phospholipids (LaDu et al., 1998; Koch et al., 2001). We and others have shown that the cholesterol transporter, ABCA1 is expressed by both glia and neurons (Wellington et al., 2002; Hirsch-Reinshagen et al., 2004; Tachikawa et al., 2005) and is required for the lipidation of apoE in the brain (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). The ABCA1-mediated lipidation of apoE is regulated by nuclear transcription factors, liver X receptors (LXR).
1.6.4.2 Liver X Receptors (LXR)

LXR have two isoforms in mammals: LXR\(\alpha\) and LXR\(\beta\), which share 80% sequence homology and have differential expression patterns (Wojcicka et al., 2007). LXR\(\alpha\) is expressed principally in the liver, intestine, kidney, spleen, and adipose tissue (Auboeuf et al., 1997) whereas LXR\(\beta\) is ubiquitously expressed albeit at lower levels (Song et al., 1994). LXRs were initially identified as orphan receptors, i.e., there were no known endogenous ligands at the time of their discovery. Later studies identified oxysterols as their endogenous ligands (Janowski et al., 1996; Janowski et al., 1999). Oxysterols are monooxygenated derivatives of cholesterol that are generated as intermediates in the cholesterol biosynthetic pathway [24(S),25-epoxycholesterol], steroid hormone biosynthesis [22(R)-hydroxycholesterol], and cholesterol metabolism pathways [24(S)-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol] (Schroepfer, 2000). 24(S)-hydroxycholesterol (cerebrosterol) is the principal cholesterol metabolite and mechanism through which cholesterol is removed from the CNS (Lund et al., 1999) whereas 27-hydroxycholesterol is the most abundant cholesterol metabolite in the periphery (Wojcicka et al., 2007). Most oxysterols have similar affinity towards both LXR isoforms. Various synthetic LXR agonists have been developed; the two most commonly used LXR agonists in experimental studies are T0901317 and GW3965. T0901317 activates both LXR isoforms with almost equal affinity (Schultz et al., 2000) as well as pregnane X receptor (Mitro et al., 2007) and farnesoid X receptor (Houck et al., 2004). GW3965 (2-[3-[3-[[2-chloro-3-(trifluoromethyl)phenyl]methyl-[2,2-di(phenyl)ethyl]amino]propoxy]phenyl]acetic acid) is a potent, orally-active synthetic LXR agonist with slightly higher affinity for LXR\(\beta\) (EC\(_{50}\): 30 nM) than LXR\(\alpha\) (EC\(_{50}\): 190
nM) (Collins et al., 2002). Unlike T0901317, GW3965 has higher selectivity for LXR over other nuclear receptors (Collins et al., 2002). The natural LXR antagonists include geranylgeranylpyrophosphate (Forman et al., 1997), polyunsaturated fatty acids (arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid and linoleic acid) (Ou et al., 2001; Yoshikawa et al., 2002) and 5α,6α-epoxycholesterol-3-sulfonate (Song et al., 2001).

1.6.4.3 LXR: Molecular Mechanism of Action

LXRs regulate gene expression by forming obligate heterodimers with another nuclear receptor, the retinoid X receptor (RXR). The LXR-RXR complex can regulate gene expression via two mechanisms: direct activation and transrepression (Gabbi et al., 2014). In the absence of agonist, the transcriptional activity of LXR/RXR is suppressed by corepressors [e.g., Nuclear Receptor Corepressor (NCoR), Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT)] (Chen and Evans, 1995; Horlein et al., 1995). During direct activation, agonist binding induces conformational change in the LXR causing dissociation of the corepressor from the LXR/RXR dimer and results in moderate level of transcription. Full transcriptional activity is gained by recruitment of coactivators [e.g., Peroxisome Proliferator Activated Receptor-γ (PPARγ) coactivator-1α (PGC-1α), Steroid Receptor Coactivator-1 (SRC-1), Activating Signal Cointegrator-2 (ASC-2)] (Oberkofler et al., 2003; Huuskonen et al., 2004; Lee et al., 2008). The activated LXR/RXR binds to the LXR-response element (LXRE) that consists of a direct repeat of the core sequence 50-AGGTCA-30 separated by 4 nucleotides (DR4) of the target gene (Wiebel and Gustafsson, 1997). LXRs, particularly LXRβ inhibit transcription.
of NF-κB-regulated proinflammatory genes that lack LXRE by an alternate mechanism called \textit{transrepression}. In this case, upon agonist binding LXRβ undergoes a specific SUMOylation by SUMO-2/3 that promotes interaction with the corepressor, NCoR and its dissociation from the LXR, which in turn blocks transcription of proinflammatory genes (Ghisletti et al., 2007; Venteclef et al., 2010).

1.6.4.4 LXR: Regulation of Cholesterol Metabolism

Much of the current understanding of the physiological role of LXRs comes from the studies of their actions in peripheral tissues. It is now well established that LXRs act as the “master regulators” of cholesterol metabolism. LXRs act as cholesterol sensors and regulate intracellular cholesterol levels via three processes: 1) by promoting the transport of excessive cholesterol in the peripheral tissues to the liver for excretion via process called reverse cholesterol transport (RCT), 2) by inhibiting intestinal cholesterol absorption and, to a lesser extent, 3) by inhibiting cellular cholesterol synthesis (Wojcicka et al., 2007). LXRs regulate the \textit{RCT} by controlling the expression of several target genes. Excessive intracellular cholesterol leads to accumulation of oxysterols, which activate LXRs that in turn increase expression of their principle target proteins, ABCA1 and ABCG1, thus leading to enhanced intracellular cholesterol efflux (Figure 1.7). This pathway has been demonstrated in a wide variety of cell types (Costet et al., 2000; Venkateswaran et al., 2000; Kennedy et al., 2001). Both ABCA1 and ABCG1 are highly expressed in macrophages and prevent excessive accumulation of intracellular cholesterol and formation of foam cells (Oram and Lawn, 2001). The cholesterol pumped out by ABC transporters is accepted by apoA-I, the principle apolipoprotein in
peripheral HDL, which transports cholesterol to liver for excretion. In addition to ABC transporters, LXRαs also increase the expression of Niemann-Pick C1 (NPC1) and Niemann-Pick C (NPC2), which are intracellular cholesterol carriers that redistribute cholesterol from the endosomal compartment to the plasma membrane (Rigamonti et al., 2005). LXRαs also increase expression of ABCG5 and ABCG8 in hepatocytes that transport cholesterol to bile canaliculi for its ultimate secretion into bile (Yu et al., 2002; Yu et al., 2003). Finally, in rats and mice synthetic LXR agonists upregulate cholesterol 7α-hydroxylase (CYP7a), a rate limiting enzyme that converts cholesterol to bile acids (Lehmann et al., 1997; Menke et al., 2002). This mechanism is unique in rats and mice as the human CYP7a gene lacks an LXRE and therefore cannot be upregulated by LXR agonists (Chiang et al., 2001; Goodwin et al., 2003) and may partly explain the susceptibility of humans to dietary-induced hypercholesterolemia (Menke et al., 2002).

In addition to induction of RCT, LXR agonists have also shown to reduce intestinal cholesterol absorption by upregulating ABCG5 and ABCG8 expression in the enterocytes that increase cholesterol recycling into the intestinal lumen (Repa et al., 2002; Yu et al., 2003).
Figure 1.7: Regulation of ApoE Lipidation by LXR.

(1) In the absence of agonist LXR/RXR are bound to corepressors (CoR) which inhibit their transcription activity. (2) Agonist (A) binding induces dissociation of corepressors and association with coactivators (CoA) turning on transcriptional activity of LXR/RXR. (3) Activated LXR activate the membrane-bound cholesterol transporter, ABCA1 that pumps free cholesterol and phospholipids out of the cell. (4) Unlipidated ApoE act as cholesterol acceptor and mature to lipidated apoE.

1.6.4.5 ABCA1: The Principal LXR Target

The principal target gene controlled by LXRs is ABCA1, a 2261 amino acid integral membrane protein. ABCA1 is almost ubiquitously expressed with high levels of expression in the macrophages, liver, intestine, heart, adrenal gland, endothelial cells, testes, and in placental trophoblasts (Langmann et al., 1999; Lawn et al., 2001; Liao et
al., 2002; Wellington et al., 2002). High ABCA1 expression levels have been also reported in mouse (Wellington et al., 2002) and rat (Fukumoto et al., 2002) brains with highest levels in neuronal layer of the cerebellum. ABCA1 belongs to subfamily A of large superfamily of ABC transporters. Although exact domain structure and topology of ABCA1 are not fully elucidated, several models have been predicted based on hydropathy analysis (analysis of hydrophobic and hydrophilic amino acids) that predict ABCA1 structure (Schmitz and Langmann, 2001; Cavelier et al., 2006). According to these models, ABCA1 is a full ABC transporter and consists of two transmembrane domains, each consisting of six membrane-spanning helices followed by two cytoplasmic nucleotide (ATP) binding domains. In addition ABCA1 also has a regulatory domain between the two halves, a feature unique to ABCA1 among other ABC transporters. The ATP-binding domains contain two conserved peptide motifs, Walker A and Walker B and a family signature region. In addition, ABCA1 has two large extracellular domains linked with two disulfide bonds and are essential for apoA-I binding and HDL formation (Tanaka et al., 2001; Nagao et al., 2012).

Cellular expression of ABCA1 is tightly regulated at both the transcriptional and post-transcriptional levels and its regulation is integrated with mechanisms that sense intracellular cholesterol content (Tall, 2008). ABCA1 transcription is controlled primarily by members of the nuclear receptor family of ligand-activated transcriptional factors, specifically LXR-RXRs and PPARs (Schmitz and Langmann, 2005). ABCA1 expression is increased by natural oxysterols along with 9-cis retinoic acid, an RXR agonist, as shown in various cell lines including macrophages, liver, intestine, and
neuronal cells in vitro (Costet et al., 2000; Schwartz et al., 2000; Kennedy et al., 2001; Murthy et al., 2002; Koldamova et al., 2003). In addition, PPARγ agonists also induce ABCA1 and associated apolipoprotein-mediated lipid efflux, although this induction is likely secondary to activation of LXR by activated PPARγ (Chawla et al., 2001; Ogata et al., 2009). The in vivo efficacy and relevance of PPARγ-mediated activation of ABCA1 via LXR remains to be determined.

Cellular levels of ABCA1 are also controlled by a fine balance of post-transcriptional modifications that either stabilize or degrade the ABCA1 protein, which has a half-life of less than 1h (Oram et al., 2000; Wang and Tall, 2003). For example, interaction of apoA-I with ABCA1 stabilizes ABCA1 protein (Wang et al., 2000; Wang et al., 2003). ABCA1 contains a PEST sequence – proline (P), glutamate (E), serine (S) and threonine (T) - in the cytoplasmic loop of ABCA1, the protein kinase A-dependent phosphorylation of which induces degradation of ABCA1 by calpain (Martinez et al., 2003; Wang et al., 2003). Interaction of ABCA1 with apoA-I prevents the phosphorylation of this PEST sequence, thus stabilizing ABCA1. ABCA1 also undergoes ubiquitin-proteosome-mediated proteolytic degradation (Feng and Tabas, 2002). Recently, a small non-coding microRNA, miR-33, has been shown to play an important role in ABCA1 expression (Moore et al., 2010). MiR-33 inhibits ABCA1 expression in mouse hepatocytes and macrophages, blocking cholesterol efflux and reducing plasma HDL levels. Conversely, inhibiting miR-33 by anti-sense oligonucleotides increases ABCA1 expression and promotes lipid efflux both in vitro in murine macrophages as well as in human THP-1, IMR-90 and HepG2 cell lines and in
vivo (Moore et al., 2010; Rayner et al., 2010). Whether CNS ABCA1 levels can also be regulated by microRNAs remains an important question to address.

ABCA1 plays the pivotal role in controlling apoA-I and apoE metabolism. The critical role of ABCA1 in HDL metabolism was first highlighted by loss-of-function mutations of chromosome 9q31 leading to defective ABCA1 that cause Tangier disease, which is characterized by extremely low plasma HDL levels (Hobbs and Rader, 1999; Rader and deGoma, 2012). ABCA1 mediates transport of free cholesterol and phospholipids to lipid-free apoA-I generating pre-β HDL, a rate-limiting step in HDL biogenesis (Oram and Vaughan, 2000). Although the role of ABCA1 in HDL biogenesis is well established, the exact mechanism of lipid transport to apoA-I by ABCA1 is still unclear and as such various models have been proposed. Vedhachalam et al. (2007) proposed a three step model of ABCA1-mediated lipidation of apoA-I in murine macrophages: in the first step, apoA-I directly binds to ABCA1 causing ABCA1 upregulation that results in enhanced translocation of phospholipids to the outer leaflet of the cell membrane. The net increase and reduction in phospholipid content leads to later compression of phospholipids in the outer half and expansion of phospholipids in the cytoplasmic leaflet of the lipid bilayer, respectively. This asymmetric packing in the phospholipids causes strain in the local cell membrane region. In the second step, the cell membrane relieves the localized strain by bulging into the extracellular space forming an exovesiculated domain to which apoA-I binds with high affinity. In the final step, binding of apoA-I initiates spontaneous microsolubilization of the membrane phospholipids and
cholesterol and creation of discoidal HDL particles containing two, three or four apoA-I molecules/particle.

A second model proposes that apoA-I binds to ABCA1 at the plasma membrane followed by internalization of apoA-I-ABCA1 complex and subsequent delivery to the late endosomes where apoA-I acquires lipids (Takahashi and Smith, 1999; Chen et al., 2001). The lipidated apoA-I is then resecreted from the cell by exocytosis.

Using state of the art single-molecule fluorescence tracking and diffusion analysis technique in HeLa cell line, Nagata et al recently proposed a novel mechanism of apoA-I lipidation by ABCA1 (Nagata et al., 2013). According to this model, ABCA1 monomers freely diffuse and translocate lipids on the plasma membrane, even in the absence of apoA-I in an ATP-dependent manner. Upon lipid acquisition, presumably in its extracellular domain or near its vicinity in the plasma membrane, ABCA1 monomer undergoes conformational changes and forms dimers. The dimers are stabilized to the plasma membrane by their interaction with the membrane-skeleton actin filaments. Lipid-free apoA-I directly binds to the extracellular domains of ABCA1 dimers but not the monomers and accepts lipids reserved by ABCA1. Upon lipid transfer to apoA-I, the ABCA1 dimers dissociate into monomers, which are released from actin filament and resumes its function to translocate lipids in an ATP-dependent manner.

Although the above models of apoA-I lipidation have been proposed based on the studies in macrophages, it is proposed that ABCA1 may mediate apoE lipidation in the CNS through similar mechanisms (Koldamova et al., 2010).
1.6.4.6 The Role of LXRs in the CNS

Although LXR function in the CNS is poorly understood, it is conceivable that like in the periphery, LXRs regulate cholesterol metabolism in the CNS. The net movement of cholesterol out of the brain is largely controlled by its conversion to the principal metabolite, 24(S)-hydroxycholesterol (Dietschy and Turley, 2004). The second route of intracellular cholesterol removal is via ABCA1 and apoE-mediated transport. Both 24(S)-hydroxycholesterol and synthetic LXR agonists increase ABCA1 expression in neurons and astrocytes in vitro as well as in vivo along with increased cholesterol efflux (Whitney et al., 2002; Liang et al., 2004; Abildayeva et al., 2006). Similarly, LXRs also increase apoE expression in astrocytes in vivo (Liang et al., 2004; Abildayeva et al., 2006). Cholesterol carried on apoE in the brain is thought to play minor role in the net cholesterol removal from the CNS but may be rather important in redistribution of cholesterol required for synaptogenesis, especially during development and following recovery from brain injury.

As discussed further apoE plays crucial role in Aβ clearance and thus in turn in AD pathophysiology. It is thus imperative to determine whether enhancing apoE function through LXR activation may be beneficial in AD. Indeed, the in vivo activation of LXRs has shown to decrease brain Aβ levels as well as improve cognition in mouse models of AD. Koldamova et al reported decreased soluable Aβ40 and Aβ42 levels following a six-day treatment with T0901317 in four-month old APP-23 mice (Koldamova et al., 2005b). These findings were replicated by Riddell et al in another AD mouse model, Tg2527 in
which a six-day treatment with T0901317 in five-month old mice selectively lowered Aβ42 but not Aβ40 in the hippocampus and reversed contextual memory deficits (Riddell et al., 2007). It should be noted that in the above two studies the LXR agonist response was assessed in young mice (4-5 months) and not in old mice (> 10 months) that develop extensive Aβ deposits. Jiang et al treated one-year old Tg2527 mice with another LXR agonist, GW3965 for four months and observed ~50% reduction in Aβ40 and Aβ42 levels with corresponding ~67% reduction in the plaque load (Jiang et al., 2008a). The authors also reported significant decrease in the number of plaque-associated activated microglia as well as IL-6 mRNA levels in GW3965-treated mice, supporting anti-inflammatory effects of LXRs in the brain (Zelcer et al., 2007). Recently Fitz et al treated 9 months old APP-23 mice with T0901317 for four months and reported a significant decrease in the amyloid plaque load, soluble and insoluble Aβ40 and Aβ42 as well as A11-positive Aβ oligomers in cortex and hippocampus along with reversal of Morris water memory deficits (Fitz et al., 2010). In all of the studies mentioned above, treatment with LXR agonists did not affect APP processing, indicating that LXR activation may affect Aβ metabolism but not its synthesis.

The beneficial effects of LXRs in Aβ metabolism are dependent on ABCA1 as shown by several studies. We and other have shown that deletion of ABCA1 in mice results in poorly-lipidated apoE and reduced apoE levels (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004), which is associated with increased amyloid burden in several mouse models of AD (Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005). Moreover, in a recent study, lack of even one copy of ABCA1 resulted increased
amyloid deposition and exacerbated memory deficits in AD mice expressing human apoE4 but not in mice expressing human apoE3 (Fitz et al., 2012). Conversely, selective overexpression of ABCA1 in PDAPP mice has been shown to increase apoE lipidation and prevent amyloid deposition (Wahrle et al., 2008). We also showed that ABCA1 is required for the beneficial effects of GW3965 in AD mice in increasing CSF apoE levels, reducing amyloid burden, and restoring novel object recognition (NOR) memory, suggesting that lipidated apoE particles may underlie the neuroprotective effects of LXR agonists (Donkin et al., 2010).

While the \textit{in vivo} studies indicate beneficial effects of LXR activation in AD mice, the results from \textit{in vitro} studies are conflicting. Fukumoto et al showed increased secreted levels of both Aβ40 and Aβ42 from neuroblastoma cell lines treated with T0901317, an effect antagonized by blocking ABCA1 expression by RNAi (Fukumoto et al., 2002). On the other hand, in two other studies treatment with the same LXR agonist resulted in decreased Aβ secretion from neuroblastoma (Sun et al., 2003) and neuroglioma (Koldamova et al., 2005b) cell lines. The reason for this discrepancy is not clear.

The role of LXRs in the CNS is further highlighted by gross anatomical abnormalities as well as excessive lipid deposits in the brain and spinal cord seen in LXR knock-out mice. Specifically, in LXRα/β double knockout mice, Wang et al reported closed lateral ventricles that were lined with lipid-laden cells, enlargement of blood vessels particularly in the substantia nigra pars reticulata and globus pallidus, excessive lipid deposits in the brain parenchyma, neuronal loss, astrocyte proliferation and myelin sheath defects.
(Wang et al., 2002). The astrocytes surrounding blood vessels also accumulated excessive lipids in aged LXRα/β null mice (Wang et al., 2002). Recently, Anderson et al reported that deletion of LXRβ in mice resulted in spinal-cord specific deficits, including motor neuron loss, axonal atrophy, astrogliosis and lipid accumulation, and impaired motor coordination; some of these features akin to that of amyotrophic lateral sclerosis (Andersson et al., 2005).

### 1.6.4.7 ApoE Structure and Polymorphisms and their Significance in AD Pathology

ApoE is a 299 amino acid glycoprotein with the molecular mass of 34 kDa and is composed of N- and C-terminal domains that are connected by a flexible hinge domain such that each domain can fold independently (Figure 1.8 A) (Aggerbeck et al., 1988; Wetterau et al., 1988). The N-terminal domain is an elongated four-helix bundle and contains receptor-binding site (22 kDa, amino acid residues 136-150) located in helix 4 (Wilson et al., 1991). The C-terminal domain contains an amphipathic α-helical lipid-binding site (10kDa, amino acid residues 244-272) that can switch between lipid-bound and lipid-free states (Hatters et al., 2006; Zhong and Weisgraber, 2009). The receptor-binding site is rich in lysine and arginine residues that are thought to interact with the ligand-binding domain of the apoE receptors, i.e. members of the LDL receptor family (Hatters et al., 2006). Lipid-free apoE has poor affinity for binding to LDL receptor (LDLR), while lipid-bound apoE binds with LDLR with high affinity.
Apolipoprotein E is encoded by the APOE gene located on chromosome 19, which is highly polymorphic. In humans, APOE gene has three alleles, ε2, ε3, and ε4 that encode three apoE isoforms, apoE2, apoE3 and apoE4, respectively (Weisgraber, 1994; Mahley, 1998). This apoE polymorphism leads to expression of six phenotypes: E2/E2, E3/E3, E4/E4, E2/E3, E2/E4 and E3/E4. The three apoE isoforms differ by single amino acid changes at residues 112 and 158: apoE 2 (Cys112, Cys158), apoE3 (Cys112, Arg158) and apoE4 (Arg112, Arg158) (Figure 1.8 B).

The single amino acid change has profound influence on the physical and physiological properties as well as stability of the three alleles (Table 1.7), which may help to understand their role in pathological conditions (Weisgraber, 1994). In apoE3 and apoE4, the Arg at position 158 forms a salt bridge with Asp154. In apoE2 the Cys at 158 cannot form such bridge with Asp154. Instead, Asp154 forms salt bridge with Arg150 which lies in the receptor binding domain of apoE2, causing conformational changes and interfering with receptor binding. As a result, apoE2 poorly binds with LDLR, while apoE3 and apoE4 have ~50-fold higher affinity for LDLR (Weisgraber et al., 1982).

Two physical properties of apoE4, viz. domain interaction and poor stability have been thought to alter its function and contribute to increased AD risk. X-ray crystallography, site-directed mutagenesis, and fluorescent resonance energy transfer studies have revealed that the amino acid at position 112 is the key to domain interaction. Thus, Arg112 in apoE4 induces conformational changes in Arg61 causing its side chain away from the four-helix bundle promoting interaction with Glu255 in the
C-terminal domain (Figure 1.8 C) (Dong et al., 1994; Dong and Weisgraber, 1996; Hatters et al., 2005). On the other hand Cys at position 112 in apoE2 and apoE3 tucks Arg61 side chain into the two-helix bundles preventing its interaction with Glu255 (Figure 1.8 C). All non-human species have threonine instead of arginine at the position 61, which does not engage in domain interaction. The mouse apoE, for example is structurally similar to human apoE4 (Arg112, Arg158) (Figure 1.8 B) but functionally behaves like apoE3 due to the absence of domain interaction (Figure 1.8 C).

Interestingly, replacing Thr61 with Arg in mouse apoE gene introduces domain interaction causing the mutated apoE behave like human apoE4 resulting in lower apoE levels (Raffai et al., 2001) along with cognitive deficits (Adeosun et al., 2014).

It is hypothesized that domain interaction alters apoE4 properties in two ways that may contribute to the negative effects of apoE4 in AD. First, domain interaction is thought to make the C-terminus of apoE4 more susceptible to cleavage by an unidentified neuronal protease yielding C-terminal truncated apoE fractions that are thought to be neurotoxic (Zhong and Weisgraber, 2009). This is supported by the observation that neuron-specific expression of apoE4 in mice leads to intraneuronal accumulation of C-terminal truncated apoE fragments, neuronal loss, increased tau phosphorylation, and cognitive deficits (Harris et al., 2003; Brecht et al., 2004). Moreover, truncated apoE4 fragments have been reported in AD brains (Huang et al., 2001). Second, domain interaction is also thought to influence the binding preference of apoE to lipid particles; apoE2 and apoE3 preferentially bind to HDL while apoE4 is principally associated with VLDL and LDL (Weisgraber, 1990; Dong and Weisgraber, 1996). The preference of
apoE4 to triglyceride-rich LDL and VLDL is thought to make it inefficient in lipid transport required for synaptogenesis and neuronal repair (Zhong and Weisgraber, 2009).

The other physical property of apoE that is postulated to contribute to its influence in AD pathology is the differences in the stability of the three isoforms. The N-terminal domain of apoE2 is the most stable, that of apoE4 the least stable while apoE3 stability is in between the two (Mahley and Huang, 2006; Zhong and Weisgraber, 2009). As a result, apoE4 acquires a unique molten globule state in which the four-helix bundle is partially unfolded exposing the proteolytic cleavage site (Morrow et al., 2002). A likely effect of molten globule formation is apoE4-mediated potentiation of Aβ42-induced lysosomal leakage and apoptosis, which has been shown in Neuro-2a cells transfected with apoE4 (Ji et al., 2002). It is proposed that the acidic pH inside lysosomes favors molten globule state of apoE4 that has increased affinity to phospholipids and membranes, which in turn can result in destabilization of the lysosomal membrane and ultimately lysosomal leakage (Mahley and Huang, 2006).
Figure 1.8: ApoE structure, polymorphism and domain interaction.

(A) Schematic of the basic structure of apoE depicting the receptor and ligand binding regions. (B) Human apoE isoforms with amino acids at key positions. Mouse apoE is shown at the bottom for comparison. (C) Domain interaction in apoE4. In human apoE2 and 3 (blue) Cys112 in the N terminal domain changes conformation of Arg61 such that it cannot engage in domain interaction with Glu225 in the C terminus. Mouse apoE (yellow) although has arginine at position 112, the Thr61 cannot engage in domain interaction with Glu225. In human apoE4, Arg112 changes conformation of Arg61 such that it engages in domain interaction with Glu225.
Table 1.7: Summary of physical and physiological properties of apoE alleles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ApoE2</th>
<th>ApoE3</th>
<th>ApoE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Allelic Frequency (%)</td>
<td>8.4</td>
<td>77.9</td>
<td>13.7</td>
</tr>
<tr>
<td>Frequency in AD Population (%)</td>
<td>3.9</td>
<td>59.4</td>
<td>36.7</td>
</tr>
<tr>
<td>Amino Acid at Position 112</td>
<td>Cys</td>
<td>Cys</td>
<td>Arg</td>
</tr>
<tr>
<td>Amino Acid at Position 158</td>
<td>Cys</td>
<td>Arg</td>
<td>Arg</td>
</tr>
<tr>
<td>Stability</td>
<td>Highest</td>
<td>Intermediate</td>
<td>Least</td>
</tr>
<tr>
<td>Folding Intermediates</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Domain Interaction</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Resistance to Molten Globule Formation</td>
<td>Highest</td>
<td>Intermediate</td>
<td>Least</td>
</tr>
<tr>
<td>LDLR Binding Affinity</td>
<td>Poor (associated with type III hyperlipoproteinemia)</td>
<td>Bind with ~ 50-fold greater affinity than apoE2</td>
<td></td>
</tr>
<tr>
<td>Lipoprotein Binding Preference</td>
<td>Preferential binding to HDL</td>
<td>Preferential binding to LDL and VLDL</td>
<td></td>
</tr>
</tbody>
</table>

1.6.4.8 ApoE and Amyloid Beta (Aβ) Metabolism

The genetic association of apoE with AD pathologies stems from its well-documented role in Aβ metabolism including clearance and BBB integrity. Data from these studies indicate that the allele, lipidation status, and levels of apoE influence three aspects of Aβ and amyloid metabolism: interaction with Aβ, Aβ degradation and Aβ clearance via cerebrovasculature. While Aβ is continually generated at a rate of 7.6%/h, it is also efficiently cleared from the CNS at an equivalent rate of 8.3%/h (Bateman et al., 2006).
Thus, the net steady state level of Aβ represents a delicate balance between its formation and degradation. Aβ is cleared through three major pathways: 1) through intracellular and extracellular enzymatic degradation including lysosomal degradation, 2) along interstitial fluid drainage pathways, and 3) through direct transport across cerebrovasculature.

1.6.4.8.1 ApoE and Aβ Interaction

The first clues on the association between apoE and Aβ came from apoE immunoreactivity found to be co-localized with parenchymal and cerebrovascular amyloid deposits in AD patients (Namba et al., 1991; Wisniewski and Frangione, 1992) and in AD mouse models (Burns et al., 2003). The lipid binding site, especially residues 244-272 located in the C terminal domain of apoE interact with residues 12-28 of Aβ (Strittmatter et al., 1993). A later epitope mapping study revealed that residues 144-148 located in the receptor binding site on the N-terminal domain of apoE can also interact with residues 13-17 of Aβ (Winkler et al., 1999). Several in vitro studies have shown that apoE can bind to Aβ in an isoform-specific manner with apoE3 forming a more stable complex with Aβ than apoE4 (LaDu et al., 1994; Tokuda et al., 2000; Petrlova et al., 2011; Tai et al., 2013). It should, however, be noted that the findings of these studies are influenced not only by the apoE isoform but also by its lipidation status and experimental conditions. For example, purified and lipid-free recombinant apoE4 was found to bind with Aβ with faster kinetics than apoE3 in vitro (Strittmatter et al., 1993). Contrary to this finding, Tokuda et al reported that delipidation of apoE caused a 5-10-fold decrease in Aβ binding without any influence of isoform (Tokuda et al., 2000). On
the other hand studies using HEK23 cell-secreted or native plasma apoE showed that apoE3 formed more abundant sodium dodecyl sulfate (SDS)-stable complexes with Aβ than apoE4 (LaDu et al., 1994; LaDu et al., 1995; Tokuda et al., 2000). To address this discrepancy, LaDu et al compared Aβ binding with purified, lipid-free apoE vs native, lipidated apoE and reported that delipidation of apoE during purification process causes conformational changes in apoE resulting in diminished interactions between apoE3 and Aβ (LaDu et al., 1995).

1.6.4.8.2 ApoE and Enzymatic Degradation of Aβ

Several metalloproteases can degrade monomeric Aβ, the most important of which are neprilysin (NEP) and insulin degrading enzyme (IDE) (Saido and Leissring, 2012). NEP, a type-II membrane-associated zinc metalloprotease is the most potent Aβ degrading enzyme in the CNS (Shirotani et al., 2001) and can degrade both monomeric and synthetic oligomeric Aβ (Kanemitsu et al., 2003). NEP is highly expressed in microglia (Iwata et al., 2000). Partial loss or decreased activity of NEP induced by intracerebral injection of a NEP inhibitor in rats (Iwata et al., 2000) or in NEP knock-out mice (Iwata et al., 2001; Rogers et al., 2002) increases Aβ levels by approximately two-fold, especially in the hippocampus, suggesting important role of NEP in Aβ catabolism. Cortical and hippocampal expression of NEP and activity decreases with aging in both rodents (Apelt et al., 2003) and humans with AD pathology (Yasojima et al., 2001b; Yasojima et al., 2001a; Russo et al., 2005; Wang et al., 2005) as well as in the cerebrovasculature in AD cases with CAA (Carpentier et al., 2002; Miners et al., 2006). Another important enzyme involved in Aβ catabolism is IDE, which is both membrane-bound as well as
secreted by microglia and mediates extracellular degradation exclusively of monomeric Aβ (Qiu et al., 1998; Vekrellis et al., 2000). The Aβ degrading activity (Perez et al., 2000) as well as expression levels (Cook et al., 2003) of IDE are significantly decreased in AD compared to control cases. Complete IDE knockdown causes > 50% reduction in Aβ degradation in mice suggesting an important role of IDE in regulation of Aβ levels (Farris et al., 2003; Miller et al., 2003). Conversely, overexpression of NEP or IDE in AD mice significantly decreases Aβ levels and retards plaque pathology suggesting that these two enzymes may be potential therapeutic targets (Leissring et al., 2003).

ApoE facilitates both intracellular and extracellular proteolytic degradation of Aβ in vitro (Jiang et al., 2008a). Further, the ability of apoE to facilitate enzymatic degradation of Aβ is dependent on the lipidation status of apoE; poorly-lipidated apoE is inefficient while lipidated apoE generated through LXR activation is efficient in stimulating proteolytic degradation (Jiang et al., 2008a). Moreover, loss of ABCA1 impairs while enhancement of ABCA1 expression through LXR activation promotes the ability of microglia to degrade Aβ. This study also showed that human apoE2 promotes maximal Aβ degradation while apoE4 is the least efficient, consistent with the isoform-dependent effect of apoE in human AD pathology.

**1.6.4.8.3 Cellular Uptake and Degradation of Aβ**

Another route of Aβ degradation is through lipoprotein receptor-mediated uptake and subsequent lysosomal degradation by the brain parenchyma and cerebrovasculature (Kanekiyo et al.; Bu, 2009). The major lipoprotein receptors involved in Aβ uptake are
low density lipoprotein (LDL) receptor and LDL receptor related protein 1 (LRP1), which are expressed in microglia, astrocytes, neurons, and smooth muscle cells at the BBB and cerebral arteries (Fan et al., 2001). Aβ binds indirectly to LDLR and LRP1 through a chaperone, mainly apoE, which can affect Aβ clearance. Recent in vitro studies have shown that Aβ can also directly bind to both LDLR (Basak et al., 2012) as well as LRP1 (Deane et al., 2004b) indicating an apoE-independent receptor-mediated clearance pathway. Overexpression of LDLR enhances cellular uptake of Aβ in vitro (Basak et al., 2012) and reduces aggregation as well as enhances clearance of Aβ in APP/PS1 transgenic mice (Kim et al., 2009), suggesting LDLR modulation as a therapeutic target for AD. Following endocytosis, the majority of endocytosed Aβ is transferred through early and late endosomes before degradation in the lysosomes (Hu et al., 2009; Li et al., 2012), while a small fraction is recycled through Ras-related protein, Rab-11-mediated mechanism (Li et al., 2012). These studies have also shown that increasing intracellular Aβ concentration through inhibition of Aβ degradation by lysosome inhibitors or by excessive transport of Aβ to lysosomes by Rab-5 and Rab-7 can be detrimental due to enhanced Aβ aggregation (Hu et al., 2009; Li et al., 2012) indicating importance of lysosomal degradation in intracellular Aβ degradation.

1.6.4.8.4 Aβ Clearance through the Cerebrovasculature

Aβ is also cleared by the cerebrovasculature through two important routes. The first clearance route is through the perivascular drainage pathway in which brain solutes, including Aβ and ISF diffuse along the basement membrane between smooth muscle cells of cerebral arterioles and ultimately drain into the CSF and lymphatic system.
The movement of Aβ and ISF along the perivascular space is in the opposite direction of the blood flow and is thought to be driven by the blood vessel pulsations (Schley et al., 2006). Aging as well as pathologies such as CAA and cardiovascular disease can stiffen the vessel walls, reducing vessel pulsation and slowing Aβ and ISF movement along the perivascular space, ultimately promoting Aβ deposition within the capillary walls (Weller et al., 2002; Schley et al., 2006). The second vascular clearance route involves receptor-mediated trafficking of Aβ from the brain parenchyma into the peripheral circulation across the BBB. The two most important receptors of this system are LRP1 and receptor for advanced glycation end products (RAGE), which are shown to have opposite actions on Aβ clearance across BBB (Deane et al., 2004a). Thus, while LRP1 mediates efflux of soluble Aβ40 into the peripheral circulation preventing Aβ accumulation in the brain, RAGE can mediate influx of Aβ from plasma to brain ISF and promote Aβ accumulation (Deane et al., 2003; Deane et al., 2004a; Zlokovic, 2004). Elegant studies by the Zolkovic group suggest that LRP1-mediated Aβ clearance across BBB may be more rapid (~6 fold faster) than ISF flow (Bell et al., 2007) and may account for about 10-15% of the total Aβ clearance (Shibata et al., 2000).

While lipidated apoE enhances enzymatic degradation of Aβ, the data on its effect on Aβ transport across the BBB is conflicting. ApoE was initially postulated to enhance Aβ clearance across the BBB through its interaction with Aβ as well as its ability to be exported to the peripheral circulation via LRP1 in vascular endothelial cells (Deane et al., 2004b). However, using in situ, real-time microdialysis, Bell et al reported that
association of Aβ40 with lipid-poor human apoE3 decreased while lipided apoE virtually blocked Aβ transport across the BBB in mice (Bell et al., 2007). The findings of Bell et al were replicated in a later study where lipidation of all three apoE isoforms significantly reduced the transport of apoE as well as apoE:Aβ complexes across the BBB (Deane et al., 2008). In the same study Deane et al also showed that apoE, in fact disrupts the transport of Aβ from brain ISF in an isoform-specific manner (apoE4 > apoE3 > apoE2). Thus, binding of apoE to Aβ redirected the rapid export of free Aβ40 and Aβ42 from LRP1 to the VLDL receptor, which in comparison to LRP1, has a much slower interaction with apoE-Aβ complex, resulting in 15- and 9-fold retention of Aβ40 and Aβ42, respectively (Deane et al., 2008). Along similar lines, apoE deficiency leads to reduction of fibrillar Aβ deposits in AD mice (Irizarry et al., 2000) which may be due in part to by reduced Aβ retention as suggested by the above microdialysis studies.

Although current evidence indicates that apoE interacts with Aβ in an isoform-specific manner, its influence on Aβ clearance has been challenged by a recent study by Holtzman and coworkers. In this study, Verghese et al mixed astrocyte-derived, human CSF-derived or reconstituted lipided apoE with cell-derived Aβ using a physiologically-relevant apoE:Aβ ratio of 50-150:1 and found negligible levels of apoE-Aβ complexes (Verghese et al., 2013). Moreover, the authors reported that apoE and Aβ competed for LRP1-dependent cellular uptake by astrocytes. *These data indicate that there is yet much to learn about the relationship between apoE and Aβ.*
Besides its influence on Aβ, apoE is also shown to serve other important role in the CNS including synaptic function and synaptogenesis and inflammation.

1.6.4.9 ApoE, Synaptic Function, and Synaptogenesis

Neuronal membranes are rich in lipids, which are crucial in neuronal homeostasis and function as well as synaptogenesis. Since adult neurons are almost entirely dependent on external source for their lipid demands it is conceivable that apoE plays a crucial role in the normal neuronal function including synaptic activity and synaptogenesis. In support of this hypothesis, apoE modulates axonal growth in vitro. Incubation of cultured fetal rabbit dorsal root ganglion cells with β-VLDL, which is rich in apoE promoted neurite outgrowth and branching via LDLR-mediated mechanism (Handelmann et al., 1992). Intriguingly, incubation with purified, lipid free apoE along with β-VLDL or cholesterol resulted in increased neurite outgrowth but decreased branching (Handelmann et al., 1992). Later studies showed that the neurotrophic effects of apoE were isoform dependent; apoE3 promoted whereas apoE4 reduced neurite extension and branching through an LDLR-mediated mechanism (Nathan et al., 1994; Holtzman et al., 1995; Fagan et al., 1996; Nathan et al., 2002). The isoform-specific effect of apoE was further demonstrated in murine neuroblastoma cells stably transfected with human apoE3 and apoE4 wherein apoE3-secreting cells promoted neurite extension and branching while apoE4 suppressed this effect (Bellosta et al., 1995). The inhibitory effect of apoE4 on the neurite outgrowth was shown to be associated with apoE4-mediated depolymerization of microtubules (Nathan et al., 1995).
In addition to synaptic remodeling, apoE is also thought to (in)directly affect learning and memory by modulating synaptic plasticity. For example, apoE-deficient mice exhibit spatial memory deficits as assessed by poor Morris water maze performance (Gordon et al., 1995) and display impaired long-term potentiation (LTP) in hippocampal CA1 neurons (Valastro et al., 2001). Moreover, recent studies indicate that apoE can alter synaptic plasticity in isoform-dependent manner. For example, LTP in the dentate gyrus neurons is significantly impaired in an apoE isoform-dependent manner with apoE4 > apoE2 and apoE-/- > apoE3 and wild-type mice (Trommer et al., 2004). Intriguingly, opposite results were reported when LTP was assessed in the Schaffer collateral synapses in the CA1 area with greatest LTP enhancement in apoE4 mice and least amount of LTP induction in apoE2 mice (Korwek et al., 2009). ApoE isoform effects on changes in LTP seems to be age-dependent with LTP enhancement seen in younger apoE4 mice that disappears in old apoE4 mice, while LTP enhancement is unaltered in the mice expressing apoE3 (Kitamura et al., 2004). The exact molecular mechanisms of isoform-dependent effects of apoE on LTP are not fully understood. Reelin, an endogenous ligand that regulates neuronal migration during embryonic development, as well as neurotransmission in the adult brain enhances LTP through clustering of the two apoE receptors, apoE receptor-2 (ApoER2) and VLDL receptors activating Src family non-receptor tyrosine kinases, which in turn phosphorylate NMDA receptors, the key receptors mediating LTP (Herz and Chen, 2006). Recently, apoE4 was shown to selectively impair Reelin-mediated LTP by reducing surface expression of ApoER2 (by locking ApoER2 in the intracellular compartment), thus interfering with Reelin-dependent phosphorylation and activation of NMDA receptors (Chen et al., 2010).
isoform-dependent effects on LTP are also reflected in hippocampal-dependent spatial memory wherein female apoE4 mice show worse memory deficits compared to apoE3 mice (Grootendorst et al., 2005).

1.6.4.10 Role of ApoE in Inflammation

Studies in apoE-deficient mice suggest that apoE may have immunomodulatory roles both in the periphery and in the CNS. ApoE-deficient mice show impaired immunity after bacterial challenge with *Listeria monocytogenes* (Roselaar and Daugherty, 1998), increased susceptibility to endotoxemia after intravenous lipopolysaccharide (LPS) administration and inoculation with *Klebsiella pneumonia* (de Bont et al., 1999) and increased systemic inflammatory response and higher mortality following LPS injection (Van Oosten et al., 2001). Further, apoE modulates inflammation in an isoform-dependent manner. Macrophages expressing apoE3 show lowest LPS-induced expression of TNF-α and IL-6 compared to apoE4 and apoE2 (Tsoi et al., 2007). Similar results were obtained in cultured primary microglia wherein apoE stimulated secretion of proinflammatory cytokines such as TNF-α, IL-1β as well as prostaglandin E2 in an isoform-specific manner with apoE4 > apoE3 > apoE2 (Chen et al., 2005; Maezawa et al., 2006). The isoform-specific modulation of inflammation has been also studied in apoE target replacement mice wherein apoE4 mice had significantly elevated TNF-α and IL6 levels in brain as well as in the periphery compared to apoE3 mice (Lynch et al., 2003). Moreover, brain autopsies revealed increased numbers of scattered microglia and microglial activation in AD patients carrying the apoE4 allele (Egensperger et al., 1998).
1.6.5 ApoE and TBI

Given the role of apoE in the lipid metabolism, synaptic function, inflammation, Aβ metabolism, and possible in tau metabolism as well as the pathophysiological similarities between TBI and AD, it is conceivable that apoE may modulate quite a few aspects of TBI pathophysiology. While the role of apoE in AD pathology is well established, its role in TBI is yet elusive. Two studies in apoE-deficient mice subjected to closed head injury (CHI) give us a glimpse of the possible contribution of apoE in TBI pathophysiology. In these studies, apoE-deficient mice showed greater cerebral edema along with increased TNF-α mRNA levels (Lynch et al., 2002) as well as had greater motor deficits, impaired spatial memory and overt neuronal death compared to wild-type mice after CHI (Chen et al., 1997).

CNS injury leads to the release of large amounts of lipids from the degenerating axonal membranes and myelin. Since CNS lipid metabolism is almost entirely based on apoE, it is possible that apoE dynamics may change in response to TBI. In humans for example, TBI results in a striking 14-fold selective reduction in CSF apoE-containing lipoproteins from 2 to 5 days following injury and a 2- and 4-fold increase in total and free cholesterol (Kay et al., 2003), reflecting a pronounced uptake of apoE particles by the injured brain. Although data on the dynamics of apoE synthesis and utilization are not yet available from human subjects, studies in animal models support a role for increased synthesis of apoE in the CNS in the hours to weeks after many types of CNS injury including TBI and spinal cord injury (SCI) (Poirier et al., 1991; Poirier et al.,
1993b; White et al., 2001a; Seitz et al., 2003; Iwata et al., 2005). For example, lesions to the entorhinal cortex have been shown to induce coordinated increases in apoE mRNA and its receptor, LDLR in parallel to clearance of lipid and membrane debris supporting the idea that apoE may accept cholesterol and lipids released from the damaged neuronal membranes and transport them back to the neurons undergoing reinnervation and taken up through LDLR (Poirier et al., 1991; Poirier et al., 1993a; White et al., 2001a). Iwata et al reported a biphasic upregulation of apoE and another CNS lipoprotein, apoJ following fluid percussion injury in rats (Iwata et al., 2005). In this model the initial peak appears at 2 days followed by return of apoE to baseline. ApoE levels then again gradually increased over 6 months post-injury. Intriguingly, the authors reported an uncoupling between the apoE protein and mRNA levels with mRNA levels showing a delayed and sustained increase following an increase in brain apoE protein levels, suggesting that apoE may enter into the injured brain by transfer through the BBB or CSF (Iwata et al., 2005). ApoE isoform may also influence synaptic plasticity after brain injury as apoE4 mice exhibit impaired synaptic plasticity compared to apoE3 mice following entorhinal cortex lesions (White et al., 2001b).

Epidemiological studies indicate that apoE may influence TBI outcomes in an isoform-dependent manner although this relation is not as clear as in AD. Many studies have reported poor post-TBI outcomes in apoE4 carriers compared to non-carriers (Sorbi et al., 1995; Teasdale et al., 1997; Lichtman et al., 2000; Fleminger and Ponsford, 2005; Smith et al., 2006) while others have found no such association (Jiang et al., 2008b; Hiekkanen et al., 2009; Pruthi et al., 2010). These conflicting data may have resulted
from various confounding factors such as short follow-up times after TBI and a large
diversity of the subject population with respect to age, gender, and injury severity.
Nonetheless, a recent meta-analysis of 14 studies with a total of 2527 subjects
concluded that apoE4 does not affect initial injury severity but rather compromises
recovery at 6 months post-injury (Zhou et al., 2008). Moreover, accumulating evidence
suggests that age at injury may alter the association of apoE4 with TBI outcome. For
example, in a study of mTBI in pediatric subjects it was observed that the apoE4-
carriers were significantly more likely to have a worse GCS score compared to non-
carriers, yet performed equivalently, if not better, than non-apoE4 carriers on
neuropsychological tests over 12 months of follow-up (Moran et al., 2009). Another
recent study of CTE cases, which are also highly representative of brain injuries
experienced in late adolescence and early adulthood, also reported that the association
of apoE4 with worse outcomes is weaker than originally anticipated (Katsnelson, 2011).

1.7 TBI: An Overview of Animal Models
Since no single animal model can replicate the entire spectrum of human TBI
pathophysiology, several large and small animal models have been developed to mimic
particular pathophysiological aspects. Large animal models (e.g., non-human primates,
pigs, dogs, sheep, cats) are useful for investigating questions related to the response of
the gyrencephalic brain to injury, and may be particularly applicable for advanced
preclinical evaluation of therapeutic agents before introduction into humans (Kwon et
al., 2010). However, the high cost of large animal models and considerable ethical
concerns associated with their use limits their wide adoption into preclinical studies. In
contrast, rodent (e.g., rat and mouse) models have emerged as the most commonly used animal models in TBI research, as they are widely available, cost-effective, amenable to many behavioral, physiological and drug discovery evaluations, and, particularly for mice, offer a wide variety of genetically modified strains.

Animal TBI models can be broadly classified into open head and closed head injury models (Figure 1.9). The following section briefly summarizes important animal TBI models along with their key advantages and disadvantages.
1.7.1 Open Head Injury Models

In open head injury (OHI) models, the mechanical force is applied directly to the dura, which is exposed by a craniotomy and as such results in almost no head movement.
The two most popular OHI models are fluid percussion (FP) and controlled cortical impact (CCI).

1.7.1.1 Fluid Percussion (FP)

FP models generate brain injury by rapidly injecting fluid onto the intact dura through a craniotomy, typically made laterally over the parietal cortex (lateral fluid percussion) or medially over the sagittal suture midway between the bregma and lambda (midline fluid percussion) (Figure 1.10) (Gurdjian et al., 1966; Thompson et al., 2005; Alder et al., 2011). Fluid propulsion is driven by dropping a pendulum onto a fluid reservoir, and pulse pressure, load duration and pulse velocity are reported. The pressure pulse of the fluid can be varied to produce more or less severe injury, thus enabling FP models to mimic a variety of human TBI injuries. FP induces mixed injury including petechial and SAH, vascular damage at the gray/white matter interface, DAI, focal necrosis, and cell loss (Povlishock and Kontos, 1985; McIntosh et al., 1987; Dixon et al., 1988; McIntosh et al., 1989; Wang et al., 1997; Thompson et al., 2005). The biomechanical studies of FP models have mainly concentrated on characterizing the input parameters such volume loading, pressure peaks, and rate of fluid flow (Stalhammar et al., 1987). High-speed X-ray imaging of the fluid pulse in rats (Dixon et al., 1988) and ferrets (Lighthall et al., 1989) shows that the complex movement of fluid in the epidural space induces gross movement of brain tissue. Data on the biomechanical response of the brain tissue to the fluid movement, however is limited to a physical model (Thibault et al., 1992). Although very widely used, particularly for rats, FP exhibits a high mortality rate due to apnea and considerable variability of outcomes among different laboratories (Cernak,
2005; Marklund and Hillered, 2011). This variability is hypothesized to be due in part to the surgical precision required to generate reproducible craniotomy position and angle as well as minimal control over injury parameter (i.e., the height of the pendulum). As a result, use of FP models often requires extensive operator training.

**Figure 1.10: Fluid percussion injury model.**
The injury is caused by a rapid pulse of saline caused by a pendulum striking the saline reservoir. The inset shows movement of fluid in the epidural space. Reproduced with permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience] (Xiong et al., 2013).

### 1.7.1.2 Controlled Cortical Impact (CCI)
CCI models use a weight drop device (Feeney et al., 1981), a pneumatic (Dixon et al., 1991; Smith et al., 1995), an electromechanic (Onyszchuk et al., 2007) or, most recently, an electromagnetic (Brody et al., 2007) piston to deliver precisely controllable tissue deformation to an exposed dura (Figure 1.11). This method induces primarily focal damage with extensive cortical loss, hippocampal and thalamic damage, intracranial hemorrhage, edema, and increased ICP (Saatman et al., 2006; Marklund
and Hillered, 2011), and is therefore used primarily to mimic severe TBI with frank tissue destruction. The injury severity and pathology are affected by several variables including craniotomy placement (midline vs lateral vs frontal), the impactor tip configuration (size and shape), impact velocity and depth, and whether or not the craniotomy is resealed after impact, which can be adjusted to produce various degrees of injury with excellent precision and reproducibility. CCI models do not suffer from “rebound injury” typically seen in weight-drop models (see below). On the other hand, CCI models tend to induce severe injury destroying large cortical areas, which is observed only in severe human TBI (Marklund and Hillered, 2011). Moreover, the cortical damage during impact is difficult to visualize if the craniotomy is barely larger than the impactor size, which may cause variation in the injury severity.

**Figure 1.11: Controlled cortical impact model.**
The injury is caused by a piston compressing the exposed dura. The inset shows direction of compression of the brain tissue. Reproduced with permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience] (Xiong et al., 2013).

### 1.7.2 Closed Head Injury Models
In closed head injury (CHI) models, the injury is induced through the intact skull by impact (e.g., dropping a weight on the intact skull or striking the intact skull with a piston), non-impact (e.g., blast) or inertial loading (by rapid rotation of head in sagittal, coronal or oblique planes). CHI models are characterized by varying degrees of head acceleration.

1.7.2.1 CHI by Impact

In impact CHI, the mechanical force is delivered to the intact skull through an impact that is delivered by various methods. The most common and simplest method is by dropping a weight of known mass from a pre-determined height that falls under gravity over the animal’s head (Marmarou et al., 1994; Chen et al., 1996; Tang et al., 1997; Beni-Adani et al., 2001; DeFord et al., 2002; Pan et al., 2003; Zohar et al., 2003; Ucar et al., 2006; Flierl et al., 2009; Zohar et al., 2011). Injury severity can be adjusted by varying the mass of the drop weight or drop height. Impact is also delivered using a pneumatically or electromagnetically-driven piston. Injury severity is usually controlled by adjusting piston speed. In another method, Kilbourne et al used a steel ball that was rolled down on a metal ramp to deliver impact (Kilbourne et al., 2009). The impact force can be directed to the vertex (Marmarou et al., 1994; Tang et al., 1997; DeFord et al., 2002; Zohar et al., 2003; Creeley et al., 2004; Wang et al., 2006), the dorso-lateral surface (Laurer et al., 2001; Shitaka et al., 2011), lateral side (Viano et al., 2009, 2012; Ren et al., 2013) or to the frontal side (Kilbourne et al., 2009) of the skull. The animal’s skull is usually surgically exposed (but without any craniotomy) to direct impact on a particular location on the skull, although in recent models (including the one developed
by us and described in the Chapter 3 of this thesis) can deliver impact to the head without any surgery (see below).

Various support systems have been used to stabilize the animal’s head during impact that also affect the degree of skull movement after impact and injury characteristics. For example, the rodent’s head is supported on a thick block of foam or gel that allows partial head acceleration causing moderate to severe brain injury with DAI as revealed by the APP histochemistry (Foda and Marmarou, 1994; Marmarou et al., 1994; Viano et al., 2012). An important variable is the spring constant of the foam used to support the head that can influence the head movement after impact. Unfortunately, physical characteristics of the foam, including the spring constant are rarely reported, challenging the replication of the model across laboratories. In another configuration, the animal head is held in a stereotaxic frame and undergoes the least amount of movement and show mild APP staining (Laurer et al., 2001; Shitaka et al., 2011).

Recently, Whalen and colleagues described a new murine impact CHI model characterized by a completely unrestrained head movement (Khuman et al., 2011; Meehan et al., 2012). In this model, isoflurane-anesthetized mice were supported on a piece of Kimwipes®, manually grasped by the tail and placed under a vertical guide tube. The impact was delivered without surgically exposing the animal skull, between coronal and lambdoid sutures with a 53 g metal bolt. Upon impact the mouse head tore the Kimwipes support with free rotation in the anterior-posterior plane. This method however, resulted in a moderately high post-CHI mortality rate (~ 20%). In a recent variant of the ‘Kimwipe model’ the animal’s body was supported on a piece of aluminum
foil suspended over an empty case with a thick foam pad at the base of the case (Kane et al., 2012). Upon impact, the aluminum foil completely tears off allowing unrestricted head and body movement of the animal as it falls into the cage. These authors reported repeatable, mild, concussive injury and suggested that the technique could be used for repeated impacts. Ren and colleagues recently developed a ‘hit and run’ TBI model in which the anesthetized mouse was hung head up vertically from its incisor teeth by a mounted metal ring and impacted by a pneumatically-driven piston over the lateral side of the head (Ren et al., 2013). Following impact the mouse fell onto a soft pad. The authors reported that driving piston at 4.8 m/s resulted in mild injury characterized by diffuse cortical astrogliosis, while impacting head at 5.2 m/s resulted in moderate injury characterized by frank tissue disruption or cavitation.

Advantages of CHI models include the concordance of the mechanism with the vast majority of human TBI that occur without skull fracture and, relative to FP and CCI, employ less-invasive and simpler surgical methods that allow rapid behavioral assessment of injury severity. Weight drop models, however, pose a number of very significant limitations. For example, high-speed videography analysis shows that many weight drop models deliver a primary and a rebound impact to the head. As well, weight drop models are limited in that velocity of head impact and head displacement cannot be varied independently as they can be when using an actuator. In addition, appropriate release of head constraint to allow human-like head kinematics is challenging with weight drop models and actuators that move downwards to create impact. A principal caveat of most current CHI animal models is that both the input parameters (e.g.,
mechanical loading, method of mechanical input, response of animal’s head to mechanical loading) as well as the cognitive, histological, and biochemical endpoints used can vary considerably among different laboratories, which has challenged the reproducibility of results and thus, the translational potential of these models. Much more needs to be learned about how these CHI models alter animal head kinematics (e.g., calculation of linear vs. angular acceleration, quantification of head acceleration), how head kinematics relate to injury severity, how brain movement relates to head movement in the species used, and to functional, molecular, and histological outcomes.

1.7.2.2 Non-Impact CHI by Blast Waves

Improvised explosive devices are a major source of TBI in military personnel. Blasts can induce TBI through several mechanisms including the blast wave itself, acceleration/deceleration forces, and particles that impact the head during the blast. To simulate non-impact, blast TBI conditions in animals, the animal is secured at one end of a long shock tube and is subjected to blast waves generated by compressed air or gas or explosives (Figure 1.12) (Rubovitch et al., 2011; Goldstein et al., 2012; Rafaels et al., 2012). The animal body is usually protected while the head is exposed to the blast waves. These injuries typically present with diffuse brain edema accompanied by hyperemia and delayed vasospasm. Again, the degree of head motion after blast appears to have a significant effect on behavioral and neuropathological outcomes as stabilizing the rodent head during blast was recently reported to be neuroprotective (Goldstein et al., 2012).
1.7.2.3 Non-Impact CHI by Inertial Loading

Many laboratories have developed inertial loading CHI models in which rotational force causes rapid acceleration of the animal head followed by a longer deceleration phase. These models have been developed for larger animals, including non-human primates (Gennarelli et al., 1981; Gennarelli et al., 1982), pigs (Smith et al., 1997; Smith et al., 2000; Browne et al., 2011), and rabbits (Gutierrez et al., 2001; Runnerstam et al., 2001; Hamberger et al., 2003; Krave et al., 2005; Krave et al., 2011), as well as for small animals such as rats (Davidsson and Risling, 2011). In these models, the animal head is secured to a mechanical system that consists of a snout clamp or a skull fixation plate. Linear motion induced by a piston is converted to rotational motion of the device, which in turn results in a rotational acceleration of the head, along either the coronal (Gennarelli et al., 1982; Smith et al., 1997; Smith et al., 2000), sagittal (Gennarelli et al., 1982; Gutierrez et al., 2001; Runnerstam et al., 2001; Davidsson and Risling, 2011;
Krave et al., 2011), axial (Smith et al., 2000; Browne et al., 2011), lateral or oblique (head turned 30° to right or left side and moving anterior or posterior with the center of rotation in the lower cervical spine) planes (Gennarelli et al., 1982). The severity of trauma can be adjusted by varying the angle of rotation or pulse duration. Studies using these models have shown that post-traumatic neurological status, such as coma, is related to the energy and form of rotation induced (Gennarelli et al., 1981; Gennarelli et al., 1982; Browne et al., 2011; Krave et al., 2011). These models have also shown that rotational forces can induce pathologies including SAH, increased ICP and elevated serum levels of an astrocyte-specific protein, S100B (Runnerstam et al., 2001; Davidsson and Risling, 2011). A distinctive neuropathological feature of these models is the DAI, as evident by the formation of axonal bulbs and accumulation of APP (Smith et al., 2000; Hamberger et al., 2003; Davidsson and Risling, 2011). Other microscopic pathologies include gliosis (Smith et al., 1997; Gutierrez et al., 2001), neuronal death (Smith et al., 1997; Runnerstam et al., 2001), and accumulation of phosphorylated heavy neurofilament subunits at neuronal cell bodies (Smith et al., 1997; Smith et al., 2000; Hamberger et al., 2003).

1.7.3 Challenges Associated With Current TBI Models

The choice of the most appropriate preclinical TBI model to use depends on what factors of human injury need to be modeled. Compared to CHI models the input parameters of FP and CCI models are well-controlled and thus induce highly reproducible injuries but have some disadvantages. First, these models represent only a small minority (0.8-3%) of human injuries that involve penetration of dura (Masson et al.,
2001; Myburgh et al., 2008; Wu et al., 2008). Second, these models, especially CCI, tend to induce severe injury through destruction of large cortical areas, which is typically observed only in severe human TBI (Marklund and Hillered, 2011). Third, these methods often require a precisely positioned craniotomy that can reduce secondary brain swelling by mimicking decompressive craniotomy often used to lower ICP (Zweckberger et al., 2006; De Bonis et al., 2010). Fourth, these methods of injury cause almost no head movement, which is rarely observed in human TBI.

CHI models, that apply impulsive (impact) loads to the head, by contrast, are increasingly attractive for modeling the majority (>95%) of human TBIs that occur without skull fracture or penetration of brain tissue. However, most CHI model systems have significant caveats with respect to their high incidence of skull fracture (necessitating immediate euthanasia of the animal) and highly variable outcomes between laboratories. We can gain further insights into the relative strengths and weaknesses of the various rodent injury models if we interpret these models in the context of what is known about the biomechanics of human, large animal, and rodent TBI. The vast majority of rodent CHI studies however, lack systematic biomechanical rigor, particularly for weight drop models where only gross mechanical input parameters such as the mass of the weight and drop height are typically reported. The reproducibility of these mechanical inputs and measures to decrease variability in mechanical input are rarely stated, and the mechanical response of animal’s head to the forces applied is even more rarely recorded. As a result, the outcomes of studies even
with similar reported mechanical inputs can vary from no detectable injury to death (Figure 1.13).

**Figure 1.13: Variability in input parameters and outcomes in weight-drop TBI studies reported in the literature.**

The figure depicts variation in two most commonly reported input parameters, drop height and drop mass (A) and associated theoretical values of input energy (B) used in mouse weight drop impact TBI studies. Panel C shows variation in mortality rate associated with input energy. Reproduced from Namjoshi et al. (2013a).

One potential source of variability especially relevant for CHI models is the relationship of head kinematics (moment of the head) following impact to both behavioral and pathological outcomes after injury. The head kinematics can vary depending on how the head is supported during impact. A few recent studies have begun to report details of head kinematics following impact CHI in rats ((Viano et al., 2009; Li et al., 2011; Viano
et al., 2012). For example, Li et al. (2011) recently studied the biomechanical parameters of the weight drop-based Marmarou impact acceleration model (Marmarou et al., 1994). Head acceleration, change in head velocity, linear and angular head acceleration were measured and compared to 1) kinematic responses predicted by a finite element model and 2) axonal damage as assessed by APP histochemistry. The authors reported that the severity of DAI was related to the linear and angular response of the rat head but, against expectation, not with the drop height. Viano and colleagues developed a rat model of CHI with unrestricted head movement to mimic concussions experienced by professional football players (Viano et al., 2009, 2012). The ability to study the detailed biomechanical responses of the rats and to allow unrestricted motion in response to impact and the proposed methods for scaling these responses to human concussions offer new insights into factors that may increase the translational potential of future CHI studies in rodents.

In animal models, classification of injury severity as mild, moderate or severe is often determined on functional outcomes such as cognitive and motor impairment that develop over hours to weeks. Importantly, these outcomes can vary widely among different research groups. For example, motor impairment may (Tang et al., 1997; DeFord et al., 2002; Andoh and Kuraishi, 2003; Creeley et al., 2004; Rubovitch et al., 2011) or may not (Tsenter et al., 2008) be observed with “mild” CHI. The severity, temporal response, and extent of secondary injury response of brain can also vary among rodent species and also within different strains of the same species. Because the severity of the primary injury at the time of impact is often unknown and may be
below the threshold of detection even when using methods such as MRI, understanding how biomechanical data affect the functional and histological outcomes of rodent CHI may offer an alternative approach to improve the predictive and translational power of preclinical TBI research in rodents.

Economic, ethical and scientific drivers have shifted the focus of preclinical TBI studies from large animals to mainly rodents, even though large animals are better mimics of the size and anatomy of the human brain (Denny-Brown and Russell, 1940; Gennarelli et al., 1981; Gennarelli et al., 1982; Duhaime, 2006). One important consideration for selecting an appropriate rodent TBI model is that the rodent and human brain differs considerably with respect to skull anatomy, brain mass and size, craniospinal angle, gray-to-white matter ratio and cortical folding, all of which influence the biomechanical parameters of brain injury. For example, rotational loading cannot be linearly scaled between human and rodent TBI (Finnie, 2001). Furthermore, the long axis of the rodent brain and spinal cord are nearly linear, as opposed to perpendicular axes of the human brain and spinal cord, which causes less rotational shearing in the rodent brain and renders rodents less vulnerable to diffuse axonal damage. Although the anatomical differences between the rodent and human brain will always pose a caveat for preclinical research, rodent-based experiments are expected to form the majority of mechanism of action and proof-of-concept studies that can then be further validated in large animal species. This progression will provide the greatest adherence to ethical principles and scientific feasibility.
A common question regarding rodent models of TBI is whether the response of the lissencephalic rodent brain to impact, acceleration or blast injury is representative of that of the gyrencephalic human brain. For example, cases of CTE, which occurs from repeated concussions, commonly show more extensive pathology in sulcal depths than elsewhere along the cortex, suggesting that shear strain may be maximal at the sulcal depths (Smith et al., 2013). Further work is warranted to investigate the role that these anatomical differences may play in the context of mTBI research. Even if the lissencephalic vs. gyrencephalic differences are found to result in an inability to reproduce anatomical injury patterns observed in humans, the vast potential of rodent models for studies investigating secondary injury pathways and for drug discovery research is a major incentive to continued use of these species.

1.8 Pharmacological and Non-pharmacological Management of TBI: An Overview of Randomized Clinical Trials

A recent review of 100 randomized clinical trials (RCT) examined efficacy of interdisciplinary interventions of 55 acute phase and 45 post-acute phase TBI trials (Lu et al., 2012). Acute phase trials were defined as interventions that occurred within 24h of TBI. These studies largely focused on patient stabilization and minimizing secondary injury, and employed standard outcomes comprised of GCS scores and mortality. By comparison, post-acute trials can be very heterogeneous in design. The post-acute trials reviewed were initiated from days-to-years post-TBI and used a wide variety of outcomes including cognitive, neuropsychological, and quality of life measures (Lu et al., 2012).
In the acute phase, only 15 of 55 trials showed a positive treatment effect and, of these, 11 evaluated non-pharmacological interventions. Furthermore, many interventions that were tested across multiple trials led to mixed results. For example, decompressive craniotomy, hyperosmotic therapy and hypothermia have shown inconsistent effects, with studies reporting positive (Cruz et al., 2001, 2002; Zhi et al., 2003; Jiang et al., 2005; Qiu et al., 2005; Jiang et al., 2006; Qiu et al., 2007), negative (Cooper et al., 2011), or no effect (Smith et al., 1986; Marion et al., 1997; Shiozaki et al., 2001; Clifton et al., 2002; Lu et al., 2003; Cooper et al., 2004; Harris et al., 2009; Clifton et al., 2011) on post-TBI outcomes. A single trial of hyperventilation to reduce ICP also revealed an adverse effect (Muizelaar et al., 1991). Other non-pharmacological interventions that have shown benefit include early nutritional supplementation with zinc within 48 hours or total parenteral nutrition within 72 hours post-TBI (Young et al., 1996). Lastly, a pre-hospital rapid sequence intubation trial also yielded positive results (Bernard et al., 2010).

The 32 acute phase pharmacological trials reviewed were grouped according to the targeted treatment mechanism. Anti-excitotoxic agents, insulin, magnesium, corticosteroids, and targeting lipid peroxidation or free radical damage either failed to show a positive treatment effect or led to adverse effects. Importantly, only 4 of 32 pharmacological acute phase RCTs for TBI showed positive treatment effects, including the psychostimulant, methylphenidate (Ritalin) (Moein et al., 2006), 2 RCTs for
progesterone (Wright et al., 2007; Xiao et al., 2008) and 1 RCT for the calcium channel blocker, nimodipine (Harders et al., 1996).

Of the 45 post-acute phase RCTs reviewed, 24 evaluated cognitive rehabilitation approaches and of these 22 showed positive treatment effects. The approaches used included comprehensive interdisciplinary rehabilitation, cognitive/academic exercises and communication skill training, compensatory techniques and computer assisted training, educational intervention, psychotherapy, and behavior modification. Six of 8 trials using physical rehabilitation also showed a positive treatment effects, and nutrition and acupuncture were found to be beneficial in the single trials conducted thus far. Potential TBI pharmacotherapies were tested in 11 post-acute RCTs, with positive treatment effects reported in 6 studies, including methylphenidate (Whyte et al., 2004; Willmott and Ponsford, 2009), CDP-choline (Calatayud Maldonado et al., 1991), and a vitamin B₆ analog, pyritinol (Kitamura, 1981). On the other hand, a single trial of phenytoin and carbamazepine was negative (Smith et al., 1994), while sertraline (Novack et al., 2009; Banos et al., 2010), rivsatigmine (Tenovuo et al., 2009) and modafinil (Jha et al., 2008) were found to have no significant treatment effects.

A number of RCTs in TBI have been completed in 2012-2013. Highlights include a Phase III randomized trial of hypothermia in children, termed the “Cool Kids” study, which recently reported that 48 h of hypothermia followed by rewarming does not reduce mortality or positively affect recovery from pediatric brain injury (Adelson et al., 2013). An intervention trial randomizing high-risk post-concussion syndrome patients to
an early visit to a physician similarly was negative, with similar outcomes to the
treatment as usual group (Matuseviciene et al., 2013). Chesnut et al recently reported
on their much anticipated trial of continuous invasive ICP monitoring following severe
TBI, showing no advantage over imaging and clinical examination alone (Chesnut et al.,
2012). Citocoline, which demonstrated promise in earlier trials as a neuroprotective
agent, failed in a subsequent Phase III trial (Zafonte et al., 2012). A trial of 30
hyperbaric oxygen therapy sessions, done in military personnel with mTBI, did not
demonstrate a significant effect (Wolf et al., 2012). Finally, in perhaps the most
influential trial of 2012, amantadine, given to TBI patients who were minimally conscious
or in a vegetative state 4 to 16 weeks following TBI, showed a highly significant effect
on accelerating the pace of functional recovery (Giacino et al., 2012).

The emerging consensus from these 100 RCTs conducted on TBI subjects is that early
intervention in the acute phase and comprehensive rehabilitation approaches in the
post-acute phase provide the most beneficial outcomes. What is far less clear is
whether a deeper understanding of the pathways involved in TBI pathogenesis may
eventually offer effective pharmacologic therapies. A critical requirement for any drug
discovery program is the availability of preclinical models that are as biofidelic to the
human condition as possible. With respect to TBI, however, a working knowledge about
neurophysiology and biomechanics is necessary to select the most appropriate model
system to address the experimental question being considered.

1.9 Study Rationale, Hypothesis, and Objectives
As discussed above, repair of damaged CNS critically depends on the lipid transport mediated by brain lipoproteins. Lipid-laden brain lipoproteins aid in neuronal membrane repair and synaptogenesis and stimulate the degradation of Aβ peptides that accumulate after TBI. Genetic and pharmacological methods to increase the cholesterol and phospholipid carried on brain lipoproteins improve cognitive function and reduce neuropathology in AD mice. However, it is not known whether similar strategies may also be beneficial for acute CNS injury.

We and others have previously demonstrated that synthetic LXR agonists effectively induce ABCA1 expression, increase apoE lipidation, reduce Aβ levels, lower inflammation and restore cognitive function in AD mouse models. Given that TBI pathology shares common features with that of AD pathology including Aβ deposition and NFT formation as well as neuroinflammation, it is of considerable interest to determine whether LXR agonists may also have potential therapeutic utility for TBI. The importance of addressing this question is two-fold. First, LXR treatment may minimize neuronal damage and promote recovery in the acute phase after injury by reducing inflammation and promoting apoE-mediated neuronal repair processes. Second, by facilitating Aβ clearance in the first weeks after injury, LXR treatment may also lessen the increased long-term risk of AD decades later.

Accordingly I hypothesized that the synthetic liver X receptor agonist, GW3965 will promote neuronal recovery following closed-head mild, repetitive traumatic brain injury in mice via apoE-mediated mechanisms.
Thus, the **first objective** of this thesis was to study therapeutic utility of the non-steroidal LXR agonist, GW3965 in mild, repetitive closed head TBI in adult male mice and whether the hypothesized beneficial effects of GW3965 were mediated through an apoE-based mechanism. I tested this hypothesis using a widely-used weight drop CHI model due to its several advantages over OHI models discussed previously. The experimental details of the first objectives are presented in Chapter 2 of this thesis.

While weight drop TBI is one of the most popular rodent CHI model, it suffers from several caveats like skull fractures and considerable experimental variation across cognitive, histological, and biochemical outcome measures. This can be due to poor control over the input parameters used to induce injury (e.g., mechanical loading, method of mechanical input, response of the animal’s head to mechanical loading). In addition, surgical manipulations required to localize the impact on the exposed skull necessitate the use analgesic cocktails, sedatives and long-term exposure to anesthetic. For example, in the weight drop CHI model used in the first objective, ethical constraints necessitated us to use a non-steroidal anti-inflammatory drug (NSAID), meloxicam for post-TBI pain suppression as well as ketamine-xylazine cocktail to keep the animal sedated during the CHI procedure. These agents can prolong the post-anesthetic recovery interfering with the immediate post-TBI assessment of neurological outcomes. In addition, NSAIDs can suppress post-TBI inflammation, while, ketamine, a NMDA receptor blocker, can suppress post-TBI glutamate-induced excitotoxicity, interfering with the secondary injury pathways. Thus, it was essential to develop a
neurotrauma model that would mitigate these limitations and thus increase its translational potential. While the second objective of this thesis was not driven by a specific hypothesis, it was derived from the caveats encountered in the first study objective.

Accordingly, the second objective of this thesis was to develop and a CHI model that:

1) offers tight control over injury parameters such as impact location and impact energy,
2) allows unrestricted head movement after impact mimicking most of the human TBI conditions, 3) allows CHI without any surgical manipulation thus reducing anesthetic analgesic and sedative requirement, 4) allows assessment of head kinematics during and following impact, and 5) allows immediate post-TBI assessment of neurological outcomes with minimal interference from anesthetics and sedatives. I further characterized acute post-TBI outcomes in mice using this new CHI model.

The experimental details of the second objective are presented in Chapter 3 of this thesis.
Chapter 2: The Liver X Receptor Agonist GW3965 Improves Recovery from Mild Repetitive Traumatic Brain Injury in Mice Partly Through Apolipoprotein E

2.1 Summary

Traumatic brain injury (TBI) increases Alzheimer’s disease (AD) risk and leads to the deposition of neurofibrillary tangles and amyloid deposits similar to those found in AD. Agonists of Liver X receptors (LXRs), which regulate the expression of many genes involved in lipid homeostasis and inflammation, improve cognition and reduce neuropathology in AD mice. One pathway by which LXR agonists exert their beneficial effects is through ABCA1-mediated lipid transport onto apolipoprotein E (apoE). To test the therapeutic utility of this pathway for TBI, we subjected male wild-type (WT) and apoE-/- mice to mild repetitive traumatic brain injury (mrTBI) followed by treatment with vehicle or the LXR agonist GW3965 at 15 mg/kg/day. GW3965 treatment restored impaired novel object recognition memory in WT but not apoE-/- mice. GW3965 did not significantly enhance the spontaneous recovery of motor deficits observed in all groups. Total soluble Aβ₄₀ and Aβ₄₂ levels were significantly elevated in WT and apoE-/- mice after injury, a response that was suppressed by GW3965 in both genotypes. WT mice showed mild but significant axonal damage at 2 d post-mrTBI, which was suppressed by GW3965. In contrast, apoE-/- mice showed severe axonal damage from 2 to 14 d after mrTBI that was unresponsive to GW3965. Because our mrTBI model does not produce significant inflammation, the beneficial effects of GW3965 we observed are unlikely to be related to reduced inflammation. Rather, our results suggest that both
apoE-dependent and apoE-independent pathways contribute to the ability of GW3965 to promote recovery from mrTBI.

2.2 Introduction
Several epidemiological studies suggest that TBI may increase the risk of dementia, particularly AD, although this association is not always observed (May et al., 2011). TBI has also been associated with an earlier onset of AD (May et al., 2011). Both neurofibrillary tau tangles and amyloid plaques are found in post-mortem TBI brain tissue. However, the widespread white matter involvement in TBI that is not present in AD results in noteworthy differences in the pattern and distribution of their common neuropathological features. For example, TBI neuropathology is largely that of tau deposition, as only approximately 30% of TBI patients also contain Aβ deposits (Roberts et al., 1994). Aβ plaques can appear within hours and remain detectable up to 47 years after a single moderate-severe TBI (Johnson et al., 2012). NFTs are also detectable after a single TBI and are remarkably prominent in mild, repetitive, concussive injuries (Johnson et al., 2012). Despite the low prevalence of amyloid deposits in the post-mortem TBI brain, APP accumulation in damaged axons is a striking histological hallmark of diffuse axonal injury (DAI) (Gentleman et al., 1993; Sherriff et al., 1994b). Increased APP in the post-TBI brain is hypothesized to trigger a burst of Aβ production that can deposit in amyloid plaques (Johnson et al., 2010). Intriguingly, microdialysis experiments in brain-injured humans have demonstrated that ISF Aβ levels correlate positively with the patient’s GCS score (Magnoni and Brody,
suggesting that Aβ release is associated with recovery of synaptic function, as has been demonstrated in animals (Schwetye et al., 2010).

ApoE also regulates Aβ metabolism, a function that underlies its genetic association with AD. The human apoE4 allele increases AD risk and hastens its onset (Corder et al., 1993; Poirier et al., 1993b), whereas apoE2 delays onset and reduces AD risk (Corder et al., 1994). Recent microdialysis studies demonstrated that apoE genotype modulates the Aβ half-life in the brain ISF, with the rate of Aβ degradation accelerated by apoE2 ($t_{1/2} = 0.56$ h) and prolonged by apoE4 ($t_{1/2} = 1.1$ h) compared to apoE3 ($t_{1/2} = 0.71$ h) (Castellano et al., 2011). Although AD risk and age of onset are indisputably modified
by apoE genotype, the relationship between apoE genotype and TBI outcome is complex. Some studies report that apoE4 carriers have significantly poorer outcomes compared to non-carriers (Teasdale et al., 1997; Lichtman et al., 2000; Smith et al., 2006), while others find no association (Jiang et al., 2008b; Pruthi et al., 2010). Many variables pose challenges to resolving these uncertainties, including relatively short follow-up times after TBI and the diversity of subjects with respect to the age, gender, and injury severity. Despite these challenges, a meta-analysis of 14 studies with 2,527 subjects concluded that apoE4 does not affect initial injury severity but rather may compromise recovery at 6 months post-injury (Zhou et al., 2008).

Improving apoE function is thought to facilitate both neuronal repair and Aβ clearance after TBI, thus potentially offering both acute and long-term benefits. One method to enhance apoE function is to promote its lipidation by ABCA1, which is the rate-limiting step in generating apoE-containing lipoprotein particles in the CNS. ABCA1 deficiency leads to poorly-lipidated apoE in the CNS (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004), and increases amyloid load in AD mice (Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005). Conversely, overexpression of ABCA1 in the CNS increases apoE lipidation and greatly decreases amyloid deposits in AD mice (Wahrle et al., 2008). Transcription of ABCA1 and apoE is induced by agonists of LXRs, which regulate many genes involved in lipid metabolism and inflammation (Jamroz-Wisniewska et al., 2007; Wojcicka et al., 2007). Genetic deficiency of LXRs increases amyloid burden in AD mice (Zelcer et al., 2007). Synthetic LXR agonists including TO901317 and GW3965 cross the BBB, induce ABCA1 and apoE expression, improve
memory and reduce Aβ levels in AD mice (Jiang et al., 2008a; Donkin et al., 2010; Fitz et al., 2010). Importantly, ABCA1 is required for several beneficial effects of GW3965 in AD mice (Donkin et al., 2010), including increased CSF apoE levels, reduced amyloid load, and improved memory. These observations provide a compelling rationale for testing the therapeutic potential of LXR agonists for TBI. Indeed, TO901317 reduces Aβ accumulation and promotes cognitive recovery in a CCI model of moderate-severe brain injury in mice (Loane et al., 2011).

Approximately 80% of human TBI are mild injuries without skull fracture and loss of consciousness (Decuypere and Klimo, 2012). It is increasingly appreciated that repetitive, mild TBI, commonly experienced by athletes in high-contact sports may lead to chronic traumatic encephalopathy (CTE), which is characterized by cognitive, executive, and motor function disturbances, tau deposition and, in some cases, amyloid deposits similar to those found in AD (Gavett et al., 2011).

To determine the therapeutic utility of LXR agonists for this type of brain injury, we established a mouse model of mild repetitive TBI (mrTBI), wherein a gravity-driven weight drop device was used to deliver two consecutive injuries 24h apart. Here we report that GW3965 improves cognitive recovery and suppresses axonal damage in an apoE-dependent manner. Surprisingly, apoE was not required for GW3965 to suppress the transient increase in Aβ levels. Our results provide additional support for the therapeutic potential of LXR agonists in TBI, and demonstrate that both apoE-dependent and apoE-independent pathways contribute to their beneficial effects.
2.3 Materials and Methods

2.3.1 Animals

Male C57Bl/6 (WT) and apoE-/- (strain name: B6.129P2-Apoetm1Unc/J) mice were obtained from Jackson Laboratories (Bar Harbor, ME). ApoE-/- mice are on C57Bl/6 background. Animals were housed with a reverse 12 h light - 12 h dark cycle for at least 10 days before TBI. The animals were fed with standard chow diet (PMI LabDiet® 5010, containing 24% protein, 5.15 fat, and 0.03% cholesterol) and had free access to water. All animal procedures were approved by the University of British Columbia Committee on Animal Care (Protocol # A07-0706) and adhered to the Canadian Council for Animal Care guidelines.

2.3.2 Mild Repetitive Traumatic Brain Injury (mrTBI)

WT (mean body weight: 29.6 ± 0.24 g) and apoE-/- (mean body weight: 30 ± 0.26 g) mice at 4 months of age were subjected to two mild CHI spaced 24 h apart using a gravity-driven weight-drop device obtained from Dr. Esther Shohami (The Hebrew University of Jerusalem). The device consists of ~ 50 cm long graduated acrylic guide tube held vertically over a metal plate (Figure 2.1A). A Teflon-tipped cone (tip diameter: 2 mm) is located at the base of the tube that can be freely raised inside the tube. A 95 g brass weight is hung inside the tube with a thread. The height of weight is manually adjusted by a thread holding lever. For inducing injury, the animal’s head is positioned under the lower end of the guide tube and the Teflon-tipped cone is rested over the exposed skull. In the original Shohami model, the animal’s head was supported by a
rigid Teflon disk. We modified this support by introducing ~ 3 mm thick computer mouse pad between the animal’s head and the Teflon disk to reduce incidence of skull fracture by partially allowing head movement following impact. This modification was made after conducting a small pilot study with and without the mouse pad. The weight is raised to the desired height and dropped over the Teflon-tipped cone, which directs the impact force to a localized area of the animal skull.

All surgical procedures were conducted using aseptic technique. One day before the TBI procedure, animals were weighed and the scalp was shaved under isoflurane anesthesia (induction: 3-4%, maintenance: 1.5% - 2%). On the day of TBI surgery, animals were anesthetized with isoflurane (induction: 3-4%, maintenance: 1.5% - 2%) in oxygen (0.9 L/min). Lubricating eye ointment was applied to prevent corneal drying. Animals were kept warm with an electric heating pad. The scalp was scrubbed three times with 4% chlorhexidine solution and 70% ethanol. Ketamine (15 mg/kg) and xylazine (1.75 mg/kg) mixture [subcutaneous (s.c.)], meloxicam (1 mg/kg, s.c.), and bupivacaine (0.125 mg/kg, s.c., under the scalp) were administered for analgesia. Sterile saline (1 ml/100 g body weight) was administered s.c. for hydration. The surgical plane of anesthesia was confirmed with the absence of toe-pinch reflex and a midline longitudinal incision was made in the scalp to expose the calvarium. After clearing the parietal bone, a sterile piece of gauze (~ 3 mm x 5 mm), pre-moistened with bupivacaine solution (2.5 mg/ml) was overlaid over the left parietal bone to minimize the incidence of skull fracture (Figure 2.1 B). Anesthesia was temporarily discontinued (not more than 30 s) and the animal was quickly moved under the weight-drop device. The
animal remained sedated with ketamine-xylazine during this temporary discontinuation of anesthesia. The head was manually held in place such that the tip of the Teflon-tipped cone rested over the piece of gauze in the left mid-parietal bone [approximately between -1 and -3 mm from bregma and 1 mm left of the sagittal suture (Paxinos and Franklin, 2001)] (Figure 2.1 B and C). A 95 g brass weight was dropped from a pre-determined height to result in injury across the left hemisphere. The precise drop height (cm) of the weight was adjusted to be one-third the weight (g) of the animal (Mean drop height: WT, 10 ± 0.08 cm; apoE-/-, 10.1± 0.09 cm). Mean body weight and drop height did not defer significantly across the genotypes, time points, and treatment groups (Figure 2.2). Anesthesia was reinstituted following injury. After removing the gauze and confirming the absence of skull fracture, the incision was closed and the animals were returned to their cages. Twenty-four hours after the first injury, the incision was reopened under isoflurane anesthesia and a second identical TBI was induced as described above. Mice suffering from skull fracture after injury were immediately euthanized. Post-TBI recovery was monitored until animals moved freely and fed normally. Sham controls received isoflurane anesthesia, pre-surgical analgesia, and skin incision only. Mice within each group were randomly assigned to one of three post-TBI assessment time points, viz., 2, 7, and 14 days after the second injury. The time of the 2nd TBI was defined as time zero.
Figure 2.1: Weight drop CHI model and impact location.

(A) Schematic of weight drop device and mouse head under the device. (B) Illustration of surgical preparation for TBI showing an anesthetized mouse with the midline scalp incision exposing the calvarium. A sterile piece of cotton gauze is overlaid across the skull to help reduce incidence of skull fracture upon impact. The location of impact is indicated by red dot. (C) Schematic diagram of murine skull showing approximate location of the weight drop impact (red circle) on the left parietal bone. The dotted rectangle represents cotton gauze.
To induce mrTBI, a 95 g brass weight was dropped on the animal’s skull using a height (cm) adjusted to one third of the animal’s body weight (g) (see Methods and Materials). The figure depicts body weight (A, B) and drop height (C, D) of WT and apoE-/- mice, respectively, across post-mrTBI time points and treatment groups. Neither drop height nor body weight differed significantly across genotypes, time points or treatment groups. Data within each genotype were analyzed by two-way ANOVA followed by a Bonferroni post-hoc test. Legend: V: untreated mice, open bars, G: GW3965-treated mice, black bars.

2.3.3 GW3965 Treatment

Injured mice were randomized into treated (GW3965) and untreated (vehicle) groups (WT mice: N=45/group, apoE-/- mice: N=30/group). Since, the animals do not become fully ambulatory and start feeding for up to 2 h following TBI, the GW3965 treatment was started with a single intraperitoneal (i.p.) bolus (20 mg/kg) 30 min after the 2nd
injury. GW3965 treatment was continued thereafter by feeding mice with standard rodent chow (Research Diets Inc., New Brunswick, NJ) in which GW3965 was compounded at 120 mg/kg until the end of the study, resulting in an average daily dose of 15 mg/kg upon determination of average daily food intake. Because we had previously demonstrated that GW3965 doses of 2.5 mg/kg/day and 33 mg/kg/day provided cognitive and neuropathological benefits in APP/PS1 mice (Donkin et al., 2010), we aimed to achieve a dose between these two extremes. A complete dose-response study was beyond the scope of this thesis project. Because GW3965 has a short plasma half-life of 2 h and high oral bioavailability (70%) (Collins et al., 2002) delivering GW3965 in chow provides more constant plasma levels than by once-daily oral gavage. Injured mice in the untreated group received a single i.p. bolus of equivalent volume of the vehicle used for dissolving GW3965, dimethyl sulfoxide 30 min after 2nd injury. These mice were thereafter fed standard rodent chow. Sham-operated mice (WT mice: N=24; apoE-/- mice: N=18) received neither GW3965 nor vehicle and were fed standard rodent chow. We have previously determined that GW3965 at a dose of 33 mg/kg/day does not cause hepatotoxicity in mice (Donkin et al., 2010).

2.3.4 Cognitive Assessment by Novel Object Recognition
Medial temporal lobe function was assessed using the novel object recognition (NOR) task performed at 2, 7, or 14 days following mrTBI (Ennaceur and Delacour, 1988). This task was selected to facilitate comparisons to our previous NOR study of GW3965 efficacy in APP/PS1 mice (Donkin et al., 2010), and because fine motor impairments observed after TBI may confound tasks such as Morris Water Maze. Each animal was
assessed with the NOR task at only one time point after sham or mrTBI surgery.

Twenty-four hours prior to the NOR task, mice were habituated to an empty open field (14” x 24” x 14”) for 10 min in a brightly-lit room. The NOR task consisted of two phases: training and testing. During training, mice were placed in the center of the open field containing two identical objects spaced equidistant from the walls (Figure 2.3). The time the animal explored each of the two objects during a 5 min epoch was quantified, with exploring defined as sniffing, climbing on, or touching the object and at a body length or less away from the object while oriented toward it. Trials were tracked using an overhead digital camera and video tracking system (ANY-maze, Stoelting Co, Wood Dale, IL). Between animals, the open field and objects were cleaned with 70% ethanol to eliminate olfactory cues. Four hours after training, mice were tested by replacing one of the identical objects with a novel object of similar size but different shape and color (Figure 2.3). The side of the open field with the novel and familiar objects was alternated for each mouse to avoid any side preference. Each animal was tracked for a period of 5 min. Novel object recognition was quantified using the discrimination index (DI) calculated as: DI = (T_N – T_F)/(T_N + T_F), where T_N and T_F represent exploration time for the novel and familiar objects, respectively. DI values range from +1 to −1, where a positive score indicates preference for the novel object, a negative score indicates preference for the familiar object, and zero indicates no discrimination (Antunes and Biala, 2012).
During the training phase the mouse is placed in a rectangular box containing two identical objects (indicated by yellow circles) and exploration time for each object is recorded for 5 min. After a retention period of 4 h, the animals are re-introduced to the same box with one familiar (yellow circle) and a novel object (red hexagon).

### 2.3.5 Motor Function Assessment by Accelerating Rotarod

Motor performance was evaluated using an accelerating rotarod (Model 7650, Ugo Basile, Collegeville, PA). Mice were first trained for two days before the 1st TBI to walk on accelerating rotarod (0 to 30 rpm in 210 s). The average of the last two trials of the second training day was used to establish baseline failing latency. Unless the animal was scheduled for sacrifice, rotarod latencies were repeatedly assessed at 1, 2, 7, and 14 d post-mrTBI. At each test day, every mouse underwent two accelerating speed trials (0 to 30 rpm in 210 s) separated by an inter-trial interval of at least 15 min. The animal was recorded as failing during the 210 s period if: (1) the animal fell completely off the rotating rod before 210 s, or (2) the animal gripped the rod and made one complete passive revolution without actively walking on the rod. The average failing latency was calculated from the two trials for each animal.

### 2.3.6 Biochemical Analyses
2.3.6.1 Tissue Collection

Mice were anesthetized with an i.p. injection of 150 mg/kg ketamine (Bimeda-MTC) and 20 mg/kg xylazine (Bayer) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) containing heparin (0.5 units/mL) for 7 min. For biochemistry, brains were longitudinally bisected and the ipsilateral half-brains were rapidly frozen over dry ice and stored at -80°C until analysis (WT mice N: injured = 10/group/time point, sham = 6/time point; apoE-/- mice N: injured = 5/group/time point, sham = 4/time point). For histology, anesthetized mice were perfused with heparinized PBS and whole brains were immersion-fixed in 10% neutral buffered formalin for 48 h followed by cryoprotection in 30% sucrose in PBS at 4°C for additional two days (N: injured = 5/treatment group/time point/genotype, sham = 2/time point/genotype).

2.3.6.2 Protein Extraction

Frozen ipsilateral half-brains were thawed over ice and homogenized in 1.5 mL of ice-cold RIPA lysis buffer (5mM EDTA, 50mM NaCl, 10mM sodium pyrophosphate, 50mM NaF, and 1% NP40 alternative, pH: 7.4) containing complete protease inhibitor cocktail (Roche Applied Science) with a Tissuemite homogenizer at full speed for 20 s. The homogenate was sonicated at 20% output for 10 s followed by centrifugation at 4°C for 10 min at 9,000 rpm in a microcentrifuge (Eppendorf). The supernatant was separated and used for analysis of ABCA1, apoE, APP, APP-CTF, LDLR, IL-6, TNF-α, MCP-1, Aβ40, and Aβ42. Aliquots of all lysates were immediately frozen at –80°C until analysis. Protein concentration was determined by DC Protein assay (Bio-Rad, Hercules, CA).
2.3.6.3 Western Blot Analyses

RIPA lysate (80 µg protein) was electrophoresed through 10% SDS-polyacrylamide gels, transferred to PVDF membrane (Millipore), and immunodetected for the detection of ABCA1, apoE, and LDLR. APP and APP-CTF were analyzed by resolving RIPA lysate through 4-12% NuPAGE Bis-Tris gradient gels (catalogue # NP0335, Invitrogen). The details of primary antibodies used for Western blotting are presented in the Table 2.1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>AC10</td>
<td>1:1000</td>
<td>Gift from Dr. M. R. Hayden, Centre for Molecular Medicine and Therapeutics, The University of British Columbia</td>
</tr>
<tr>
<td>ApoE</td>
<td>M-20</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>APP Holoprotein</td>
<td>22C11</td>
<td>1:2000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>APP-CTF</td>
<td>C1/6.1</td>
<td>1:1000</td>
<td>Covance</td>
</tr>
<tr>
<td>LDLR</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>R&amp;D Systems</td>
</tr>
</tbody>
</table>

Table 2.1: Details of primary antibodies used for Western blotting.

All membranes were probed with anti-GAPDH antibody (clone 6C5, 1:5000, Chemicon) as a loading control. Blots were developed using enhanced chemiluminescence (Amersham ECL, GE Healthcare) according to the manufacturer’s recommendations. Films were scanned into TIFF format at 600 dpi resolution and pixel counts were determined using ImageJ (version 1.46, NIH). Levels of ABCA1, apoE, APP, APP-CTF, and LDLR were normalized to GAPDH and expressed as fold difference compared to sham controls.
2.3.6.4 Quantitative Assessment of Endogenous Aβ by ELISA

Endogenous Aβ40 and Aβ42 levels in ipsilateral half-brain lysates were quantified by ELISA (KMB3481 and KMB3431, Invitrogen) following the manufacturer's instructions. Levels of soluble Aβ40 and Aβ42 were normalized to total protein.

2.3.6.5 Quantitative Assessment of IL-6, TNFα, and MCP-1 by ELISA

IL-6, TNFα and monocyte chemotactic protein-1 (MCP-1) levels in ipsilateral half-brain lysates were determined by ELISA. Briefly, 96-well plates were coated and incubated overnight at 4°C with primary antibody against murine IL-6, TNFα, and MCP-1. Brain lysates were incubated with primary antibody at room temperature for 1 h. Following washing, samples were incubated with biotinylated secondary antibodies at room temperature for 1h. All the primary and secondary antibodies were obtained from eBioscience. The details of primary and secondary antibodies are presented in the Table 2.2. Samples were developed with avidin-horseradish peroxidase (eBioscience) and TMB substrate.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clone</td>
<td>Concentration</td>
</tr>
<tr>
<td>IL-6</td>
<td>MP5-20F3</td>
<td>1:1000</td>
</tr>
<tr>
<td>TNFα</td>
<td>TN3-19.12</td>
<td>1:1000</td>
</tr>
<tr>
<td>MCP-1</td>
<td>4E2</td>
<td>1:1000</td>
</tr>
<tr>
<td>-------</td>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>4E2</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2: Details of primary and secondary antibodies used for cytokine ELISA.

2.3.7 Histology

Brains were cut into coronal sections (40 µm) on a cryotome and all sections starting from the genu of corpus callosum (~1.1 mm anterior to bregma) to the posterior hippocampus (~3.3 mm posterior to Bregma) were collected in 1X PBS with sodium azide.

2.3.7.1 Assessment of Axonal Injury by Silver Staining

Three to four coronal brain sections, separated by 400 µm, from the bregma to the posterior hippocampus, were stained using FD NeuroSilver Kit II (FD NeuroTechnologies Inc, Columbia, MD) according to the manufacturer’s protocol, which is based on the method originally described by Gallyas et al (1990). The manufacturer’s protocol was further optimized with the following modifications: 1) Sections were stored in 4% paraformaldehyde in saline-free phosphate buffer at 4°C for a minimum of 5 d prior to staining. 2) Step 4 was decreased from 2x2 min incubations to 1x2 min incubation to increase staining intensity. 3) Steps 3, 4, and 5 were performed with vigorous shaking. From each section, 40X-magnified images of ipsilateral corpus callosum, cingulum, external capsule, internal capsule, and optic tracts were captured with a Zeiss Axio Imager A1 microscope attached to an Olympus DP72 camera and digitized using cellSens® Standard digital imaging software (version 1.4.1, Build 8624, OLYMPUS). A semiquantitative scale was developed to score staining intensity, ranging
from 0 (0 to <10% staining) to 3 (> 70% staining). The scale was based on the extent and intensity of argyrophilic structures (cell bodies and axonal fibers) within an image field. Every image was independently scored by two trained raters who were blinded to the injury, genotype, and treatment status. Scores from the two raters were averaged. A minimum of 3 sections per brain were scored and combined to define the average silver stain score of each brain region. The silver staining was carried out on samples containing 5 injured brains/genotype/time point/group and 2 sham brains/genotype/time point.

### 2.3.7.2 Assessment of Microglial Activation by Iba1 Immunohistochemistry

Microglial activation induced by MrTBI was assessed with Iba1 immunohistochemistry. Briefly, 3-4 coronal brain sections, separated by 400 µm, starting from the bregma to the posterior hippocampus were washed in Tris-buffered saline (TBS), treated with 0.3% hydrogen peroxide for 10 min and blocked with 3% normal goat serum (NGS) in TBS containing 0.25% Triton-X (TBS-X) for 30 min. Sections were incubated overnight in TBS-X containing anti-Iba1 polyclonal antibody (Catalogue # 019-19741, 1:1000, Wako Chemicals, Richmond, VA) and 1% NGS. Following washing, sections were incubated in biotinylated goat-anti-rabbit secondary antibody (1:1000 in TBS-X) for 1 h. Sections were visualized with horseradish peroxidase (Vectastain Elite ABC Kit PK-6120, Vector Laboratories (Canada) Inc, Burlington, ON) and DAB substrate. Sections were mounted on Superfrost® Plus (Fisher Scientific) slides, dehydrated in ascending concentrations (50, 70, 90, and 95%) of alcohol, cleared with xylene, and coverslipped in DPX.
mounting medium (Electron Microscopy Sciences, Hatfield, PA). Images were captured and digitized as above.

The mrTBI and GW3965 treatment protocol is summarized in Figure 2.4.

**Figure 2.4: Summary of mrTBI, Gw3965 treatment and post-injury outcomes.**
The post-TBI time points are relative to the second TBI. GW: GW3965, NOR: Novel object recognition, V: Vehicle (DMSO) used for dissolving GW3965 for single i.p. bolus, WT: Wild-type mice.

### 2.3.8 Statistical Analyses

All data are presented as mean ± SEM. For analysis of NOR data, because all mice equally explored two similar objects during the training phase (DI ~ 0), DI scores during training for animals of the same genotype were pooled. Similarly, DI scores during testing were pooled for sham mice within each genotype. DI scores were compared
using two-way ANOVA followed by a Bonferroni post-hoc test. For rotarod analysis, baseline latencies of mice from all time points within genotype and treatment groups were pooled. Rotarod data were analyzed for time and treatment as well as time and genotype effects using two-way repeated measures ANOVA followed by a Bonferroni post-hoc test, as animals were tested repeatedly until sacrifice. For biochemical and histological analyses, data from sham mice from all time points within each genotype were pooled. Biochemistry and silver stain data were analyzed for time and treatment as well as for time and genotype effects using two-way ANOVA followed by a Bonferroni post-hoc test. A $p$ value of < 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism (version 5.04, GraphPad Software Inc).

2.4 Results

2.4.1 ApoE is Required for GW3965 to Improve Cognitive Function After mrTBI

Non-spatial, working memory was assessed using the novel object recognition (NOR) task at 2, 7, and 14 days following mrTBI. Discrimination between the novel and familiar objects was determined using the discrimination index (DI) (Antunes and Biala, 2012). During training, all animals displayed an equivalent time exploring each of the two identical objects (DI ~ 0) (Figure 2.5) indicating that genotype, surgical procedures, treatment, and time did not affect baseline exploratory behavior. Sham-operated WT (Figure 2.5 A, gray bar, $p < 0.001$ compared to training, two-way ANOVA) and apoE-/- (Figure 2.5 B, gray bar, $p < 0.01$ compared to training, two-way ANOVA) mice retained preference to novelty as indicated by positive DI values. Untreated WT (Figure 2.5 A, open bars) and apoE-/- (Figure 2.5 B, open bars) failed to discriminate between the
novel and familiar objects ($p > 0.05$ compared to training, two-way ANOVA) at all post-mrTBI time points. Although GW3965-treated WT mice showed impaired memory at 2d post-mrTBI (Figure 2.5 A, black bars, $p > 0.05$ compared to training, two-way ANOVA) object recognition was restored by day 7 and 14 post-mrTBI ($p < 0.001$ compared to training, two-way ANOVA). Finally, GW3965 treatment failed to improve NOR performance in apoE-/- mice at any post-mrTBI time points (Figure 2.5 B, black bars, $p > 0.05$ compared to training, two-way ANOVA). Gross motor impairment can affect NOR performance. To rule out this possibility, we assessed the spontaneous activity of the animal in the open field by comparing the total distance covered by the animal in the 5 min epochs of training and testing phases. We found that the total path length covered by sham and injured WT as well as apoE-/- mice were not significantly different indicating no gross motor impairment (Figure 2.6, $p > 0.05$, two-way ANOVA).
Figure 2.5: ApoE is required for GW3965 to improve NOR performance after mrTBI.

NOR memory was evaluated in untreated (V) and GW3965-treated (G) WT (A) and apoE -/- (B) mice at 2, 7, and 14 days post-mrTBI. Bars represent discrimination index (DI) scores. Cohorts were: sham (WT, n=23, pooled; apoE-/-, n=18, pooled) and 2 day (WT: untreated, n=18, GW3965-treated, n=15; apoE-/-: untreated, n=10, GW3965-treated, n=10), 7 day (WT: untreated, n=13, GW3965-treated, n=10; apoE-/-: untreated, n=11, GW3965-treated, n=11), and 14 day (WT: untreated, n=14, GW3965-treated,
n=14; apoE-/-: untreated, n=9, GW3965-treated, n=9) post-mrTBI. DI scores were analyzed using two-way ANOVA and Bonferroni post hoc test. **: $p<0.01$, ***: $p<0.001$, ###: $p<0.001$.

Figure 2.6: NOR performance was not affected by motor impairment.

To assess whether NOR performance was affected by motor impairment; the total path length (m) covered by WT and apoE-/- mice during testing and training was measured. (A, B), total path length covered by WT mice during training and testing, respectively. (C, D), total path length covered by apoE-/- mice during training and testing, respectively. The path lengths covered by WT and apoE-/- mice were not significantly different from sham animals during testing, indicating that NOR was not affected by motor impairment. *: $p<0.05$ and **: $p<0.01$. Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test. Legend: V- untreated mice, open bars, G- GW3965-treated mice, black bars.
2.4.2 Spontaneous Recovery of Motor Dysfunction After mrTBI is Unaffected by GW3965

We next assessed post-mrTBI fine motor functions with accelerating rotarod. mrTBI significantly impaired the motor performance of WT (Figure 2.7 A, $p < 0.05$, two-way repeated measures ANOVA) and apoE-/- (Figure 2.7 B, $p < 0.05$, two-way repeated measures ANOVA) mice at 1, 2, and 7 d post-injury as indicated by reduced failing latencies on an accelerating rotarod, compared to the baseline performance. By 14 d, however, motor performance had fully recovered in WT and apoE-/- animals (Figure 2.7 A and B, $p > 0.05$, two-way repeated measures ANOVA). GW3965 treatment of WT or apoE-/- mice did not significantly reduce the severity of motor impairment at any time point, nor did it accelerate the rate of spontaneous recovery in either genotype (Figure 2.7 A and B, curly brackets). In contrast to GW3965, loss of apoE-/- led to significantly more severe motor impairment compared to WT animals at 1 and 2 d post-mrTBI in both untreated and treated groups (Figure 2.7 C and D, $p < 0.05$, two-way repeated measures ANOVA).
Figure 2.7: mrTBI-induced motor impairment recovers spontaneously independent of GW3965 and apoE.

Motor performance of WT (n=38) and apoE-/- (n=30) mice was evaluated by the accelerating rotarod task (0 to 30 rpm in 210 s). (A) Rotarod latencies of untreated (V, open squares) and GW3965-treated (G, black filled squares) WT mice before and following mrTBI. (B) Rotarod latencies of untreated (V, open circles) and GW3965-treated (G, black filled circles) apoE-/- mice before and following mrTBI. Asterisks in A and B denote significant differences between baseline and post-mrTBI latencies within each group. For both genotypes, the treatment effect was not significant at any post-TBI time point (curly brackets). (C) Rotarod latencies of untreated WT (open squares) and apoE-/- (open circles) mice before and following mrTBI. (D) Rotarod latencies of GW3965-treated WT (black filled squares) and apoE-/- (black filled circles) mice before and following mrTBI. # and § in C and D denote significant differences between the latencies of WT and apoE-/- mice at the respective time points. Cohorts were: Untreated WT mice: (1 d=34, 2 d=34, 7 d=24; 14 d=14); GW3965-treated WT mice: (1 d=35, 2 d=35, 7 d=25, 14 d=14); untreated apoE-/- mice: (1 d=24, 2 d=24, 7 d=20, 14 d=9), and
GW3965-treated apoE/- mice: (1 d=27, 2 d=22, 7 d=20, 14 d=9). Data were analyzed using two-way repeated measures ANOVA followed by a Bonferroni post hoc test. *: \( p<0.05 \), **: \( p<0.01 \), ***: \( p<0.001 \), ****: \( p<0.0001 \), #: \( p<0.05 \), ##: \( p<0.01 \), §§: \( p<0.01 \).

### 2.4.3 GW3965 Prevents mrTBI-Induced Elevation of Endogenous Aβ Levels

We determined levels of soluble endogenous Aβ40 and Aβ42 species from ipsilateral half brains of WT and apoE/- mice at 2, 7, and 14 d after mrTBI. Compared to sham controls, injured WT mice showed a sustained ~1.5-fold increase in Aβ40 (Figure 2.8 A, open bars, \( p < 0.05 \), two-way ANOVA), and a transient ~1.7 fold increase in Aβ42 that returned to baseline by 14 d post-injury (Figure 2.8 B, open bars, \( p < 0.05 \), two-way ANOVA). In contrast to untreated controls, GW3965 suppressed the increase of both Aβ40 and Aβ42 in WT mice (Figure 2.8 A and B, black bars, \( p > 0.05 \), two-way ANOVA) as compared to the sham. Similarly, apoE/- mice exhibited a sustained ~1.5-fold increase in Aβ40 (Figure 2.8 C, open bars, \( p < 0.05 \), two-way ANOVA), and a transient ~1.4-fold increase in Aβ42 after mrTBI that returned to baseline by 14 d post-injury (Figure 2.8 D, open bars, \( p < 0.05 \), two-way ANOVA). Surprisingly, GW3965 effectively suppressed the increase in both Aβ40 and Aβ42 in apoE/- mice (Figure 2.8 C and D, black bars, \( p > 0.05 \), two-way ANOVA) as compared to the sham. The GW3965 treatment effect was significant in both, WT and apoE/- mice for Aβ40 (Figure 2.8 A and C, \( p < 0.05 \), two-way ANOVA), but only in WT mice for Aβ42 (Figure 2.8 B, \( p < 0.05 \), two-way ANOVA). Furthermore, compared to WT mice, loss of apoE did not lead to significantly greater accumulation of Aβ species, with the single exception of Aβ40 levels 7 d post-mrTBI in both vehicle (Figure 2.8 A and C, #, \( p < 0.05 \), two-way ANOVA) and GW3965-treated groups (Figure 2.8 A and C, §, \( p < 0.05 \), two-way ANOVA). APP
holoprotein and APP-CTFα levels remained unchanged in both untreated and treated WT and apoE-/- mice following mrTBI (Figure 2.9, $p > 0.05$, two-way ANOVA), indicating that increased APP processing does not account for the changes in Aβ levels.
**Figure 2.8: GW3965 prevents mrTBI-induced accumulation of endogenous Aβ in WT and apoE-/- mice.**

Total, soluble murine Aβ40 and Aβ42 levels were measured from ipsilateral half brains of WT (A, B) and apoE-/- (C, D) mice, respectively, at 2, 7, and 14 d post-mrTBI. Data from sham animals within each genotype were pooled (grey bars). Numbers inside the bars indicate sample size. Asterisks above individual bars indicate significant difference compared to the respective sham levels. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. # indicates a significant difference ($p < 0.05$) between Aβ40 levels of untreated WT and apoE-/- brains at 7 d post-mrTBI. § indicates a significant difference ($p < 0.05$) between Aβ40 levels of GW3965-treated WT and apoE-/- brains at 7 d post-mrTBI. Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test.
Legend: S: sham-operated mice, gray bars, V: untreated mice, open bars, G: GW3965-treated mice, black bars.

Figure 2.9: APP and APP-CTFα levels remain unchanged following mrTBI.

APP holoprotein (A, C) and APP-CTFα (B, D) protein levels in ipsilateral half brains were analyzed by Western blotting, with representative blots shown for WT (A, B) and apoE/- (C, D) mice. Data are expressed as fold difference relative to sham values. Data from sham animals within each genotype were pooled (grey bars). Numbers inside bars
indicate sample size. Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test. Legend: S: sham-operated mice, gray bars, V: untreated mice, open bars, G: GW3965-treated mice, black bars.

2.4.4 GW3965 Enhances ABCA1 Induction After mrTBI:

To determine whether lipid mobilization pathways are induced in our mrTBI model, we determined the levels of ABCA1, apoE, and LDLR in treated and untreated WT and apoE-/- mice. Compared to sham controls, untreated WT brains showed a transient 1.6-fold increase in ABCA1 levels on post-mrTBI day 7 (Figure 2.10 A, open bars, *p* < 0.01, two-way ANOVA) that returned to baseline by 14 d. This finding is consistent to the findings reported in a FP injury model (Cartagena et al., 2008) and suggests that endogenous LXR agonists may be transiently induced after TBI. As ABCA1 is a sensitive LXR target, GW3965 augmented this response, leading to a 1.5-2.4-fold increase in ABCA1 protein levels over the time points examined (Figure 2.10 A, black bars, *p* < 0.05, two-way ANOVA). These results are consistent with our previous observations of ABCA1 upregulation in GW3965-treated APP/PS1 mice (Donkin et al., 2010). In contrast to WT animals, untreated apoE-/- mice did not show a transient elevation of ABCA1 after mrTBI (Figure 2.10 B, open bars, *p* > 0.05, two-way ANOVA), suggesting that the endogenous signals that lead to ABCA1 induction after TBI may be compromised in the absence of apoE. Nevertheless, similar to the WT mice GW3965 treatment significantly increased ABCA1 levels in injured apoE-/- mice (Figure 2.10 B, black bars, *p* < 0.05, two-way ANOVA), suggesting that GW3965 may bypass apoE and directly increase ABCA1 expression after mrTBI. Neither mrTBI nor GW3965 treatment led to significant changes in apoE levels in WT mice (Figure 2.11 A, *p* > 0.05, two-way
ANOVA), a result consistent with the relative insensitivity of apoE compared to ABCA1 as a LXR target (Koldamova et al., 2005b; Zelcer et al., 2007; Donkin et al., 2010). Similar to apoE, LDLR levels remained unaffected after mrTBI in both treated and untreated WT (Figure 2.11 B) and apoE-/-(Figure 2.11 C) mice ($p > 0.05$, two-way ANOVA).

**Figure 2.10: GW3965 augments ABCA1 levels in WT and apoE-/− mice following mrTBI.** ABCA1 protein levels were determined in ipsilateral half brains of WT (A) and apoE-/− (B) mice following mrTBI using Western blots, with representative blots shown below the graphs. Data are expressed as fold difference normalized to sham values. Data from sham animals within each genotype were pooled (grey bars). Numbers inside the bars indicate sample size. Asterisks above individual bars indicate significant difference compared to the respective sham levels. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$. Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test. Legend: S: sham V: untreated mice, open bars, G: GW3965-treated mice, black bars.
Levels of apoE and LDLR protein in WT mice (A, B) and LDLR protein in apoE-/- mice (C) were determined in ipsilateral half brains following mrTBI using Western blotting, with representative blots shown on the right. Data are expressed as fold difference normalized to sham values. Data from sham animals within each genotype were pooled. Numbers inside bars indicate sample size. Data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. Legend: S: sham-operated mice, gray bars, V: untreated mice, G: GW3965-treated mice.

Figure 2.11: ApoE and LDLR levels are unaffected by mrTBI or GW3965.
2.4.5 ApoE is Required for GW3965-Mediated Suppression of Axonal Damage After mrTBI

Histological assessment of axonal damage using silver staining revealed argyrophilic structures in cell bodies and axons in several ipsilateral white matter regions, including the corpus callosum, cingulum, external and internal capsules, and optic tracts, with strikingly greater accumulation observed in apoE-/- mice (Figures 2.12, 2.13, and 2.14 arrows). Notably, intense argyrophilia was observed in the bilateral optic tracts of WT and apoE-/- mice, suggesting the involvement of strong coup-contrecoup forces in our model. In comparison, very mild silver staining was observed in grey matter regions including the sensory-motor cortex directly under the impact site as well as the piriform cortex, farthest from the impact site (not shown).
Figure 2.12: Loss of apoE exacerbates axonal injury after mrTBI.
Axonal damage following mrTBI was assessed with silver staining (arrows). The left panel depicts representative images of silver-stained coronal sections at approximately -1.58 mm from bregma (Paxinos and Franklin, 2001) of untreated WT (A) and apoE-/- (B) mouse brains harvested at 7 d post-mrTBI. White matter areas with prominent silver staining are indicated by the black squares. Injury location is indicated by the arrowhead. The right panel (C-L) depicts representative 40x-magnified images of silver staining in five white matter areas in the brains of untreated WT (C-G) and apoE-/- (H-L) mice harvested at 7 d post-mrTBI.
Figure 2.13: mrTBI leads to mild axonal damage in WT mice.

Axonal damage following mrTBI was assessed with silver staining. The far left column depicts representative images of silver-stained coronal sections at approximately -1.82 mm from bregma (Paxinos and Franklin, 2001) of sham (A), untreated (B), and GW3965-treated (C) WT brains harvested at 2 d post-mrTBI. Locations of the white matter areas where silver staining was most prominent are indicated by black squares. Injury location is indicated by the arrowhead. Columns on the right depict representative 40X-magnified images of silver staining in five white matter areas in sham-operated (D-H), untreated TBI (I-M), and GW3965-treated (N-R) brains of WT mice harvested at 2 d post-mrTBI.
Figure 2.14: Loss of apoE exacerbates axonal damage after mrTBI. Axonal damage following mrTBI was assessed with silver staining (arrows). The far left column depicts representative images of silver-stained coronal sections at approximately -1.82 mm from bregma (Paxinos and Franklin, 2001) of sham (A), untreated (B), and GW3965-treated (C) apoE-/- brains harvested at 7 d post-mrTBI. Locations of the white matter areas where silver staining was most prominent are indicated by black squares. Injury location is indicated by the arrowhead. The columns on the right depict representative 40X-magnified images of silver staining in five white matter areas in sham-operated (D-H), untreated (I-M), and GW3965-treated (N-R) brains of apoE-/- mice harvested at 7 d post-mrTBI.
In untreated WT mice, axonal damage was significantly elevated in the corpus callosum, cingulum, external capsule, and internal capsule at 2 d post mrTBI as revealed by the semiquantitative analysis of silver-stained images (Figure 2.15 A-D, open bars, $p < 0.05$, two-way ANOVA). In this group, silver staining intensity returned to baseline by 7 d, suggesting that endogenous neuronal repair pathways were efficiently activated in these regions. More robust and sustained axonal damage was observed in the optic tracts of untreated WT mice (Figure 2.15 E, open bars, $p < 0.05$, two-way ANOVA). In WT mice, GW3965 effectively suppressed axonal damage in all regions examined (Figure 2.15 A-E, black bars, $p < 0.05$, two-way ANOVA). Untreated apoE-/- mice showed extensive axonal damage that peaked at 7 d post-injury and remained elevated at 14 d in the corpus callosum, external and internal capsules, and optic tract (Figure 2.15 F-J, open bars, $p < 0.05$, two-way ANOVA). In contrast to WT mice, GW3965 failed to suppress axonal damage in apoE-/- animals (Figure 2.15 F-J, black bars, $p < 0.05$, two-way ANOVA). In all regions examined, axonal damage was significantly more extensive in apoE-/- mice compared to WT controls (Figure 2.15 F-J, $p < 0.05$, two-way ANOVA; # and § denote comparison of WT and apoE-/- mice within untreated and treated groups, respectively).
Figure 2.15: ApoE is required for GW3965 to suppress axonal damage after mTBI. Silver-stained images of white matter areas in WT and apoE-/- brains were analyzed semiquantitatively using an arbitrary silver staining scale extending from 0 (<10% argyrophilic structures covering the image field) to 3 (>70% argyrophilic structures covering the image field). The bar graphs represent mean ± SEM silver stain intensity score (arbitrary value) of WT (A-E) and apoE-/- (F-J) brains in the corpus callosum (A,
2.4.6 Weight Drop TBI Model Produces Negligible Neuroinflammation

To investigate the extent to which our mrTBI model induces inflammation, we examined post-injury microglial activation using Iba1 immunohistochemistry and measured IL-6, TNFα, and MCP-1 levels in ipsilateral half brains. Examination of coronal sections revealed uniform distribution of Iba1-immunoreactive microglia with negligible changes in the immunoreactivity in the injured WT and apoE-/- mice in either the ipsilateral cortex (Figure 2.16) or hippocampus (Figure 2.17) compared to sham controls. Further, GW3965 treatment did not reduce Iba1 staining below baseline (Figure 2.16 and 2.17).
Figure 2.16: mrTBI does not induce microglial activation in the cortex.

Microglial activation in sham and injured WT and apoE/- mice was assessed with Iba1 immunohistochemistry. The top panel depicts representative images of Iba1-stained coronal sections at approximately -1.82 mm from bregma (Paxinos and Franklin, 2001). The bottom panel depicts 10X-magnified images of ipsilateral cortex underlying the injury site (indicated by the black rectangle). Legend: V- untreated mice, G- GW3965-treated mice.
**Figure 2.17: mrTBI induces negligible hippocampal microglial activation.**

Microglial activation in sham and injured WT and apoE/- hippocampi was assessed with Iba1 immunohistochemistry. The top panel depicts representative images of Iba1-stained coronal sections at approximately -1.82 mm from bregma (Paxinos and Franklin, 2001). The bottom panel depicts 10X-magnified images ipsilateral
Although no animal in this study had any obvious skull fracture, approximately 4-8% of mice showed evidence of microcontusions in the cortex directly under the impact site (Figure 2.18, arrows). Increased Iba1 immunoreactivity was clearly associated with these localized areas of more severe injury (Figure 2.18).

Figure 2.18: Pronounced microglial activation is localized only around contused areas. In this study, approximately 4-8% of brains subjected to mrTBI showed micro contusions in the absence of gross skull fracture. The left panel shows a Iba1-stained coronal section at approximately -1.82 mm from bregma (Paxinos and Franklin, 2001) with a micro contusion in the cortex below impact site (black square). The right panel shows representative 10X-magnified images of Iba1-stained untreated WT and apoE-/- contused cortices. Microcontusions are denoted by black arrows. Note the pronounced localized activation of microglia around the contusion.

Consistent with this model inducing negligible microglial activation, IL-6 levels in the ipsilateral brains were unchanged in both WT and apoE-/- mice, with or without
GW3965 (Figure 2.19, p > 0.05, two-way ANOVA). TNFα and MCP-1 levels were below the detection limit (not shown).

Figure 2.19: Pronounced microglial activation is localized only around contused areas. Interleukin-6 (IL-6) levels were measured from ipsilateral half brains of WT and apoE-/- mice harvested at 2, 7, and 14 d post-mrTBI. Data from sham animals within each genotype were pooled. Cohorts were: sham (WT, n=9, pooled; apoE-/-, n=10, pooled), 2 day (WT: untreated, n=5, GW3965-treated, n=5; apoE-/-: untreated, n=5, GW3965-treated, n=5), 7 day (WT: untreated, n=4, GW3965-treated, n=4; apoE-/-: untreated, n=6, GW3965-treated, n=6), and 14 day (WT: untreated, n=5, GW3965-treated, n=5; apoE-/-: untreated, n=5, GW3965-treated, n=5) post-mrTBI. Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test. Legend: Sham WT: open bars, sham apoE-/-: grey bars, vehicle-treated WT: open striped bars, vehicle-treated apoE-/-: grey striped bars, GW3965-treated WT: open stippled bars, GW3965-treated apoE-/-: grey stippled bars.

2.4.7 Retrospective Power Analysis

In this study we did not perform prospective power calculations to determine the sample size. A retrospective power analysis was thus carried out based on the collected data.
using an online post-hoc power calculator (http://clincalc.com/stats/power.aspx). For power calculations it was assumed that the post-TBI outcomes in apoE-/- mice will be worse compared to those in the WT mice, hence any significant change in the WT mice will likely indicate similar or more change in apoE-/- mice. In this study we there were four factors that could influence the study outcome: genotype, injury status, treatment and time point. Of these we hypothesized that the smallest effect will be of TBI and TBI x treatment interaction in WT mice and thus a significant difference for these interactions will likely indicate significant results for the other group comparisons. Based on these assumptions the retrospective power analysis was carried for TBI effect and treatment x TBI effect at 7 d time point.

The retrospective power analysis revealed that the study was adequately powered to detect TBI effect in WT mice and was underpowered to detect treatment x TBI effect although all the observed changes were statistically significant.

The results of power calculations for various post-TBI outcomes are summarized in the Table 2.3.

<table>
<thead>
<tr>
<th>Post-TBI Outcome</th>
<th>Time Point</th>
<th>TBI Effect</th>
<th>Treatment x TBI Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed Change (%)</td>
<td>N</td>
<td>Power</td>
</tr>
<tr>
<td>NOR 7d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.3: Summary of retrospective power calculations

The table summarizes results of retrospective power calculations for various post-TBI outcomes that were assessed in the present study. All the observed changes reported in this table were statistically significant.

2.5 Discussion

The goal of this study was to evaluate the ability of GW3965 to promote recovery in a model of mrTBI specifically designed to mimic repeated concussion. We found that therapeutic administration of GW3965 improved NOR performance, suppressed Aβ accumulation, and reduced axonal damage after mrTBI. Loss of apoE exacerbated the severity of motor impairment and axonal damage and eliminated the ability of GW3965 to restore NOR performance and to promote axonal recovery. These results are consistent with the role of apoE in neuronal repair and synaptic restoration. ApoE levels did not change after TBI or after GW3965 treatment, which suggests that injury severity
was not sufficient to elevate apoE as well as reflects the poor sensitivity of apoE as an LXR target compared to ABCA1 (Donkin et al., 2010). However, it is possible that apoE may show localized upregulation in regions with more severe damage where microglial activation is pronounced. Moreover, it is also possible that GW3965-mediated lipidation of the available apoE mass may have been sufficient to promote post-TBI recovery in our model. Although we did not assess post-TBI lipidation status of apoE with or without GW3965 treatment in the present study, future studies will test whether ABCA1-mediated lipidation of apoE contributes to the beneficial effects of GW3965 after mrTBI.

We used a commonly-used weight drop model to induce CHI in mice. The rationale for using this model was less invasive surgical manipulations including craniotomy that is required for FPI and CCI models, and relative simplicity compared to FPI and CCI model, and high throughput. In addition, being a CHI model, it is clinically more relevant as about 90% of human TBI do not involve penetration of dura. Using the weight drop device we established a TBI protocol wherein every mouse received two CHI spaced at 24 h. In our initial pilot studies, we found that for mice weighing 25 to 32 g, a drop height (in cm) equal to the one third the body weight (in g) resulted in mild TBI with low (<10%) incidence of skull fracture.

Surprisingly, apoE was not required for GW3965 to suppress the transient increase in Aβ levels induced in our model. Further studies will be required to characterize these apoE-independent pathways that promote Aβ clearance after TBI. This will be an important endeavor, as axonal APP accumulation is a hallmark of TBI (Gentleman et al.,
Theoretically, the Aβ produced after TBI could trigger Aβ-dependent toxic pathways that exacerbate damage (Johnson et al., 2010). In support of this γ-secretase inhibitors, which block Aβ production, reduce cognitive and pathological changes following CCI in mice (Loane et al., 2009). However, the relationship between Aβ levels and TBI recovery is complex. In WT and AD mice, PBS-soluble Aβ levels in brain ISF microdialysates decrease by up to 50% within 90 min after CCI with impact depths ranging from 1-2 mm (Schwetye et al., 2010). Intriguingly, Schwetye et al. (2010) found no evidence of transient increases in Aβ levels after CCI, which differs from our findings and those from other investigators using relatively crude tissue lysates (see references in the Schwetye et al. (Schwetye et al., 2010) paper/discussion). Intra-axonal Aβ accumulation is observed in 3XTg-AD and APP/PS1 mice from 1-24 h after moderate-severe CCI (Tran et al., 2011b; Tran et al., 2011c), again suggesting that distinct pools of Aβ may differ in their responses after injury. Future studies are needed to clarify the factors that influence Aβ dynamics after TBI, including apoE.

Although our mrTBI model clearly leads to behavioral deficits and axonal damage, it is not severe enough to trigger significant inflammation except in localized regions near microcontusions. Importantly, inclusion or exclusion of mice with microcontusions did not influence the group outcomes for NOR, rotarod, and silver stain data. These results suggest that Iba1-positive staining of reactive microglia may be a potent secondary effect of structural brain damage, although it is possible that the time points examined in this study may have missed a peak of more global inflammation in our model.
Interest in understanding the pathophysiology of mild closed-head TBI has led to the development of various impact and blast models. However, there is considerable variation in outcomes. For example, many groups have reported mild cognitive deficits without motor impairment in weight drop-based mild TBI mouse models (Tang et al., 1997; DeFord et al., 2002; Pan et al., 2003; Creeley et al., 2004; Zohar et al., 2011). In contrast, the Shohami group observed motor deficits in both severe and mild TBI, but found that severe injury was required for cognitive impairment (Tsenter et al., 2008).

Using an electromagnetically-controlled piston to produce mrTBI, Shitaka et al observed cognitive deficits, white matter damage, and robust microglial activation without significant structural damage or APP immunoreactivity (Shitaka et al., 2011). Our mrTBI model also shows sustained cognitive and transient motor deficits, axonal damage, and transient Aβ accumulation. The most noteworthy difference between our model and that of Shitaka et al is that we observed very little microglial activation, suggesting that marked inflammation is not required to lead to behavioral and axonal changes. Our model also does not lead to significant increases in apoE or LDLR levels, which have been observed in models of moderate-severe TBI (Poirier, 1994; Iwata et al., 2005; Petit-Turcotte et al., 2007; Loane et al., 2011).

We and others have demonstrated that synthetic LXR agonists effectively enhance the ability of lipidated apoE to reduce Aβ levels and restore cognitive function in AD mice (Riddell et al., 2007; Donkin et al., 2010). Given that TBI also involves altered apoE and Aβ metabolism, it is of considerable interest to determine whether LXR agonists also have potential therapeutic benefits for TBI. The importance of addressing this question...
is two-fold. First, LXR treatment may minimize neuronal damage and promote acute recovery by reducing inflammation and promoting neuronal repair. Second, by facilitating Aβ clearance in the first weeks after injury, LXR treatment may also reduce the increased long-term risk of AD years or decades later. Although current LXR agonists have safety issues such as hypertriglyceridemia and hepatic steatosis that preclude their present clinical use (Grefhorst et al., 2002; Groot et al., 2005), ongoing drug discovery efforts may lead to a safe and effective compound. Furthermore, the metabolic risks of LXR agonists may be clinically tolerable if short-term treatment could improve functional recovery as well as decrease long-term AD risk. Building on the findings of Loane and colleagues (Loane et al., 2011), our study provides additional support for the potential of LXR agonists to treat mrTBI.

Our study also illustrates that the mechanisms by which LXR agonists promote recovery after TBI may not directly correspond to their effects in AD models. For example, outcomes such as cognitive recovery and axonal damage are apoE-dependent, but surprisingly, Aβ clearance is independent of apoE in our model. Additionally, because our model produces negligible inflammation, the beneficial effects of LXR agonists on TBI recovery may be independent from their established anti-inflammatory effects. Future investigations will be designed to identify the LXR targets operative in our mrTBI model, assess their efficacy to reduce tau pathology, and evaluate their therapeutic utility in models of moderate-severe TBI.
One caveat of this study was the lack of prospective power analysis to determine adequate sample size. We therefore performed retrospective power calculations and found that the study was adequately powered to capture TBI effect. It should however be noted that the use retrospective power calculations is controversial (Thomas, 1997). For example, retrospective power analysis is generally carried out only for the negative study results and as such will produce a low post-hoc power result. This may be misinterpreted as the study having inadequate power, which is reflected by the low power observed in the present study to detect TBI x treatment effect, although the interaction was significant. Based on the results of the present studies future studies will be designed to include prospective power analysis.

A caveat of the weight drop TBI model used in this study is the absence of a systematic biomechanical assessment of injury. Although input parameters such as weight mass and drop height are reported in most CHI studies including ours, the reproducibility of these mechanical inputs and the response of the animal's head to the forces applied are not well understood. Li and colleagues recently studied the biomechanical parameters of the weight-drop based Marmarou rat TBI model (Marmarou et al., 1994) and found that DAI severity was related to the linear and angular response of the rat head but not with the drop height (Li et al., 2011). The importance of free head acceleration after application of mechanical forces was further underlined by a recent study by Goldstein and colleagues who demonstrated that a single blast injury in mice can generate significantly impaired memory performance, long-term potentiation, and axonal conductance accompanied by tau pathology, myelinated axonopathy,
microvasculopathy, and neuroinflammation (Goldstein et al., 2012). Intriguingly, immobilizing the animal’s head to prevent blast-induced head oscillations prevented memory deficits (Goldstein et al., 2012). It is possible that the very mild pathology in our model is due to relatively little head movement after impact.

The caveats associated with the weight drop model inspired us to develop a better TBI model which can allow unrestricted head movement and integrate biomechanics with TBI outcomes. In the next chapter of this thesis I have described the novel rodent neurotrauma model called CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) that we developed in collaboration with the biomechanical engineers at the International Collaboration on Repair Disorders (ICORD), The University of British Columbia.
3.1 Summary

Traumatic brain injury (TBI) is a major health care concern that currently lacks any effective treatment. Despite promising outcomes from many preclinical studies, clinical evaluations have failed to identify effective pharmacological therapies, suggesting that the translational potential of preclinical models may require improvement. Rodents continue to be the most widely used species for preclinical TBI research. As most human TBIs result from impact to an intact skull, closed head injury (CHI) models are highly relevant, however, traditional CHI models suffer from extensive experimental variability that may be due to poor control over biomechanical inputs. Here we describe a novel CHI model called CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) that fully integrates biomechanical, behavioral, and neuropathological analyses. CHIMERA is distinct from existing neurotrauma model systems in that it uses a completely non-surgical procedure to precisely deliver impacts of prescribed dynamic characteristics to a closed skull while enabling kinematic analysis of unconstrained head movement. In this study we characterized head kinematics as well as functional, neuropathological, and biochemical outcomes up to 14d following repeated TBI (rTBI) in adult C57BL/6 mice using CHIMERA. Head kinematic analysis showed excellent repeatability over two closed head impacts separated at 24h. Injured mice showed significantly prolonged loss of righting reflex and displayed neurological, motor, and cognitive deficits along with anxiety-like behavior. Repeated TBI led to
diffuse axonal injury with extensive microgliosis in white matter from 2-14d post-rTBI. Injured mouse brains also showed significantly increased levels of TNF-α and IL-1β and increased endogenous tau phosphorylation. Repeated TBI using CHIMERA mimics many of the functional and pathological characteristics of human TBI with a reliable biomechanical response of the head. This makes CHIMERA well suited to investigate the pathophysiology of TBI and for drug development programs.

3.2 Introduction

Traumatic brain injury (TBI) is a leading worldwide cause of death and disability for persons under 45 years of age with a cost to society of over 17 billion USD per year. In the United States, the overall incidence of TBI is estimated to be 538 per 100,000 persons, which represents at least 1.7 million new cases per year since 2003 (Gerberding and Binder, 2003; Langlois et al., 2006; Faul et al., 2010). TBI incidence is reportedly lower in Europe (235 per 100,000) and Australia (322 per 100,000) (Cassidy et al., 2004; Tagliaferri et al., 2006) although recent epidemiological data suggests far greater incidence (749 per 100,000) (Feigin et al., 2013). Mild TBI (mTBI), which includes concussion, comprises over 75% of all TBIs (Gerberding and Binder, 2003). The reported incidence of mTBI is rising and mTBI is increasingly recognized as an injury for which medical attention should be sought.

Falls are the most prevalent cause of TBI, although motor vehicle accidents and impacts against objects are also common causes (Cassidy et al., 2004; Faul et al., 2010; Roozenbeek et al., 2013). TBI resulting from high-contact sports such as boxing,
American football, ice hockey, soccer, and rugby account for almost 21% of all head injuries among children and adolescents, particularly for mTBI (Centers for Disease Control and Prevention, 2007). In these situations, the skull experiences an impact resulting in brain deformation and resulting injury that most often occurs without skull fracture. TBI is also considered a “signature injury” in modern warfare, as approximately 20% of veterans from the Iraq or Afghanistan wars are reported to have experienced a TBI, 80% of which involve both blunt impact and overpressure mechanisms (Taber et al., 2006; Hoge et al., 2008; Elder and Cristian, 2009; Sevagan et al., 2013).

Furthermore, the growing awareness that mTBI may have long-lasting and severe consequences (Gavett et al., 2011; Jordan, 2013; Rusnak, 2013; Smith et al., 2013) highlights the urgency to understand much more about the acute and long-term consequences of brain injury.

The rapid accelerations and rotations during impact TBI lead to vigorous movement and deformation of brain tissue within the skull that can result in contusions as the brain contacts the interior of the bony skull. These inertial and contact forces directly affect neurons, glia, and blood vessels producing a primary injury that initiates secondary processes within hours to weeks after the initial injury (McIntosh et al., 1996; Blumbergs, 1997; Davis, 2000; Giza and Hovda, 2001; Werner and Engelhard, 2007; McAllister, 2011). These secondary changes lead to a plethora of events including edema, raised ICP, impaired CBF, increased BBB permeability, inflammation, axonal injury, calcium influx, elevated oxidative stress, free radical-mediated damage, excitatory neurotransmitter release, and cell death (McIntosh et al., 1996; Blumbergs,
1997; Davis, 2000; Giza and Hovda, 2001; Werner and Engelhard, 2007; McAllister, 2011). Although very few treatment options are available for the primary injury, secondary injury pathways are potentially modifiable (Graham et al., 2000). An increasingly wide variety of experimental animal models are therefore being developed to characterize secondary injury processes and for the evaluation of candidate therapeutic approaches.

No single animal model can replicate the entire spectrum of human TBI pathophysiology; therefore, several large and small animal models have been developed to mimic particular aspects. Popular rodent models include OHI models including FP and CCI systems, and CHI models that use either gravity or mechanical methods to impact the intact skull (reviewed in Chapter 1). Although FP and CCI models employ highly reproducible mechanical inputs and can mimic many pathological features of human TBI, the prominent tissue destruction and lack of head movement decreases the resemblance to the majority of human injuries that occur due to impact and/or acceleration on an intact skull. In contrast, CHI models employ methods that do not generally cause overt brain tissue loss and can also allow rapid behavioral assessment of injury severity. As such, CHI models are considered by some to better mimic the majority of human TBI. However, a major limitation of most current CHI animal models is that the input parameters used to induce injury (e.g., mechanical loading, method of mechanical input, response of the animal’s head to mechanical loading) are often poorly controlled and this can lead to considerable experimental
variation across cognitive, histological, and biochemical outcome measures (Namjoshi et al., 2013a).

Here we report a novel neurotrauma model called CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration). CHIMERA was developed to address the absence of a simple and reliable model of rodent CHI that mimics the majority of human TBI cases. CHIMERA is distinct from existing neurotrauma model systems in that it is completely surgery-free and fully integrates biomechanical, behavioral, and neuropathological analyses after delivering impacts of defined energy to a closed skull with unconstrained head motion after impact. We show that that repeated TBI (rTBI) in mice using CHIMERA reliably induces motor deficits and anxiety-like behavior and leads to diffuse axonal injury (DAI) with extensive white matter inflammation and increased phosphorylation of endogenous tau.

3.3 Materials and Methods

3.3.1 CHIMERA Impactor

The CHIMERA impactor consists of an aluminum frame that supports an animal holding platform above a pneumatic system platform (Figure 3.1 A).

3.3.1.1 Animal Holding Platform

The animal holding platform is composed of a fixed head plate that supports the animal's head in a supine position and a body plate that positions and secures the animal's torso. The head plate has a hole through which the tip of the impactor piston
projects to impact the animal’s head. A cushion made from closed-cell foam surrounds the hole to minimize rebound impact when the animal’s head falls back on the head plate following impacted by the piston. Two perpendicular lines across the piston hole act as crosshairs for aligning the animal’s head over the hole. The body plate holds a restraint system consisting of an animal bed of closed-cell foam contoured to the shape of the animal’s body and two Velcro straps. The animal holding platform is attached to the frame by hinges and its angle of inclination can be adjusted. In this study, the angle was set to approximately 32° such that the frontal and parietal bones laid flat over the hole in the head plate, thus delivering impact to the dorsal cortical region.

3.3.1.2 Pneumatic Impactor System

The pneumatic impactor system includes a 1.9 L accumulator air tank (Model F9124, Firestone Industrial Products, Indianapolis, IN), pressure regulator (Model 700-BF, ControlAir Inc., Amherst, NH), a digital pressure gauge (Model DPG409-150G, Omega, Laval, QC), a two-way solenoid valve (Model 21HN2KY110, Granzow Inc., Charlotte, NC), and a trigger button. The pressure regulator and digital pressure gauge allow precise adjustment of air pressure with minimum increments of 0.1 psi (0.69 kPa), enabling accurate delivery of piston velocity and impact energy (Figure 3.2).

3.3.1.3 Impact Piston and Barrel System

Impact is induced with a free-floating metal impact piston with a body diameter of 15 mm and a tip diameter of 5 mm whose trajectory is constrained to linear motion by a stainless steel barrel (internal diameter: 16 mm, external diameter: 20 mm). Four
different masses of impact pistons are available with the current system: 50 g, 75 g and 100 g pistons machined out of a chrome-coated steel rod, and a 25 g piston machined out of an aluminum rod, to allow impact velocity or impact energy to be independently adjusted (Figure 3.1 B). Only the piston tip (length: 2 cm, diameter: 0.5 cm) extrudes from the barrel. The barrel design includes a series of holes drilled along the length to vent air as the piston moves towards the open end. The piston is accelerated by a controlled pulse of compressed air along the length of the barrel until it clears the venting holes. The barrel can be mounted in either a vertical or horizontal configuration relative to the animal holding platform. In the vertical configuration, the barrel is located below the supine animal's head such that the impact cylinder strikes the head to initiate flexion. In this configuration the animal holding platform can be inclined relative to the head plate such that the frontal and parietal bones lie flat over the hole in the head plate, thus delivering impact to the dorsal cortical region. Alternatively, the animal holding platform can be adjusted to lie parallel to the head plate, such that the impact is directed towards the interparietal and occipital bones. In the lateral configuration the barrel is mounted orthogonally relative to the head plate and the body is plate is inclined to keep the head flat over the hole. In this configuration, the piston travels horizontally to impact the left side of the head and initiate lateral and twisting head movements.
Figure 3.1: CHIMERA impactor and impact pistons.
(A) CHIMERA with various parts labeled as follows: 1. head plate, 2. body plate, 3. animal bed, 4. Velcro straps, 5. air tank, 6. air pressure regulator, 7. digital pressure gauge, 8. two-way solenoid valve, 9 vertical piston barrel. B) Impact pistons. CHIMERA impactor can use impact pistons of four different masses, a 25 g piston constructed of aluminum and 50 g, 75 g and 100 g pistons constructed of chrome-coated steel.

3.3.1.4 CHIMERA Calibration

The impactor was first calibrated by determining the air pressure-piston velocity relationship by measuring the exit velocity of the piston at various air pressures (0.5, 1, 1.5, 2, 3, 5, 7, 10, 15, 20, 30, 50, 70, 100, and 130 psi). The velocity was measured in triplicate at each pressure value for all four piston masses in both horizontal and vertical orientations. Each impact event was recorded by a high-speed video camera at 10,000 fps. Video motion analysis software (TEMA Motion, Image Systems AB, Sweden) was used to track the trajectory of a specific point on the impact piston. The calibration curves for all four piston masses showed an inflection point between 10 and 15 psi such
that they were concave upwards before this point and concave downwards after the point. The cause of this is unknown, but it is believed to be due to the interaction between friction, drag, and piston velocity. Because of this effect, two 2nd-order polynomial curves were used to fit the data. An $R^2$ value of $>0.995$ was found for all curves. A representative calibration curve of impact energy for the 50 g piston at air pressure values between 0 and 12 psi is shown in the Figure 3.2. Using these curves, the desired impact velocity or energy can be independently interpolated. By choosing the appropriate combination of piston mass and air pressure, impacts of input energy ranging from 0.01 J to 18 J can be precisely generated.

![Figure 3.2: Piston energy-air pressure calibration curve.](image)

Air pressure-energy calibration curve was obtained by driving a 50 g piston at increasing air pressure values and calculating the resultant impact energy. The graph depicts energy measurements in triplicates for each air pressure value.

All experiments reported in the present Chapter were performed using a 50 g piston and a vertically configured barrel. We initially performed a pilot experiment using three
impact energies (0.4, 0.5 and 0.6 J) to determine the maximum allowable impact energy without leading to animal mortality. In the experiments described in this Chapter we used impact energy of 0.5 J with the corresponding piston velocity of 4.5 m/s.

3.3.2 CHIMERA Repetitive TBI (rTBI) Procedure

All animal procedures were approved by the University of British Columbia Committee on Animal Care (protocol # A11-0225) and were carried out in strict accordance with the Canadian Council on Animal Care guidelines. Male C57Bl/6 mice (mean ± SD body weight 34 ± 4 g) at 4-5 months of age were housed with a reversed 12 h light-12 h dark cycle for at least 10 days and were subjected to repetitive TBI (rTBI) using CHIMERA impactor as follows.

The animal was first anesthetized in an anesthesia induction chamber with 5% isoflurane in oxygen (0.9 L/min). Once the animal lost righting reflex, it was quickly transferred to the CHIMERA device and kept in a prone position on the animal bed. The anesthesia was maintained with 2.5-3% isoflurane. Lubricating eye ointment was applied to prevent corneal drying. Meloxicam (1 mg/kg) and saline (1 mL/100 g body weight) were administered by subcutaneous injections for pain control and hydration, respectively. The animal was then reoriented in a supine position in the animal bed. The body plate was inclined such that the top of the animal’s head laid flat over the piston hole in the head plate and the head was aligned using crosshairs such that the piston struck the vertex of the head covering a 5 mm area surrounding the bregma (Figure 3.3 B and C). The animal’s body was secured with two Velcro straps (Figure 3.3 A).
Figure 3.3: Mouse head and impact position.

(A) Close-up view of animal strapped on the holding platform. (B) Location of impact relative to the mouse head and brain in sagittal plane. P: impact piston. (C) Estimated location of impact on the mouse skull (left) and brain (right) based on mouse brain atlas (Paxinos and Franklin, 2001). The area covered by the impactor tip is shown by black circle.

Impact was induced by pressing a trigger button that fires the piston and when connected, simultaneously activates a high-speed camera to record the resulting head trajectory. Isoflurane delivery was immediately stopped following the impact, the animal was transferred to recovery cage and continuously monitored until fully ambulatory. Twenty-four hours after the first impact, a second identical impact (including same drug regimen) was delivered. Sham animals underwent all of these procedures except for the
impact. Isoflurane exposure time was measured for each impact from the start of isoflurane delivery till isoflurane discontinuation. Approximately ~ 4 % of animals displayed hind limb paralysis that lasted for several hours after TBI and were euthanized. These animals were excluded from the data analysis. Sham-operated and rTBI mice were randomly assigned to one of three post-TBI assessment time points, viz., 2, 7, and 14 days after the second injury. The time of the 2\textsuperscript{nd} TBI was defined as time zero.

### 3.3.3 High-Speed Videography and Kinematic Analyses

For kinematic analysis, a cohort of 8 mice was subjected to rTBI and impact events were recorded at 5,000 frames per second using a high-speed video camera (Q-PRI, AOS Technologies, Switzerland). Head motion was tracked using two markers, one being non-toxic paint applied on lateral side of head to mark the cheek area. Because the skin is loose over the bony skull, we also marked the position of the maxilla by wrapping dental floss positioned just caudal to the upper incisors around the animal's snout (Figure 3.4). Videos were analyzed using ProAnalyst Professional motion analysis software (v 1.5.6.8, Xcitex Inc., Woburn, MA) as follows. The origin was set using the paint marker in the Cartesian coordinates (Figure 3.4 A). The X and Y coordinates of the position of each marker were tracked on a frame-by-frame basis and were processed with a 400-Hz low-pass Butterworth filter to mitigate the noise in the recorded measurements. Linear kinematic parameters were assessed from trajectories obtained by tracking the paint mark, which was closest to the CG of the animal's head. Velocities and accelerations were determined by discrete differentiation of the marker position.
data. Resultant linear velocity and acceleration were calculated as the magnitude of their respective X and Y components using the following equations.

\[
\text{Linear Head Displacement} = \sqrt{X_{pos}^2 + Y_{pos}^2}
\]

Where, \(X_{pos}\) and \(Y_{pos}\) represent X and Y positions of the paint mark from the origin, respectively.

\[
\text{Linear Head Velocity} = \sqrt{X_{vel}^2 + Y_{vel}^2}
\]

Where, \(X_{vel}\) and \(Y_{vel}\) represent velocities at X and Y positions, respectively.

\[
\text{Linear Head Acceleration} = \sqrt{X_{acc}^2 + Y_{acc}^2}
\]

Where, \(X_{acc}\) and \(Y_{acc}\) represent acceleration at X and Y positions, respectively.

Angular rotation of the head during TBI was determined by the angle of the line joining the dental floss and the paint mark with the horizon (Figure 3.4 B). Immediately after video capture, the dental floss was removed. The kinetic energy (KE) transferred from the piston to the head was determined by \(KE = 0.5 \times M_e \times \Delta V^2\) (Viano et al., 2012), where \(M_e\) is the effective mass and approximated by head mass measured from the 8 mice (3.4 g) and \(\Delta V\) is change in head velocity. A scaling factor \(\lambda\) \([\lambda = (\text{mass of human brain} / \text{mass of mouse brain})^{1/3} = 13.8]\) was used to estimate the human head-equivalent kinematic parameters from the animal data (Viano et al., 2009).
Figure 3.4: **External markers used for mouse head tracking.**

(A) Head movement was tracked using two markers: a dental floss (red arrow in the first image) wrapped around the maxilla and a non-toxic paint (yellow arrow in the first image) applied at the lateral size of the head. Head trajectories and linear kinematic parameters were obtained by tracking the paint (yellow arrow) marker. Two dots separated by 10 mm were marked on the CHIMERA frame and were used to calibrate the distance. (B) Angle of head deflection and angular kinematic parameters were obtained by determining the angle of the line joining the two tracking markers with the horizon.

### 3.3.4 Behavioral Analyses

Duration of loss of righting reflex (LRR) was calculated as the time interval from isoflurane discontinuation to the first sign of righting after each impact. Neurological impairment was assessed using the neurological severity score (NSS, Table 3.1) (Flierl et al., 2009) determined at 1 h and at 1, 2, and 7 d after the second TBI. Motor performance was evaluated at 1, 2, 7, and 14 d after the second TBI using an accelerating rotarod as described in Chapter 2. Open field activity was assessed at 1, 7
and 14 d after the second TBI using a Plexiglas box (14” x 24” x 14”). The floor of the box was virtually divided into 60 equal squares using an overhead digital camera and video tracking software (ANY-maze, v. 4.99, Stoelting Co, Wood Dale, IL). The field was further subdivided into a peripheral zone along the walls of the open field consisting of 28 squares that surrounded a central zone consisting of 32 squares (Figure 3.5). The animal was placed in the center of the box and spontaneous activity was recorded over a 10 min epoch, including quantification of the total distance traveled, number of line crossings, and immobile time. The thigmotaxis index (TI) was calculated as: $TI = (T_P - T_C)/(T_P + T_C)$ where $T_P$ and $T_C$ represent time spent in the peripheral and central zones, respectively. Working memory was assessed by the passive avoidance task from 7 to 10 days after second TBI. Passive avoidance testing was conducted in a device that consisted of two adjoining compartments, one illuminated (20.3 x 15.9 x 21.3 cm) and one darkened (20.3 x 15.9 x 21.3 cm), divided by a guillotine-style door (Med Associates Inc., St. Albans, VT). The floor of the compartments consisted of steel rods capable of delivering an electric foot-shock. The electric shock was delivered by a programmable animal shoker (Med Associates Inc.). Each session consisted of placing mice into the illuminated compartment and using a timer to record the latency of the mice to cross into the darkened compartment. On day 7 after the second TBI (training) mice received an electric foot-shock (0.3 mA, 2 s) as soon as they crossed from the illuminated into the darkened compartment. Following foot-shock, mice were removed from the apparatus and returned to their home cage. On days 8-10 after the second TBI mice were tested for memory retention. The latency for the mice to cross into the darkened compartment was recorded. No shock was delivered during testing. Mice that
did not cross over into the darkened compartment were allowed to remain in the illuminated compartment for the full 5 min and assigned a latency of 300 s. Non-working memory was assessed with the Barnes maze from 8-13 d post-rTBI. Barnes maze testing was conducted on a grey circular platform (91 cm diameter) with 20 circular holes (5 cm diameter; Stoelting Co) at its periphery located in a 3.0 x 3.6 m room. An escape box was positioned beneath one of the holes. Extra-maze visual cues consisted of black and white images (cross, lightning bolt, smiley face) printed on glossy white paper and placed on the walls surrounding the maze. Other visual cues included a cart with a laptop and the experimenter. For motivational purposes, mice were subjected to mild food deprivation (1 g of food per day) from days 8-13 after the second TBI and maintained at 90% of free feeding body weight. During testing mice were exposed to aversive stimuli in the form of two lights (100 W) positioned at either side of the maze and a buzzer (2.9 kHz, 65 dB) which hung 10 cm above the maze. On day 8 mice completed one 5-min habituation trial to become familiar with the maze environment and to practice descending into the escape box. On days 9-13 mice were tested for memory retention. Mice were given four trials per day (15-min inter-trial interval) for five days. For each trial the animal was placed in a black plastic start tube (7 cm diameter, 12 cm height) at the center of the maze. After 10 s, the start tube was raised and the buzzer was turned on to start the trial. Mice that were unable to locate the escape hole in 90 s were gently guided to it. Mice remained in the escape box for 10 s before being returned to their home cage. Mice were tracked during each trial using a digital camera placed 4 m above the center of the maze and ANY-maze tracking system.
Figure 3.5: Open field thigmotaxis.

The floor of the open field was virtually divided into central (gray area) and peripheral (white area) zones. Thigmotaxis was assessed by placing the mouse in the open field and measuring time spent in central and peripheral zones.
### Table 3.1: Neurological severity score (NSS) tasks.

Post-TBI neurological impairment is assessed by NSS, which is a composite of ten different tasks that mice perform. Failure in each task earns one point, while passing the task gets no point. Total NSS is obtained by adding scores of individual tasks. Based on the total NSS, injury can be classified as mild (NSS: < 5), moderate (NSS: 5-7) and severe/fatal (NSS: 8-10). Adapted from (Flierl et al., 2009).

<table>
<thead>
<tr>
<th>Task</th>
<th>Description</th>
<th>Success/Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exit circle</td>
<td>Ability to exit a circle of 30 cm diameter within 3 min</td>
<td>0/1</td>
</tr>
<tr>
<td>Monoparesis/hemiparesis</td>
<td>Paresis of front and/or back limbs</td>
<td>0/1</td>
</tr>
<tr>
<td>Straight walk</td>
<td>Alertness, initiative and motor ability to walk straight</td>
<td>0/1</td>
</tr>
<tr>
<td>Startle reflex</td>
<td>Innate reflex; the mouse will bounce in response to a loud hand clap</td>
<td>0/1</td>
</tr>
<tr>
<td>Seeking behavior</td>
<td>Physiological behavior as a sign of 'interest' in the environment</td>
<td>0/1</td>
</tr>
<tr>
<td>Beam balancing</td>
<td>Ability to balance on a beam of 7 mm width for at least 10 s</td>
<td>0/1</td>
</tr>
<tr>
<td>Round stick balancing</td>
<td>Ability to balance on a round stick of 5 mm diameter for at least 10 s</td>
<td>0/1</td>
</tr>
<tr>
<td>3 cm wide beam walk</td>
<td>Ability to cross a 30-cm long beam of 3 cm width</td>
<td>0/1</td>
</tr>
<tr>
<td>2 cm wide beam walk</td>
<td>Same task, increased difficulty on a 2-cm wide beam</td>
<td>0/1</td>
</tr>
<tr>
<td>1 cm wide beam walk</td>
<td>Same task, increased difficulty on a 1-cm wide beam</td>
<td>0/1</td>
</tr>
<tr>
<td>Maximal Score</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

3.3.5 Tissue Collection and Processing

For histological analyses, mice were anesthetized with an i.p. injection of 150 mg/kg ketamine (Zoetis) and 20 mg/kg xylazine (Bayer) at 2, 7, and 14 d post-rTBI, and brains were collected from perfused animals as described in the Chapter 2 except that 4%
paraformaldehyde rather than 10% neutral buffered formalin was used to post-fix hemisected brain tissue for histology. For biochemical analyses, brains were harvested as described in the Chapter 2 at 6 and 12 d and at 2, 7, and 14 d post-rTBI, longitudinally hemisected and rapidly frozen over dry ice and stored at -80°C until analysis.

3.3.6 Iba1 Immunohistochemistry and Silver Staining

Microglial activation and axonal injury were assessed using Iba-1 immunohistochemistry and silver staining, respectively as described in Chapter 2. Microglial activation was quantified using fractal analysis using ImageJ (NIH). Three to four microglia were randomly chosen from 40X-magnified Iba-1 stained images, converted to 8-bit images, and thresholded. Thresholded images were converted to outline images and analyzed using the FracLac plugin (Karperien, A., FracLac for ImageJ. http://rsb.info.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm. 1999-2013.). The box counting method was used and the mean fractal dimension was analyzed. The number of microglia in each white matter regions was quantified by scanning the entire area using a Olympus BX61 microscope at 10X magnification with a ProScan motorized XY stage (Prior Scientific Inc, Rockland, MA). The component images were stitched together into a montage using ImagePro Plus image analysis software (Media Cybernetics Inc., Rockville, MD). The number of Iba1-positive cells was manually counted in the entire white matter track ROI. The area of ROI was measured by ImageJ as pixels and scaled to mm². Cell density was finally expressed as number of Iba-1 positive cells per mm². Silver staining intensity was quantified using ImageJ (version
1.48, NIH) on 40X-magnified images. The images were thresholded and the region of interest (ROI) was selected. The ratio of area of positive signal in ROI to total ROI area was reported as percent positive.

3.3.7 Biochemical Analyses

3.3.7.1 Tissue Processing

For protein determination, half-brains were homogenized in RIPA lysis buffer as described in Chapter 2.

3.3.7.2 Assessment of TNF-α and IL-1β by ELISA

Endogenous TNF-α and IL-1β protein levels in the half-brain homogenates were quantified by commercial ELISA kits (BD Biosciences OptEIA 559603 and 555268, respectively) following the manufacturer’s instructions.

3.3.7.3 Quantitative Assessment of Phosphorylated and Total Tau by Simple Western Analysis

Phosphorylated and total tau were assessed using a completely automated capillary electrophoresis-sized-based Simple Western system (O’Neill et al., 2006) using the WES machine (ProteinSimple, San Jose, CA). Simple Western is a gel-free, blot-free, capillary-based, automated protein immunodetection system that automates all the steps following sample preparation including sample loading, size-based protein separation, immunoprobing, washing, detection, and data analysis. All procedures were performed with manufacturer's reagents according to the user manual. Briefly, 5 µl of
RIPA lysate (2 µg of protein) was mixed with 1.2 µl of 5x fluorescent master mix and heated at 95°C for 5 min. The samples, blocking reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in the manufacturer provided microplate. Following plate loading the separation and immunodetection were performed automatically using default settings. Data were analyzed with Compass software (ProteinSimple). All tau antibodies were kind gifts from Dr. Peter Davies (Albert Einstein College of Medicine, Manhasset, NY, USA). The details of tau antibodies are presented in the Table 3.2. GAPDH (clone 6C5, 1:25000, Chemicon) was used as a loading control. Levels of p-tau and total tau were normalized to GAPDH. Levels of phosphorylated tau were expressed as fold difference compared to sham controls at the respective time points.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Tau Form</th>
<th>Epitope(s)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RZ3</td>
<td>p-Tau</td>
<td>Thr231</td>
<td>1:25</td>
</tr>
<tr>
<td>PHF1</td>
<td>p-Tau</td>
<td>Ser396 &amp; Ser404</td>
<td>1:25</td>
</tr>
<tr>
<td>CP13</td>
<td>p-Tau</td>
<td>Ser202 &amp; Thr404</td>
<td>1:25</td>
</tr>
<tr>
<td>DA9</td>
<td>Total Tau</td>
<td>102 to 140</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

*Table 3.2: Details of monoclonal antibodies for probing p-tau and total tau.*

The rTBI protocol and post-rTBI end points are summarized in Figure 3.6.
Figure 3.6: CHIMERA-rTBI procedure and post-rTBI endpoints

The figure indicates time line for rTBI/sham procedure and behavioral, biochemical and histological end points at various post-rTBI time points.

BM: Barnes maze, Iba-1: Iba-1 immunohistochemistry, NSS: neurological severity score, OF: open field behavior, PA: passive avoidance, RR: rotarod, SS: silver stain

3.3.8 Statistical Analyses

The head kinematics data and graphs are presented as mean ± 95% CI. Behavioral data and graphs are presented as mean ± SEM. All other data and graphs are presented as mean ± SD unless otherwise specified. NSS, LRR, thigmotaxis, and rotarod data were analyzed using repeated measures two-way ANOVA followed by the Holm-Sidak post-hoc test, as animals were tested repeatedly until sacrifice. Passive avoidance and Barnes maze data were analyzed by repeated measures two-way ANOVA. Iba-1 and silver staining data were analyzed by two-way ANOVA followed by Tukey’s post-hoc test. For all the above statistical analyses, a $p$ value of <0.05 was considered significant. Tau phosphorylation and cytokine protein expression at each post-rTBI time point was compared to the respective sham values by $t$ test followed by
Bonferroni correction of multiple comparison, with $p$ value set to <0.01 (5 comparisons) for detecting statistical significance. Statistical analyses of behavioral data were performed using SigmaPlot (version 12.5, Systat Software Inc.). Statistical analyses for rest of the data were performed using GraphPad Prism (version 6.04, GraphPad Software Inc).

3.4 Results

3.4.1 Head Kinematics Following CHIMERA rTBI

Analysis of high-speed videography (5000 fps) was used to assess the biomechanical responses of the head in a group of 8 mice during CHIMERA rTBI at impact energy of 0.5 J. Peak kinematic parameters are depicted in Figure 3.7 and Table 3.3. Following a vertical upward impact of a pneumatically-accelerated piston onto an intact skull of a supine mouse, the head follows a looped trajectory in the sagittal plane (Figures 3.7 A and 3.8). The average head trajectories following two TBIs were highly consistent (Figure 3.7 A). The head traveled a peak linear displacement of 49.6 ± 3.5 mm in 15.7 ± 2.4 ms (Figure 3.7 B) and a peak angular rotation of 2.6 ± 0.28 rad in 24.8 ± 3.1 ms (Figure 3.7 C). The peak linear velocity was 6.6 ± 0.8 m/s at 3.4 ± 1.0 ms (Figure 3.7 D), and the peak angular velocity was 305.8 ± 73.7 rad/s at 2.8 ± 1.9 ms following initial impactor contact (Figure 3.7 F). The head experienced large linear and angular accelerations following impact, achieving a peak linear acceleration of 385.3 ± 52 g in 1.5 ± 0.3 ms (Figure 3.7 E) and peak angular acceleration of 253.6 ± 69.0 krad/s² in 0.8 ± 1.1 ms (Figure 3.7 G). As the head was stationary before impact, the change in head velocity ($\Delta V$) equals peak head velocity and was found to be 6.6 m/s. The energy
transferred from the piston to the head was 0.07 J, which equals to about 86% loss of the input energy (0.5 J).
Figure 3.7: Head kinematics following CHIMERA rTBI.

Head kinematic parameters during impacts were assessed in 8 mice subjected to rTBI. Data are represented as the mean ± SD for each impact. (A) Head trajectory in the sagittal plane following impact. (B) Head displacement-time graph following impact. (C) Head deflection is measured as the angle between the snout, side marker and the horizontal plane. Linear head velocity and linear head acceleration are depicted in (D).
and (E), respectively. (F) and (G) show angular head velocity and angular acceleration, respectively. All graphs are presented as mean ± SD.

Figure 3.8: CHIMERA allows unrestricted head motion during TBI.

Before impact, the mouse head was freely supported on a foam pad in the supine position. Velcro straps were applied to the torso. Impact from the piston deflects the head, which then subsequently returns to its original position on the foam pad. The images were taken at 5,000 fps, at an angle perpendicular to the direction of impact and along the mouse sagittal plane. Each image shown was 12 ms apart.
Table 3.3: Summary of peak values of head kinematic parameters.
The table depicts average peak values of head kinematic parameters for rTBI from 8 rTBI mice. The coefficient of variation (CV) was calculated as the average of day 1 and day 2 peak values from all available recordings.

Using the equal stress/equal velocity approach (Holbourn, 1943; Gutierrez et al., 2001; Viano et al., 2009) to scale our murine kinematic data to human-equivalent values, ΔV was found to be comparable to National Football League (NFL) values and higher than Olympic boxing values, whereas scaled linear and angular velocity and acceleration parameters were lower than NFL values but comparable to Olympic boxing values Table 3.4.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Impact-acceleration</td>
<td>Mouse</td>
<td>Blast</td>
<td>6.6</td>
<td>22.6</td>
<td>27.9</td>
<td>1.4</td>
<td>40</td>
<td>NA</td>
<td>NA</td>
<td>36.2</td>
<td>NA</td>
<td>34.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Non-impact rotation</td>
<td>Rat</td>
<td>NA</td>
<td>72.9</td>
<td>14.2</td>
<td>16.9</td>
<td>NA</td>
<td>3.0</td>
<td>NA</td>
<td>NA</td>
<td>32.8-68.6</td>
<td>60.5-82.5</td>
<td>2.5-17.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Impact-acceleration</td>
<td>Rat</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>93.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>4.3-5.7</td>
<td>1.4-1.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Non-impact rotation</td>
<td>Rat</td>
<td>NA</td>
<td>NA</td>
<td>6.4</td>
<td>NA</td>
<td>1.1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>32.8-68.6</td>
<td>1.4-1.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Weight drop</td>
<td>Rat</td>
<td>NA</td>
<td>4.3-5.7</td>
<td>60.5-82.5</td>
<td>1.4-1.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>34.8</td>
<td>97.8</td>
<td>6.4</td>
<td>9.3</td>
</tr>
<tr>
<td>NFL concussion</td>
<td>Human</td>
<td>NA</td>
<td>NA</td>
<td>34.8</td>
<td>97.8</td>
<td>6.4</td>
<td>250</td>
<td>NA</td>
<td>NA</td>
<td>29.3</td>
<td>71.2</td>
<td>3.2</td>
<td>79</td>
</tr>
<tr>
<td>Olympic boxing – hook punch</td>
<td>Hybrid III dummy</td>
<td>3.1</td>
<td>29.3</td>
<td>71.2</td>
<td>9.3</td>
<td>79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olympic boxing – uppercut</td>
<td>Hybrid III dummy</td>
<td>2.8</td>
<td>17.5</td>
<td>24.1</td>
<td>3.2</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVA involving pedestrian head impact (50% probability resulting in moderate injury)</td>
<td>Hybrid III dummy and FEM</td>
<td>NA</td>
<td>NA</td>
<td>116</td>
<td>11.4</td>
<td>825</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.4: Comparison of head kinematic parameters between rodent TBI models and human TBI.**

The table compares most commonly-reported head kinematic parameters between different TBI models in mice and rats. All the kinematic parameters are scaled to human
values according to the equal velocity/equal stress approach. According to this approach, $\Delta V$ is unscaled, linear acceleration and angular velocity are scaled by 1 scale factor ($\lambda$), and angular acceleration is scaled by $\lambda^2$. The $\lambda$ values for mice and rats were 13.8 and 11.0, respectively. National Football League (NFL) concussion values and data from punches by Olympic boxers are included for comparison. Head Injury Criterion for 15 ms (HIC15) is included to compare the likelihood of head injury. HIC15 for the CHIMERA was calculated based on the definition:

$$HIC = \left[ \frac{1}{t_2 - t_1} \int_{t_1}^{t_2} a(t)dt \right]^{2.5} (t_2 - t_1)_{\text{max}}$$

where linear acceleration ($a$) is in $g$ and scaled by $\lambda$, time ($t$) is in seconds and scaled by $1/\lambda$, and $t_1$ and $t_2$ are determined to give maximum value to the HIC function such that $t_2 - t_1 = 15$ ms.

FEM: Finite element model

NA: Data not available/not applicable

HIC15: Head Injury Criterion for 15 ms

$\Delta V$: Change in velocity

MVA: Motor vehicle accidents

### 3.4.2 CHIMERA rTBI Induces Behavioral Deficits

Loss of righting reflex (LRR) in animals after TBI is considered analogous to loss of consciousness in humans after TBI and can be considered as a behavioral indicator of injury severity (Dewitt et al., 2013). Mice subjected to CHIMERA rTBI showed significantly increased LRR duration compared to sham animals (Figure 3.9 A; TBI effect: $F(1, 65) = 59.61, p < 0.001$). LRR duration was consistent between the first and
second impacts (Figure 3.9 A). We further assessed injury severity using the NSS, which is a composite of ten tasks that assess motor reflexes, alertness, and physiological behavior (Flierl et al., 2009). The NSS of injured animals was significantly higher than sham mice from 1 h to 7d post-procedure (Figure 3.9 B, TBI effect: $F(1, 191) = 44.12, p < 0.001$). In injured mice, the NSS score showed maximum deficits at 1h post-procedure ($p < 0.001$) followed by steady spontaneous improvement over 1-7d, albeit remaining significantly higher than sham animals at each post-rTBI time point (Figure 3.9 B, $p < 0.001$). Similarly, rTBI significantly impaired motor performance from 1-7d post-injury as indicated by reduced fall latencies on an accelerating rotarod compared to sham controls (Figure 3.9 C, TBI effect: $F(1, 220) = 11.99, p < 0.001$). Fall latencies showed both time effects ($F(4, 220) = 13.70, p < 0.001$) and TBI x Time interaction ($F(4, 220) = 11.22, p < 0.001$). Motor deficits in injured mice peaked at 1d ($p < 0.001$) and returned to baseline conditions by 14d post-injury ($p = 0.22$), whereas sham mice did not show any motor deficit ($p > 0.82$). Injured mice showed anxiety-like behavior as indicated by significantly increased thigmotaxis in an open field test (Figure 3.9 D, TBI effect: $F(1, 53) = 12.30, p < 0.001$). The thigmotactic behavior of both groups significantly declined over time in similar trend (Figure 3.9 D, Time effect: $F(2, 53) = 5.45, p = 0.007$; TBI x Time interaction insignificant). Open field thigmotaxis was not affected by gross motor activity as no significant differences in total distance traveled or time immobile were observed between injured and sham-operated mice (Figure 3.10). Repeated TBI also induced working memory impairment as indicated by decreased latencies to enter the darkened compartment on the passive avoidance task (Figure 3.9 E, TBI effect: $F(1, 28) = 4.6, p = 0.041$). For all mice, a main effect of Day indicated that
mouse behavior changed over the training and testing sessions evaluated in this study 
\( F(3, 84) = 58.55, p < 0.001 \). For the main effect of Day, pairwise comparisons 
indicated that mice entered the darkened compartment significantly faster on Day 7 (\( p < 
0.001 \)) than on Days 8–10 (\( p > 0.05 \)) signifying that all mice remembered the shock to 
some degree (Figure 3.9 E). Injured mice also showed spatial reference memory 
impairment as indicated by increased latencies to locate the escape hole on the Barnes 
maze (Figure 3.9 F, TBI effect: \( F(1, 28) = 6.27, p = 0.018 \)). Under our experimental 
conditions, cognitive performance did not spontaneously resolve by the end of our 
testing period.
Figure 3.9: CHIMERA rTBI induces behavioral deficits.

(A) Duration of loss of righting reflex (LRR) was assessed immediately following the sham or TBI procedure. Cohort size: Sham, N = 31; rTBI, N = 39. (B) Neurological severity score (NSS) was assessed at 1 h, 1 d, and 2 d and 7 d post-rTBI. Cohort size: Sham (1h: N = 34, 1d: N = 31, 2d: N = 35, 7d: N = 21); rTBI (1h, 1d and 2d: N = 42, 7d: N = 25). (C) Motor performance was assessed on an accelerating rotarod at 1, 2, 7 and 14 d post-rTBI. Cohort size: Sham (1d and 2d: N = 35, 7d: N = 21, 14d: N = 15); rTBI (1d and 2d: N = 41, 7d: N = 25, 14d: N = 15). (D) Thigmotaxis was quantified at 1, 7 and 14 d post-rTBI. Cohort size: Sham (1d: N = 23, 7d: N = 16, 14d: N = 15); rTBI (1d: N = 24, 7d: N = 10, 14d: N = 15). LRR, NSS, rotarod and tigmotaxis data were analyzed by repeated measures two-way ANOVA followed by Holm-Sidak post-hoc test. Working memory was assessed by passive avoidance test (E) from 7 to 10 d post-rTBI. Non-working memory was assessed by Barnes maze (F) from 9 to 13 d post-rTBI. Data at each time point represent mean of four trials. Barnes maze and passive avoidance data
(Cohort size: sham, N = 15; rTBI, N = 15) were analyzed by repeated measures two-way ANOVA. For all graphs, * indicates significant rTBI effect within time point and # indicates significant time effect within each group. *, **, *** and ***** indicate \( p<0.05 \), \( p<0.01 \), \( p<0.001 \), and \( p<0.0001 \), respectively. #, ### and #### indicate \( p<0.05 \), \( p<0.001 \), and \( p<0.0001 \), respectively.

Figure 3.10: CHIMERA rTBI does not significantly affect general mobility.
General mobility was tested by the open field test at 1, 7 and 14 d post-injury. There was no rTBI effect at any time point for the total distance travelled (A) and time spent immobile (B). Data are presented as the mean ± SEM and analyzed by repeated measures two-way ANOVA followed by Holm-Sidak post-hoc test. In all graphs, *** and # indicate significant time effect for rTBI (\( p<0.001 \)) and sham (\( p<0.05 \)) mice, respectively.

3.4.3 CHIMERA rTBI Induces Widespread Diffuse Axonal Injury
Silver staining was used to assess post-rTBI axonal damage at 2, 7, and 14 d (Figure 3.11). rTBI brains revealed widespread axonal injury, as indicated by intense punctate and fiber-associated argyrophilic structures in white matter tracts including the olfactory nerve layer of the olfactory bulb, corpus callosum, and optic tracts (Figure 3.11 A and B). Axonal injury was observed at both coup (corpus callosum) and contra-coup (optic
tract) regions, indicating a diffuse injury pattern. High-magnification of the affected areas at 100X revealed numerous axonal varicosities (Figure 3.11 C), which is a characteristic histological feature of human axonal pathology after TBI (Johnson et al., 2013).

Quantitative analysis revealed significant silver uptake in the injured olfactory nerve layer (TBI effect: $F(1, 27) = 16.89, p < 0.0001$), which was maximum at 2d ($p < 0.001$) and returned to sham levels over 7-14d (Figure 3.12 A). On the other hand, rTBI induced persistent silver stain uptake in the corpus callosum (Figure 3.12 B, TBI effect: $F(1, 37) = 41.54, p < 0.0001$; Time effect insignificant) (Figure 3.12 C). In optic tract, silver uptake was most intense (Figures 3.12 B and C TBI effect: $F(1, 36) = 107.4$) and there was a significant time and injury interaction (TBI x Time interaction: $F(2, 36) = 11.66$), indicating persistent increase in axonal degeneration in contrecoup regions.
Figure 3.11: CHIMERA rTBI induces diffuse axonal injury.
Axonal degeneration was assessed by silver staining at 2, 7 and 14 d post-rTBI. (A)
Coronal sections showing white matter areas including olfactory nerve layer, corpus
callosum and optic tract with prominent silver staining indicated by black rectangles. (B) Representative 40X-magnified images of the same brain regions in sham-operated (upper panel) or rTBI-induced (lower panel) animals. (C) 100X-magnified images of the same brain regions in rTBI-induced animals. Axonal varicosities are indicated by arrows.

Figure 3.12: CHIMERA rTBI induces sustained axonal injury

Silver stained images were quantified by calculating % of region of interest (ROI) in the white matter tract area stained with silver stain. The bars indicate mean ± SEM percent of ROI showing positive signal in sham and rTBI-induced animals in (A) olfactory nerve layer, (B) corpus callosum and (C) optic tract. Data were analyzed using two-way ANOVA followed Tukey post-hoc test. For all graphs * indicates rTBI effect within each time point and # indicates time effect within each group. For all graphs, *, **, *** and **** indicate $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively and ### and #### indicate $p<0.001$, $p<0.0001$, respectively.

3.4.4 CHIMERA rTBI Induces Widespread Microglial Activation

Using Iba-1 immunohistochemistry, we observed significantly increased activated microglia throughout several white matter tracts including the olfactory nerve layer, corpus callosum, optic tracts, and brachium of superior colliculus of injured brains compared to the sham controls as assessed using both fractal analysis and microglial
density (Figures 3.13 and 3.14). Quantification of microglial morphology by fractal analysis revealed that microglia in sham animals displayed highly ramified and extensively branched processes that are characteristic of the resting state (Figure 3.13 C, upper row, Figure 3.14 A-D, open bars). By contrast, microglia in the corpus callosum, brachium of superior colliculus, and olfactory nerve layer of injured animals had predominantly hypertrophic to bushy morphology with primary branches only, whereas those in the optic tract showed amoeboid morphology characteristic of highly activated microglia (Figure 3.13 C, lower row, Figure 3.14 A-D, black bars). Quantitative analysis showed significant and persistent microglial activation in the injured olfactory nerve layer (TBI effect: $F(1, 35) = 13.64, p = 0.0008$), optic tract (TBI effect: $F(1, 37) = 9.77, p = 0.0034$), corpus callosum (TBI effect: $F(1, 38) = 29.51, p < 0.0001$), and brachium of superior colliculus (TBI effect: $F(1, 38) = 24.5, p < 0.0001$) as soon as 2d (Figure 3.14 A-D).

In addition to changes in microglial morphology, we observed significant increases in the number of microglia in the same white matter regions including the olfactory nerve layer (Figure 3.14 E, TBI effect: $F(1, 16) = 21.53, p = 0.0003$), optic tract (Figure 3.14 G, TBI effect: $F(1, 16) = 90.30, p = 0.0001$), corpus callosum (Figure 3.14 F, TBI effect: $F(1, 19) = 22.25, p = 0.0002$) and brachium of superior colliculus (Figure 3.14 H, TBI effect: $F(1, 18) = 34.85, p < 0.0001$), indicating that injury induced proliferation or recruitment of immune cells. In olfactory bulb (Time effect insignificant), corpus callosum (Time effect insignificant) and optic tract, microglia cell number was persistently increased from 2d up to 14d ($p < 0.05$). In the brachium of superior
colliculus, a delayed but persistent increase in the Iba-1 positive cell number was observed from 7d to 14d ($p < 0.01$).
Figure 3.13: CHIMERA rTBI induces widespread microglial activation.

Microglial activation was assessed using Iba-1 immunohistochemistry at 2 d post-rTBI. 
(A) Representative images of Iba-1 stained coronal sections of olfactory bulb and brain. Areas with prominent microglial activation are indicated by black rectangles. 
(B) Representative 40X-magnified images of olfactory never layer, corpus callosum, optic
tract, and brachium of superior colliculus showing resting microglia in sham brains (upper row) and activated microglia in injured brains (lower row). **(C)** Representative 100X-magnified images showing the morphology of Iba-1-stained resting microglia in sham (upper row) and activated microglia in rTBI (lower row) brains.
Figure 3.14: Quantitative analysis of microglial response to rTBI.

Bar graphs in the left column (A-D) indicate mean ± SD fractal dimension for microglial morphology in (A) olfactory nerve layer, (B) corpus callosum, (C) brachium of superior
colliculus, and (D) optic tract. Bar graphs in the right column (E-H) show mean ± SD number of Iba-1 positive cells per mm² in the same white matter regions. Data were analyzed by two-way ANOVA followed by a Tukey post-hoc test. Numbers inside the bars indicate sample size. For all graphs, * indicates a significant rTBI effect within a particular time point while # indicates a significant time effect within rTBI group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. ##: $p < 0.01$, ####: $p < 0.0001$.

### 3.4.5 CHIMERA rTBI Increases Proinflammatory Cytokine Levels

In addition to the microglial response, we also measured protein levels of the proinflammatory cytokines TNFα and IL-1β in the half brain homogenates. Protein levels TNFα (Figure 3.15 A) and IL-1β (Figure 3.15 B) were significantly higher at 2 d post-TBI compared to the respective sham levels ($p < 0.01$, t tests with Bonferroni corrections).

![Figure 3.15: CHIMERA rTBI increases proinflammatory cytokine levels.](image)

Values are expressed as mean % fold change in TNFα (A) and IL-1β (B) levels in rTBI brain lysates compared to cytokine levels in the sham brain lysates at respective time points. For both graphs, ** indicates $p < 0.01$ in comparison of rTBI vs sham, using t-test with Bonferroni correction of multiple comparisons ($p = 0.05/5 = 0.01$).
3.4.6 CHIMERA rTBI Increases Endogenous Tau Phosphorylation

We next assessed the phosphorylation levels of endogenous murine tau using three antibodies directed against different phosphorylation sites, namely CP13 (pSer202), RZ3 (pThr231), and PHF1 (pSer396 and pSer404). In addition, total murine tau levels were determined by the antibody DA9. Western blot analysis showed significantly increased phosphorylation of all the probed epitopes in rTBI brain lysates at 6 h, 12 h and 2d compared to the respective sham brain lysates (Figure 3.16 A-C and G-I, \( p<0.01 \), t tests with Bonferroni correction). The change in tau phosphorylation was due to a significant increase in ratio of phosphorylated tau:total tau, but not as a result of change in total tau level (Figure 3.16 D-F and G-I, \( p<0.01 \), t tests with Bonferroni corrections).
Figure 3.16: CHIMERA rTBI increases endogenous tau phosphorylation.

The graphs in the left column (A-C) depict fold change in endogenous phosphorylated tau levels in rTBI half-brain homogenates compared to the sham brains using antibodies CP13 (pSer202 and pThr205), RZ3 (pThr231) and PHF1 (pSer396 and pSer404), respectively. Graphs in the middle column (D-F) depict quantitation of phosphorylated tau as a proportion of total tau (DA9). Representative immunoblots of phosphorylated and corresponding total tau are depicted in the right column (G-I). Arrows on the left of the blots indicate position of 66 kDa marker. Data are presented as the mean ± SEM.
fold change in rTBI compared to the respective shams at each time point. For all graphs, *, ** and *** indicate \( p < 0.01 \) for the comparison of rTBI vs respective sham values, using multiple t-tests with Bonferroni correction (\( p = 0.05/5 = 0.01 \)).

3.5 Discussion

The major goal of this study was to develop a simple, reliable model of murine CHI that replicates fundamental aspects of human impact TBI through precise delivery of known biomechanical inputs. CHIMERA fulfills these criteria and offers several key advantages over comparable rodent TBI models.

The first and foremost advantage is CHIMERA allows completely surgery-free induction of TBI eliminating the need of longer anesthesia duration as well as sedatives such as ketamine and xylazine (that were used in the weight drop model), as well as learning complex surgery such as craniotomy that is required for FP and CCI models. Almost all of the currently available TBI models require some surgery to deliver the mechanical force to either exposed skull (e.g. in weight drop TBI) or to the dura (as in FP and CCI models) that increases the learning curve of the operator. For example, a novice learning FP injury may struggle at the beginning to perform the craniotomy without disrupting the dura as well as gluing the hub for attaching fluid injection tube without introducing the glue into the craniotomy site (Alder et al., 2011). Moreover, head securing method such as a stereotaxic setup used for CCI model and surgery themselves increase the duration of injury procedure. For example, the duration required for weight drop injury model discussed in Chapter 2 is ~ 20 min. In contrast, using CHIMERA a fully-trained individual can finish the whole procedure in ~ 4 and ~ 8
minutes with and without high speed videography, respectively. Shorter injury procedure also reduces the duration of anesthesia exposure. Duration of anesthesia exposure is important as general anesthetic may influence injury outcomes. For example, isoflurane, the most commonly used general anesthetic for rodent surgery is shown to exert neuroprotective actions in TBI (Statler et al., 2000; Statler et al., 2006).

A second advantage of CHIMERA is that it allows unrestricted head movement after impact replicating clinically encountered head impact situations as opposed to other TBI models (e.g. weight drop) in which head support systems restrict head movement to varying degree (Namjoshi et al., 2013a). As discussed in Chapter 1, head acceleration, rather the impact itself is thought to induce more brain damage. In support of this, CHIMERA-rTBI induces robust and sustained axonal damage and significant neuroinflammation compared to the weight drop rTBI as discussed in Chapter 2.

Third advantage is the very high reproducibility of mechanical inputs (i.e. piston velocity and impact energy) as exemplified by high $R^2$ values (~ 0.99) of the piston velocity vs air pressure calibration curve (Figure 3.2). This reflected in the high reproducibility in the head kinematic parameters with a reasonable cohort size ($N = 8$). By fine adjustment in the air pressure, CHIMERA allows precise control over mechanical input. Moreover, the biomechanical inputs can be delivered by changing variety of parameters (impact energy, location, and direction) that make CHIMERA a highly versatile model to study TBI under a variety of mechanical input conditions. Thus, by choosing appropriate combination of piston mass (25 g, 50 g, 75 g or 100 g) and air pressure, CHIMERA can
deliver impacts over a wide energy range from 0.01 J to 19 J. The impact can be delivered either in vertical direction that results in head acceleration in the sagittal plane or in the horizontal direction resulting head movement in an oblique plane. By adjusting the angle between body and the head, the impact can be delivered at the vertex or on the posterior aspect of the skull. By using the horizontal configuration, the impact can be delivered to the lateral side of the head.

Lastly, the build-in mechanism of CHIMERA allows simultaneous high-speed video recording that can be used to measure head kinematics following impact. This is very important for the validation of impact procedures.

In this study, a 50 g piston delivered an input impact with a kinetic energy of 0.5 J. Calibration curves show that the 50 g piston has an energy range of 0.01 J to 14.0 J (useful range for mouse TBI is 0.1 J to 1 J) in minimum steps of 0.01 J, with highly reproducible performance (Figure 3.2). Adjusting piston mass and dimensions will allow other biomechanical parameters to be controlled, so that it may be possible to experimentally model the biomechanical conditions observed under different types of human impact TBI.

High-speed video integrated into CHIMERA enables measurement of mouse head kinematic parameters that can be scaled to humans. The most-commonly used method for scaling kinematic parameters between humans and animals is based on the equal stress/equal velocity approach (Holbourn, 1943; Gutierrez et al., 2001; Viano et al.,
2009). For this certain assumptions are made such as: 1) neurons, blood and CSF all have about the same density as water, 2) brain tissue is extremely incompressible, 3) brain tissue has small modulus of rigidity and thus it is extremely easy to change shape of the brain, and 4) human and animal brains have similar density and geometry, are elastic and are isotropic (i.e. uniform in all directions) (Holbourn, 1943). According to this principle, velocity does not scale and is the same for human and animal data. We used a scaling factor $\lambda$ [$\lambda = (\text{mass of human brain} / \text{mass of mouse brain})^{1/3} = 13.8$] to estimate the human head-equivalent kinematic parameters from our animal data. Although this scaling approach has been widely used in the study of impact biomechanics (Holbourn, 1943; Gutierrez et al., 2001; Viano et al., 2009), and has been applied to a rat model (Viano et al., 2009), it is important to be cautious in its extrapolation as the human and rodent brains differ in geometry, white : grey matter ratio, ventricular volume and position, and cortical folding. All these factors render the “scaling” of rodent data to human to be an approximation at best. Under our experimental conditions, behavioral and neuropathological changes reliably occurred at lower scaled values of all kinematic parameters (except for impact duration) than those reported for NFL concussions (Pellman et al., 2003a; Pellman et al., 2003b). Because clinical mTBI can occur under many different circumstances (e.g., falls, passengers and pedestrians in motor vehicle accidents, non-NFL sports), an important area for future research will be to determine how various impact characteristics lead to functional and biochemical changes. The precision and flexibility CHIMERA offers with respect to impact parameters will help to refine the relationship between impact characteristics and physiological outcomes.
An additional advantage of CHIMERA is that it overcomes much of the variability observed with most closed-head injury models. Typical weight-drop models (Marmarou et al., 1994; Flierl et al., 2009) have poor control over biomechanical input parameters, such as friction and air resistance inside the guide tube that may contribute to variable outcomes, high incidence of skull fractures, and limited dynamic range, thereby posing challenges in comparing results from different laboratories (Namjoshi et al., 2013a). Head movement during many CHI impact models is often at least partially restricted by anchoring the head within a stereotaxic frame or by resting the animal on a foam support. In a recent modification, Kane et al supported mice on a piece of aluminum foil that ruptures upon impact and leads to a 180° rotation of the animal (Kane et al., 2012). While this modification allows unrestricted head movement, it still includes possible sources of variability including stretching of the aluminum foil before yielding to the force generated by the weight-drop and less reliable and adjustable positioning of impact location compared to CHIMERA.

Loss of consciousness for <30 min is one of the clinical criteria for mTBI (Carroll et al., 2004) and the analogous measure in mice is LRR. Interestingly, a second TBI occurring 24h after the first impact did not prolong LRR time. Post-concussion patients may also show balance difficulties or postural instability up to several days (Guskiewicz, 2003; Blume et al., 2011; Putukian, 2011), as well as mood changes such as irritability or anxiety (Blume et al., 2011; Daneshvar et al., 2011). Though general locomotor activities were not severely affected, CHIMERA TBI resulted in deficits in fine motor
coordination and neurological performance. Similar changes have also been reported by other groups (Tsenter et al., 2008; Mouzon et al., 2012). CHIMERA also increased thigmotaxis in TBI animals, suggesting an anxiety-like behavior (Simon et al., 1994). Our model also revealed deficits in both working and non-working memory as assessed by passive avoidance test and Barnes maze, respectively.

Human and experimental TBI induces rapid neuroinflammatory responses as demonstrated by changes in cytokine levels (e.g., IL-1β and IL-6) and microglial activation (Taupin et al., 1993; Shohami et al., 1997; Rooker et al., 2006; Shein et al., 2007; Shitaka et al., 2011; Perez-Polo et al., 2013). Under our conditions, rTBI led to elevated IL-1β and TNF-α levels at 2 d after injury, which was accompanied by histological evidence of microglial activation. Because our animal ethics committee required the use of meloxicam for pre-emptive pain control, it is possible that an inflammatory response occurring during the first few hours after impact (Taupin et al., 1993; Shohami et al., 1997; Shein et al., 2007) was suppressed (Engelhardt, 1996). Iba-1-positive activated microglia were particularly evident along white matter tracts throughout the brain, whereas grey matter was essentially spared. Microglial activation as assessed by fractal analysis and cell density was significant at 2d and persistent until 14d. However, as Iba-1 does not distinguish the source of immune cells, the increase in cell number in this study may be due to proliferation of resident immune cells in the brain or recruitment of immune cells from the periphery, or both.
Diffuse axonal injury (DAI) is one of the characteristic pathologies of TBI (Adams et al., 1989; Johnson et al., 2013). Using silver staining, we observed increased argyrophilic fibers and punctate structures in several white matter tracts across the brain, suggesting a diffuse pattern of damaged axons. Axonal varicosities, a classical feature of DAI in humans, were also present (Johnson et al., 2013). Interestingly, both white matter areas that were close to (e.g., corpus callosum) and distant from (e.g., optic tract and olfactory nerve layer) the impact site were affected, suggesting coup and contra-coup injuries are present in our model. Affected white matter areas, including the corpus callosum, optic tract, and the olfactory system, have been reported in other CHI models that induce impact at the superior side of skull (Foda and Marmarou, 1994; Creeley et al., 2004; Shitaka et al., 2011; Namjoshi et al., 2013b). Several white matter areas, including olfactory nerve layer, corpus callosum and optic tract showed both increased argyrophilic staining and microglial activation, suggesting a possible relationship between axonal damage and neuroinflammation, in agreement with previous reports (Ciallella et al., 2002; Shitaka et al., 2011; Lin and Wen, 2013). Interestingly, axonal injury in corpus callosum and optic tract continued to increase over 14 days while microglial activation resolved in all white matter tract areas over 2-7 days. On the other hand, axonal injury was resolved in the olfactory neuronal layer within 7 days suggesting efficient neural repair/neuroregeneration in this white matter region. Future studies will characterize the dynamics of post-rTBI axonal damage over long-term follow up (up to 6 months).
Hyperphosphorylation of the cytoskeletal protein tau is a pathological event observed in many neurodegenerative diseases including AD (Ballatore et al., 2007) and CTE (McKee et al., 2013). In our model, we demonstrated that tau hyperphosphorylation is an early event after rTBI in wild-type mice, again in agreement with other models of CHI (Genis et al., 2000; Goldstein et al., 2012). It should, however be noted that post-TBI changes in phosphorylation of murine tau does not predict whether human tau will show similar dynamics. Further experiments using transgenic human tau mice will be required to investigate the influence of rTBI on tau deposition.

Future studies will be conducted to characterize the relationships between kinematics and the resulting behavioral and neuropathological responses across a variety of impact parameters. The significant advantages CHIMERA offers over comparable rodent TBI models are expected to facilitate the acquisition of preclinical data with improved relevance to human TBI, thereby accelerating the pace of successful research to understand the mechanisms of TBI and to develop effective therapeutic approaches for this devastating condition.
Chapter 4: Conclusions and Future Directions

The experiments described in Chapter 2 of this thesis were designed to assess the role of apoE in the recovery from TBI. As discussed in the introductory chapter, TBI, while a pathological entity in itself, is also suggested to increase risk of dementia in TBI survivors. Although the cause and effect mechanisms linking these two pathological conditions are still unclear, several observations common to both diseases may help to understand the relationship between the two. For example, post-mortem analyses of traumatized human brains have revealed formation of diffuse Aβ deposits and NFTs, which are the two pathological hallmarks of AD. Neurons are rich in lipids, which play crucial role in neuronal function as well as synaptogenesis. Thus it is conceivable that the brain lipid metabolism, which is based on apoE may serve important roles in neuronal recovery following TBI. While the apoE4 isoform is the best established genetic risk factor for AD, the association between apoE and TBI is still unclear. Epidemiological data suggests that apoE may influence post-TBI recovery. The role of apoE in AD pathogenesis stems from its role in Aβ metabolism, inflammation, and synaptic remodeling. We and other groups have previously shown that ABCA1-mediated lipidation of apoE is crucial for its role in Aβ clearance. Thus enhancing apoE lipidation and function may be beneficial in AD pathology which is reflected by the beneficial effects of LXR agonists on facilitating Aβ clearance and improving cognitive function in AD mouse model.
Based on these observations we hypothesized that LXR agonists may have improved recovery from TBI through apoE-based mechanisms. As described in Chapter 2 of this thesis, we tested this hypothesis by treating wild-type and apoE-/- mice that were subjected to mrTBI using popular weight drop method with a synthetic LXR agonist GW3965.

4.1 Chapter 2: Conclusions

The first objective of this thesis was to test the therapeutic utility of synthetic LXR agonist, GW3965 in mild, repetitive TBI (mrTBI), which is described in Chapter 2. I found that GW3965 treatment improves NOR performance as well as promotes axonal recovery following mrTBI and these outcomes require apoE. I further found that GW3965 can also suppress the elevation of Aβ following TBI, although surprisingly this outcome was apoE-independent. The overall results from this study suggest that synthetic LXR agonists may be a promising pharmacological treatment for TBI. Moreover, we observed treatment effects on the above-mentioned the post-TBI deficits as early as two days following GW3965 administration, suggesting that LXR agonists may be beneficial in the management of acute post-TBI phase.

We found that mrTBI induced significant NOR memory deficits in untreated WT as well as apoE-/- mice that persisted from 2 to 14 days. While GW3965 treatment restored NOR memory deficits in WT animals, it failed to show this beneficial effect in apoE-/- mice. This indicates that apoE is required for the beneficial effect of GW3965 in post-TBI cognitive deficits. Previously thought to assess pure working memory, NOR task is
now suggested to test episodic memory of the animal (Ennaceur, 2010). Although the exact neural circuits for NOR task are not fully understood, studies indicate that ventromedial prefrontal cortex (Akirav and Maroun, 2006) and hippocampus (Broadbent et al., 2010) are essential for object recognition.

We tested TBI-induced motor deficits on an accelerating rotarod. The rationale for using accelerating vs constant speed rotarod was that in the former method, the accelerating rod constantly challenges the animal requiring motor coordination and planning while the latter may suffer from testing endurance rather than motor coordination (Deacon, 2013). Our TBI model induced impairment in finer motor tasks, such as motor coordination and planning. While the exact mechanism for spontaneous recovery of motor function observed in this study is presently unknown, it is possible that with the repetitive rotarod testing after TBI we may have inadvertently introduced motor training akin to post-TBI rehabilitation program in humans. It is well known that repeated motor tasks promote development of spinal neural circuits, called central pattern generators that when activated can produce rhythmic motor patterns such as walking in the absence of sensory or descending inputs from brain (Marder and Bucher, 2001). The above-mentioned possibility is further supported by a recent study, in which a 30 min daily bilateral movement training in mice using the rotarod for 33 days improved motor function and promoted axonal remodeling and rewiring of the corticospinal tract (the major motor pathway that controls fine movements and posture) after unilateral contusion of the motor cortex (Nakagawa et al., 2013). The authors further showed that rotarod training increased neural activity in the spinal cord laminae VII, VIII and X. The
commissural interneurons located in these laminae of the rodent spinal cord are involved in generating locomotor-like activity (Kjaerulff and Kiehn, 1996; Kiehn and Kjaerulff, 1998). We further observed that loss of apoE further exacerbated the magnitude of motor impairment following mrTBI. Intriguingly, GW3965 treatment neither affected the severity of motor deficits nor the kinetics of spontaneous recovery. Future studies will be necessary to assess specific motor pathways affected in our TBI model, changes in the plasticity and remodeling of these neurons and role of apoE in these processes.

Untreated WT brains showed a transient increase in ABCA1, the principal LXR target protein that peaked at 7 days and dropped back to sham levels by day 14. It is hypothesized that the damaged neurons release lipids that are taken up by the viable cells as shown by increased levels of LDLR, a major apoE receptor with no change in ABCA1 in first 3 days after CCI injury (Loane et al., 2011). In the same model, ABCA1 levels peaked at 7 days with corresponding decrease in LDLR (Loane et al., 2011). In our study, while the LDLR levels were not affected at any post-TBI time point, the ABCA1 dynamics was similar to that reported in the CCI model, i.e. with no change at 2 day post-TBI and peaking by day 7. This delayed increase ABCA1 may indicate cholesterol efflux by the viable cells that can be used for synaptic repair and remodeling during post-TBI recovery. Interestingly, unlike WT mice we did not see any changes in ABCA1 levels in untreated apoE-/- mice following TBI. This suggests that the endogenous signals leading to upregulation of ABCA1 might be compromised by the absence of apoE. Tall and coworkers have shown that interaction of ABCA1 with apoA-
I, the peripheral counterpart of apoE, at the cell membrane inhibits calpain-mediated degradation of ABCA1 that results in increase in the both cell surface expression and activity of ABCA1 (Martinez et al., 2003). Extending this concept, it is also possible that ABCA1 turnover is increased in the CNS leading to its rapid degradation in the absence of apoE (Krimbou et al., 2004). As expected, GW3965 treatment caused sustained increase in ABCA1 in both WT and apoE-/- mice.

We further found that neither TBI alone nor GW3965 treatment had any effect on apoE levels in WT mice. This result is consistent with the relative insensitivity of apoE compared to ABCA1 as a LXR target (Donkin et al., 2010). Since we determined apoE levels in the whole half-brain extracts, it is possible that regional changes in the apoE levels, if any may have been missed. While we did not assess the lipidation status of apoE, it is also possible that even though the total apoE pool does not change per say the ABCA1-mediated lipidation of the available apoE may have been sufficient to promote recovery mechanisms after mild injury. More studies can be done to assess whether the same holds true in case of moderate-to-severe TBI. Moreover, the contribution of ABCA1 in LXR agonist-mediated recovery may be more important as recently shown by our group that ABCA1 is required for the beneficial effects of GW3965 in AD mice (Donkin et al., 2010). This hypothesis can be tested in future studies using ABCA1-/- mice.

Surprisingly, contrary to our hypothesis GW3965 treatment blocked Aβ elevation even in the absence of apoE. While, it is generally agreed that apoE modulates Aβ clearance
through enzymatic degradation as well as clearance through cerebrovasculature recent data suggests that the interaction of apoE with Aβ and its influence on Aβ metabolism is complex than thought (see section 1.6.4.7). Our observation indicates that LXR activation may engage compensatory mechanisms in the absence of apoE to enhance Aβ clearance. A potential candidate for such compensatory mechanism is apoA-I, the peripheral counterpart of apoE. Although apoA-I is not synthesized in the brain, it is present in the CSF at relatively high levels of 0.1%-0.5% relative to plasma levels, suggesting that it crosses BBB (Stukas et al., 2014). A recent study from our lab has shown that apoA-I levels remain significantly higher in the CNS than in plasma in ABCA1-/- mice and that 8-week treatment of AD mice with GW3965 causes a dramatic increase in the cerebral apoA-I levels (Stukas et al., 2011). In the present study we did not observe any changes in brain as well as CSF apoA-I levels (data not shown) either following TBI or with GW3965 treatment, which may be due to short duration of treatment compared to long-term treatment (Stukas et al., 2011). Nonetheless, recent studies suggest apoA-I as another apolipoprotein that may play important role in injured brain for several reasons. First, AD mice that lack apoA-I show increased cerebrovascular amyloid deposits and impaired spatial learning and memory function (Lefterov et al., 2010) whereas overexpression of human apoA-I protects age-associated cognitive deficits and lowers neuroinflammation and CAA (Lewis et al., 2010). Interestingly, deletion or overexpression of apoA-I seem to affect Aβ and amyloid deposits only in the cerebrovasculature and not in the brain parenchyma (Lefterov et al., 2010; Lewis et al., 2010) suggesting that role of apoA-I may be more prominent in the maintenance of the cerebrovasculature (Stukas et al., 2014). Second, in vitro studies
have shown that apoA-I binds to Aβ (Koldamova et al., 2001), prevents Aβ aggregation and reduces Aβ-induced cytotoxicity, oxidative stress, and neurodegeneration (Lefterov et al., 2010; Lewis et al., 2010). Lastly, serum apoA-I levels were recently shown to be elevated in patients with mTBI and assessment serum apoA-I levels in combination with S100B, an astrocyte-specific protein has been proposed to be a useful biomarker for classification of mTBI (Bazarian et al., 2013). ApoA-I therefore may have more important role in the CNS function and lipid metabolism than it is appreciated (Stukas et al., 2014). Thus, future studies will be designed to get a deeper insight into the role of apoA-I in the brain, which may offer novel approaches to promote neuronal recovery after TBI.

4.2 Chapter 2: Study Limitations and Future Directions

While the results from this study indicate that synthetic LXR agonists may offer beneficial therapeutic effects in TBI partly through apoE-mediated mechanism there are several limitations to this study that may help guide future studies.

Although the LXR stimulation offers beneficial effects in mrTBI, current LXR agonists have safety issues such as hypertriglyceridemia and hepatic steatosis (Grefhorst et al., 2002; Groot et al., 2005) that has virtually precluded the development of LXR agonists for chronic diseases. To date, only one Phase I study of a synthetic LXR agonist, LXR-623, in humans has been reported (Katz et al., 2009). In this study, the safety, pharmacokinetic and pharmacodynamic profile of LXR-623, was studied in healthy human volunteers after a single ascending dose. Although the authors reported a
favorable safety profile with dose-dependent increase in ABCA1 and ABCG1 expression, further development of this compound was discontinued due to the development of adverse CNS events at the highest doses used (150 and 300 mg), including confusion, forgetfulness, decreased comprehension, lightheadedness, drowsiness, paresthesias, palpitations, decreased concentration, altered time perception and paranoid ideation (Katz et al., 2009). It is therefore of interest to develop strategies that can retain the beneficial effects of LXR activation and circumvent their unwanted side effects. For example, development of LXRβ-selective agonists is an active area of research, as LXRα underlies much of the undesired lipogenic effects (Quinet et al., 2006). Another promising approach is to develop tissue-specific LXR agonists or antagonists, as the magnitude of induction of LXR response genes varies considerably among tissues (Brunham et al., 2006). As shown in this study, the beneficial effects of LXR agonist were seen even in the acute post-TBI phase, which suggests that these agents may be useful for short-term rather than chronic treatment, which could improve functional recovery as well as decrease long-term AD risk. While studying long-term effects of LXR treatment was beyond the scope of this thesis project, future studies could be designed for this. Finally, less potent compounds or compounds with LXR-agonist like activity may offer another approach, as proton pump inhibitors have some LXR activity in murine primary astrocytes and human glial-derived cell lines (Cronican et al., 2010).

Our study suggests that apoE is not necessary for LXR-mediated suppression of endogenous Aβ. While the amyloid plaques are one of the pathological hallmarks of AD,
the overall influence of Aβ in TBI pathology is questionable as amyloid deposits are found in only 30% of the clinical TBI cases. On the other hand, tau hyperphosphorylation is common neuropathology of TBI, which suggests that the cascade of events that occur following TBI may lead directly to tau pathogenesis independent of Aβ. This hypothesis is supported by a recent study where tau pathology still occurs when Aβ production is blocked (Tran et al., 2011a). Although in the present study we did not undertake systematic examination tauopathy using specific anti-tau antibodies, the possibility of tauopathy may come from indirect evidence. For example, silver uptake is associated with both 3-repeat and 4-repeat tau deposits (Uchihara et al., 2005). Silver uptake into damaged axons in white matter tracts is by far the most sensitive histological marker of injury in our weight drop mrTBI model as it is detectable even in the absence of marked microglial activation or cell death. This suggests that these white matter tracts may be sites where tau pathology begins after mrTBI. This possibility is further supported by robust silver uptake along with increased tau phosphorylation observed in CHIMERA-TBI (discussed in the next section). Moreover, apoE-/- mice clearly show profoundly greater and prolonged silver staining of damaged axons, suggesting that apoE’s role in repair of damaged neuronal membranes may (in)directly influence pathways that lead to tau phosphorylation. Supporting this hypothesis is our observation that promoting apoE function with GW3965 suppresses silver uptake in WT but not in apoE-/- mice. The influence of apoE on tau phosphorylation is unclear although the current evidence suggests that apoE may inhibit tau phosphorylation by inhibition of tau kinases, including GSK3β (Ohkubo et al., 2003;
Hoe et al., 2006). Thus, it will be now important to determine the interaction between murine as well as human apoE on tau phosphorylation in mrTBI.

Last but not least, although we used the widely-used weight drop model in this study, this model has several caveats, including poor control over mechanical parameters and variable injury outcomes (discussed in Chapter 1). Although currently underappreciated, rigorous biomechanical validation of TBI models is essential to minimize variations in the outcomes and increase their translational potential (Namjoshi et al., 2013a). As described in the Discussion section of Chapter 2, we did not conduct a systematic biomechanical evaluation of the weight drop model. This strongly motivated us to develop a novel TBI model that can mitigate the caveats associated with the weight drop model, as well as allow us to study injury biomechanics. Accordingly in collaboration with the biomechanical engineers at the University of British Columbia, we developed a novel neurotrauma model called CHIMERA that uses completely non-surgical procedure to induce closed head injury in mice. In the Chapter 3 of this thesis, I have discussed the biomechanical, functional as well as neuropathological characterization of CHIMERA.

As will be discussed in the next section of this Chapter, we now have a novel, simple yet highly versatile TBI model that can reliably mimic some neuropathological features of human TBI with high experimental reproducibility. Based on the results discussed in this thesis, we believe that CHIMERA will prove a highly useful and biofidelic model for conducting future studies including those proposed above.
4.3 Chapter 3: Conclusions

The study described in Chapter 3 was not driven by a specific hypothesis but rather describes characterization of a new, simple, and reliable model of murine CHI, CHIMERA that replicates fundamental aspects of human impact TBI through precise delivery of known biomechanical inputs.

CHIMERA is completely nonsurgical and requires only isoflurane anesthesia, therefore enabling immediate neurological severity assessments using LRR and NSS measures. Being nonsurgical, CHIMERA is ideal for studies investigating multiple impacts as well the long-term consequences of impact TBI. These advantages overcome many limitations of surgically-induced TBI models, including longer anesthetic exposure as well as inclusion of opioid analgesics (e.g., buprenorphine) and sedatives like xylazine that can interfere with rapid neurological assessment, low throughput, extensive operator training, and limited suitability for studies involving repetitive TBI or long-term TBI outcomes. CHIMERA produces injury using a simple, reliable and semi-automated procedure that requires < 10 min per animal to produce defined injury. As the biomechanical input parameters are highly adjustable, i.e., across impact energy, velocity, and direction, CHIMERA offers a wide dynamic range of precisely controllable inputs to reproduce the conditions that occur in human impact TBI. Importantly, the kinematic analyses facilitated by CHIMERA enable head motion parameters to be integrated with behavioral and neuropathological outcome measures, potentially enabling improved relevance to human TBI. CHIMERA produces diffuse injury
characterized by activation of inflammatory reactions, axonal damage, and tau phosphorylation, replicating many aspects of human impact TBI without overt focal damage. Taken together, these attributes make CHIMERA a valuable new model to investigate the mechanisms of TBI and for use in preclinical drug discovery and development programs.

We found several differences in the neuropathological outcomes of CHIMERA-rTBI compared to the weight drop rTBI. Thus, silver stain data revealed that the axonal injury in CHIMERA-rTBI was different than that in weight drop rTBI in the terms of extent, intensity and dynamics. In the weight drop rTBI, for example the axonal injury was restricted in the coup and contrecoup white matter tracts below the impact site. In the CHIMERA-rTBI in addition to the coup-contrecoup spread, we found significant axonal pathology at the site distant from the impact site, such as olfactory nerve layer. Qualitative comparison revealed robust axonal pathology in CHIMERA-rTBI compared to mild axonal injury in weight drop rTBI. Lastly, while the axonal pathology in weight drop rTBI resolved over 7-14 days, it continued to increase over the same duration in CHIMERA-rTBI suggesting ongoing secondary injury pathways that may have overwhelmed endogenous recovery mechanisms. CHIMERA-rTBI induced robust microglial activation in the white matter tracts along with moderate increase in the cytokine levels. On the other hand both microglial activation and elevation of cytokine levels were absent in weight drop rTBI. There are several possibilities for these differences. For example the mechanical inputs as well as head support system used in these two models were different that may have affected head kinematics. For example,
while we did not systematically assessed head kinematics in the weight drop rTBI model, a preliminary high-speed video analysis revealed almost no head moment but a crush type of injury following weight drop (data not presented). On the other hand CHIMERA-rTBI allows free head movement that translates into robust, widespread and sustained axonal injury coupled with significant microglial activation and elevated cytokine levels. Moreover, we cannot rule out influence of longer duration of anesthesia as well as effects of ketamine, xylazine, and bupivacaine on the secondary injury pathways in weight drop rTBI. Lastly, while CHIMERA-rTBI induced a transient increase in phosphorylation of endogenous tau, tau phosphorylation in weight drop rTBI was variable (data not shown).

Comparisons of weight drop rTBI and CHIMERA-rTBI models are summarized in the Table 4.1
<table>
<thead>
<tr>
<th>Weight Drop rTBI</th>
<th>CHIMERA rTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TBI Procedure:</strong></td>
<td><strong>TBI Procedure:</strong></td>
</tr>
<tr>
<td>Surgery required</td>
<td>Surgery-free</td>
</tr>
<tr>
<td>Total duration of isoflurane anesthesia: ~ 20 min</td>
<td>Total duration of isoflurane anesthesia: ~ 4 min (without videography) and ~ 8 min (with videography)</td>
</tr>
<tr>
<td>Local anesthetic used: Bupivacaine</td>
<td>No local anesthetic required</td>
</tr>
<tr>
<td>Analgesics used: Meloxicam</td>
<td>Analgesics used: Meloxicam</td>
</tr>
<tr>
<td>Sedatives used: Ketamine and xylazine</td>
<td>No sedatives required</td>
</tr>
<tr>
<td>Total duration of injury procedure: ~ 20 min/animal</td>
<td>Total duration of injury procedure: 4-8 min/animal</td>
</tr>
<tr>
<td>Duration of recovery from anesthesia: 60-90 min</td>
<td>Duration of recovery from anesthesia: 6-10 min</td>
</tr>
<tr>
<td>Mortality rate: 10-15 %</td>
<td>Mortality rate: 3-4 %</td>
</tr>
</tbody>
</table>

**Input Parameters and Head Kinematics:**

<table>
<thead>
<tr>
<th>Weight Drop rTBI</th>
<th>CHIMERA rTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drop height is controlled, however friction and air resistance can have unpredictable influence on the velocity of gravity-driven mass</td>
<td>Input parameters are precisely controlled. Piston velocity is maintained throughout the piston travel by constant air pressure.</td>
</tr>
<tr>
<td>The head support system allows virtually no head movement following impact</td>
<td>The head support system allows free head movement following impact</td>
</tr>
<tr>
<td>Head requires to be held manually under the guide tube that can influence head kinematics following impact</td>
<td>Head does not require manual holding</td>
</tr>
<tr>
<td>High-speed videography requires manual trigger and cannot be easily integrated into the injury procedure</td>
<td>High-speed videography is triggered by CHIMERA impactor and is easily integrated into the injury procedure</td>
</tr>
</tbody>
</table>

**Proposed Injury Mechanism**

<table>
<thead>
<tr>
<th>Weight Drop rTBI</th>
<th>CHIMERA rTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impact-head crush/compression</td>
<td>Impact-head acceleration</td>
</tr>
</tbody>
</table>

**Behavioral Outcomes**

<table>
<thead>
<tr>
<th>Weight Drop rTBI</th>
<th>CHIMERA rTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury induced cognitive deficits from 2-14 d as assessed by NOR</td>
<td>Injury induced cognitive deficits from 2-14 days as assessed by passive avoidance test and Barnes maze</td>
</tr>
<tr>
<td>Injury induced motor deficits from 1-7 d with spontaneous recovery by 14 d.</td>
<td>Injury induced motor deficits from 1-7 d with spontaneous recovery by 14 d.</td>
</tr>
</tbody>
</table>

**Neuropathological Outcomes**

<table>
<thead>
<tr>
<th>Weight Drop rTBI</th>
<th>CHIMERA rTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonal injury restricted to coup-contrecoup white matter under the impact site</td>
<td>Widespread axonal injury including coup-contrecoup white matter as well as site distant from the impact (olfactory bulb)</td>
</tr>
<tr>
<td>Mild axonal injury</td>
<td>Robust axonal injury</td>
</tr>
<tr>
<td>Axonal injury in most of white matter resolves over 7-14 days</td>
<td>Axonal injury continues to increase over 2-14 days</td>
</tr>
<tr>
<td>No microglial activation</td>
<td>Robust microglial activation</td>
</tr>
</tbody>
</table>

**Biochemical Outcomes**

<table>
<thead>
<tr>
<th>Weight Drop rTBI</th>
<th>CHIMERA rTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No change in the proinflammatory cytokines</td>
<td>Moderate and transient increase in the proinflammatory cytokine levels</td>
</tr>
<tr>
<td>Variable changes in phosphorylation of endogenous tau</td>
<td>Transient but significant increase in endogenous tau phosphorylation</td>
</tr>
</tbody>
</table>

(Table 4.1: Comparison of weight drop-rTBI and CHIMERA-rTBI models.)
4.4 Chapter 3: Study Limitations and Future Directions

While CHIMERA offers several advantages over traditional TBI models, we also acknowledge several limitations of the present study, which stem principally from the very nascent nature of the model. An interesting aspect of this study was although several behavioral, inflammatory, and tau responses mostly returned to baseline by 14 days post-injury using the injury parameters used in this study, cognitive performance and axonal pathology remained robust. Understanding the temporal response of the various behavioral, biochemical and neuropathological outcomes used in this study under a variety of injury conditions is now a very important future topic. Thus, the injury characteristics using CHIMERA can be varied by independently adjusting various parameters (e.g., impact energy, number of impacts, duration between successive impacts, impact location and impact direction). Such assessment was beyond the scope of this thesis.

While CHIMERA-TBI allows free head movement following TBI, high speed videos revealed that the impact induced significant hyperflexion of the animal’s neck. It is possible the frontal Velcro straps used to restrain the animal body may lead to significant strain in the neck during such hyperflexion that can result in the spinal cord injury. Indeed during the pilot study, post-mortem examination revealed that approximately 30% of mice injured by CHIMERA develop visible contusions of the cervical and thoracic spinal cord and these animals have significantly worse motor behavior. Moreover the most common reason for the post-TBI mortality (~3.75%)
associated with CHIMERA was the development of hind limb paralysis. While seemingly a caveat of the model, this can be exploited to develop a neurotrauma model that induces either TBI alone or TBI along with spinal cord injury (SCI). Accordingly, future studies are designed to control neck hyperflexion. One way to achieve this is by gradually slowing head acceleration after peak acceleration phase (that is thought to cause TBI) using soft foam. The utility of such model is further highlighted by recent data indicating that TBI is a common co-occurring injury with SCI (Macciocchi et al., 2008). Moreover, up to 30% of neurotrauma cases may also have some degree of combined TBI/SCI, which can go undiagnosed and untreated relative to the most obvious injury (Povolny and Kaplan, 1993). To our knowledge, there is only one animal model of combined TBI/SCI that uses a double surgical method to induce separate TBI and SCI (Inoue et al., 2013). By controlling neck flexion, we will be in a unique position to study either TBI or combined TBI and SCI without any surgical manipulations in a single model.

A major limitation of the present study is short post-TBI follow-up time. While inclusion of longer post-injury time points was beyond the scope of this thesis, ongoing and future studies are designed to address this. While thigmotaxis indicates only anxiety-like behavior, more appropriate tests such as elevated plus maze will be used in future to assess post-TBI anxiety.

Ongoing and future studies are also designed to undertake a detailed injury characterization of CHIMERA to test influence of various input parameters such as full
dynamic range of CHIMERA, comparison of vertical vs lateral head impact and varying the number or intervals of repetitive impacts. As mentioned previously, the wide impact energy range and relatively simple design makes CHIMERA easily adaptable for studying TBI in large rodents such as rats, which have been the traditional rodent model of choice for most TBI studies due to their superior behavioral repertoire and larger brains for imaging. Future studies are planned to compare injury responses in mice and rats using CHIMERA.

Notwithstanding these limitations, the present study indicates that CHIMERA will be a valuable neurotrauma model for conducting both the mechanistic as well as pharmacological studies.

Taken together, the first study described in this thesis shows that the synthetic LXR agonists may be further developed as a potential treatment for mild TBI. While this study indicates that the beneficial effects of LXR in TBI are mediated partially through apoE-dependent mechanisms there are several unanswered questions related to the mechanism. We believe that these questions can be addressed in future studies using a highly innovative neurotrauma model, CHIMERA described in the second study of this thesis.
Bibliography


GA: Centers for Disease Control and Prevention, National Center for Injury Prevention and Control.


261


pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J Biol Chem 277:34182-34190.


Irizarry MC, Cheung BS, Rebeck GW, Paul SM, Bales KR, Hyman BT (2000) Apolipoprotein E affects the amount, form, and anatomical distribution of amyloid beta-


Neubuerger KT, Sinton DW, Denst J (1959) Cerebral atrophy associated with boxing. AMA Arch Neurol Psychiatry 81:403-408.


of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's

Scalfani MT, Dhar R, Zazulia AR, Videen TO, Diringer MN (2012) Effect of osmotic
27:526.e527-512.

Scheuner D et al. (1996) Secreted amyloid beta-protein similar to that in the senile
plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP

explain the reverse perivascular transport of solutes out of the brain. J Theor Biol
238:962-974.

profile and phosphorylation state in dementia pugilistica recapitulate Alzheimer's

Schmitz G, Langmann T (2001) Structure, function and regulation of the ABC1 gene


Tai LM, Bilousova T, Jungbauer L, Roeske SK, Youmans KL, Yu C, Poon WW,
Cornwell LB, Miller CA, Vinters HV, Van Eldik LJ, Fardo DW, Estus S, Bu G, Gylys KH,
Ladu MJ (2013) Levels of soluble apolipoprotein E/amyloid-beta (Abeta) complex are
reduced and oligomeric Abeta increased with APOE4 and Alzheimer disease in a

concentration in cortex and brain stem during the acute phase of experimental closed

Takahashi Y, Smith JD (1999) Cholesterol efflux to apolipoprotein A1 involves
endocytosis and resecretion in a calcium-dependent pathway. Proc Natl Acad Sci USA
96 11358-11363.

Takhounts EG, Crandall JR, Darvish K (2003) On the importance of nonlinearity of brain

Tall AR (2008) Cholesterol efflux pathways and other potential mechanisms involved in

Tanaka AR, Ikeda Y, Abe-Dohmae S, Arakawa R, Sadanami K, Kidera A, Nakagawa S,
Contains a Large Amino-Terminal Extracellular Domain Homologous to an Epitope of


mRNA and protein distribution patterns predict multiple different roles and levels of regulation. Lab Invest 82:273-283.


Zuccarello M, Iavicoli R, Pardatscher K, Cervellini P, Fiore D, Mingrino S, Gerosa M