THE INTERACTION OF REPETITIVE MILD TRAUMATIC BRAIN INJURY, ALZHEIMER DISEASE AND AGEING

by

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Abstract

Traumatic brain injury (TBI) is an important public health issue worldwide. It is strongly linked to neurodegenerative conditions such as Alzheimer’s Disease (AD) and Chronic Traumatic Encephalopathy. Despite numerous promising pre-clinical studies, there is no effective clinical treatment for TBI, indicating inefficiency in translation of scientific research from bench to bedside.

This thesis thus attempts to address the issue through two approaches. Firstly, we reviewed the commonly-used pre-clinical TBI models, and found that many lack thorough biomechanical considerations. We thus developed a new mouse TBI model, Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA). CHIMERA reproducibly induces clinically-relevant TBI, both in terms of biomechanics and neuropathology. CHIMERA mild TBI (mTBI) induces behavioural (e.g. neurological, motor, and cognitive), histological and biochemical changes (e.g. diffuse axonal injury, white matter microgliosis, and brain cytokine induction). We further demonstrated that CHIMERA mTBI outcomes are scalable by varying the mechanical inputs. These observations demonstrate that CHIMERA is a novel and valuable platform for TBI research.

Next, we induced CHIMERA mTBI in APP/PS1 mice, a transgenic model of AD amyloidosis, and characterised both acute and long-term consequences. Here we included two age groups of animals, as TBI occur in both the young and the old populations. In the acute phase, mTBI led to subtle and transient age-dependent changes in Aβ deposits. Age-at-injury and genotype showed complex interactions in determining microglial and cytokine outcomes, such that neuroinflammation was increased in old wildtype mice and young APP/PS1 mice. Age-at-injury also markedly affected neurofilament response, as neurofilament-positive axonal bulbs and plasma neurofilament-light levels were elevated in young mice, but not old mice, of both genotypes. In the chronic phase, mTBI led to prolonged white matter microgliosis and axonal injury up to 8-mo post-injury. MTBI also intensified long-term fear memory in APP/PS1 mice, reminiscent of post-traumatic stress disorder phenotypes.
In summary, we have developed a reproducible and clinically-relevant TBI model. We showed that genetic predisposition to AD and age-at-injury are both significant modifiers of acute and long-term mTBI outcomes. These findings may provide insights for future attempts in understanding the mechanistic pathways of TBI pathogenesis.
Lay Summary

Traumatic brain injury (TBI) is an important public health issue. In the US, its annual incidence is 715 per 100,000. TBI can occur to people of all ages, including infants (e.g. abuse), young adults (e.g. sports injury, motor vehicle accident), and the elderly (e.g. fall). Moderate and severe TBI may result in devastating physical and cognitive disabilities. Recent studies also showed that repetitive exposure to mild TBI may lead to brain degeneration. However, it is unclear how TBI outcomes can be affected by age or genetic factors. In this thesis we first developed a research TBI model that integrates biomechanical considerations and produces reproducible and clinically-relevant pathologies. We then characterised the acute and long-term consequences of repetitive mild TBI using a transgenic mouse model of Alzheimer’s disease. We find that age-at-injury and Alzheimer’s disease genetics can modify TBI outcomes including brain inflammation, white matter injury, and post-traumatic stress disorder-like phenotypes.
Preface

All of the work presented henceforth was conducted at the Djavad Mowafaghian Centre for Brain Health, Centre for Disease Modeling, or Child and Family Research Institute at the University of British Columbia. All projects and associated methods were approved by the University of British Columbia’s Research Ethics Board:

- Biosafety approval: B14-0136 (Factors affecting the risk of Alzheimer’s Disease)
- Animal care approval: A13-0036 (Breeding Protocol); A15-0096 and A11-0225 (ABCA1 and ApoE function in Traumatic Brain Injury)
- Personal animal care and safety training certifications: Canadian Council on Animal Care (5668-12); Rodent Husbandry (RBH-228-12); Rodent Anaesthesia (RA-140-12); Rodent Surgery (RSx-104-12); Biological Safety (LB-2012-125709); Chemical Safety (CS-2012-125709)

Portions of Chapter 1 are reproduced from the published review article: Namjoshi DR, Good C, Cheng WH, Panenka W, Richards D, Cripton PA, Wellington CL (2013) Towards clinical management of traumatic brain injury: a review of models and mechanisms from a biomechanical perspective. Disease Models & Mechanisms 6: 1325-1338. I am a co-author of this article and contributed to paragraphs reviewing different traumatic brain injury models. 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.

as Dhananjay R Namjoshi. The CHIMERA design was conceived by Cheryl L. Wellington, Peter A. Cripton, Dhananjay R. Namjoshi, Wai Hang Cheng, and Kurt McInnes. Dhananjay R Namjoshi and Wai Hang Cheng were responsible for designing and conducting experiments and analyzing the results. Support for animal head injury procedures 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 kinematic analysis was performed by Wai Hang Cheng, with expert advice from Kurt McInnes and Peter A. Cripton. Biochemical assays were conducted by Anna Wilkinson and Jianjia Fan. Histology experiments were conducted by Dhananjay R. Namjoshi and Wai Hang Cheng, with support from Michael Carr, Jerome Roberts and Arooj Hayat. The manuscript was prepared by Dhananjay R. Namjoshi, Wai Hang Cheng, and Cheryl L. Wellington.

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List of Symbols

cm  Centimetre

\(d\)  Day

fps  Frames per Second

\(g\)  Gram

\(g\)  Earth Gravitational Acceleration

\(h\)  Hour

\(J\)  Joules

kHz  Kilo Hertz

kPa  Kilo Pascal

\(\text{krad/s}^{2}\)  Kilo Radian per Second

\(\text{m/s}\)  Metre per Second

\(\text{min}\)  Minute

\(\text{mo}\)  Month

\(\text{mm}\)  Millimetre

\(\text{ms}\)  Millisecond

\(\text{rad}\)  Radian

\(\text{rad/s}\)  Radian per Second

\(\text{s}\)  Second

\(\text{yr}\)  Year
List of Abbreviations

$[^{11}C]$-PiB $[^{11}C]$ Pittsburgh Compound B
Aβ Amyloid beta
ABCA1 ATP-Binding Cassette Transporter A1
AD Alzheimer Disease
AICD APP Intracellular Domain
AIS Abbreviated Injury Score
ANOVA Analysis of Variance
AOS APP/PS1-Old-Sham Group
AOT APP/PS1-Old-TBI Group
APOE Apolipoprotein E
APP Amyloid Precursor Protein
AYS APP/PS1-Young-Sham Group
AYT APP/PS1-Young-TBI Group
BBB Blood Brain Barrier
BDNF Brain-derived Neurotrophic Factor
BM Barnes Maze
BrIC Brain Injury Criteria
BSC Brachium of Superior Colliculus
CA Cornu Ammonis
CAA Cerebral amyloid angiopathy
CBF Cerebrovascular Blood Flow
CC Corpus Callosum
CCI Controlled-Cortical Impact
CD Cluster of Differentiation
CDC Centers for Disease Control
CHI Closed-Head Injury
CHIMERA Closed-Head Impact Model of Engineered Rotational Acceleration
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<td>CTE</td>
<td>Chronic Traumatic Encephalopathy</td>
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<td>CTF</td>
<td>C-terminal Fragment</td>
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<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
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<td>Cx</td>
<td>Cortex</td>
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<tr>
<td>DAI</td>
<td>Diffuse Axonal Injury</td>
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<tr>
<td>DAMP</td>
<td>Danger-Associated Molecular Patterns</td>
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<tr>
<td>DoD/VA</td>
<td>Department of Defence / Veteran Affairs</td>
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<tr>
<td>DTI</td>
<td>Diffusion Tensor Imaging</td>
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<tr>
<td>EDH</td>
<td>Epidural Hemorrhage</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
<td>EPM</td>
<td>Elevated Plus Maze</td>
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<tr>
<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<tr>
<td>FPI</td>
<td>Fluid Percussion Injury</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
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<tr>
<td>FDDNP</td>
<td>2-[(6-[(2-[(fluorine-18]fluoroethyl)(methyl)amino]-2-naphthyl)-ethylidene]malononitrile</td>
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<tr>
<td>FPS</td>
<td>Frames per Second</td>
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<tr>
<td>GCS</td>
<td>Glasgow Coma Scale</td>
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<td>GFAP</td>
<td>Glial Acidic Fibrillary Protein</td>
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<tr>
<td>GOS</td>
<td>Glasgow Outcome Scale</td>
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<tr>
<td>GOS-E</td>
<td>Glasgow Outcome Scale Extended</td>
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<tr>
<td>GuHCl</td>
<td>Guanidinium Hydrochloride</td>
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<tr>
<td>GWAS</td>
<td>Genome-wide Association Studies</td>
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<tr>
<td>HIC</td>
<td>Head Injury Criterion</td>
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<tr>
<td>Iba1</td>
<td>Ionized Calcium Binding Adapter Molecule 1</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<td>ISF</td>
<td>Interstitial Fluid</td>
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<tr>
<td>JNK</td>
<td>c-Jun-N-terminal</td>
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<tr>
<td>LGN</td>
<td>Lateral Geniculate Nucleus</td>
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<tr>
<td>LOC</td>
<td>Loss of Consciousness</td>
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<tr>
<td>LRP1</td>
<td>Low-Density Lipoprotein Receptor-Related Protein 1</td>
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<td>LRR</td>
<td>Loss of Righting Reflex</td>
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<td>LXR</td>
<td>Liver X Receptor</td>
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<td>MAPT</td>
<td>Microtubule-Associated Protein Tau</td>
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<td>MCI</td>
<td>Mild Cognitive Impairment</td>
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<td>MG</td>
<td>Microglia</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<td>MMSE</td>
<td>Mini-Mental State Score</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>mTBI</td>
<td>Mild Traumatic Brain Injury</td>
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<tr>
<td>MVA</td>
<td>Motor Vehicle Accident</td>
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<tr>
<td>NFL</td>
<td>National Football League</td>
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<td>NFT</td>
<td>Neurofibrillary Tangle</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<td>NGS</td>
<td>Normal Goat Serum</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>NSS</td>
<td>Neurological Severity Score</td>
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<tr>
<td>ON</td>
<td>Olfactory nerve layer</td>
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<tr>
<td>OR</td>
<td>Odd Ratio</td>
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<tr>
<td>OT</td>
<td>Optic Tract</td>
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<tr>
<td>PA</td>
<td>Passive Avoidance</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Patterns</td>
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<tr>
<td>PD</td>
<td>Parkinson Disease</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCS</td>
<td>Postconcussive Syndrome</td>
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<tr>
<td>PECAM</td>
<td>Platelet Endothelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired Helical Filament</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
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<td>PND</td>
<td>Postnatal Day</td>
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<td>PS</td>
<td>Presenilin</td>
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<tr>
<td>PTSD</td>
<td>Post-Traumatic Stress Disorder</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmoprecipitation Assay</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>rad</td>
<td>Radiance</td>
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<tr>
<td>rTBI</td>
<td>Repetitive Traumatic Brain Injury</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SAH</td>
<td>Subarachnoid Hemorrhage</td>
</tr>
<tr>
<td>SDH</td>
<td>Subdural Hematoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
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<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
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<tr>
<td>SDH</td>
<td>Subdural Hematoma</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic Brain Injury</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>ThioS</td>
<td>Thioflavin S</td>
</tr>
<tr>
<td>rTBI</td>
<td>Repeated Traumatic Brain Injury</td>
</tr>
<tr>
<td>TDP-43</td>
<td>Transactive Response DNA Binding Protein 43</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>vmPFC</td>
<td>ventromedial prefrontal cortex</td>
</tr>
<tr>
<td>WD</td>
<td>Weight Drop</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>WOS</td>
<td>Wildtype-Old-Sham Group</td>
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<td>WOT</td>
<td>Wildtype-Old-TBI Group</td>
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<tr>
<td>WSTC</td>
<td>Wayne State Tolerance Curve</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>WYS</td>
<td>Wildtype-Young-Sham Group</td>
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<tr>
<td>WYT</td>
<td>Wildtype-Young-TBI Group</td>
</tr>
<tr>
<td>VLDLR</td>
<td>Very low density lipoprotein receptor</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula Occludens</td>
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</table>
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Words can hardly express my gratitude for all the help and encouragement I have received during my PhD research and during the writing of this thesis. It would not have been possible for me to finish my studies without all the supports I have obtained, be they academic, technical, financial, or emotional.

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Wai Hang Cheng, Tom

“The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way,
Where many paths and errands meet.
And whither then? I cannot say.’’

by J.R.R. Tolkien
For my mother,

and all people with neurodegeneration or brain injury, including my grandaunt,

and their loved ones,

and the caretakers
Chapter 1: Introduction

1.1 Overview of epidemiology, classification, and research of TBI

1.1.1 Definition, epidemiology and significance of TBI

A commonly cited definition of Traumatic Brain injury (TBI) is “an alteration in brain function, or other evidence of brain pathology, caused by an external force”, where the “alteration in brain function” includes a decreased level of consciousness, which may not necessarily be loss of consciousness, post-traumatic amnesia, neurological deficits, or any alteration in mental state at the time of injury. (Menon, Schwab et al. 2010) Similar definitions are used by other organisations. For example, according to the Centers for Disease Control and Prevention, “a TBI is caused by a bump, blow or jolt to the head or a penetrating head injury that disrupts the normal function of the brain.” (Centers for Disease Control and Prevention 2016)

TBI has a high incidence rate and incurs a tremendous socio-economic cost worldwide. In North America, the annual incidence of TBI is over 3 million. (Statistics Canada 2015, Centers for Disease Control and Prevention 2016) Every year in the US alone, TBI results in over 2 million emergency department visits (715 per 100,000). A similar magnitude of annual TBI incidence is reported in other regions including Europe (262 per 100,000), India (160 per 100,000), Korea (467 per 100,000) and New Zealand (790 per 100,000). (Gururaj 2002, Feigin, Theadom et al. 2013, Peeters, van den Brande et al. 2015, Kim, Chung et al. 2016) In addition to the high incidence rate, TBI may also result in long-term disabilities and may incur huge socio-economic cost. Annually in the US, there are 120,000 cases of TBI-induced long-term disability (43 per 100,000) (Selassie, Zaloshnja et al. 2008), and 50,000 deaths (17 per 100,000) (Centers for Disease Control and Prevention 2016). The annual economic cost of TBI is over $60 billion in the US (lifetime medical cost: $9 billion; total productivity loss: $51 billion) (Finkelstein 2006) and over €33 billion in Europe (Olesen, Gustavsson et al. 2012). Furthermore, it is very likely that these reported numbers are underestimations of the real situation. TBI is often referred to as a “silent epidemic” (Goldstein 1990, Rusnak 2013), because, until recently, many cases of TBI (particularly mild TBI (mTBI)) were not recognized as an important medical condition by both
the general public and medical professionals. Taken all together these observations demonstrate that TBI is an immediate and alarming global public health concern.

Despite the high incidence and enormous socio-economic cost, currently there are no effective pharmacological treatments for TBI. (Menon and Maas 2015) Thus far, all TBI therapeutic agents have failed phase III clinical trials, including the highly anticipated progesterone studies. (Skolnick, Maas et al. 2014, Wright, Yeatts et al. 2014) The lack of any effective pharmacological intervention further emphasizes the dire need to improve our understanding on the pathophysiology of TBI for both acute and chronic time periods.

The work presented in this thesis is thus aimed at addressing several key knowledge gaps that must be solved to make meaningful progress in TBI research. The first step, which is covered in Chapters 2 and 3, is to develop a TBI research animal model that accurately reflects human TBI and can act as a platform for future TBI research. The second knowledge gap, which is covered in Chapters 4 and 5, concerns our insufficient understanding of both acute and long-term results of repetitive mTBI, particularly in an animal model with genetics predisposed to the development of neurodegenerative disease. The third gap, which is also covered in Chapter 4 and 5, encompasses the interactions of common confounding factors in TBI research.

To be more specific with our hypotheses, in Chapter 2, we hypothesized that by developing a novel experimental TBI model that can reproducibly perform TBI in animals through clinically relevant injury mechanisms, we will be able to improve our understanding on TBI pathophysiology, in various aspects including post-injury behavioral changes, inflammation and axonal injury. In Chapter 3, we further hypothesized that the severity of injury outcomes of our TBI model (e.g. behavioral, inflammation, and axonal injury) is scalable and can be adjusted by varying the level of biomechanical inputs when the TBI is performed. In Chapter 4, we hypothesized that acute TBI outcomes can be significantly affected by factors including age-at-injury and genetic predisposition to neurodegeneration, by using our new TBI platform with an AD transgenic mouse model. In Chapter 5, using the same TBI model and the same mouse strain, we hypothesized that chronic TBI outcomes can be significantly affected by genetic predisposition to neurodegeneration.

The depth, breadth, and innovation in this thesis makes a substantial contribution to our understanding of TBI pathophysiology and delineates several areas for future studies that sheds
may one day lead to effective treatments. The following sections discuss the etiology and classification of TBI and provide an introduction to the acute and chronic TBI-related neuropathologies and neurodegenerative changes.

1.1.2 Classification of TBI
TBI is an umbrella term that includes all forms of injuries to the brain as a result of a physical force. As a result, TBI is an intrinsically heterogeneous entity. Depending on the context, there are a number of non-exclusive systems that the clinical and scientific communities have used to classify TBI.

1.1.2.1 Classification of TBI by injury severity
In the clinical setting, TBI severity is traditionally classified using the Glasgow Coma Scale (GCS) within 48 h of injury. Although widely used, it is important to note that the GCS was developed as a clinical tool to mainly assess the level of consciousness only. The GCS is composed of three categories, including eye opening, motor response and verbal response (Teasdale 1974), each of which containing 4 to 6 items, resulting in a minimum possible score of 3 and a maximum possible score of 15. A lower GCS means more extensive loss of responses and suggests more severe injury. A TBI resulting in GCS of 3-8 is considered severe TBI, 9-12 as moderate TBI, and 13-15 as mild TBI (mTBI). (Rimel, Jane et al. 1979, Rimel, Giordani et al. 1982) Despite its widespread use, the GCS has been criticized for its low inter-observer reliability, and failure to provide valid measurement for patients with comorbid physiological impairments common in TBI such as facial injuries or intoxication. (Marion and Carlier 1994, Teasdale, Maas et al. 2014) The GCS is thus now widely recognized as imprecise and insufficiently granular to assess severity, particularly for mTBI, a gap that is driving considerable efforts to define neuroimaging or fluid biomarkers to assist in diagnostic classification. (See Table 1.1)

Another commonly used scale in the clinical setting, particularly for cases that involve multiple traumas such as motor vehicle accidents, is the head portion of the Abbreviated Injury Score (AIS). (Baker, O'Neill et al. 1974, Copes, Lawnick et al. 1988, Gennarelli and Wodzin 2006)
The AIS is an anatomically based system that uses a separate score for each body region (head, face, neck, or lower extremities), and scores each region from 1-6 based on neuroradiology or operative findings. (Savitsky, Givon et al. 2016) An AIS of 1 is classified as minor injury, 2 as moderate injury, 3 as serious injury, 4 as severe injury, 5 as critical injury and 6 as maximum (fatal) injury. An AIS_{head} of 1 (excluding superficial contusion to the head) is often considered as mTBI, AIS_{head} 2 as moderate TBI, and 3 or above as severe TBI. However, the use of AIS is limited by the fact that there is no absolute standardization on injury severity. (Savitsky, Givon et al. 2016)

Other systems have been developed to expand TBI severity scales. A frequently used one is based on the definition by the American Congress of Rehabilitation (American Congress of Rehabilitation Medicine 1993) and the Department of Defence / Veteran Affairs (DoD/VA) Definition and Symptomatic Taxonomy Working Group (O'Neil 2013). In this system, TBI is classified based on both GCS and supplemented with clinical signs such as duration of loss of consciousness and amnesia. (See Table 1.3)

Most recently, there are several schemes that attempt to further refine and sub-categorize mTBI. (Kamins and Giza 2016) For example, “complicated mTBI” is defined as mTBI combined with intracranial imaging findings. “Concussion” is defined as a clinical syndrome that “transiently disturbs normal brain function, causing neurological-cognitive-behavioral signs and symptoms”. (Kamins and Giza 2016) “Subconcussion” is a condition that causes “subclinical injury in the absence of overt acute signs and symptoms”. (Kamins and Giza 2016) These definitions are in flux, and many expect that the current clinical classification scheme will eventually be replaced by one based on biomarkers that more closely reflects the underlying pathophysiology. (Saatman, Duhaime et al. 2008, Diaz-Arrastia, Kochanek et al. 2014)

In summary, there is no definitive biomarker for TBI, and more particularly so in mild cases. Therefore, clinically TBI is often classified based on severity, as mild, moderate, or severe. However such definitions are neither objective nor quantitative. Regardless of the differences in definition, however, all epidemiological studies show that mTBI is the most common form of TBI (58%-80%), and moderate and severe TBI are of comparable frequency (8-25% and 10-20%, respectively). (Bruns and Hauser 2003)
1.1.2.2 Classification of TBI by injury mechanism and injury pattern

In addition to classification based on severity, TBI can also be classified based on injury patterns (open head vs. closed head; focal vs. diffuse), or by injury mechanisms (penetrating, blunt, inertial, or blast). These non-mutually exclusive classifications provide some information to the mode in which the external force is applied, and is particularly useful to TBI research as injury mechanisms are often independent of injury severity and may result in different injury outcomes.

- **By injury pattern**
  - an open head injury occurs when the integrity of the skull and dura is breached;
  - a closed head injury occurs when there is no dural breach;
  - a focal TBI is one that results in damage or compression of tissue under the cranium at the site of impact (coup) or of tissue opposite to the impact (contre-coup); (Andriessen, Jacobs et al. 2010)
  - a diffuse or multifocal TBI is one that involves widespread damages in the brain, such as in the form of diffuse axonal injury, diffuse vascular injury, hypoxic ischemic injury, or brain swelling; (Andriessen, Jacobs et al. 2010)

- **By injury mechanism**
  - a penetrating TBI is an open head injury in which a foreign body, such as a knife or a bullet (ballistic injury), enters the brain parenchyma; (Chandra and Sundaramurthy 2015, de Lanerolle, Kim et al. 2015)
  - a blunt TBI involves direct contact between the head and a moving or stationary object; (Chandra and Sundaramurthy 2015, de Lanerolle, Kim et al. 2015)
  - an inertial injury occurs when the injury results from sudden acceleration or deceleration of the brain; (McAllister 2011)
  - a blast injury occurs when a person is exposed to an explosion, receiving injuries directly due to blast waves, or from subsequent flying fragments or collision. (de Lanerolle, Kim et al. 2015, Mathews and Koyfman 2015)
In addition, TBI can also be classified based on the presence or absence of clinical symptoms, such as neuroimaging results by computed tomography (CT), which may indicate the presence of mass lesions and structural damage, and a raise in intra-cranial pressure. (Maas, Stocchetti et al. 2008) However, since mass structural lesions are not common features of TBI, and CT scans may not reveal the more intricate damages such as axonal injury, this classification is only auxiliary to the standard classifications.

1.1.3 Etiology and age/sex distribution of TBI occurrences
The cause of TBI is highly heterogeneous. According to the classification system used in by the Centers for Disease Control and Prevention (CDC) (Faul, Xu et al. 2010), “falls” are the major cause that contributes to 35.2% of all TBI cases. The second major cause is “motor vehicle-traffic” accidents, which contributes to 17.3% of TBI cases. Other causes included “struck by/against”, which contributes to 16.5%, and “assault”, which contributes to 10% of TBI cases. Sports-related TBI, which can be classified as “falls” or “struck by/against” in the CDC system, has been reported to contribute roughly 10% of all TBI. (Nolan 2005, Selassie, Wilson et al. 2013)

Although TBI can occur to any person at any age, studies have shown that three particular age groups are at highest risk. Very young children (0-4 yr) have the highest incidence of TBI, followed by older adolescents (15-24 yr) and older adults (65 yr and above). (Faul 2010) However, the highest rates of TBI-related hospitalization and death are found among older adults (65 yr and above), indicating that the aged brain may be far less able to mount a repair and recovery response to TBI than the young brain. Support for this hypothesis is covered in Chapters 4 in this thesis. Falls are the most common cause of TBI for both very young children and older adult groups (64.2% and 60.7%, respectively), while motor vehicle-traffic accidents and assaults account for most of the TBI in older adolescents (30.8% and 20.3%). Reports have also shown that TBI occurs 1.4 times more in males than in females. (Faul 2010) This is true for almost all age groups with the exception of the older adults group.
1.1.4 Risk factors that affect TBI outcomes

1.1.4.1 Age at injury

Several clinical studies report that age at injury plays a significant role in affecting TBI outcomes and recovery. Despite a lower rate of TBI among people older than 75 yr of age, this group of patients have the highest rates of post-TBI hospitalizations and mortality. (Faul 2010)

Longitudinal studies show that, compared to younger patients, persons over age 65 yr have poorer functional outcomes at discharge and at 6-month post-injury. (Mosenthal, Lavery et al. 2002, Mosenthal, Livingston et al. 2004) Other studies have also shown that older patients may show worse health status, more disability and more behavioral impairments, up to 1-2 yr post-TBI. (Testa, Malec et al. 2005, Forsslund, Roe et al. 2013, Scholten, Haagsma et al. 2015) A systematic review involving 35 publications on 14 TBI cohorts has concluded that that older age at injury is a strong predictor for ongoing disability. (Willemse-van Son, Ribbers et al. 2007)

However, TBI outcomes can be at least partly improved in the elderly by using appropriate clinical management (i.e. timely MRI) (Stocchetti, Paterno et al. 2012), suggesting that even in the elderly TBI patients may return to independent living.

On the other hand, clinical studies on mTBI show that children or young adolescents may also show prolonged resolution of acute TBI symptoms and recovery of impaired memory and processing speeds compared to young adults (> 7 days vs 3-5 days). (Field, Collins et al. 2003, Sim, Terryberry-Spoehr et al. 2008, Zuckerman, Lee et al. 2012) Children and youth with TBI also have higher frequencies of complications such as hematomas, or delayed development of secondary pathologies such as seizure. (Snoek, Minderhoud et al. 1984, Harmon, Drezner et al. 2013) These studies highlight the fact that developmental maturation is an important variable for TBI outcomes, which may encompass both physiological (i.e. completion of myelination) and psychosocial (i.e. achievement of executive function) components.

Cho et al. recently performed gene expression analysis on the acute phase peripheral blood from TBI patients (mostly mTBI), either between 19-35 yr or 60-89 yr of age (n = 33 per age group). (Cho, Latour et al. 2016) They found that the younger group had higher expression of genes related to inflammatory regulation (e.g. basic leucine zipper transcription factor 2, leucine-rich repeat neuronal 3, and lymphoid enhancer-binding factor 1). On the other hand, the older group had a higher expression of S100 proteins, which are linked to poor recovery from TBI, and a
reduced expression of noggin, which is linked to neuroregeneration. This study provides further support that age-at-injury is an important factor in determining TBI outcomes.

In experimental TBI, reports using fluid percussion injury (FPI, a TBI model that involves direct impact of a fluid pulse on the dura, see Section 1.1.6.1.1) have shown that TBI in aged rats (20-mo vs 3-mo) induced greater mortality, longer suppression of righting and escape behavior, and more exacerbated motor and cognitive deficits. (Hamm, Jenkins et al. 1991, Hamm, White-Gbadebo et al. 1992) Other studies using controlled cortical impact models (CCI, a focal contusion model, see Section 1.1.6.1.1) showed that TBI in aged mice (22-mo vs. 6-mo) induced more pronounced and prolonged activation of microglia and astrocytes in hippocampus (Sandhir, Onyszchuk et al. 2008), as well as greater motor deficits and prolonged compromise of the blood brain barrier (BBB) (Onyszchuk, He et al. 2008).

Experimental TBI studies with immature animals suggest that sparing of TBI-induced behavioral deficits may occur in younger animals. CCI in postnatal day (PND) 17 rats (juvenile) vs PND28 rats (adolescent) showed that at 14 days post-injury, Morris water maze testing revealed spatial learning and memory deficits in the PND28 cohort, but not the PND17 cohort. (Prins and Hovda 1998) Adelson et al. compared different severities of CCI in PND7 (infantile) vs PND17 rats. They showed that up to post-injury day 17, the PND7 group may have higher injury threshold (less Morris water maze learning deficits at low severity injury) than the PND 17 group. (Adelson, Fellows-Mayle et al. 2013) Another study compared suction injury in PND35 or PND2, PND5, or PND9 rats, and showed that at 3-4 mo post-injury, spatial reversal and delayed responses tasks were impaired in the PND35 cohort, but not in the young cohorts.

Histological deficits in young animals were less consistent. Studies comparing closed head injury (CHI) in PND11 vs PND17 rats showed at 14 days to 28 days post-injury, greater hippocampal atrophy, larger ventricles, white matter injury and gliosis were found in the PND11 group but not the PND17 group. In addition, the hemorrhagic cortical contusion in PND11 group subsequently atrophied, but in the PND17 group it developed into a cavity with glial scar. (Huh and Raghupathi 2007, Raghupathi and Huh 2007) In contrast, Casella et al. showed that CCI induced a greater decrease of dendritic length and branching of hippocampal CA1 neurons in PND17 rats but not PND7 rats, at 28 days post-injury. (Casella, Thomas et al. 2014) All together, these
studies suggest that while the immature brain may vulnerable to TBI, they may also have greater plasticity or compensatory capacities for functional recovery.

1.1.4.2 Pre-injury functioning
Cognitive reserve refers to individual differences in recruiting neural networks or cognitive processes that are employed to complete a task. It has been used to explain individual differences to TBI outcome. Cognitive reserve is often evaluated using measures of cognitive ability such as educational or occupational achievement and premorbid intelligence quotient. The passive reserve model emphasizes on the amount of damage that one can sustain before showing functional impairments. The active model of reserve describes the ability of the brain to flexibly utilize resources after injury, thus compensating for damages. (Stern 2002, Bartres-Faz and Arenaza-Urquijo 2011, Bigler 2015, Mathias and Wheaton 2015) A meta-analysis of 90 existing human non-penetrating TBI studies, with 8856 human participants, has shown that both higher pre-injury education level and IQ contribute to a small, but significant, improvement in recovery. (Mathias and Wheaton 2015)

1.1.4.3 Sex
The influence of sex on TBI outcomes is so far inconclusive. However, a number of studies have shown that female patients have better post-TBI outcomes including cognitive performance. (Saban, Smith et al. 2011, Rigon, Turkstra et al. 2016) However, the results have been contradicted by other studies based on post-injury symptoms, such as headache, concentration difficulty and light sensitivity. (Bazarian 2010, Covassin, Elbin et al. 2013, Scholten, Haagsma et al. 2016, Dillard, Ditchman et al. 2017) A systematic review that involves 35 publication from 14 human TBI cohorts concluded that there was insufficient evidence to support sex as a significantly predictor of TBI outcomes at 1 yr post-injury. (Willemse-van Son, Ribbers et al. 2007) A more recent meta-analysis involving 91 publications with 3801 concussion athletes and 5631 controls reported that females showed more neuropsychological deficits than males after TBI. (Dougan, Horswill et al. 2014) Another recent systematic review involving 16 publications from 14 human TBI cohorts reported that sex may differentially affect mTBI symptoms (Cancelliere, Donovan et al. 2016), as young females have higher rates of epilepsy, suicide risk
and pain sensitivity, whereas males have a higher risk for schizophrenia. However, there was no sex difference found for postconcussion symptoms or dementia risk.

1.1.4.4 Repetitive exposures to TBI

Studies have shown that exposure to TBI may have cumulative effects. Many groups have compared TBI cases (mostly concussions in young athletes) with a prior history of multiple previous concussions to control cases (TBI with no previous history of concussion). (Gronwall and Wrightson 1975, Gaetz, Goodman et al. 2000, Collins, Lovell et al. 2002, Guskiewicz, McCrea et al. 2003, De Beaumont, Lassonde et al. 2007, Covassin, Moran et al. 2013) They found that cases with previous concussions had a higher chance of sustaining a new concussion, and when concussed, displayed more symptoms and neurological / electrophysiological deficits (e.g. loss of consciousness, amnesia, confusion, migraine-cognitive-fatigue symptoms, verbal memory and reaction time deficits, etc). They also had a slower recovery after a new concussion. In boxing (Jordan, Relkin et al. 1997), football (McKee, Stern et al. 2013) and military personnel (Meabon, Huber et al. 2016), exposure to TBI (number of bouts, number of years played, and number of blast or other TBI experienced, respectively) has been associated with worsened chronic pathologies. In addition, in some occasions, the “second impact syndrome” may occur to a human sustaining a second concussion. This condition refers to the situation when a second concussion, which happens before a prior concussion has resolved, causes a catastrophic brain injury (often cerebral edema or death). There is no specific time interval between the two concussions, but it is often stated as within a few weeks. Such incidences have mainly been reported in case studies on sports athletes (e.g. football, ice hockey, and boxing). However, the exact incidence or mortality rate of such occurrences is currently unknown. (Stovitz, Weseman et al. 2017)

Repetitive TBI in animal models (such as CHI or blast) has also been shown to induce greater pathological changes, including neurological and motor deficits, axonal injury, microglia activation, astrogliosis, degenerating neurons, disruption of BBB integrity, disruption of brain glucose metabolism. (Laurer, Bareyre et al. 2001, Shitaka, Tran et al. 2011, Bolton and Saatman 2014, Luo, Nguyen et al. 2014, Meabon, Huber et al. 2016)
1.1.4.5 Other socio-environmental factors
A number of prospective studies have shown that increased number of social-environmental risks, such as parental mental health or low income, is associated with poorer behavioral and cognitive recovery, particularly in pediatric TBI cases (Gerring and Wade 2012).

1.1.4.6 Apolipoprotein E
Apolipoprotein E (apoE) the major lipoprotein in the central nervous system (CNS) is composed of 299 amino acids and is responsible for the transport of phospholipids and cholesterol. It is mainly synthesized by astrocytes, pericytes and, to a less extent, microglia. (Fan, Donkin et al. 2009, Hirsch-Reinshagen, Donkin et al. 2009, Kim, Basak et al. 2009, Gibbons, Udawela et al. 2011) ApoE is important in synapse formation, cytoskeleton restructuring, and repair of damaged neuronal membranes. Humans have three major apoE isoforms; apoE2, apoE3, and apoE4. They are transcribed by 3 alleles of the APOE gene: APOEε2, APOEε3, and APOEε4. (Zhao, Liu et al. 2017) Though structurally different by only one or two amino acid residues, these isoforms differ functionally in terms of protein folding and binding affinity towards interacting partners. APOEε4 is the strongest genetic risk factors for Alzheimer Disease (AD) (see Section 1.3.1.1), and may increase risk of other neurological diseases, such as vascular dementia (Rohn 2014) and multiple sclerosis (Shi, Han et al. 2014), and mood disorders (Gibbons, Udawela et al. 2011).

Numerous studies have investigated the association between APOE polymorphism and TBI outcomes (Padgett, Summers et al. 2016), but the results have been inconsistent. Some studies have shown that APOEε4 is associated with unfavorable TBI outcomes: For example, in a study involving 651 mild, moderate, and severe TBI patients, Ponsford et al. showed that APOEε4 increased risks for poorer long-term functional outcome, but it did not associate with acute GCS score or post-trauma amnesia. (Ponsford, McLaren et al. 2011) In a prospective study of 1094 mild, moderate, and severe TBI patients, APOEε4 was associated with worse outcomes, but only in children and young adults. (Teasdale, Murray et al. 2005) In a cohort of 30 boxers, APOEε4 was associated with more severe impairments among the high-exposure boxers. (Jordan, Relkin et al. 1997) In contrast, other studies did not show an association of APOEε4 with poorer TBI outcomes.(Liberman, Stewart et al. 2002, Chamelian, Reis et al. 2004, Hiekkanen, Kurki et al. 2009, Shadli, Pieter et al. 2011) In particular, the prospective Rotterdam Study showed that while
APOEε4 increased risk for AD (in 6645 elderly), TBI and APOEε4 had no interaction with respect to AD risk. (Mehta, Ott et al. 1999) In a study on 78 mild to moderate TBI patients from the military, Han et al. (Han, Drake et al. 2007) showed that APOEε4 carriers had better cognitive measures such as attention and episodic memory than non-carriers. In a sample of 110 black Zulu mild, moderate or severe TBI patients, no significant association of APOEε4 and TBI outcomes was observed. (Nathoo, Chetry et al. 2003) In an Indian sample of 98 mild to moderate TBI patients, Prurhi et al. reported no significant differences in neuropsychological tests between APOEε4 carriers and non-carriers. (Pruthi, Chandramouli et al. 2010)

The above discrepancies may be due to study variables such as sample size, heterogeneity of TBI types and severities, age of patients, and time-points of follow-up. Subsequently, several groups performed meta-analyses on these data. Zhou et al. analyzed 14 cohorts with 2527 participants, and reported that APOEε4 is not associated with acute TBI severity, but is associated with poorer functional outcomes after 6-mo post-injury (Odd ratio (OR) = 1.36, CI 1.04 – 1.78). (Zhou, Xu et al. 2008) Li et al. analyzed 12 studies and reported that APOEε4 is associated with a 1.36-fold increased risk of unfavorable functional outcomes after 6 mo. (Li, Bao et al. 2015) Another recent meta-analysis focusing on pediatric non-penetrating TBI analyzed 6 cohorts (a total of 358 cases) and observed that APOEε4 is associated with poorer outcomes at 6-month post-injury, with an increased risk of 2.36. (Kassam, Gagnon et al. 2016)

These reports suggest that apoE may play a role in overall functional recovery after TBI, and that apoE isoforms may differentially affect the recovery trajectory in a small but significant manner. However, compared to the role of apoE in AD, the contribution of apoE in TBI may be considerably less potent. It should be noted that many of these reports are based on outcome measurements such as the Glasgow Outcome Scale (GOS) or Glasgow Outcome Scale Extended (GOS-E). These scales measure the global functional outcome ranging from death, vegetative state, disability, to recovery (see Table 1.6 and Table 1.7), and were developed for description of the more persistent and devastating disabilities as a result of moderate-severe brain injuries. Therefore, elucidation of the role of apoE4 in functional recovery of TBI, particularly in mTBI, may require more sensitive tests. Interestingly, a recent study that used magnetic resonance imaging (MRI) to study cortical thickness in a cohort of 160 military personnel (Hayes, Logue et al. 2017) showed that mTBI personnel with high polygenic risk for AD (which includes APOE)
are associated with reduced cortical thickness in AD-vulnerable regions. However, mTBI with APOE alone was not significantly associated with reduced cortical thickness in this study.

Many animal studies have shown that apoE deficiency worsens TBI outcomes, and that apoE-based therapeutics may be beneficial. Hong et al. showed that CCI in wildtype (WT) mice induced proliferation of neural stem/progenitor cell in hippocampus. However, this induction was abolished in apoE-deficient mice. (Hong, Washington et al. 2016) Our group showed that weight drop-TBI (WD, see Section 1.1.6.1.2) induced a greater extent of axonal injury in apoE-deficient mice. In addition, the LXR agonist GW3965 (which induces the level of apoE and its lipidation through ATP-binding cassette transporter A1 (ABCA1)) was able to ameliorate axonal injury in WT mice but not apoE-deficient mice. (Namjoshi, Martin et al. 2013) Loane et al. showed that CCI induced Aβ in mice, and another LXR agonist T0901317, was able to suppress such Aβ increase and reduce brain lesion volume. (Loane, Washington et al. 2011) In addition, the apoE mimetic COG1410 (which is a modified peptide base on amino residue 138-149 of apoE) has been shown to reduce cortical tissue loss, improve memory performance, reduce axonal injury (amyloid precursor protein (APP) varicosities), reduce number of activated microglia, decrease matrix metalloproteinase 9 (MMP9) activity, and improve BBB disruption after FPI or CCI in rodents. (Hoane, Kaufman et al. 2009, Kaufman, Beare et al. 2010, Jiang and Brody 2012, Cao, Jiang et al. 2016)

Numerous studies have investigated the functions of apoE3 and apoE4 by using transgenic mice that express different isoforms of human apoE on a mouse apoE-deficient background. Bennett et al. showed that CCI induced more severe axonal injuries (amyloid precursor protein (APP) varicosities) in apoE4-expressing mice than apoE2 or apoE3-expressing mice. (Bennett, Esparza et al. 2013) Ferguson et al. showed that while CCI induced oxidative stress markers in mouse hippocampus, a greater induction of antioxidant genes were observed in post-injury apoE3 mice than apoE4. (Ferguson, Mouzon et al. 2010) Mannix et al. found that CCI induced worse cognitive performance in mature mice expressing apoE4 under the control of the glial acidic fibrillary protein (GFAP) promoter relative to mature WT mice. (Mannix, Zhang et al. 2011) When injury was performed at PND20, apoE4 mice performed similarly to WT mice at 2 wk post-injury, but were significantly worse than WT animals at 6-mo post-injury. In vitro studies inducing mechanical injuries to cultured neurons from an apoE-deficient background also
showed that the application of recombinant apoE4, but not apoE3, increased intracellular Ca\textsuperscript{2+} level and apoptosis. (Jiang, Zhong et al. 2015)

### 1.1.4.2 Other genetic factors
Polymorphisms in several other genes have been linked or suggested to affect brain injury outcomes such as survival and recovery. (Dardiotis, Fountas et al. 2010, Dardiotis, Grigoriadis et al. 2012, Davidson, Cusimano et al. 2015) These genes include:

- **Genes implicated in neurodegeneration**, such as neprilysin (an enzyme that is essential in the degradation of Aβ) (Johnson, Stewart et al. 2009), microtubule-associated protein tau (the major component of neurofibrillary tangles, which is a pathological hallmark of many neurodegenerative disease including AD) (Bennett, Reuter-Rice et al. 2016), neurofilament (composes the intermediate filament in neurons, and is a promising biomarker for axonal injury and neurological diseases) (Panenka, Gardner et al. 2017);

- **Genes implicated in growth, repair and inflammation**, such as brain-derived neurotrophic factor (BDNF, a growth factor important in axon remodelling and synaptic formation) (Krueger, Pardini et al. 2011, McAllister 2011), cytokines (mediators of inflammatory response or its resolution, including tumor necrosis factor (TNF)α, interleukin (IL)-1, IL-6, and transforming growth factor (TGF) β) (Liberman, Stewart et al. 2002, Uzan, Tanriverdi et al. 2005, Bennett, Reuter-Rice et al. 2016), p53 (a protein important in regulation of apoptosis, neuronal damage and repair) (Martinez-Lucas, Moreno-Cuesta et al. 2005);

- **Others**, including endothelial nitric oxide synthase (eNOS) (an enzyme that regulates vascular dilation and immune cell adhesion) (Robertson, Gopinath et al. 2011), angiotensin converting enzyme (which regulates the production of angiotensin II and the degradation of bradykinin) (Ariza, Matarin et al. 2006), etc.

However, the effects of these genetic factors on TBI outcomes are generally less well characterised than apoE polymorphism, and the sample size in many of these studies are much smaller and often limited to moderate-severe TBI.

Regarding genetic risk factors specifically for mTBI, Panenka et al. reviewed 6 studies and suggested that polymorphism of the apoE promoter (-219G/T) and BDNF (met/met) may have
greater risk of sustaining a mTBI. (Panenka, Gardner et al. 2017) Although the clinical significance of these factors remains to be confirmed, it is important to note that genetic factors may be involved in both the risk of sustaining a TBI, as well as in the repair and recovery pathways after TBI. For example, variants in genes that affect reward and executive function circuitry may be associated with participation in activities that increase TBI risk.

1.1.5 Injury biomechanics of TBI

Injury biomechanics is defined as a field of research that “uses the principles of mechanics to investigate and explain the physical and physiological responses to impact that result in injury. (Viano, King et al. 1989) In the next sections, the physical properties of the brain and the injury biomechanics of blunt and inertia TBI will be discussed. Penetrating and blast TBI, which are not the focus of this thesis, will only be discussed briefly.

1.1.5.1 The unique material properties of the brain

The brain has notable material properties that make it a biomechanically unique tissue. (Holbourn 1943) First, the brain has a density close to water, and its density is quite uniform as nerve tissue, blood, and cerebrospinal fluid all have similar densities. Second, the brain is extremely incompressible to hydrostatic pressure (a pressure that is uniformly applied in all directions). Third, the brain has a very small modulus of rigidity and can easily change in shape. Fourth, the brain is protected by the skull, which has a very high rigidity. For these reasons, Holbourn concluded that (1) the shape of the skull and the brain are important in determining the location of injuries, and (2) brain tissues are most susceptible to injuries due to shear-strain deformation, but less to uniform compression.

1.1.5.2 Blunt and inertia TBI

When an object impacts on head, elastic (reversible) or plastic (irreversible) deformation of the skull will occur. Regardless of whether integrity of the skull and dura are breached (open head injury) or not (closed head injury), the skull indentation will create shear-strains to the local superficial region that cause focal contusion at the impact site. (Holbourn 1943) A blunt injury
may induce further damage by creating hematoma, which may occur when the dura becomes separated from the skull during skull deformation or skull fracture. (Young, Rule et al. 2015) (See Section 1.2.1.1) Bleeding from the meningeal arteries or the bridging veins will create epidural and subdural hematoma, respectively. Depending on the extent of the hematoma, it may induce a focal or diffuse injury.

Apart from skull deformation and hematoma, brain injury can be caused by sudden motion of the brain. Since the brain is suspended inside the skull, an impact to the head may induce desynchronized motion between the brain and the skull. As the brain moves to and fro, it may be impacted by the inside of the skull at both the impact site (coup injury) and the opposite side to the impact site (contre-coup injury). (Bigler 1990, Young, Rule et al. 2015) In addition to relative motion, other injury mechanisms have been proposed as the major contributor to contact injuries, such as differential pressure gradients, cavitation phenomena, or pressure wave propagation. (Gurdjian, Lissner et al. 1954, Hardy 2006, Schmitt 2014). (Illustration 1.1) Impact-induced contusion injuries are often found at coup and contre-coup sites, with particularly high frequencies at locations where the inner surface of the skull is not smooth, such as the ventral frontal and temporal regions, or along the longitudinal fissure, such as near the falx cerebri. (Illustration 1.2)

In addition, a sudden change in the velocity of the brain will result in shear-strain inside the tissue. If the force inducing the motion is perfectly aligned with the centre of gravity of the head, the change in velocity will only be linear and does not involve any rotational component (i.e. no spinning, turning, or change in direction). In such theoretical cases, the shear-strains will be small, except near foramens or blood vessels. (Holbourn 1943, Ommaya, Yarnell et al. 1967, Ommaya and Hirsch 1971) On the other hand, in most realistic scenarios, impacts on the head will result in some head rotation. When such rotation occurs, tissue closest to the centre of mass will have the least rotational (tangential) motion, whereas tissue closest to the surface will be farthest away from the centre and thus have the greatest rotational (tangential) motion. As tissues are travelling at different rates relative to one another, significant shear-strains will occur in large parts of the brain, creating a diffuse injury. (Ommaya, Faas et al. 1968, Unterharnscheidt, De Beukelaer et al. 1969, Smith, Chen et al. 1997, Smith, Nonaka et al. 2000) Therefore, head

In summary, during blunt TBI, an object impacts on the head and will induce skull deformation, linear acceleration, and rotational motion. All of these will contribute to shear-strains on the brain tissue and thus cause focal and diffuse injuries (or multifocal injuries). During pure inertial TBI (as in whiplash injury), shear-strains created by rotational motion are the major contributor to tissue damage.

Certain regions of the brains are more vulnerable to such rotation motion-induced injury than others. For example, white matter tracts are generally more vulnerable to TBI, and diffuse axonal injury is a common occurrence. This is due to the specific viscoelastic properties of the brain and the highly anisotropic (directional) organization of white matter axons. In normal situations, axons are a compliant and elastic material – they are able to elongate without breakage during stretch, and will restore to their original length when the stretch is removed. However, when the tissue experiences deformation with a high strain rate, i.e. the tissue strain (or deformation per unit length) occurs at a very short time interval – such as during TBI where the acceleration phase of most events are in the millisecond (ms) range - the high strain rate will render the axons very brittle, resulting in structural and functional damages to axons. (Smith, Wolf et al. 1999, Smith 2000, Johnson, Stewart et al. 2013) Another frequent site of such injuries is at the depth of sulci. Studies suggest that both tissue stress (internal force per unit area) and strain are concentrated at the depth of the sulci, due to their distinct geometry as well as the presence of blood vessels. (Jin, Lee et al. 2006, Cloots, Gervaise et al. 2008)

Attempts have been made to establish a relationship between head acceleration and injury outcome. The Wayne State Tolerance Curve (WSTC) (Illustration 1.3) was proposed by Lissner et al. (Lissner, Lebow et al. 1960) and later expanded by McElhaney et al. (McElhaney 1976) and confirmed by Ono et al. (Ono 1980) This curve is based on human cadaver and animal experiments and shows that the brain can tolerate higher accelerations if the duration is shorter.
Based on these data, the Head Injury Criterion (HIC) was proposed (Versace 1971), which is shown below. Essentially this function calculates the 2.5 exponent of the integral of acceleration over a defined time interval, which essentially means that HIC is a function of the change of velocity over that period of time. A common variation of the HIC is HIC-15, which is the maximum value when an interval of 15 ms is considered. The automobile industry uses HIC-15 of 700 as the safety cut off for 5% risk of severe injury in adults.

\[ HIC_{15} = \left\{ \left[ \frac{1}{t_2 - t_1} \int_{t_1}^{t_2} a(t)dt \right]^{2.5} (t_2 - t_1) \right\}_{\text{max}} \]

where \( t_1 - t_2 \) refers to the 15 ms time interval and \( a \) refers to the acceleration at that instance.

Despite its very widespread application, the WSTC has several limitations. Apart from technical issues (as in instrumentation and scaling of animal data), the WTSC only includes the linear acceleration component of head motion, which, as discussed above, is very unlikely to be the sole component in most human TBI cases. Furthermore, the primary outcome of the WSTC is skull fractures in cadaver heads. However, skull fracture is not a valid functional assessment of TBI or its outcomes, as TBI can occur without skull fracture, and the tolerance of brain tissue to acceleration/deceleration forces is not fully understood. Finally, the material properties of brain tissue from cadavers are likely to be significantly different from living tissue, due to post-mortem tissue preparations, and lack of blood pressure or flow of other tissue fluids (e.g. cerebrospinal and interstitial fluids). (Schmitt 2014) These factors have attracted criticism on the sensitivity and practicality of the WSTC to capture a true threshold for acceleration tolerance for TBI.

More recently, the brain injury criteria (BrIC), shown below, was suggested to complement the HIC. (Takhounts, Craig et al. 2013) The BrIC measurement estimates injury risk based on rotational velocity in all three independent axes (e.g. sagittal, coronal, transverse). It has been established using anthropomorphic test devices and college football data, and has been shown to correlate with other mathematical models. However, like all other injury threshold scales, it cannot be directly validated using induced human injury in living subjects due to ethical reasons. Its usefulness therefore remains to be confirmed.
\[ BrIC = \sqrt{\left( \frac{\omega_x}{\omega_{xC}} \right)^2 + \left( \frac{\omega_y}{\omega_{yC}} \right)^2 + \left( \frac{\omega_z}{\omega_{zC}} \right)^2} \]

where \( \omega_x, \omega_y, \) and \( \omega_z \) refer to the maximum angular velocities about X-, Y-, and Z-axes, respectively, and \( \omega_{xC}, \omega_{yC}, \) and \( \omega_{zC} \) refer to the critical angular velocities in the respective directions, and equals 66.25, 56.45, 42.87 rad/s, respectively.

### 1.1.5.3 Penetrating TBI

In penetrating TBI, the injuries are often categorized as caused by low velocity projectiles (e.g. knife) or high velocity projectiles (e.g. bullet), although there is no consensus on the boundary of low vs. high velocities. (Young, Rule et al. 2015) This velocity-based categorization is more for convenience than precision, as penetration is also dependent on the mass and shape of the object, and the contact location of the head. Generally speaking, in low-velocity penetrations, the injury is usually due to crushing, cutting, and shearing of brain tissue along the travel path of the object, resulting in localized damage comparable to the dimension of the object. On the other hand, in high-velocity penetrations, the crushing and cutting of brain tissue by the penetrating object will result in a wound (permanent cavity) whose size is dependent on both the object dimension and its velocity. These wounds are typically a few times greater than the object diameter. In addition, the adjacent area surrounding the permanent cavity will also be damaged by the resultant compression, shearing, and dislocation of brain tissue (temporary cavity). Studies have shown that the temporary cavity is the major contributor to pathology in penetrating TBI.

### 1.1.5.4 Blast TBI

Blast injury is further categorized into primary, secondary, tertiary, or quaternary injuries. (Chandra and Sundaramurthy 2015, Mathews and Koyfman 2015) A primary blast injury refers to the injury due to direct exposure to blast waves. Blast waves in the form of air overpressure can directly impact the head. In addition, its energy can be transmitted through the skull and into the brain, generating pressure gradients on the skull and the brain, and induce shear, spallation, implosion, and inertial effects of the neuronal and cerebrovascular tissue. (McAllister 2011, Young, Rule et al. 2015) Secondary and tertiary blast injuries refer to the injuries due to fast-
moving fragments of the explosive devices (penetrating), or collision of the body with hard surfaces (blunt), respectively. Quaternary injury refers to the exposure to toxic gas or chemicals as a result of the explosion.

1.1.6 Common research models of TBI
Currently there are many different TBI models available in vivo and in vitro. In vivo models use animals such as non-human primates, pigs, goats, rats, mice, and even fruit flies to investigate the effects of a controlled blunt, blast or penetrating injury. In contrast, in vitro models do not induce injury on research animals per se, but perform experiments on cultured organs, tissues, primary cells, or cell lines. Compared to in vitro models, the injury parameters and research findings in in vivo studies can be more readily translated into human settings. However, the use of animals to model human TBI has been challenged with practical issues such as ethics and expensive cost (especially for large animal), and technical issues. Some examples of the technical difficulties include animal-to-animal variation, anatomical and structural differences between the animal and human brain, and complexity due to the simultaneous involvement of many cell types and interactions of many pathophysiological processes. On the other hand, while in vitro models do not actually model TBI but rather traumatic cellular injury, they do provide an invaluable approach to supplement in vivo TBI research with a more simplified and controlled setting, thus allowing mechanistic studies and investigation of cell signalling pathways to be performed in one cell type at a time. The following section is intended to summarize the existing common research models in the TBI field. More focus will be put on the in vivo models, which is the focus of this thesis.

1.1.6.1 In vivo models

1.1.6.1.1 Open head models
These models typically involve creating a cranial window and directly delivering an impact onto the dura and brain parenchyma. Two of the most common open head models are discussed below.
Control cortical impact model

The controlled cortical impact (CCI) model is currently one of the most commonly used experimental TBI model. Using an electromagnetically or pneumatically-driven metal piston, classical CCI induces impacts onto the intact dura of a craniotomy-exposed and stereotaxically secured head. (Lighthall 1988, Dixon, Clifton et al. 1991) Injury severity of CCI can be varied by changing the depth of depression of neural tissue (typically 0.2 – 1 mm), the impact velocity (typically 0.5 – 10 m/s), and the dwell time (typically 25 – 250 ms) of the piston. (Cernak 2005, Levin and Robertson 2013) CCI was first developed for use in ferrets (Lighthall 1988), and was subsequently expanded to rats (Dixon, Clifton et al. 1991), mice (Smith, Soares et al. 1995), pigs, non-human primates, and other animals. (Xiong, Mahmood et al. 2013, Osier, Korpon et al. 2015) Depending on impact location and severity, CCI often induces large lesion volumes in the cortex and hippocampus. It is also reported to induce ventriculomegaly, atrophy of grey and white matter, cell death, axonal injury, BBB damage, and inflammation, resulting in neurological, motor, and cognitive deficits. (Levin and Robertson 2013, Xiong, Mahmood et al. 2013, Osier, Korpon et al. 2015) CCI has the advantage of having high reproducibility due to its precise control of impact parameters and head positioning. Importantly, however, fixing the head into a stereotactic frame by definition prevents head motion induced by impact. Thus, CCI mainly induces focal injuries without head motion, which limits its relevance to the most typical TBI cases in the clinic. (Namjoshi, Cheng et al. 2014)

Fluid percussion injury model

The fluid percussion injury (FPI) model is the oldest experimental TBI model developed in the late 1960s, and remains the most widely used. In FPI, a gravity-driven pendulum strikes onto a piston connected to a reservoir of fluid, which generates a fluid pressure pulse onto the intact dura of a craniotomy-exposed head. (Dixon, Lyeth et al. 1987, McIntosh, Noble et al. 1987) Injury severity of FPI can be adjusted by changing the drop height of the pendulum, and thus the magnitude of the fluid pressure pulse (typically 0.2 – 2.1 atmospheric pressure). (Alder, Fujioka et al. 2011) FPI was first developed for use in rabbits (Lindgren and Rinder 1965), and was subsequently translated to cats (Sullivan, Martinez et al. 1976), rats (Dixon, Lyeth et al. 1987), mice (Carbonell, Maris et al. 1998), pigs (Zink, Walsh et al. 1993), and other animals. (Osier,
Korpon et al. 2015) FPI has been shown to induce ventriculomegaly, atrophy of grey and white matter, cell death, axonal injury, and cerebrovascular injuries, inducing neurological, motor, and cognitive deficits. (Levin and Robertson 2013, Osier, Korpon et al. 2015) By opening the cranial window at different locations, FPI allows adjustable impact site (e.g. midline or lateral FPI), and some minimal head motion during impact. Thus FPI induces a focal injuries with mixed features of diffuse injuries. However, the control of impact parameters and head motion is often inadequate, as the only adjustable variable in this model is the drop height of the pendulum, and small variations in the surgical preparation (i.e. angle of craniotomy) can lead to large differences in outcome.

In summary, both CCI and FPI model TBI by impacting the intact exposed dura. Gene expression profiling has shown that injuries resulting from these two approaches share similar patterns of gene expression changes, including inflammation and cell adhesion. (Natale, Ahmed et al. 2003) These models also allow a certain degree of control over injury severity and injury site. However, the shear-stress generated by these models is mostly generated from direct impact of the piston on the dura, which compresses the brain. In addition, control of head motion (if present at all) in these models is either absent or poor. Therefore, both CCI and FPI primarily model a focal cortical contusion, with only some involvement of diffuse injury patterns. For these reasons, CCI and FPI do not fully capture the acceleration-induced injury seen in the vast majority of clinical mTBI cases. (Namjoshi, Good et al. 2013)

1.1.6.1.2 Closed head models

The closed head models are generally designed to model mild, and possibly repetitive, TBI. Among the many models developed over the years, the most commonly used two closed head models are discussed below.
• **Weight drop model**

The weight drop (WD) model involves dropping a metal weight from a defined height through a guide tube onto the skull under different configurations. The severity of WD injury can be varied by adjusting the weight of the drop mass (typically 10 – 150 g), or the height from which the mass is dropped (typically 5 – 100 cm), although the relationships among drop height, drop mass and phenotype are highly variable. There are several variations of WD, which are mostly different at the impact setting. In Marmarou’s WD, the weight is dropped onto the exposed skull, which is covered by steel helmet. (Marmarou, Foda et al. 1994) In Feeney’s WD, the weight is dropped onto intact dura after craniotomy. (Feeney 1981) In Shohami’s WD, the weight is dropped directly on the intact skull. (Shapira, Shohami et al. 1988, Shohami, Shapira et al. 1988) Depending on the variation, WD can induce focal (Feeney’s WD) or diffuse injury (Marmarou’s WD). WD has been shown to induce pathologies including axonal injury, glial activation, and cerebrovascular disruption, resulting in neurological, motor, and cognitive deficits. (Xiong, Mahmood et al. 2013, Osier, Korpon et al. 2015) In all forms of WD, the animal head usually rests on a solid or compressible surface, and thus the impact induces minimal motion and frequently results in skull compression. In addition, there is minimal control over impact parameters, and the mechanical aspects of WD are often highly variable (e.g. friction between weight and guide tube, shape of piston, air cushion and air drag effects). These factors render WD studies to be prone to extraordinarily poor reproducibility. (Namjoshi, Good et al. 2013)

• **Closed head injury model**

The “Closed head injury” model (CHI) is a recent modification of CCI. Similar to CCI, CHI employs an electromagnetically or pneumatically driven piston to impact a stereotaxically secured head. However, only scalp incision, but no craniotomy, is involved. (Laurer, Bareyre et al. 2001, Bayly, Dikranian et al. 2006, Huh and Raghupathi 2007, Raghupathi and Huh 2007, Luo, Nguyen et al. 2014) CHI has been shown to induce cell death (Bayly, Dikranian et al. 2006), BBB damage (Laurer, Bareyre et al. 2001), gliosis, and axonal injury (Shitaka, Tran et al. 2011, Bennett, Mac Donald et al. 2012, Bolton and Saatman 2014), resulting in neurological, motor, and cognitive deficits. (Raghupathi and Huh 2007, Bolton Hall, Joseph et al. 2016) Its main advantage is that it retains precise control over injury parameters and flexibility in impact
site, while inducing a more diffused injury instead of open head contusion. It has been recently employed to model mTBI in rats and mice, particularly in the context of repeated injury. However, this model still only allows minimal head motion due to fixation of the head within a stereotactic frame.

1.1.6.1.3 Rotational injury models
In contrast to open head and closed head impact models, non-impact rotational models do not involve direct contact between head and impactor. Instead these models induce TBI by generating fast rotational motion around the sagittal (Gennarelli, Thibault et al. 1982, Gutierrez, Huang et al. 2001, Runnerstam, Bao et al. 2001, Davidsson and Risling 2011, Krave, Al-Olama et al. 2011), coronal (Gennarelli, Thibault et al. 1982, Smith, Chen et al. 1997, Smith, Nonaka et al. 2000), axial (Smith, Nonaka et al. 2000, Browne, Chen et al. 2011), or oblique axes (Gennarelli, Thibault et al. 1982) of the head. The rotational models have been developed primarily for larger animals, including non-human primates (Gennarelli, Adams et al. 1981, Gennarelli, Thibault et al. 1982), pigs (Smith, Chen et al. 1997, Smith, Nonaka et al. 2000, Browne, Chen et al. 2011), and rabbits (Gutierrez, Huang et al. 2001, Runnerstam, Bao et al. 2001, Krave, Al-Olama et al. 2011), as well as for smaller animals such as rodents (Davidsson and Risling 2011). These devices often cause a short rotational acceleration followed by a longer deceleration of the head, usually by firmly securing a mechanical system to the animal head through a snout clamp, or by a skull fixation plate connected to a lever system. The degree of trauma can be adjusted by varying the angle of rotation, the magnitude of rotational acceleration (typical in $10^3 - 10^6$ rad/s$^2$), or the duration of the pulse (typically <10 ms). 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, Adams et al. 1981, Gennarelli, Thibault et al. 1982, Browne, Chen et al. 2011, Krave, Al-Olama et al. 2011) In addition, these models have demonstrated clinically relevant macroscopic pathologies, such as subarachnoid hemorrhage and intracranial pressure increase (Runnerstam, Bao et al. 2001), and microscopic pathologies, such as axonal injury (Smith, Chen et al. 1997, Smith, Nonaka et al. 2000, Hamberger, Huang et al. 2003, Davidsson and Risling 2011), glial responses (Smith, Chen et al. 1997, Gutierrez, Huang et al. 2001, Davidsson and Risling 2011), and neuronal cell death (Smith, Chen et al. 1997,
Runnerstam, Bao et al. 2001). However, as these models are used by only a few laboratories each of which have developed their own non-commercial system, it is difficult to compare results across studies, as there is no standardization over the injury parameters (e.g. the axis, duration, magnitude, and centre of rotation, and the firmness of attachment between the head and the device). In addition, in real-life scenario, a pure impact-free rotational TBI is rare in the clinical setting.

1.1.6.1.4 Other in vivo models
There are many more other types of TBI models. One major group includes the blast models. The blast models are designed to investigate the effects of primary blast waves to animals, such as rodents and pigs. (Xiong, Mahmood et al. 2013, Osier, Korpon et al. 2015) They recapitulate TBI encountered in the military setting by using compression-driven shock tubes. Blast models have been shown to induce axonal injury, cerebral edema, inflammation, cerebrovascular changes and tauopathy. (Goldstein, Fisher et al. 2012) However, as the shock tube is usually an expensive homemade device, and there are often a lot of adjustable variables (e.g. fixation of head, application of protective gear to body, duration and magnitude of pressure pulse, etc), direct comparison between models is again difficult. Another type of TBI model is the cryogenic injury model, which induces a focal injury by applying a cold rod (by dry ice or liquid nitrogen) to the exposure dura or skull in rodents. This model has been shown to induce cell death, glial response, inflammation, and most importantly, BBB damage. While it can produce lesions that are reproducible in size and location, its clinical relevance to the more common forms of clinical TBI is questionable, as this model does not involve the mechanical stress components that are responsible for contre-coup and diffuse axonal injuries (Albert-Weissenberger and Siren 2010).

(See Table 1.4)

1.1.6.2 In vitro models
In addition to the many in vivo models discussed above, a considerable amount TBI research utilizes in vitro models. Some of the common in vitro models of TBI include axonal transection (in which the axons of neuronal cultures are cut) (Mukhin, Ivanova et al. 1998), fluid shear stress (in which cells are deformed with the shear force produced by fluid movement) (LaPlaca and
Thibault 1997), hydrostatic pressure (in which a transient or static pressure is applied to cells) (Shepard, Ghajar et al. 1991, Murphy and Horrocks 1993), stretch injury models (in which the cells are grown on an elastic surface, which is then elongated) (Ellis, McKinney et al. 1995, McKinney, Willoughby et al. 1996), and blast models (Morrison, Elkin et al. 2011). Apart from the axonal transection model and the blast model, most other in vitro TBI models are based on the rapid application of a stretch or a shear stress to the neurons or part of the neurons (axons). While there is no standardization on the extent of the stretch (strain) or the speed at which it was applied (strain rate), most studies employ a strain value of 2%-30%, at a strain rate of 0.5-33/sec. These mechanical values are based on previous brain surrogates experiments. (Margulies, Thibault et al. 1990, Thibault, Meaney et al. 1992, Meaney, Smith et al. 1995) The severity of the injury can be adjusted by adjusting the strain and the strain rate.

1.1.6.3 Ex vivo and in silico models
In addition to in vivo and in vitro models, ex vivo and in silico models have been developed to study TBI. These models are particularly useful for biomechanical studies on material properties of the brain during impact or rotation, rather than its biological or functional consequences.

Holbourn was among the first that investigated deformation of brain tissue during rotational accelerations using ex vivo methods (Holbourn 1943). He used 5% gelatin and paraffin-wax as surrogates for brain and skull, and demonstrated that rotational acceleration can induce intense shear-strain to the multiple regions of the brain. Subsequent studies utilized high speed videography to analyze deformation of brain surrogates under rapid acceleration, inside the skulls of humans and other primates (Margulies, Thibault et al. 1990, Parnaik, Beillas et al. 2004), cats (Thibault, Meaney et al. 1992), and pigs (Meaney, Smith et al. 1995). They have shown that during the acceleration phase, the brain surrogates can experience high strain rate (10 – 30/sec) over a short duration (<10 ms).

Other studies have demonstrated brain motion during rapid acceleration using cadaver human skulls and brains, and visualization with neutral density targets and high speed x-ray videography. (Hardy, Foster et al. 2001, Hardy, Mason et al. 2007, King, Yang et al. 2011) These studies have become the gold standard of the biomechanics field regarding TBI research.
In addition to measuring TBI biomechanics physically, computer finite element models of the human head have been constructed based on the size, geometry, and material properties of its anatomical components, including the scalp, skull, pia mater, dura, cerebral spinal fluid, tentorium, falx, and brain. A finite element model is one that has been discretized into simple-shaped elements so that their related mathematical equations can be easily and accurately solved, and implemented for the whole complex structure. (Rezaei 2014) Computer simulations can then be performed to model the interactions of the different parts when the head experiences different types of loading. (Rezaei 2014) Though these finite element models are much simplified models of the human head, they have been verified against cadaveric experiments (Horgan 2003) and close agreement of results has been obtained. With the advancement of computation power and the aggregation of knowledge based on physical models, these finite element models may become increasingly important in the understanding of the biomechanics of TBI.

In summary, there are currently a large number of TBI models that are designed to model different aspects of TBI. Due to the complexity of the brain and the inherent heterogeneity of TBI, we wished to enhance our in vivo research using a model that offers high reproducibility as well as broad versatility. In order to best recapitulate the injury biomechanics of TBI, we formed a synergistic partnership between neuroscientists and mechanical engineers, to produce a biomechanically relevant animal model of TBI, called Closed Head Impact Model of Engineered Rotational Acceleration (CHIMERA). A full description of CHIMERA is given in Chapter 2.
1.2 Overview of TBI pathologies: acute and subacute biological responses

In this section, I will outline the common acute and subacute pathophysiologies and behavioral changes observed after TBI, including both primary injuries (immediate injuries that occur due to mechanical force) and secondary injuries (delayed injuries that developed afterwards). I will discuss gross pathologies, microscopic features and functional deficits. Outcomes from TBI ranging from severe to mild will be included, but the emphasis will be put on mTBI. Neurodegenerative and chronic changes induced by TBI will be included in the next section (Section 1.3). It should be noted that this grouping is only for convenience of discussion; it does not imply that the two acute/sub-acute and the chronic aspects of TBI are discrete events.

1.2.1 General gross pathophysiology of TBI

The mechanical force occurring during TBI may result in immediate (primary) or delayed (secondary) macroscopic or microscopic tissue damages in the skull, brain, or blood vessels. Intensive tissue stress or strains due to blunt or inertial TBI may induce gross pathologies such as skull fracture, epidural hemorrhage, subarachnoid hemorrhage, cerebral contusion, and/or intracerebral hemorrhage. These gross pathologies are prevalent in severe and fatal TBI, but less common in mTBI. On the other hand, microscopic pathologies such as diffuse axonal injury and inflammatory changes (Section 1.2.2 to 1.2.4 below) are frequent occurrences in all forms of TBI.

1.2.1.1 Skull fracture

Skull fractures are present in 80% of fatal TBI cases (Graham 1996) but are found in only 3% of mTBI cases. (Smith 2011) They usually occur in blunt and penetrating TBI, when deformation of the skull exceeds its elastic properties. Skull fracture can be classified by the involvement and tearing of dura and scalp (penetrating and external compound fracture, respectively). It can also be classified based on the fracture pattern. Linear fracture is the most frequent type when impacted by a hard, intermediate-sized object, whereas depressed fractures are common in impacts by smaller objects. (Pearl 1998) While skull fracture itself may be of little clinical
significance, it may induce complications, such as laceration of neuronal, vascular, or dural tissues, hemorrhage, or infection. (Silver 1994, Pearl 1998)

1.2.1.2 Meningeal hemorrhages
Epidural hemorrhage (EDH), which is present in 5% to 15% of fatal TBI, is commonly caused by falls or motor vehicle accidents. EDH often occurs as a result of laceration of the middle meningeal artery induced by skull fracture. It can also be caused by laceration of the middle meningeal vein or diploic veins. Arterial EDH can be acute and fatal when untreated, by causing increased intracranial pressure and brain herniation. (Silver 1994, Pearl 1998)

Subarachnoid hemorrhage (SAH) can be caused by blunt TBI or inertial TBI. It is often caused by avulsion of the vertebral artery at the base of the brain, resulting in diffuse spreading of blood in the subarachnoid space, causing vasospasm and acute hydrocephalus, and leading to death. (Silver 1994, Pearl 1998)

Subdural hematoma (SDH) is commonly observed in TBI caused by a fall (particularly among the elderly) or assault (particularly among child abuse cases). The presence of SDH is associated with worse TBI outcomes. (Lee, Segar et al. 2017) In contrast to EDH and SAH, SDH are caused by rapid rotational injuries with high strain rate. Thus, blunt impact is not necessary to cause SDH, provided that sufficiently high rotational acceleration occurs. In addition, in blunt TBI-induced SDH, the site of occurrence of may not match the site of impact. SDH are results of tearing of bridging veins between cortical surface and dural sinuses, which may lead to diffuse spreading of blood in the subdural space, covering the hemispheres unilaterally or bilaterally. (Silver 1994, Pearl 1998)

1.2.1.3 Cerebral contusions
Cerebral contusions are focal injuries caused by blunt TBI. Contusions are bruises of the brain surface, such as crests of gyri. Coup contusions are caused by in-bending of the skull at the site of impact, or its subsequent rebound-suction effect. Contre-coup contusions occur at locations distant to the site of impact, and are often found at frontal and temporal areas. Contusions can also occur secondary to other TBI pathologies. Increased intracranial pressure can induce
herniation contusions, when the brain is pressed against rigid surfaces such as ridges and protrusions of the skull, or compartmentations such as the falx. Skull fracture can also induce contusion of underlying brain tissue, resulting in fracture contusions. (Silver 1994, Pearl 1998)

1.2.1.4 Edema
Cerebral edema is one of the most life-threatening clinical symptoms of TBI. (Marmarou 2003) Cytotoxic edema, which is the more common form, occurs when water accumulates inside cells, as a result of a disruption of sodium and potassium pumps on the cell membrane. This can be caused by failure of energy metabolism or membrane damage, such as by reactive oxygen species (ROS). On the other hand, vasogenic edema refers to the accumulation of water in the extracellular space. This is caused by increased cerebrovascular permeability, allowing infiltration of plasma-derived, osmotically active solutes into the interstitial space. (Smith 2011, Salehi, Zhang et al. 2017) Factors that contribute to BBB dysfunction and thus vasogenic edema include reactive oxygen species (ROS) and matrix metalloproteinases (MMP) -2 and -9. (Price, Wilson et al. 2016) (See Section 1.2.4.2) If left untreated, brain edema can raise intracranial pressure, causing herniation of brain tissue and decreasing blood perfusion, leading to ischemia.

1.2.2 Diffuse axonal injury
Due to the viscoelastic properties of axons, white matter is particularly vulnerable to diffuse (or multifocal) axonal injury (DAI). It is particularly common in TBI that involves rapid acceleration, deceleration or rotation. Primary axotomy, which is relatively less common in the clinical setting except in severe cases, occurs when the mechanical force exceeds the elasticity limit of axons, resulting in breakage.(Povlishock, Becker et al. 1983, Maxwell, Watt et al. 1993, Christman, Grady et al. 1994, Maxwell, Povlishock et al. 1997, Johnson, Stewart et al. 2013) Secondary axotomy or axonal degeneration, which are more common in most cases, occurs when the initial damage triggers biological sequelae. (Hill, Coleman et al. 2016) Some of the major secondary injury mechanisms are discussed below.
1.2.2.1 Excitotoxicity

Excitotoxicity refers to the cascade of intracellular events caused by over-stimulation due to excessive neurotransmitters, resulting in calcium overload and subsequent neuronal injury. (Reviewed in (Krishnamurthy and Laskowitz 2016)) Glutamate, a major excitatory neurotransmitter, can initiate cell injury through ionotropic receptors, primarily N-methyl-D-aspartic acid (NMDA) receptors, and other metabotropic receptors. In physiological conditions, glutamate regulates synaptic transmission, neuronal growth and axonal guidance. Its presence is regulated by ATP-dependent transporters in neurons and glia. However, shearing and stretching during TBI can lead to mechanoporation of neuronal and axonal membranes, resulting in dysfunction of sodium channel and excessive sodium influx. This causes membrane depolarization and opens voltage-gated calcium channels, triggering neurotransmitter release. This process will result in depolarization of postsynaptic neurons and form a positive-feedback loop, leading to further neurotransmitter release. (Krishnamurthy and Laskowitz 2016, Smith 2016) Excitotoxicity is also aggravated by impaired neurotransmitter reuptake caused by metabolic disturbances or ischemic conditions following injury. (Quillinan, Herson et al. 2016)

1.2.2.2 Disruption of calcium homeostasis

The increase of intracellular cytoplasmic calcium can trigger further release of calcium from intracellular calcium stores (e.g. endoplasmic reticulum). Calcium increase can lead to massive activation of proteases and enzymes, including calpains, caspases and cysteine proteases. (Buki and Povlishock 2006) In particular, calpain activation can result in proteolysis of cytoskeletal proteins, including tubulin, microtubule-associated proteins, neurofilament and spectrin. It is important in different forms of axonal injury, such as axonal undulations, disruption of axonal transport, and degranulation and segmentation of distal injured axons during Wallerian degeneration. Calpain activation may also further aggravate permeability of cellular membranes. (Billger, Wallin et al. 1988, Caner, Can et al. 2004, Ma, Ferguson et al. 2013) In addition, calcium overload is involved in mitochondrial dysfunction, generation of reactive oxygen species, and apoptotic and necrotic cell death. (Pinton, Giorgi et al. 2008, Hill, Coleman et al. 2016)
1.2.2.3 Pathologies of DAI

Due to the orientation and location of axonal fibres, common sites of DAI include corpus callosum, brain stem (superior cerebellar peduncle), fornices, and generally junctions between grey and white matter or between anatomic boundaries of brain structures. (Adams, Graham et al. 1981, Adams, Doyle et al. 1989, Silver 1994, Pearl 1998) Pathologically, axonal swellings, which consist of accumulation of transported materials, may be developed within hours to days after injury. (Smith, Chen et al. 1999) These structures often appear as multiple swellings along the length of axons (beads-on-a-string structures) known as “axonal varicosities”. (Tang-Schomer, Johnson et al. 2012, Johnson, Stewart et al. 2013) Axonal swellings can also appear in the form of a single large “axonal bulbs” (or previously “retraction balls”). These axonal structures can be detected using silver staining (Blumbergs, Jones et al. 1989) or by more specific immunohistochemistry against targets such as β-amyloid precursor protein (APP) (Sherriff, Bridges et al. 1994) or neurofilament (Grady, McLaughlin et al. 1993), which indicate interrupted fast axonal transport (APP) or slow transport (neurofilament), respectively. (Smith 2000) The discovery that many proteins (Smith, Uryu et al. 2003, Uryu, Chen et al. 2007), including APP and its processing enzymes BACE and PS1 (see Section 1.3.2.1.1), co-accumulate at axonal interruptions after TBI, has led to the hypothesis that the aberrant accumulation, processing, and subsequent release of axonal APP may act as the source of parenchymal Aβ plaques. (Johnson, Stewart et al. 2010) (See Section 1.2.5)

Though immunohistochemistry remains a “gold standard” of DAI, recent advances in neuroimaging techniques such as diffusion tensor imaging (DTI), which measures the unequal diffusion of water across or along axons, has shown much promise in detection of DAI in vivo, even in mTBI cases. (Alhilali, Yaeger et al. 2014, Eierud, Craddock et al. 2014, Aoki and Inokuchi 2016, Khong, Odenwald et al. 2016) Using DTI tractography analysis, reports have shown tract volume reduction in moderate to severe TBI patients more than one year after injury (Haberg, Olsen et al. 2015), suggesting TBI may induce chronic axonal degeneration even after a single TBI (Johnson, Stewart et al. 2013). Myelin water imaging is another MRI imaging technique that is being investigated as a DAI marker. (Wright, Jarrett et al. 2016)
1.2.3 Neuroinflammatory responses

The brain was previously thought to be an immunologically privileged organ. However, it is now recognized that inflammation of the brain is central to a many neural pathologies, including TBI. Neuroinflammation is characterized by changes in expression of cell signalling molecules, such as cytokines and chemokines, and in initiation of cellular responses, such as microglial, glial and endothelial cell activation. (Perry, Nicoll et al. 2010, Helmy, De Simoni et al. 2011) TBI has been shown to induce acute and chronic changes in all of these components.

1.2.3.1 Cytokines

Cytokines are a group of intercellular signalling molecules produced by the immune system, as well as many types of brain cells including microglia, astrocytes, endothelial cells and neurons. They often act synergistically in cascades, and are involved in multiple physiological, neuromodulatory and restorative processes. (Helmy, De Simoni et al. 2011) Clinically, TBI patients have shown increased levels of pro-inflammatory cytokines, such as interleukin (IL) 1-β, IL-6, and tumor necrosis factor (TNF)-α, in tissues including blood, cerebrospinal fluid (CSF), brain interstitial fluid, and post-mortem brain. (Helmy, De Simoni et al. 2011) Both clinical and experimental studies have demonstrated that the acute response of these pro-inflammatory cytokines in brain is a very rapid event, usually peaking within hours of injury, and may resolve within days. (Helmy, Carpenter et al. 2011, Helmy, De Simoni et al. 2011, Bennett, Reuter-Rice et al. 2016) Cytokine levels have also been correlated with the severity of pathology in TBI patients. (Hayakata, Shiozaki et al. 2004, Shiozaki, Hayakata et al. 2005). In experimental studies, antagonism of IL-1 receptor has been shown to reduce contusion volume (Toulmond and Rothwell 1995, Jones, Prior et al. 2005) and improve cognitive deficits (Sanderson, Raghupathi et al. 1999, Clausen, Hanell et al. 2009). In contrast, genetic deletion of IL-6 has been shown to increase oxidative stress damage (Penkowa, Giralt et al. 2000), whereas overexpression of IL-6 reduces apoptotic cell death (Penkowa, Giralt et al. 2003). Both genetic deletion and pharmacological studies show that tumor necrosis factor (TNF)-α has a dual function in TBI, as it is functionally and histopathologically detrimental in the acute phase (Shohami, Bass et al. 1996, Bermpohl, You et al. 2007, Chio, Lin et al. 2010), but required for subsequent regeneration
and healing. (Scherbel, Raghupathi et al. 1999, Shohami, Ginis et al. 1999, Sullivan, Bruce-Keller et al. 1999). In short, cytokines play an important and very complex role in TBI.

1.2.3.2 Microglia

Microglia are resident immune cells in the brain that share a common yolk sac origin with macrophages. In physiological conditions, the adult pool of microglia is separate from the peripheral myeloid progenitors. (Saijo and Glass 2011, Prinz and Priller 2014) Morphologically, microglia assume a ramified morphology in normal physiological conditions (resting state). They are important in maintaining central nervous system (CNS) homeostasis by neuronal surveillance and synaptic pruning. (Tremblay, Stevens et al. 2011, Katsumoto, Lu et al. 2014) Upon detection of signals such as danger-associated molecular patterns (DAMP) or pathogen-associated molecular patterns (PAMP), microglia become activated and undergo a range of morphological changes by adopting a “hypertrophic” or ‘bushy” phenotype with fewer cellular processes and enlarged cell bodies. Under strong stimulation they may even revert to an amoeboid appearance. During activation, the morphological changes of microglia are often accompanied by changes in gene expression (such as cytokine and growth factors) and functional properties (such as phagocytosis). (Kettenmann, Hanisch et al. 2011)

Clinical and experimental studies have shown that TBI may induce a widespread microglia response within hours to days of TBI (Clark 1974, Folkersma, Boellaard et al. 2011, Velazquez, Ortega et al. 2015, Wofford, Harris et al. 2017), which may persist for months or years after TBI. (Nagamoto-Combs, McNeal et al. 2007, Ramlackhansingh, Brooks et al. 2011, Acosta, Tajiri et al. 2013, Glushakova, Johnson et al. 2014, Loane, Kumar et al. 2014) In addition to morphological change and activation, microgliosis is also a common phenomenon, which is characterized by an expansion in the number of immune cells at the injury site. The origin of these immune cells is widely debated, but it is most likely that both local microglia and circulating sources of monocytes contribute to such immune responses, particularly when the integrity of BBB is breached. (Li and Zhang 2016)

Apart from the categorization of microglia into resting or activated status, different states of microglia activation have been proposed. For example, microglial polarization refers to the proposition that activated microglia may display a spectrum of characteristics, ranging from pro-
inflammatory or “M1-like” to anti-inflammatory or “M2-like”. (Kumar, Alvarez-Croda et al. 2016) It should be noted that more recent evidences have shown that such a classification system may have serious limitations and does not truly capture the complexity and variety of microglial activation states. (Reviewed in (Ransohoff 2016)) However, due to the large body of work published using this categorization of M1/M2 microglia, it may be beneficial to first briefly review this categorization and then its limitations.

The M1/M2 categorization of microglia was originally borrowed from the M1/M2 macrophage polarization. (Martinez and Gordon 2014) In TBI, the activation and polarization of brain microglia/infiltrated monocytes is possibly quite different from the injury response of peripheral macrophages. The polarization of peripheral macrophages is often characterized by a distinct sequential response of M1 (pro-inflammatory: IL-1β, TNFα, iNOS), M2a (anti-inflammatory and wound-healing: Arg-1, Ym1) and M2c (immunosuppressive and remodeling: IL-4Rα, SOCS3, TGFβ) phenotypes. In contrast, TBI involves a chronic induction of microglia/monocytes with M1- or Mtran- phenotypes, and its M2 response is only transient. This imbalance of M1/M2 phenotypes has been reported in other forms of CNS injury, such as spinal cord injury (David and Kroner 2011), and a chronic mixed phenotype of microglial/macrophages have been reported in late-stage AD patients (Sudduth, Schmitt et al. 2013) and APP/PS1 mouse models (Weekman, Sudduth et al. 2014). These evidences show that the cellular immune response in the CNS can be very different from systemic immune response.

The effect of TBI on microglia polarization is further complicated by factors such as age at injury. The activation of immune cells is usually a highly regulated event that tends to decrease cell-to-cell variability. However, such strong orchestration is compromised by ageing. (Martinez-Jimenez, Eling et al. 2017) In TBI, several lines of evidence have suggested that the immune response in young animals may be quite different to that of old animals. Using the CCI model, Kumar et al. (Kumar, Stoica et al. 2013) showed that while TBI in both young (3-mo) and aged (24-mo) C57/B6 mice induced M1, M2a, and M2c responses after 24 h, the M1 and M2a phenotypes were exacerbated in old mice, whereas the M2c response was impaired in aged mice. In addition, the microglia/monocytes in aged mice also appeared more activated, in terms of cellular morphology and protein marker (MHC-II). This effect of age at injury on
microglia/monocyte activation and polarization was thought to be associated with greater lesion size and neuronal loss in the aged mice.

Though a large body of work have used the M1/M2 microglia categorization system in study of brain diseases including TBI, more recent advances in whole-genome transcriptomic and epigenomic studies have argued that such a system is artificial and may not reflect the true complex nature of microglial activation status. (Ransohoff 2016) Instead of a linear spectrum of polarization, transcriptome analysis has shown that macrophages display a three-dimensional network of activation states, and the M1 and M2 activation states do not fall into hubs of such organization. (Xue, Schmidt et al. 2014) In addition, a lot of M1/M2 classification is built on *in vitro* work using circulating monocytes or bone marrow-derived macrophages. There is a big gap in extrapolating such findings to *in vivo* microglia, which are separate from the hematopoietic lineage and are exposed to a vastly different environment. Finally, recent expression profile analyses do not support the idea that the use of a few M1 and M2 markers is informative in classifying activation status of microglia. (Hickman, Kingery et al. 2013, Morganti, Riparip et al. 2016, Ransohoff 2016) Therefore it may appear that future microglia studies will be more facilitated by high-throughput genome-wide analyses of transcriptomics, epigenomics, and proteomic, rather than by more simplistic classification of activation status into M1/M2.

In summary, after TBI, microglia often display a robust change in cell morphology and gene expression profiles. However, it should be noted that the cellular immune response in the brain is not a simple duplicate of the immune response in periphery. Furthermore, the effects of TBI on microglia activation is further complicated by inherent differences between white matter and grey matter microglia, and subject factors such as age. (Kettenmann, Hanisch et al. 2011) The chronic effects of persistent microglial activation, which is associated with neurodegenerative changes such as axonal atrophy and tauopathy, is further elaborated in Section 1.3.3.2.

### 1.2.3.3 Endothelial cell activation and immune cell infiltration

Experimental TBI can activate stress-sensitive signalling pathways including c-Jun N-terminal kinase (JNK) and NFκB (Yang, Mu et al. 1995, Ahmad, Crupi et al. 2013, Sun, Dai et al. 2013) in the brain, both of which are important regulators of the expression of endothelial cell adhesion molecules including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule
(VCAM)-1, and E-selectin (Kim, Moon et al. 2001). In fact, preclinical studies have shown that in addition to activation of resident immune cells, TBI can increase infiltration of peripheral neutrophil and T-cell infiltration to the brain through upregulation of proteins such as endothelial ICAM-1, VCAM-1, platelet endothelial cell adhesion molecule (PECAM)-1, and E-selectin. (Carlos, Clark et al. 1997, Balabanov, Goldman et al. 2001, Kalinowska and Losy 2006, Clausen, Lorant et al. 2007) Infiltrating leukocytes can then activate resident microglia through the release of pro-inflammatory cytokines and reactive oxygen species, which in turn propagates the local response to further recruit more immune cell from the periphery. In fact, other models of microglia activation have been directly correlated with local induction of endothelial ICAM-1. (Huber, Campos et al. 2006) Thus, TBI may directly or indirectly induce endothelial activation, which promotes recruitment of peripheral immune cells and perpetuates neuroinflammation.

1.2.4 Cerebrovascular changes

1.2.4.1 Intracerebral haemorrhage

Intracerebral haemorrhages can occur as a primary injury after TBI due to the direct rupture of intracerebral vessels, or as secondary injury in association with contusions (progressive intracerebral haemorrhage or haemorrhagic progression of contusion). In immediately fatal TBI, the strong mechanical force can result in diffuse vascular injury by causing simultaneous shearing of small blood vessels at multiple regions, such as thalamus, brain stem, and white matter. In non-immediately fatal TBI, a contusive injury may lead to loss of structural integrity of the surrounding vasculature, resulting in a delayed (usually within hours) expansion of a lesion, or remote progression of a lesion non-contiguous or contre-coup to the injury site. (Kurland, Hong et al. 2012, Cepeda, Gomez et al. 2016) Clinically, this type of haemorrhage is mostly found at the frontal and temporal lobes, and may lead to persistent residual blood products up to 6 mo post-injury and contribute to long-term neuropsychological deficits. (Martin, Wright et al. 2017)
1.2.4.2 Cerebrovascular permeability

While haemorrhage may be more prevalent in the more severe types of TBI, other features of cerebrovascular function, such as decreased BBB integrity, can be compromised in the absence of hemorrhage or after its resolution. The BBB is the highly selective interface that separates the central nervous system (CNS) from the circulatory system. This physical barrier is important in maintaining the local homeostasis of the CNS, and is achieved by the formation of continuous tight junctions between cerebrovascular endothelial cells, and supported by other components of the neurovascular unit including astrocytes, pericytes, and neurons. (Alves 2014) Using imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) of peripherally-injected agents, the disruption of BBB integrity has been clinically observed even in mTBI cases (Korn, Golan et al. 2005, Tomkins, Shelef et al. 2008), and may last for years after injury. In experimental TBI, increased BBB permeability has been functionally demonstrated by the extravasation of peripheral proteins such as IgG (Pop and Badaut 2011) or extrinsic agents such as Evans Blue (Lin, Pan et al. 2012, Kawoos, Gu et al. 2016, Li, Watts et al. 2016). At the molecular level, TBI has been shown to reduce the levels of proteins involved in tight junction formation, such as zonula occludens (ZO), claudins, and occludins. (Higashida, Kreipke et al. 2011, Abdul-Muneer, Schuetz et al. 2013, Blixt, Svensson et al. 2015) Ultrastructural changes to the BBB have been reported in experimental mild to moderate TBI, including features such as membrane blebbing, the formation of large vacuoles, and pinocytotic vesicles in endothelial cells (Povlishock, Becker et al. 1978, Wei, Dietrich et al. 1980), migration of pericytes, and changes in the thickness of the basal lamina (Dore-Duffy, Owen et al. 2000). These experimental studies have shown that BBB breakdown is a biphasic event. The initial phase of increased permeability occurs as soon as hours after TBI and may partially recover. The second phase is delayed and may occur starting three to seven days post-injury. (Baskaya, Rao et al. 1997, Shlosberg, Benifla et al. 2010) Factors that have been proposed to contribute to BBB dysfunction include metalloproteinases, oxidative stress, inflammation and immune cell infiltration, excitotoxicity, etc. (Higashida, Kreipke et al. 2011, Alves 2014)
1.2.4.3 Cerebrovascular reactivity and cerebrovascular blood flow

Similar to CBF disruption in human TBI, experimental TBI shows that cerebrovascular function can be disrupted though alterations in the level of cellular signalling molecules important in vascular health, such as nitric oxide (NO) (Lamas 2006), which is also a potent cerebrovascular dilator. Experimental TBI may upregulate endothelial NO synthase (eNOS) (Cobbs, Fenoy et al. 1997, Lu, Moochhala et al. 2001, DeWitt and Prough 2003) and inducible NO synthase (iNOS) (Wada, Chatzipanteli et al. 1998), leading to a transient increase in NO after injury followed by a subsequent reduction (Cherian, Goodman et al. 2000). This may lead to the dysregulation of reactive oxygen species (ROS), e.g. superoxide anion radicals, which can result in direct oxidative damage and further interfere with NO signalling and disrupt vasodilation and vasoconstriction (Elliott, Lacey et al. 1998).

1.2.5 Amyloid-beta changes
Beta-amyloid (Aβ) is a species of peptides (typically 38 -42 amino acid residues) generated by proteolytic cleavage of amyloid precursor protein (APP). Amyloid plaques, one of the two pathological hallmarks of AD, is mainly composed of fibrillary Aβ deposits. (See Section 1.3.2.1.1)

Among patients that died after a moderate to severe TBI, Aβ deposits have been observed in their brains in 30% to 50% out of all cases, at a higher frequency than age-matched controls. (Roberts, Gentleman et al. 1994, Ikonomovic, Uryu et al. 2004)(152 TBI and 44 normal controls in Roberts 1994 and 18 TBI and 10 normal controls in Ikonomovic 2004, respectively)
addition, Aβ deposits were found in TBI patients as acute as 2 h after injury, and as young as 10 to 30 yr old, which are not expected to have spontaneous Aβ deposits at this age. (Ikonomovic, Uryu et al. 2004) In TBI patients, the Aβ deposits generally appear as diffuse plaques rather than the neuritic plaques, which are the most commonly seen form in confirmed AD cases. (See Section 1.3.1.1)

One study (Graham, Gentleman et al. 1995) performed comprehensive histological analysis on fatal TBI brains, by sampling 44 representative areas per sample (ranging from cerebrum, cerebellum, and brainstem), using 14 samples that were less than 65 yr old. Aβ depositions were found in all cases, but the amount of deposition varied greatly. In 7 cases there were only minimal deposition, 5 had moderate deposition, and 2 had extensive deposition. The Aβ depositions appeared bilaterally on grey matter including frontal cortex and the limbic system, but were less frequent in cerebellum and pons, and totally absent in white matter. In addition, there was no association between their pattern and the sites of focal or diffuse pathologies such as contusion or hematoma. Further immunohistochemical characterization (Gentleman, Greenberg et al. 1997) showed that parenchymal Aβ42 was the predominant species deposited in these samples. Aβ40 deposition was less common and mostly restricted to vascular deposits.

Another study also showed that TBI induced Aβ species-specific changes. (Gentleman, Greenberg et al. 1997) In a cohort of 19 severe TBI patients, 6 resected brain samples showed cortical Aβ deposits. These deposit-positive samples had an elevated level of soluble Aβ42, but not Aβ40, compared to deposit-negative cases. In addition, 3 of these 6 cases were apoE4 carriers (50%), which is higher than the expected frequency of 25%, suggesting Aβ deposition after TBI may be associated with apoE isoform status.

Recent advances in imaging techniques has allowed in vivo imaging of amyloid in TBI patients. Using Positron emission tomography – computer tomography (PET-CT) with amyloid binding agent [11C] Pittsburgh Compound B ([11C]-PiB), Hong et al. (Hong, Veenith et al. 2014) has shown that survivors of moderate and severe TBI had increased amyloid burden in grey matter and striatum. (15 TBI vs 11 normal controls, median post-injury interval = 11 days) In a second cohort, they used 11C-PiB and immunohistochemistry to analyse brain samples from patients who died after TBI (16 TBI vs 7 normal control at 3 h to 56 d post-TBI), and found amyloid burden in neocortex grey matter but not cerebellum.
In summary, these clinical observations suggest that moderate and severe TBI may acutely induce Aβ deposition. However, it is quite likely that this process is very dynamic, and is further influenced by factors such as age at injury as well as genetic factors. Chronic Aβ changes induced by TBI are further discussed in Section 1.3.3.

Numerous experimental studies have been performed to investigate the effects of TBI on animal models. Due to the differences in amino acid sequence, endogenous rodent Aβ is not prone to aggregation. Thus in order to study the effects of TBI on Aβ pathologies, many researchers utilize transgenic rodent models, which are genetically modified with human APP (normal or with AD mutations), or species that have an Aβ sequence closer to human, such as pigs and guinea pigs. Due to the many variables encountered in the design of these researches, including animal model (different forms of APP, and different levels and regulation of expression), age-at-injury, TBI model, TBI severity, etc., it is not surprising that the study results have not been consistent. (See Table 1.5) On one hand, many studies have shown that TBI increased levels of axonal APP, cortical soluble and insoluble Aβ, and Aβ deposits. (Bird, Sohrabi et al. 2016, Ikonomovic, Mi et al. 2017) These studies have generally induced CCI to animals at an age before the development of extensive plaques, and typically monitored Aβ levels from hours to 2 mo post-injury. On the other hand, a few studies (Nakagawa, Nakamura et al. 1999, Nakagawa, Reed et al. 2000, Miszczuk, Debski et al. 2016) have shown that TBI actually lowered Aβ levels. Of note, these 3 studies are different from many other in that all 3 studies monitored Aβ deposits for long-term (4 to 8 mo post-injury), and in one study, animals with extensive level of pre-injury plaques were used (Nakagawa, Reed et al. 2000). These evidences suggest that the dynamics of TBI-induced Aβ pathology may be complex, and the outcomes may be affected by factors such as level of pre-injury plaques, time point of investigation, extensiveness of tissue destruction due to TBI, and induction of genes important in Aβ generation, clearance, or degradation. (Miszczuk, Debski et al. 2016, Ikonomovic, Mi et al. 2017) One of the main objectives of this thesis is thus to delineate some of these interacting factors. (See Chapter 4 and 5).

1.2.6 Behavioral changes
Immediately following TBI, patients may show signs such as loss of consciousness, alteration of consciousness, or post-traumatic amnesia (retrograde or anterograde). These signs are used to
categorize TBI severity. (Blyth and Bazarian 2010) Within days of injury, patients with mild to moderate TBI usually develop multiple neuropsychiatric symptoms. These signs are collectively known as postconcussive symptoms, include cognitive, somatic, and affective components. Examples of the cognitive symptoms include decreased attention, slowed speed of information processing, and memory issues. Examples of somatic symptoms include hypersensitivity to noise or light, tinnitus, headaches, fatigue, dizziness, vertigo, nausea, and sleep disturbances. Examples of affective symptoms include depression, irritability, and anxiety. (Jorge and Robinson 2011) Other symptoms such as motor components (such as postural stability and gait, including static and dynamic balance deficits, and decreased gait velocity and stride length. (Guskiewicz, Ross et al. 2001, Parker, Osternig et al. 2006, Broglio and Puetz 2008, Covassin, Elbin et al. 2013) Recently, specific changes related to the visual functions have been recognized as part of the symptoms that can happen after TBI. These symptoms include indirect traumatic optic neuropathy (reduced visual acuity or field) (Singman, Daphalapurkar et al. 2016), changes in pupillary light reflex, and deficits in eye movements (Ventura, Jancuska et al. 2015), such as vergence (coordinated movement of eyes to focus objects moving in or out), vestibular-ocular reflex (eye movements to compensate for head movements), saccades (rapidly shifting horizontal gaze), and pursuit (tracking moving objects).

Usually at least one of the above symptoms are described by mTBI patients in the immediate post-injury period. (Jorge and Robinson 2011) In most mTBI cases, many of these signs decay exponentially over time and are resolved within weeks of injury. (Barlow 2016) However, studies have shown that over 45% mTBI patients may still present at least one symptom at 3 months post injury (most commonly headache, memory issues, fatigue, irritability, sleep problems, etc). (Rimel, Giordani et al. 1981, Levin, Mattis et al. 1987, Lundin, de Boussard et al. 2006, Schwab, Terrio et al. 2017) Clinically, postconcussive syndrome (PCS) is a condition that describes the mTBI cases with lingering or worsening symptoms beyond the acute phase. The diagnostic criteria of PCS vary according to guidelines, but are generally defined as the presence of at least 3 symptoms at 3 mo after mTBI. (Barlow 2016) PCS has been associated with TBI severity (such as acute symptoms including loss of consciousness and amnesia), TBI biomarkers (such as axonal injury, glial changes, or Aβ levels) (Khong, Odenwald et al. 2016, Shahim, Gren et al. 2016), and age of injury. (Barlow 2016)
In addition, TBI, particularly military cases, is associated with the development of post-traumatic stress disorder (PTSD). There are considerable overlapping symptoms between symptoms of TBI and PTSD. (Jaffee 2011) However, PTSD is primarily defined as a disorder that is developed after exposure to an intense traumatic event. It involves intrusive thoughts, re-experiencing avoidance of the traumatic event, and increased arousal (such as hypersensitivity, irritability, and sleep disturbances), beyond 1 month post-injury. (Kennedy, Jaffee et al. 2007) Reports have shown that TBI may increase the risk for PTSD by 1.92 to 2.37 fold. (Schneiderman, Braver et al. 2008, Bryant, O'Donnell et al. 2010, Howlett and Stein 2016) Different neurological mechanisms have been proposed to explain the relationship between TBI and PTSD, including structural changes of hippocampus and amygdala, disruption to hypothalamic-pituitary-adrenal axis, and neurochemical and neurotransmitter changes. (Kennedy, Jaffee et al. 2007)

1.2.7 Other changes
Diffuse axonal injuries caused by TBI may lead to disruption of functional brain networks, and impaired connectivity has been reported in TBI patients. Using resting-state functional magnetic resonant imaging (fMRI), a study (Zhou 2016) showed that the brains of mTBI patients had altered small-world properties during the acute to subacute phase (3 - 53 d post-injury), such that they have lower global efficiency (centrality) and high local efficiency (clustering), suggesting functional disruption of long-distance communication of brain networks and resultant compensatory mechanisms at the local level. Another study (Han, Mac Donald et al. 2014) performing fMRI on military personnel exposed to blast TBI showed that, compared to military personnel without TBI, they have lower global efficiency of connectivity in the initial scan (<90 or 30 d post-TBI, in 2 cohorts), and such connectivity disruption was resolved or reduced in the follow-up scan (6 or 12 mo after the initial scan). These studies show that even mTBI may induce diffuse axonal injuries that have significant functional consequences.
1.3 Overview of TBI: chronic and neurodegenerative changes

The chronic and neurodegenerative changes after TBI will be discussed in the following sections. The focus of Section 1.3.1 will be put on long-term pathological changes of a single moderate or severe TBI. The pathological changes associated with repetitive mTBI will be the focus of Section 1.3.2.

1.3.1 Chronic outcomes of moderate and severe TBI

Moderate and severe TBI are well known to have a high mortality rate and long-term disability. In a Swedish epidemiological study that involved 1,143,470 individuals (Sariaslan, Sharp et al. 2016), 9.1% (104,290) had at least one TBI before 25 yr of age. Among these individuals with TBI, 23,614 had one or more moderate to severe TBI. After a median follow-up of 8 yr, compared to their unaffected siblings, these individuals with moderate to severe TBI had an increased risk of premature mortality (OR 1.92, CI 1.34 - 2.74), developing disability (defined by receiving disability pension, OR 2.06, CI 1.78 - 2.38), psychiatric visits (OR 1.31, CI 1.02 – 1.68), and welfare recipiency (OR 1.21, CI 1.12 - 1.32). Mild TBI only slightly increased risk of premature mortality (OR 1.26, CI 1.02 – 1.55), disability (OR 1.36, CI 1.25 - 1.47), psychiatric visits (OR 1.20, CI 1.06 – 1.37), and welfare recipiency (OR 1.18, CI 1.13 – 1.23). An Australian and New Zealand study (Myburgh, Cooper et al. 2008) followed 363 severe TBI patients who were admitted to the intensive care unit and found that the mortality rate at 6-mo and 12-mo was 31.6% and 35.1%, respectively. TBI outcome was assessed by Glasgow Outcome Scale Extended (GOS-E). (See Table 1.6 and Table 1.7) The rate of favourable outcome (GOS-E 5-8: moderate disability to good recovery) at 6-mo and 12-mo was 44.9% and 48.5%, respectively. A study in the Netherlands recruited TBI patients (339 severe and 169 moderate) and showed that 6-mo mortality rate was 46% and 21%, respectively. The proportion of unfavourable outcome (GOS-E 2-6: vegetative state to moderate disability) at 1 yr among survivors were 52% and 45%, respectively. This is equivalent to saying that a total of 74% of severe TBI and 57% of moderate TBI patients died or continue to show unfavorable outcome at 1 yr. In a Finnish study (Raj, Siironen et al. 2014) that included 890 TBI patients (including moderate, severe, or complicated mTBI), 6-mo mortality rate was 22% and unfavorable outcome (GOS 1-3: death to severe disability) was 47%.
In summary, for moderate and severe TBI, the 6-mo mortality rate across different studies was approximately 30%, and the rate of long-term unfavourable outcome was approximately 50%.

1.3.1.1 Chronic neurodegenerative changes after moderate or severe TBI

Many studies have shown that TBI, particularly moderate and severe TBI, may increase long-term risk for AD (Guo, Cupples et al. 2000, Plassman, Havlik et al. 2000, Wang, Lin et al. 2012) or dementia (Wang, Lin et al. 2012, Barnes, Kaup et al. 2014). Specifically, Plassman et al. recruited 548 military veterans and 1228 matched control patients, and found that AD risk was increased in patients with moderate TBI (OR 2.32, CI 1.04 – 5.17) or severe TBI (OR 4.51, CI 1.77 – 11.47), but not mTBI (OR 0.76, CI 0.18 – 3.29). (Plassman, Havlik et al. 2000) Though such associations were not replicated in all studies (Mehta, Ott et al. 1999), generally it is agreed that there are sufficient data suggesting an association between moderate or severe TBI with AD. (Bazarian, Cernak et al. 2009)

Recent advances in imaging techniques have made it possible to study the long-term amyloid changes in living TBI patients. Using positron emission tomography-computed tomography (PET-CT) with amyloid-binding agents, such as $^{11}$C-Pittsburgh compound B (PiB), a study showed that moderate and severe TBI patients had a significantly higher Aβ burden than age-matched controls (9 TBI vs. 9 normal controls vs. 10 AD, at 11 mo to 17 yr after injury). (Scott, Ramlackhansingh et al. 2016) Most interestingly, while the regional distribution of signals from TBI patients partly overlaps with that of AD, such as in the cortical regions (Scott, Ramlackhansingh et al. 2016), there were also striking regional differences. In TBI patients, there were areas of involvement that were atypical to AD, such as cerebellum, suggesting that the amyloid pathophysiology of TBI and AD may be related but not identical.

It should be noted that the development of post-TBI amyloid may be a very dynamic event and can subject to much patient-to-patient variation. A study showed that only some patients exposed to a moderate to severe TBI might show increased amyloid burden in long-term (3 out of 12 patients, at 5-129 mo post-injury).(Kawai, Kawanishi et al. 2013) Another case report used $^{[18}\text{F}]$-florbetapir (Gatson, Stebbins et al. 2016) to study one severe TBI patient and one severe TBI patient with stroke. In these cases, they found that at 12 mo post-injury, amyloid level was decreased when compared with 1 mo post-injury in a region-specific manner (at hippocampus,
and at caudate nucleus and occipital cortex, respectively), suggesting clearance of initial amyloid accumulation. At 24 mo post injury, amyloid burden increased in the first case and decreased in the second case, when compared to the 12 mo scan. In another study that involved 27 mTBI patients and 10 controls (Yang, Hsiao et al. 2015), $^{18}$F-florbetapir was used to quantify brain amyloid at >6 yr post-injury. They found that mTBI patients with cognitive impairment (6 out of 27), but not those without (21 out of 27), had increased amyloid accumulation. In addition, the patients with cognitive impairment had a higher-than-expected allele frequency of apoE4 (0.25, vs 0.05 in control).

Apart from in vivo imaging studies, immunohistological studies have also shown that a single moderate or severe TBI may lead to the development of Aβ and tau pathologies in long-term. Using brain samples from 39 moderate to severe TBI survivors (survival time = 1-47 yr, median = 8 yr, mean age = 53 yr) and 47 controls (mean age = 47 yr), Johnson et al. found that neurofibrillary tangles (NFT) were more prevalent and widespread in TBI than control samples, particularly in cases less than 60 yr of age (34% vs 9%). (Johnson, Stewart et al. 2012) In addition, NFT found in TBI survivors were more extensive than that of controls. Using immunohistochemistry (IHC), the occurrence of Aβ pathology was not significantly different between TBI and controls, though the TBI cases seemed to have more extensive Aβ pathology. However, using thioflavin S (a specific dye to stain fibrillary amyloid), they revealed that fibrillary amyloid plaques were significantly more frequent in the TBI cases than the control cases (64% vs 0%). In short, this study showed that a single TBI may be sufficient to induce more severe tau and amyloid pathologies.

A systematic review by Bazarian et al. analyzed 75 studies on the long-term outcomes (≥ 6 mo) of TBI, and concluded that there is sufficient evidence of an association between moderate or severe TBI with parkinsonism. (Bazarian, Cernak et al. 2009) (OR ranging from 3.0 to 11.0 depending on study). A recent US study (Crane, Gibbons et al. 2016) analyzed 3 cohorts of participants (7130 in total, 865 reported a history of TBI with loss of consciousness (LOC)) with a median follow-up period of 4.7 – 6.2 yr. In this study no significant association between TBI with LOC and AD was found, but they did report significant associations between TBI and parkinsonism (OR ranging from 1.65 to 3.56 depending on cohorts and LOC duration). In addition, TBI with LOC ≤ 1 h was associated with Lewy bodies in frontal or temporal cortex.
(overall OR 1.59, CI 1.06 – 2.39), and TBI with LOC > 1 h was associated with cerebral cortical microinfarcts (overall OR 1.58, CI 1.06 – 2.35).

Altogether summary, these studies suggest that moderate or severe TBI may induce chronic neurodegenerative changes that include Aβ, tau, or Lewy body neuropathologies. However, these pathologies may be modified by factors such as injury severity, disease progression and genetics, and requires further investigation.

1.3.1.2 Chronic microglial activation after moderate or severe TBI
Clinical studies using PET-CT with a ligand for activated glia/immune cells, (R)-PK11195 (1-[2-chlorophenyl]-N-methyl-N-[1-methylpropyl]-3-isoquinoline carboxamide), have shown widespread persistent signals in brains of living moderate-to-severe TBI patients, even months and years after injury (Folkersma, Boellaard et al. 2011, Ramlackhansingh, Brooks et al. 2011). In immunohistochemical studies, Johnson et al. (Johnson 2013) have shown densely packed activated microglia (CR3/43 and CD68-positive) in white matter of TBI survivors, at >1 yr and up to 18 yr post-injury. These observations further strengthen the idea that TBI may induce extensive and prolonged neuroinflammation through the microglial response, which is associated with chronic cerebrovascular and white matter damages (Glushakova, Johnson et al. 2014, Kou and VandeVord 2014), and tau pathology (Cherry, Tripodis et al. 2016).

1.3.2 Neurodegenerative changes after repetitive mild TBI:
1.3.2.1 General introduction to neurodegenerative diseases
1.3.2.1.1 Alzheimer’s Disease

• Introduction

Alzheimer’s Disease (AD) is a progressive neurodegenerative disease and is the most common form of dementia (accounting for 60 % - 80% of all cases), affecting over 35 million people worldwide. (Querfurth and LaFerla 2010) It is one of the leading causes of death in high-income countries. (Alzheimer's Association 2016, WHO 2017) In US, the annual costs of health care and long-term care for individuals with AD was over $200 billion.(Alzheimer's Association 2017) The clinical features of AD involve progressive cognitive decline and an eventual loss of
cognitive functions, such as memory impairment, loss of planning and executive functions, loss of word comprehension ability (aphasia), or loss of muscle coordination required to perform complex tasks (apraxia). (Castellani 2010).

• Clinical diagnosis and pathological features

Currently the clinical diagnosis of AD identifies two stages: mild cognitive impairment (MCI) due to AD and dementia due to AD. In the MCI stage, patients show cognitive decline greater than expected for their age and education, but daily activities are not significantly affected. In the dementia stage, patients show noticeable cognitive and behavioural symptoms that affect daily function. (Alzheimer's Association 2016)

Histopathologically, AD is characterized by two hallmarks: β-amyloid deposits and neurofibrillary tangles (NFT). β-amyloid deposits are extracellular aggregates of Aβ peptides, and NFT are intracellular aggregation of hyperphosphorylated tau protein in neurons. These two pathological hallmarks are often found in the cerebral cortex and limbic system, and their extensiveness and distribution are useful in the staging of AD (Section “Staging of AD” below). In addition to β-amyloid and NFT, cerebral amyloid angiopathy (CAA) is a common finding in AD brains (80%). CAA refers β-amyloid deposits within the vascular media of leptomeningial and penetrating cortical arteries.

• Disease genes and etiology

AD can be classified as sporadic (late onset), in which no causal disease genes are identified, or familial, in which mutations in disease genes are involved. Currently, familial AD accounts for 1% of all AD cases and is associated with multiple mutations in three disease genes coding for amyloid precursor protein (APP), presenilin 1 and presenilin 2 proteins. (Goate, Chartier-Harlin et al. 1991, Hendriks, van Duijn et al. 1992, Mullan, Crawford et al. 1992, Scheuner, Eckman et al. 1996) In most familial forms of AD, patients typically develop clinical signs as early as 30 to 40 yr of age, which is termed early-onset AD. In contrast, the sporadic forms of AD, which typically display signs around 60 yr of age, are known as late-onset AD. Though familial AD are
far less common than the sporadic forms, they are pivotal in the understanding of AD, and currently the vast majority of transgenic research models of AD are based on genetic mutations found in these cases.

The exact disease mechanism of AD is still not fully understood. However, many studies are focusing on the pathological roles of Aβ and tau. This is due to two reasons: firstly, aggregation of Aβ and tau are the defining pathological features of AD; secondly, mutations in APP gene (which encodes for the precursor of Aβ), microtubule-associated protein tau (MAPT) gene (which encodes different isoforms of tau for tau), or in genes related to their metabolism can lead to familial AD or related dementia. Thirdly, Down Syndrome patients, who have trisomy 21 and thus an extra copy of APP gene, also develop Aβ deposits and tauopathy, but at a much faster pace. (Olson and Shaw 1969) Based on these observation, it has been hypothesized that the dysregulation of Aβ and tau metabolism may be central to AD pathogenesis. Other hypotheses of AD include neuroinflammation (McGeer and McGeer 1992, McGeer and McGeer 2004) and disruption of energy metabolism. The Aβ and tau hypotheses are discussed below.

- **Aβ and amyloid cascade hypothesis**

Aβ are a species of peptides consisting of 36 to 43 amino acids. Aβ peptides are generated by proteolytic processing of the amyloid precursor protein (APP), which is a type I transmembrane protein that has been implicated in neurogenesis, neuronal migration and synaptic formation. (Zheng and Koo 2006, Lazarov, Demars et al. 2012) After synthesis, APP undergoes post-translational modification and then is routed through complex intracellular trafficking. (Thinakaran and Koo 2008, Querfurth and LaFerla 2010) It is initially directed to cell surface, and then rapidly internalized due to its YENPTY motif and trafficked through endocytic and recycling compartments. Finally, APP is released back to cell surface or degraded in lysosomes. APP can undergo proteolytic cleavage at different sites by different enzyme protein complexes, including α- or β- secretase, and γ-secretases. The action of α- and β-secretases are exclusive to each other. The latter initiates the amyloidogenic pathway of APP processing, whereas the former initiates the non-amyloidogenic pathway, as α-secretase cuts within the Aβ sequence and precludes the generation of Aβ. Both pathways involve a final cleavage by γ-secretase.
The first step of the amyloidogenic pathway occurs at endocytic organelles (Kinoshita, Shah et al. 2003), where the N-terminal ectodomain of APP is cleaved by β-secretase, generating the soluble N-terminal fragment sAPP\textsubscript{β} and the transmembrane β- C-terminal fragment (CTF). In contrast, the non-amyloidogenic pathway mainly occurs at cell surface, where the ectodomain of APP undergoes cleavage by α-secretase, releasing the soluble fragment sAPP\textsubscript{α} and the membrane bound α-CTF (De Strooper and Annaert 2000, Asai, Hattori et al. 2003, Jorissen, Prox et al. 2010). At both cell surface and endosomal compartments, the α- and β-CTF are then further cleaved by γ-secretase at the transmembrane domain (Parvathy, Hussain et al. 1999, Fukumori, Okochi et al. 2006). This generates the cytosolic C-terminal APP intracellular domain (AICD) and a middle hydrophobic fragment, P3 or Aβ, depending on whether APP has been previously cleaved by α- or β- secretase. Since γ-secretase can cut at multiple sites, the Aβ fragment may be of a variable number of residues, thus generating multiple possible species. (Illustration 1.4)

The most common Aβ species are Aβ40 (90%) and Aβ42 (<10%). (Burdick, Soreghan et al. 1992) Aβ42 is considered to be the more toxic species, as it is more prone to aggregation and forms the seeding core of β-amyloid deposits. (Jarrett, Berger et al. 1993, Iwatsubo, Odaka et al. 1994) Aβ40 is thought to be more soluble and is the predominant form found in CAA vessels and in cerebrospinal fluid. Physiologically, Aβ is essential for the survival of neuronal cultures. (Plant, Boyle et al. 2003) Aβ production is associated with synaptic activity (Kamenetz, Tomita et al. 2003, Lesne, Ali et al. 2005) and may modulate synaptic functions. (Plant, Webster et al. 2006) A recent study also suggest that Aβ may act as a natural anti-microbial agent in the brain. (Kumar, Alvarez-Croda et al. 2016) The pathological functions of Aβ include inhibition of synaptic transmission, neuronal toxicity, induction of inflammation, oxidative stress, and disruption of cerebrovascular function, etc. (Thomas, McLendon et al. 1997, Chow, Mattson et al. 2010, Dietrich, Xiang et al. 2010, Sadigh-Eteghad, Sabermanouf et al. 2015).

Though genetic mutations involved in familial AD are often related to aberrant production or processing of APP, the absence of such mutations in the sporadic, and more common, forms of AD suggests that disruption of Aβ clearance, instead of production, may be the main reason behind the imbalance of Aβ levels. Aβ is cleared from the brain through several pathways, including receptor-mediated cellular and vascular clearance, such as by low-density lipoprotein
receptor-related protein 1 (LRP1) and P-glycoprotein, and through peptidase degradation, such as by neprilysin, insulin-degrading enzyme, and endothelin-converting enzyme. (Sadigh-Eteghad, Sabermarouf et al. 2015) In mice, 75% of Aβ is cleared through the BBB and 10% through the interstitial fluid (ISF). (Shibata, Yamada et al. 2000, Zlokovic 2004) In young healthy humans, each hour 8.3% of Aβ is cleared from CSF. (Bateman, Munsell et al. 2006) Recently the brain glymphatic system has been discovered as one major brain clearance system. It describes how CSF from the subarachnoid space can flow along the periarterial space, and subsequently transported into the brain parachyma and joins the ISF. This fluid flux will then drive ISF to be collected in the perivenous space and drained out of the brain through the cervical lymphatic system. (Jessen, Munk et al. 2015) It is suggested that lower glymphatic system activity during ageing may be one of the triggers that leads to interstitial accumulation of Aβ and its subsequent deposition.

Regardless of overproduction or disrupted clearance, excessive human Aβ is prone to aggregation due to its hydrophobicity. Aβ monomers can assemble into more toxic Aβ oligomers (two to six peptides) (Walsh and Selkoe 2007), which may then grow into protofibrils and fibrils and finally arrange themselves into a β-pleated sheet formation. This highly fibrillary structure then becomes the core of mature “neuritic plaques” or “senile plaques”, which is surrounded by radiating fibrils. (Hardy and Allsop 1991) In contrast, Aβ aggregates with an amorphous structure are called “diffuse plaques”. Neuritic plaques are surrounded by dystrophic neurites containing paired helical filaments, and activated microglia and astrocytes. Histologically they can be detected using staining techniques such as thioflavin and silver staining. On the other hand, diffuse plaques do not associate with abnormal neurites or glial cells, and are only detectable by immunochemical techniques. They are considered pre-amyloid, as clinical observations in Down syndrome patients (who overexpress APP and accumulates Aβ) show that diffuse plaques occur at a much earlier age than neuritic plaques.

In summary, the amyloid cascade hypothesis (Hardy and Selkoe 2002) postulates that an accumulation of Aβ (such as due to genetic mutations) will lead to Aβ oligomerization and deposition, and cause synaptic dysfunction, glial activation, oxidative injury. This will then drive development of NFT and result in neuronal dysfunction and death, resulting in dementia.
• **Tau hypothesis**

In humans there are six isoforms of tau, which are products of alternative splicing from the MAPT gene. The tau isoforms differ from each other in having either 3 or 4 repeat sequences in Exon 10 (3R or 4R), and having either 0, 1, or 2 inserts on Exon 2 and 3 (0N, 1N, 2N), rendering a total of 6 combinations. (Higuchi 2002) Tau is also subjected to post-translational modifications including phosphorylation, acetylation, glycosylation, glycation, etc. *In vitro* normal tau binds to microtubules and promotes their assembly and stability. Phosphorylation of tau lowers its affinity and destabilizes microtubule assembly. *In vivo*, tau colocalizes to the dynamic ends of microtubules, regulates axonal transport, modulates trafficking of postsynaptic proteins, promotes neurite growth, and may connect the microtubule and actin filament networks. Tau is also involved in signalling pathways such as nerve growth factor (NGF) signalling and phospholipase C (PLC) γ. Many mutations in the MAPT gene are linked to neurodegenerative diseases including AD, frontotemporal dementia, progressive supranuclear palsy, Pick disease, and many others. Most of these mutations tend to reduce the affinity of tau towards microtubules, and causes tau hyperphosphorylation and aggregation. (Trojanowski and Lee 2005, Querfurth and LaFerla 2010, Morris, Maeda et al. 2011, Wang and Mandelkow 2016)

Physiologically, the isoform and phosphorylation status of tau are highly regulated. For example, fetal and early post-natal infants have higher levels of 3R0N tau, and a higher extent of tau phosphorylation. In contrast, in adult brain, tau is more heterogeneous with respect to isoform composition and is less phosphorylated. However, during pathological conditions, tau undergoes abnormal phosphorylation at various sites that can be recognized by different antibodies, such as Ser202/Thr205 (AT8) and Ser396/Ser404 (PHF1). These hyperphosphorylated tau proteins become misfolded and insoluble, and tend to associate into paired helical filament (PHF) structures, forming the NFT found in AD brains. Hyperphosphorylation of different tau isoforms is associated with several different neurodegenerative conditions. For example, Pick’s disease and argyrophilic grain disease contains mostly 3R tau and progressive supranuclear palsy contains 4R tau. In AD, a mixture of 3R and 4R tau is found. (Castellani 2010)

Since hyperphosphorylated tau loses its function in stabilizing microtubules, it has been proposed that microtubule depolymerisation and subsequent disruption in axonal transport and
formation of dystrophic neurites would induce neuronal dysfunction, synaptic degeneration, and eventually neuronal death. (Trojanowski and Lee 2005, Castellani 2010) Excess kinase activity (such as cyclin-dependent kinase or cdk5 and glycogen synthase kinase 3 or GSK3β) or reduced phosphatase activity (such as protein phosphatase 2 or PP2A) has been proposed to induce hyperphosphorylation of tau.

In summary, the tau hypothesis postulates that tau is central to AD. It suggests that as tau becomes aberrantly hyperphosphorylated and aggregates, it loses its normal function, such as microtubule stabilization, and causes neuronal death, which eventually leads to dementia. (Mudher and Lovestone 2002)

- The interactive roles of Aβ and tau in AD

Some recent hypotheses have proposed that instead of a serial causality, the Aβ and tau pathways may be linked by separate mechanisms. (Small and Duff 2008) In fact, primary culture studies show that tau is essential for the neuronal toxic effect of Aβ. (Rapoport, Dawson et al. 2002) In vivo studies also show that transgenic mice with MAPT mutations will have exacerbated tauopathy when injected with Aβ to the brain (Gotz, Chen et al. 2001), or when crossed with mice with APP mutation (Lewis, Dickson et al. 2001). In addition, another study used a mouse model that has both APP and MAPT mutations, and showed that passive immunotherapy against Aβ sequentially cleared Aβ deposits and then tau pathology. (Oddo, Billings et al. 2004) Other neuropathologies, such as oxidative phosphorylation, are also synergistically disrupted in mice with both APP and MAPT mutations (Rhein, Song et al. 2009). These studies suggest that Aβ and tau may have a complex interactive relationship in AD pathogenesis. (LaFerla 2010)

- Risk factors
  - ApoE

Compared to the APOEε3 (the most common allele), APOEε4 is well-established to increase risk of Alzheimer Disease (AD) by 3 fold (with one APOEε4 allele) or 12-fold (with two APOEε 4 alleles), and is also associated with an earlier age of AD onset. Conversely, APOEε 2 is
considered the protective allele and lowers AD risk. Apart from increasing AD risk and hastening its onset, the APOEε4 allele may be associated with worse cognitive performance (Lipnicki, Crawford et al. 2017), greater amyloid burden (McNamara, Gomez-Isla et al. 1998, Drzezga, Grimmer et al. 2009) and increased cerebrovascular pathology (Premkumar, Cohen et al. 1996, Drzezga, Grimmer et al. 2009, Hultman, Strickland et al. 2013, Ossenkoppele, van der Flier et al. 2013).

The apoE lipoproteins bind to Aβ, with the affinity of apoE4 < apoE3. (LaDu, Falduto et al. 1994) In AD brain samples, apoE colocalizes to neuritic plaques and NFT. (Namba, Tomonaga et al. 1991) In addition to regulating cholesterol level and thus modifying the activity of γ-secretases (Ossenkoppele, van der Flier et al. 2013), one of the major possible roles of apoE in AD is facilitating Aβ clearance across BBB. (Deane, Sagare et al. 2008) In mice, exogenous apoE2 and apoE3 facilitated Aβ clearance across BBB through both VLDLR (very low density lipoprotein receptor) and LRP1 (Low density lipoprotein receptor-related protein 1). However, apoE4 cleared Aβ mainly through VLDLR only. Since VLDLR has a slower kinetics in Aβ internalization, Aβ clearance was impaired in the presence of apoE. (Deane, Sagare et al. 2008) In mouse models expressing human apoE, apoE4 was less efficient in Aβ clearance than apoE3 or apoE2, leading to greater retention of Aβ in ISF and hippocampus of apoE4-expressing mice.(Castellano, Kim et al. 2011) Similar findings were also reported in cognitively normal human. When compared to the ε2/ε3 genotype, the ε4/ε4 genotype had higher brain amyloid and lower CSF Aβ, suggesting greater brain Aβ accumulation in the ε4/ε4 carriers.(Castellano, Kim et al. 2011) In addition to clearance through BBB, microglial phagocytosis and degradation of Aβ was also more efficient in apoE3 than apoE4 mice. (Jiang, Lee et al. 2008) Finally, therapeutic stimulation of apoE level and its lipidation status in mice, such as by agonists of liver X receptor (LXR) (Riddell, Zhou et al. 2007, Vanmierlo, Rutten et al. 2011) or retinoid X receptor (RXR) (Cramer, Cirrito et al. 2012) facilitated Aβ clearance. Interestingly, in mice overexpressing APP, apoE-deficiency lowered the amount of Aβ deposits. (Bales, Verina et al. 1997, DeMattos, Cirrito et al. 2004) A more recent in vivo study showed that APP/PS1 mice lacking ABCA1 had worse Aβ pathology and Aβ clearance. However, APP/PS1 mice lacking apoE or both apoE and apoAI had less Aβ pathology, faster Aβ clearance, but worse cognitive behavior. (Fitz, Tapias et al. 2015) These evidences suggest that the level, isoform, and lipidation status of apoE may influence the clearance, degradation, and deposition of Aβ.
Recent genome-wide association studies (GWAS) (Harold, Abraham et al. 2009, Lambert, Ibrahim-Verbaas et al. 2013) have found over 20 additional genetic loci that are associated with late-onset AD. Though not all of these genes have been confirmed or well characterized for their roles in AD pathogenesis, many of them are involved in cholesterol metabolism, inflammation, and cellular vesicle transport. A summary of these genes and their major known function is listed below (Giri, Zhang et al. 2016):

- Cholesterol metabolism: SORL1, ABα7, CLU
- Immune response: CR1, CD33, MS4A, ABα7, EPHa1, TREM2, CLU, HLA-DRb5/HLA-DRB1, INPP5D, MEF2C
- Endocytosis and vesicle-mediated transport: BIN1, CD2AP, PICALM, EPHa1, SORL1
- Cytoskeletal function and axonal transport: NMEB, CELF1, CASS4
- Tau pathology and angiogenesis: FERMT2
- Hippocampal synaptic function: PTK2B
- Epigenetic regulation: ZCWPW1
- Others: SLC24H4-RIN3

The principal risk factor for AD is age. The incidence of AD doubles every 5 yr after 65 yr of age. Epidemiological studies have shown that AD affects one-third of people older than 85 yr of age. Other risk factors that increase AD risk include lifestyle factors such as obesity (OR 1.8, CI 1.00 – 3.29), and medical conditions such as diabetes (OR 1.39, CI 1.16 – 1.66). Factors such as physical activity (OR 0.55, CI 0.36 – 0.84) and cognitive reserve (OR for dementia 0.54, CI 0.49 – 0.59) have beneficial effects and lower AD risks. (Ballard, Gauthier et al. 2011) Finally, as mentioned above, moderate-severe TBI is also an established risk factor for AD.
• **Staging of AD**

The development and progression of NFT is highly associated with the severity of AD. Based on this, the Braak staging is a system that categorizes AD into six stages, based on both the extensiveness and the distribution of neurofibrillary tangles and neuropil threads found in the autopsy samples. (Braak and Braak 1991) Six stages of NFT progression were proposed, with increasing intensity of NFT from Stage I to Stage VI. More importantly, the areas of involvement also expand as the stages progress. Stage I and II are the “transentorhinal stages. These stages are aligned with the preclinical phases of AD, and there are only minimal neurofibrillary changes of the hippocampus. Stage III and IV and the “limbic stages”, which shows some neurofibrillary changes at the hippocampus but not neocortex (or isocortex). These stages represent the time when clinical signs manifest themselves. Stage V and IV are the “isocortical stages”. They are the final stages with substantial involvement of both the hippocampus and neocortex, and correspond to fully developed AD. This staging system based on NFT neuropathology has been shown to correlate with mini-mental state score (MMSE) and other clinical measurements. (Bancher, Braak et al. 1993, Bancher, Jellinger et al. 1996, Gertz, Xuereb et al. 1998)

Initially, amyloid plaques were considered not useful in staging AD, as the size and shape of amyloid deposits generally show large heterogeneity among AD patients. In addition, amyloid deposits may appear in brains of non-dementia samples. (Braak and Braak 1991) However, subsequent studies have shown that the regions of involvement of Aβ deposition also show a sequential spreading pattern. (Thal, Rub et al. 2002) In the first phase of this five phase system, the neocortex is the initially affected area. As the phase progresses, the allocortical brain regions (entorhinal and insular cortex), the subcortical nuclei (putamen, caudate nucleus), the brainstem, and the cerebellum becomes sequentially and additionally involved. In addition, non-dementia samples with amyloid pathology all belonged to the early stages of the Thal system, whereas the clinically proven AD cases belonged to the later stages.
• Seeding and self-propagation of Aβ and tau

The findings that AD pathologies manifest a hierarchical spreading sequence has stimulated the idea that AD may have a prion-like induction and spreading mechanism. (Jucker and Walker 2011) Indeed, in vitro and in vivo studies have shown that Aβ and tau pathologies can self-propagate (Guo and Lee 2011), and different strains of Aβ or tau seeds can lead to spreading of distinct pathological and conformational features. (Sanders, Kaufman et al. 2014, Stohr, Condello et al. 2014, Watts, Condello et al. 2014) He et al. showed that human Aβ injected to the mouse olfactory bulb could spread to distal connected regions, such as amygdala and piriform cortex, in 3 days (He, Zheng et al. 2017). Recently, using advanced spectroscopic techniques, two independent groups revealed much information about the structural conformation of Aβ peptides and showed how their structures can induce amyloid propagation in AD. (Xiao, Cali et al. 2015, Walti, Ravotti et al. 2016) The spreading properties of tau have also been demonstrated in multiple studies. Using a mouse model that expresses mutant tau specifically in the entorhinal cortex, de Calignon et al. showed that tau can propagate to downstream brain circuits in the dentate gyrus and CA field of hippocampus. (de Calignon, Polydoro et al. 2012) Dujardin et al. also showed that injection of wild-type tau-expressing lentivirus to mouse hippocampus can result in tau spreading to distant connected neurons in the olfactory and limbic systems (Dujardin, Lecolle et al. 2014). Recent longitudinal imaging studies (Cho, Choi et al. 2016) have provided more evidences for spreading of Aβ and tau in living AD patients. Different mechanism have been proposed for the spreading of such prion-like agents, including exocytosis/endocytosis, exosomes, free release and micropinocytosis, etc. (Holmes and Diamond 2014, Wang, Balaji et al. 2017)

• AD treatment

Currently only five drugs (4 cholinesterase and 1 N-methyl-D-aspartate receptor antagonist) are approved by the US Food and Drug Administration (FDA) for the treatment of AD. However, all these drugs are symptomatic treatments, and do not treat underlying cause of AD or slow the rate of AD progression. Within the decade 2002-2012, 244 drugs were tested for AD in 413 clinical trials, but only one was successfully completed and obtained approval by FDA. This extremely low successful rate reflects the complexity in AD research, and point to the need to improve
1.3.2.2 Pathological and behavioral features of Chronic Traumatic Encephalopathy

1.3.2.2.1 Pathologies of Chronic Traumatic Encephalopathy

Repetitive exposure to concussive or subconcussive TBI has long been proposed to be associated with neurodegeneration. Based on clinical observations of boxers, in 1928 Martland coined the term “punch drunk” (Martland 1928) and reported a spectrum of progressive neurological conditions commonly seen among many long-term boxers. He noted that these patients may display mild to severe forms of gait and facial features similar to Parkinsonism, tremors, deafness, and eventually mental deterioration. In autopsy samples, he found perivascular punctate “ring hemorrhages” in the deep brain regions such as the corpus striatum. In 1973 Corsellis et al. (Corsellis, Bruton et al. 1973) reported the finding of neurofibrillary tangles in brain samples of these “dementia pugilisitca” cases. Amyloid pathology was not observed by Corsellis et al. However, subsequent studies have reported amyloid pathology, including diffuse plaques, senile plaques, and CAA, in all of the re-examined samples from Corsellis et al., using improved methods. (Roberts, Allsop et al. 1990, Tokuda, Ikeda et al. 1991)

More recently, interest on neurodegeneration induced by repetitive concussion revived when Omalu et al. (Omalu, DeKosky et al. 2005) reported finding neurodegenerative changes, including diffuse amyloid plaques and NFT, in the brain of a retired National Football League player. Importantly, he noted differences between these pathological features with that of AD, as NFT were found in the neocortex but not the entorhinal cortex or hippocampus.

In 2009 McKee et al. published a review (McKee, Cantu et al. 2009) of these “chronic traumatic encephalopathy” (CTE) cases based on 48 published samples and 3 additional samples from deceased retired athletes. In summary, neuropathological analysis showed that CTE samples show macroscopic signs characteristic of neurodegeneration, such as ventricular dilation, and atrophy of cerebral hemispheres, medial temporal lobe, mammillary bodies, and brainstem. CTE brains also show microscopic changes including NFT that are immunochemically identical to, but spatially different from, AD. Specifically, NFT found in CTE are prominent at perivascular
sites, around sulcal depths, and at superficial cortical layers, whereas this distribution is not observed in AD.

In McKee’s report, 40% of CTE cases show amyloid depositions, and most of them are in the form of diffuse plaques. Abnormalities in other proteins linked to neurodegenerative diseases, such as transactive response DNA binding protein 43 (TDP-43) and α-synuclein positive Lewy bodies, were found in 85% and 22% of the CTE cases, respectively. McKee et al. (McKee, Stern et al. 2013) subsequently proposed a system of staging CTE progression, based on pathological analysis of 68 CTE subjects, which is summarized in Section 1.3.2.2.3. (See Table 1.8)

In addition to post-mortem analysis, in vivo imaging studies have been performed in suspected CTE cases. In one study, PET scans were performed on retired football players with suspected CTE using 2-[1-[6-[(2-[fluorine-18]fluoroethyl)(methyl)amino]-2-naphthyl]-ethylidene]malononitrile (FDDNP), a ligand that binds non-specifically to aggregated proteins, including both neurofibrillary tangles and amyloid (14 CTE, 28 normal controls, 24 AD). (Barrio, Small et al. 2015) These retired football players showed a significantly higher level of aggregated protein in the brains compared to cognitively normal controls, though the overall severity was lower than that of diagnosed AD patients. In addition, brain regions not primarily affected by AD, such as striatum, thalamus, and pons, were involved in these suspected CTE patients. (Barrio, Small et al. 2015) Another in vivo imaging case study (Mitsis, Riggio et al. 2014) analyzed one retired NFL player, and found increased [18F]-T807 binding (a tau ligand) but no [18F]-florbetapir binding, suggesting the presence of tauopathy changes without amyloid deposition.

In summary, based on both post-mortem analyses and in vivo imaging studies, it is highly speculated that repetitive exposure to mTBI may induce neurodegenerative changes characterised by a distinct spatial pattern of tauopathy. In addition, while CTE and AD may have partly overlapping disease mechanisms, they are likely to be different clinical entities.

1.3.2.2.2 Behavioral deficits in CTE

CTE patients may show clinical signs including speech and gait problems, personality changes, and memory disturbances. It is reported that as many as one third of these patients may present
clinical symptoms within 4 yr of retirement. Mood disturbances were reported in 30% of the cohort, and motor abnormalities (such as Parkinsonism and ataxia) were reported in 41%. (McKee, Cantu et al. 2009) Cognitive problems (such as attention and memory) and emotion and mood issues (such as irritability, aggressive behavior, high frequency of suicide, substance abuse, and depression) are also common findings. (Omalu, Hammers et al. 2011) The progression of behavioral systems appeared to start with cognitive and emotional decline, and subsequent loss of motor functioning. (Saulle and Greenwald 2012)

1.3.2.2.3 Staging of CTE
In 2013, McKee et al. (McKee, Stern et al. 2013) further reported a systematic analysis of CTE brains, based on IHC analysis of 85 brain samples with known history of repetitive exposure to mTBI (due to contact sport, military service, and a case of self-injurious head-banging). Among these samples, 68 showed various severity of neuropathological changes, including NFT, axonal injury and axonal loss, and aggregation of proteins such as Aβ, TDP43, and α-synuclein. They further classified CTE severity from Stage I to Stage IV, based on these pathological findings. Macroscopically, Stage I CTE brains show mild enlargement of lateral ventricles, which progresses over time. In Stage IV, in addition to progressive enlargement of both the lateral and the third ventricles, CTE brains also show marked atrophy of the cerebral cortex, medial temporal lobe, and thalamus, as well as cavum septum pellucidum. Depigmentation of locus coeruleus and substantia nigra are also found in all Stage IV samples. Microscopically, Stage I CTE samples are characterized by sparse focal perivascular NFT, particularly at sulcal depths of frontal cortices. In Stage II, NFT and pre-tangles are found in multiple foci of frontal, temporal, and parietal cortices, particularly at the superficial laminae, affecting structures including amygdala, hippocampus, entorhinal cortex, thalamus, and the midbrain. In Stage III CTE, NFT are widespread in frontal, parietal and temporal cortices, and are observed in additional structures including olfactory bulbs, mammillary bodies, and substantia nigra. In Stage IV, tau pathology is widespread thought the cerebrum, basal ganglia, brainstem, and spinal cord. Regarding axonal changes, in Stage I CTE, scattered phosphorylated neurofilament-immunoreactive axonal varicosities are found in frontal cortex, subcortical white matter, and deep white matter. In Stage II, axonal varicosities are found in white matter tracts. In Stage III
severe axonal loss is observed in subcortical white matter. In Stage IV, marked axonal loss is found in white matter tracts.

As for TDP-43 pathology, in Stage I CTE, TDP-43-immunoreactive neurites are found in half of the cases, at frontal subcortical white matter and fornix. In Stage II, additional TDP-43-immunoreactive neurites and inclusions are observed in brainstem and medial temporal lobe, with a perivascular and periventricular distribution. In Stage III, TDP-43-immunoreactive neurites are generally found in the cerebral cortex, medial temporal lobe, and brainstem. In Stage IV, TDP-43 immunoreactive inclusions or neurites are severe and widespread in all cases.

Clinically, a progression of neurological symptoms is also noted in the patients classified from Stage I to Stage IV. Stage I patients generally show headache and loss of attention and concentration. Stage II patients show additional symptoms including depression, explosivity, and memory deficits. Stage III patients also show executive dysfunction, and Stage IV patients show additional deficits including language and visuospatial difficulties, and gait problems.

In summary, different aspects of neuropathologies found in these CTE cases generally showed a progressive deterioration from Stage I to Stage IV. More importantly, the pathological stages correlated with duration of exposure to the sport, survival after retirement, and age at death. These evidences further support the idea that repetitive exposure to TBI may induce progressive neurodegenerative changes. In addition, it also supported the idea that propagation of pathological proteins through neuronal connections may be one of the disease mechanisms of CTE. (Kondo, Shahpasand et al. 2015)

1.4 Failures in clinical trials for treatment of TBI

Currently there are no effective treatment for TBI. Although several agents that performed well in preclinical studies, none showed beneficial effects in clinical trials. In 2014, two phase 3 clinical trials were published showing that progesterone, a neurosteroid that has shown promising results in experimental TBI, did not improve 6 mo outcomes (by GOS/GOS-E) in moderate to severe TBI patients. (Skolnick, Maas et al. 2014, Wright, Yeatts et al. 2014) The use of erythropoietin was tested in a phase 2 (Robertson, Hannay et al. 2014) and a phase 3 trial (Nichol, French et al. 2015), and no beneficial effect was observed at 6 mo by GOS-E. Other

1.5 Summary, research hypothesis, and study aims

TBI has a high incidence world-wide, and may lead to dire consequences. However, it has been referred to as a “silent epidemic”, due to the previous lack of awareness from the public as well as the medical sector. Recently it is more recognized as an important public health concern, particularly among people who may be exposed to multiple concussions during their career. Nevertheless, currently there is no effective treatment for TBI, and its disease mechanism is still poorly understood. This lag of a scientific understanding of TBI is partly due to its complicated and heterogeneous nature. But it also points towards the possibility that existing TBI research models may not fully reflect the complex clinical scenarios.

Therefore, the overall hypothesis of this thesis is that a novel experimental TBI model that can reproducibly perform TBI through clinically relevant injury mechanisms will be pivotal in improving our current understanding of TBI pathophysiology, and in understanding how different factors such as age-at-injury and genetic predisposition to neurodegeneration, can modify the acute and chronic outcomes of TBI.

The specific aims of this thesis include, firstly, to establish a clinically-relevant TBI research model that allows integrated analysis of biomechanics and pathology. Secondly, this thesis aims to use this novel TBI model to characterize the acute, subacute, and chronic behavioral, histological, and biochemical outcomes of repetitive mTBI, in a mouse model of AD. By doing so, this thesis aims to delineate the interactive effects of several common confounding factors in TBI studies, including age of injury, genetic pre-disposition to amyloid deposition, and time after injury. This study will thus shed light on future TBI research, and may pave the avenue for better translation of TBI therapeutic research from bench to bedside.
1.6 Thesis organization and study designs

In Chapter 2, I will discuss the development of the Closed Head Injury Model of Rotational Acceleration (CHIMERA). This is an adjustable rodent pneumatically-driven impact acceleration system that induces surgery-free TBI without restraining head movement. In this study 4 month male C57/B6 mice received two mTBI (0.5J impact energy, 2 days apart). High speed videography was performed to ensure reproducibility of head motion. Behavioral, histological, and biochemical analyses were performed to evaluate injury outcomes up to 14 days post-injury. The major findings include neurological and motor deficits, diffuse axonal injuries, microgliosis at corresponding white matter, and cytokine induction.

In Chapter 3, I will discuss further characterization of CHIMERA in the induction of mTBI. In this study 5 mo male C57/B6 mice received a single TBI ranging from 0.1 to 0.7J, and behavioral, histological, biochemical assessment were performed up to 14 days post-injury. The injury conditions were then grouped into 3 clusters based on kinematic and pathological analysis: 0.1 to 0.4J (sub-threshold), 0.5J (threshold), and 0.6 to 0.7J (mTBI). Injury severity-dependent responses were observed in neurological and motor deficits, diffuse axonal injury, white matter microgliosis, and cytokine induction. More importantly, strong correlations were found between head kinematic parameters and injury outcomes.

In Chapter 4, I will discuss the acute outcomes of CHIMERA-induced repetitive mTBI in a model of AD. Male APP/PS1 (6 mo or 13 mo) mice and age-matched wildtype littermates received 2 mTBI (0.5J, 24 h apart), and behavioral, histological, biochemical assessment were performed up to 14 days post-injury. The major finding was that age at injury markedly altered the neurofilament response to injury independent of genotype, a finding with considerable implication for the use of neurofilament as a TBI biomarker. Age at injury and genotype had an interactive effect on the neuroinflammatory response. Finally, age at injury had subtle and transient alterations in the pattern of Aβ deposits without affecting Aβ levels in any biochemical fraction examined.

In Chapter 5, I will discuss the chronic effects of repetitive mTBI in APP/PS1 mice. Male 6 mo APP/PS1 mice and age-matched wildtype littermates received 2 mTBI (0.5J, 24 h apart). Behavioral assessments were followed up to 8 mo post-injury, and histological and biochemical assays were performed at 8 mo post-injury. The major findings included prolonged white matter
microgliosis and reduction in anxiety-like behavior in both genotypes. Interestingly, TBI in APP/PS1 mice specifically intensified long-term fear memory and impaired reversal spatial learning.

In Chapter 6, I will discuss the limitations of the studies presented in this thesis and suggest possible future study directions to better understand the neurodegenerative consequences of TBI.
Illustration 1.1 Different injury mechanisms of blunt TBI

These schematic diagrams illustrate different possible scenarios when a fast-moving head impact onto a solid surface. (A) The skull undergoes momentary deformation. (B) Stress waves induced by the impact may propagate in the skull or brain. (C) The skull comes to an abrupt stop, but the brain attempts to keep moving due to inertia, causing relative motion between the brain and the skull. (D) The motion of the brain induces positive at the impact site and negative pressure at the contre-coup site. Adapted from (Schmitt 2014)
Illustration 1.2  Frequencies of contusion sites

This illustration indicates the frequency of contusion sites in TBI. Even though impacts may theoretically occur to the head at any location and in any direction, contusion at certain sites occur more frequently than others due to anatomical structures such as bony protrusions of the skull. The illustration on the left shows the brain in the lateral view, and the illustration on the right the basal view. Adapted from (Holbourn 1943, Bigler 1990)
Illustration 1.3 Wayne State tolerance curve

The WSTC shows that when the acceleration duration is short, the tolerance for head injuries (in terms of linear acceleration) is greater. Conversely, a linear acceleration of lower magnitude may still cause injuries if the acceleration duration is extended. Adapted from (Young, Rule et al. 2015)
Illustration 1.4  
APP processing, Aβ clearance and polymerization

This illustration shows the key steps and products in both the amyloidogenic processing and non-amyloidogenic processing of APP. In physiological conditions, a large proportion of Aβ generated during amyloidogenic processing is either degraded enzymatically (e.g. by endothelin-converting enzyme, neprilysin, insulin-degrading enzyme, etc) or transported and drained into cerebrospinal fluid, or to lymphatic or vascular circulation. Adapted from (Querfurth and LaFerla 2010, Baranello, Bharani et al. 2015).
### Table 1.1  The Glasgow coma scale.

<table>
<thead>
<tr>
<th>Category</th>
<th>Assessment</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye Opening</td>
<td>Spontaneous</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>To speech</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>To pain</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Best Verbal Response</td>
<td>Orientated and converses normally</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Confused</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Inappropriate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Incomprehensible</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Best Motor Response</td>
<td>Obeying commands</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Moves to localising pain</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Flexing to withdraw from pain</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Abnormal flexion (decorticate)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Abnormal extension (decerebrate)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Maximum score</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

Note: Adapted from Teasdale et al. 1974 and CDC Mass Trauma Resources.

### Table 1.2  The Abbreviated injury scale.

<table>
<thead>
<tr>
<th>Categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Minor</td>
</tr>
<tr>
<td>2 Moderate</td>
</tr>
<tr>
<td>3 Serious (not life-threatening)</td>
</tr>
<tr>
<td>4 Severe (life-threatening, survival probable)</td>
</tr>
<tr>
<td>5 Critical (survival uncertain)</td>
</tr>
<tr>
<td>6 Maximum (fatal within 24 h)</td>
</tr>
</tbody>
</table>

Note: Adapted from Committee on Medical Aspects of Automotive Safety, 1971.
Table 1.3  Classification of TBI severity.

<table>
<thead>
<tr>
<th>Severity Criteria</th>
<th>Mild TBI</th>
<th>Moderate TBI</th>
<th>Severe TBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural imaging</td>
<td>Normal</td>
<td>Normal or abnormal</td>
<td>Normal or abnormal</td>
</tr>
<tr>
<td>Loss of consciousness</td>
<td>0 – 30 min</td>
<td>30 min – 24 h</td>
<td>&gt; 24 h</td>
</tr>
<tr>
<td>Altered mental state</td>
<td>≤ 24 h</td>
<td>&gt; 24 h s; severity based on other criteria</td>
<td></td>
</tr>
<tr>
<td>Post-trauma amnesia</td>
<td>≤ 1 d</td>
<td>1 – 7 d</td>
<td>&gt; 7 d</td>
</tr>
<tr>
<td>Glasgow Coma Scale score</td>
<td>13 - 15</td>
<td>9 - 12</td>
<td>&lt;9</td>
</tr>
</tbody>
</table>

Note:

1. This table is adapted from the DoD/VA Clinical Practice Guideline for the Management of Concussion-mild Traumatic Brain Injury.

2. If criteria of more than one severity level is met, the higher severity level is assigned.
Table 1.4  Summary of common *in vivo* experimental TBI models.

<table>
<thead>
<tr>
<th>TBI Model</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open head models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control cortical impact</td>
<td>• High precision in impact location and adjustable impact parameters</td>
<td>• Craniotomy required</td>
</tr>
<tr>
<td>Fluid percussion injury</td>
<td>• Adjustable impact location and injury parameters</td>
<td>• Mostly focal injury without head motion (low clinical relevance)</td>
</tr>
<tr>
<td></td>
<td>• Displays certain characteristics of diffuse injury</td>
<td></td>
</tr>
<tr>
<td><strong>Closed head models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight drop</td>
<td>• Inexpensive to set up</td>
<td>• Craniotomy required</td>
</tr>
<tr>
<td></td>
<td>• Displays certain characteristics of diffuse injury</td>
<td>• Variable experimental outcomes across laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• No control over head motion</td>
</tr>
<tr>
<td>Closed head injury</td>
<td>• Adjustable impact location and impact parameters</td>
<td>• Hugely variable experimental outcomes across laboratories</td>
</tr>
<tr>
<td></td>
<td>• Displays certain characteristics of diffuse injury</td>
<td>• Head motion during impact is restricted and poorly controlled</td>
</tr>
<tr>
<td>Rotational injury models</td>
<td>• Applicable to large (pigs) and small (rat) animals</td>
<td>• Head motion during impact is restricted and poorly controlled</td>
</tr>
<tr>
<td></td>
<td>• Purely rotational injury (no impact or risk of skull fracture, contusion, or other complications)</td>
<td>• Many different home-made designs, making comparison across laboratory difficult</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Purely rotational injury (a rare case clinically)</td>
</tr>
</tbody>
</table>
Table 1.5   Literature review of TBI research articles using AD-related animal models.

All available research articles performing TBI research using AD transgenic animal models or animals capable of developing Alzheimer-like pathologies were listed below. The search was performed using PubMed with key words including “AD”, “Alzheimer’s Disease”, “Traumatic brain injury”, and “TBI”, and was updated until 2017.

<table>
<thead>
<tr>
<th>Year</th>
<th>First Author; Last Author</th>
<th>Animal Model (Age at Injury)</th>
<th>Extensive plaque at baseline?</th>
<th>TBI Model</th>
<th>Study Time Point (Post-injury)</th>
<th>TBI-induced Aβ Changes</th>
<th>Other Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>Murai; McIntosh</td>
<td>APP-YAC (13 mo) N</td>
<td>N</td>
<td>CCI</td>
<td>1d, 7d</td>
<td>Whole brain: ↓AB40 (7d; ELISA); no amyloid plaques</td>
<td></td>
</tr>
<tr>
<td>1999</td>
<td>Nakagawa; Trojanowski</td>
<td>PDAPP (4 mo); N</td>
<td>N</td>
<td>CCI</td>
<td>2 mo, 5 mo, 8 mo</td>
<td>Ipsi Hippo &amp; cingulate: ↓Aβ deposits vs contra (5 -8 mo; IHC)</td>
<td>Ipsi hippo atrophy</td>
</tr>
<tr>
<td>2000</td>
<td>Nakagawa; Trojanowski</td>
<td>PDAPP (24 mo); Y</td>
<td>Y</td>
<td>CCI</td>
<td>1wk, 9wk, 4 mo</td>
<td>Ipsi Hippo: ↓Aβ plaques (4 mo; IHC)</td>
<td>Peri-lesion neuron loss, gliosis, atrophy</td>
</tr>
<tr>
<td>2002</td>
<td>Hartman; Holtzman</td>
<td>PDAPP x ApoE3/E4/E-KO (9 mo); N</td>
<td>N</td>
<td>CCI</td>
<td>Up to 3 mo</td>
<td>Hippo: E3/E-KO: ↑Aβ diffuse plaques; E4: ↑Aβ neuritic plaques (3mo; IHC)</td>
<td>Hippo &amp; cortex tissue loss, cell loss</td>
</tr>
<tr>
<td>Year</td>
<td>First Author; Last Author</td>
<td>Animal Model (Age at Injury)</td>
<td>Extensive plaque at baseline?</td>
<td>TBI Model</td>
<td>Study Time Point (Post-injury)</td>
<td>TBI-induced Aβ Changes</td>
<td>Other Findings</td>
</tr>
<tr>
<td>------</td>
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<td>-----------------------------</td>
<td>-------------------------------</td>
<td>-----------</td>
<td>-----------------------------</td>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>2002</td>
<td>Uryu; Trojanowski</td>
<td>Tg2576 (9 mo); N</td>
<td>Single or repetitive closed-head CCI</td>
<td>2d, 9wk, 4 mo</td>
<td>Cx, Hippo, Peri-Hippo: ↑Aβ deposition (4 mo; IHC)</td>
<td>Cognitive impairment, lipid peroxidation</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Iwata; Smith</td>
<td>SD rat (3-4 mo)</td>
<td>parasagittal FP</td>
<td>2d, 4d, 7d, 14d, 1mo, 2mo, 6mo, 12mo</td>
<td>Thalamus axons &amp; WM; ↑ weak neuronal Aβ (2 wk); strong Aβ (1 mo to 1-yr); (IHC)</td>
<td>APP 751/770 gene expression increased from 2d to 7d</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>Chen; Smith</td>
<td>Pigs (6 mo); N</td>
<td>Rotational acceleration</td>
<td>3h, 3d, 7d, 6 mo</td>
<td>Sub-cx WM &amp; basal ganglia: ↑axonal Aβ &amp; APP accumulation (3d, 7d, 6 mo; IHC); GM/WM, perivascular: Aβ deposits and plaques (3d, 7d, 6m; IHC and histochemistry)</td>
<td>BACE1, PS1, activated caspase3, CCA and kinesin coaccumulate with APP and Aβ in damaged axons; ↑C99 and activated caspase 3 (3h to 7d)</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Yoshiyama; Trojanowski</td>
<td>hTau (12 mo); NA</td>
<td>16 TBI (4 x 4 in 4wk)</td>
<td>9 mo</td>
<td>Hippo, entorhinal cx: ↑NFT in 1 out of 18 mice (9 mo; IHC; not stat. sign.)</td>
<td>cerebral atrophy</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>Abrahamson; DeKosky</td>
<td>APPNLh (3 mo); N</td>
<td>CCI</td>
<td>3hr - 14d</td>
<td>Hippo: ↑Aβ40 (3h-24h) and Aβ42 (3h -14d; ELISA); ↑APP (6h-3d; WB)</td>
<td>↑Aβ40, Aβ42, activated caspase 3, and caspase-cleaved APP are inhibited by caspase inhibitor BAF</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>First Author; Last Author</td>
<td>Animal Model (Age at Injury)</td>
<td>TBI Model</td>
<td>Study Time Point (Post-injury)</td>
<td>TBI-induced Aβ Changes</td>
<td>Other Findings</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
<td>-----------</td>
<td>-------------------------------</td>
<td>----------------------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Crawford; Mullan</td>
<td>APPsw (22 mo); Y</td>
<td>CCI</td>
<td>24hr</td>
<td>caspase-cleaved APP (3h-24h; WB)</td>
<td>Altered biological pathways include immune response, cell cycle, cell death, etc</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>Abrahamson; Dekosky</td>
<td>APPNLh (3 mo); N</td>
<td>CCI</td>
<td>3d, 7d</td>
<td>Cx and Hippo: Aβ40 and 42 (3wk; ELISA)</td>
<td>Simvastatin (3-HMG CoA reductase inhibitor) ↓TBI-induced Aβ, hippo damage (CA3 synaptophysin), microglia activation, improved spatial memory; CA1 and spatial learning not improved by drug</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>Laskowitz; Dawson</td>
<td>PDAPP x ApoE3/4TR (3-4mo); N</td>
<td>Closed-head CCI</td>
<td>1wk - 12wk</td>
<td>Whole brain: Total Aβ (4 wk); Aβ 40 (1 wk - 4 wk) (ELISA) Hippo &amp; whole brain: Intracellular Aβ (1 wk - 12 wk; IHC)</td>
<td>Effects of ApoE4 &gt; ApoE3; apoE-mimetic ↓pathology in E3 and E3/E4</td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Tran; Brody</td>
<td>3xTg-AD (5-7 mo); N</td>
<td>CCI</td>
<td>24hr</td>
<td>Axonal activation of JNK; JNK inhibitor reduces total/p-Tau</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>First Author; Last Author</td>
<td>Animal Model (Age at Injury)</td>
<td>Extensive plaque at baseline?</td>
<td>TBI Model</td>
<td>Study Time Point (Post-injury)</td>
<td>TBI-induced Aβ Changes</td>
<td>Other Findings</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------</td>
<td>-----------------------------</td>
<td>-------------------------------</td>
<td>-----------</td>
<td>-------------------------------</td>
<td>------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>2013</td>
<td>Abrahamson; Ikonomovic</td>
<td>APPNLh (3-5 mo); N</td>
<td></td>
<td>CCI</td>
<td>3d, 3wk</td>
<td>Cx and Hippo: ↑Aβ40 &amp; 42 (3wk; ELISA)</td>
<td>J-CBF in ipsi cx (vs WT); CBF, Aβ and tissue loss suppressed by simvastatin</td>
</tr>
<tr>
<td>2013</td>
<td>Tajiri; Borlongan</td>
<td>APP/PS1 (3 mo); N</td>
<td></td>
<td>CCI</td>
<td>2wk, 6wk</td>
<td>Cx: ↑Aβ deposit (6wk; IHC)</td>
<td>Neuron loss, spatial task error; Retrograde amnesia (2-6 wk)</td>
</tr>
<tr>
<td>2013</td>
<td>Bennett; Brody</td>
<td>3xTg-AD x ApoE2/3/4 (6-8 mo); N</td>
<td></td>
<td>CCI</td>
<td>24hr</td>
<td>Fimbria: ↑axonal APP in 3xTg-AD x Apoe2 (24hr; IHC)</td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>Bates; Harvey</td>
<td>Guinea pig (3 - 48 mo); N</td>
<td></td>
<td>FPI</td>
<td>24hr - 7d</td>
<td>Cx and Hippo; ↑APP and tau (24h - 7d, IHC)</td>
<td>GSK-3 activation, p-Tau, cytokines</td>
</tr>
<tr>
<td>2014</td>
<td>Sawmillier; Tan</td>
<td>Tg2576 (age not mentioned)</td>
<td></td>
<td>CCI</td>
<td>3d</td>
<td>Whole brain: ↑soluble Aβ40 and 42 (3d; ELISA)</td>
<td>Luteolin (flavonoid) abolishes these pathologies</td>
</tr>
<tr>
<td>2016</td>
<td>Miszczuk; Pitkanen</td>
<td>APPPS1 (3 mo) N</td>
<td></td>
<td>CCI</td>
<td>2d - 4 mo</td>
<td>Cx: ↓Aβ deposits (4 mo; IHC)</td>
<td>AD-TBI ↓ spatial memory; ↑epileptogenesis, ↑cortical apoE, ABCA1, clu, Nos1, Mapk3</td>
</tr>
</tbody>
</table>
Table 1.6  The Glasgow outcome scale

<table>
<thead>
<tr>
<th>Categories</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Death</td>
</tr>
<tr>
<td>2</td>
<td>Vegetative State</td>
</tr>
<tr>
<td>3</td>
<td>Severe Disability (conscious but disabled)</td>
</tr>
<tr>
<td>4</td>
<td>Moderate Disability (disabled but independent)</td>
</tr>
<tr>
<td>5</td>
<td>Good Recovery</td>
</tr>
</tbody>
</table>

Note: This table is adapted from (Jennett and Bond 1975).

Table 1.7  The Glasgow outcome scale extended

<table>
<thead>
<tr>
<th>Categories</th>
<th>Sub-categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Death</td>
</tr>
<tr>
<td>2</td>
<td>Vegetative State</td>
</tr>
<tr>
<td>3</td>
<td>Severe Disability</td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Moderate Disability</td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Good Recovery</td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Note: This table is adapted from (Wilson, Pettigrew et al. 1998).
Table 1.8  Similarities and differences between CTE and AD

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>CTE</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cognitive (early)</td>
<td>Impaired attention and memory</td>
<td></td>
</tr>
<tr>
<td>Mood &amp; Behavior (middle)</td>
<td>Depression, aggression, suicides</td>
<td>Anxiety, restlessness</td>
</tr>
<tr>
<td>Motor (late)</td>
<td>Impaired coordination, tremor</td>
<td>Loss of motor functions</td>
</tr>
<tr>
<td></td>
<td>Brain atrophy, ventricle dilation</td>
<td></td>
</tr>
<tr>
<td>Gross</td>
<td>Cavum septum pellucidum</td>
<td>No common finding</td>
</tr>
<tr>
<td>Pathologies</td>
<td>Aβ</td>
<td>All have amyloid deposition (senile plaques in late stage)</td>
</tr>
<tr>
<td></td>
<td>50% develop amyloid deposition (diffuse plaques)</td>
<td></td>
</tr>
<tr>
<td>Tau</td>
<td>• NFT in superficial cortical laminae</td>
<td>• NFT in deeper layer projection neurons</td>
</tr>
<tr>
<td></td>
<td>• Most concentrated at frontotemporal lobes around sulci</td>
<td>• Uniform distribution, starting from entorhinal cx</td>
</tr>
</tbody>
</table>
Chapter 2: Development of the Closed Head Impact Model of Engineered Rotational Acceleration (CHIMERA) as a novel and clinically relevant model of TBI

2.1 Introduction

Traumatic brain injury (TBI) is a leading worldwide cause of death and disability for persons under 45 yr 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, Rutland-Brown et al. 2006, Faul, Xu et al. 2010) TBI incidence is reportedly lower in Europe (235 per 100,000) and Australia (322 per 100,000) (Cassidy, Carroll et al. 2004, Tagliaferri, Compagnone et al. 2006) although recent epidemiological data suggests far greater incidence (749 per 100,000). (Feigin, Theadom et al. 2013) Mild TBI (mTBI), which includes concussion, comprises over 75% of all TBIs. (Gerberding and Binder 2003) As mTBI is increasingly recognized as an injury for which medical attention should be sought, the reported incidence of mTBI is rising.

Falls are the most prevalent cause of TBI, and motor vehicle accidents and impacts against objects are also common causes. (Cassidy, Carroll et al. 2004, Faul, Xu et al. 2010, Roozenbeek, Maas et al. 2013) TBI resulting from 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, Warden et al. 2006, Hoge, McGurk et al. 2008, Elder and Cristian 2009, Sevagan, Zhu et al. 2013) Furthermore, the growing awareness that mTBI may have long-lasting and severe
consequences (Gavett, Stern et al. 2011, Jordan 2013, Rusnak 2013, Smith, Johnson et al. 2013) highlights the urgency to understand much more about the acute and long-term consequences of brain injury.

Rapid acceleration and rotation during impact TBI leads 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, blood vessels and glia, producing a primary injury that initiates secondary processes within hours to weeks after the initial injury. (McIntosh, Smith 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 intracranial pressure, impaired cerebral blood flow, increased blood-brain barrier permeability, inflammation, axonal injury, calcium influx, elevated oxidative stress, free radical-mediated damage, excitatory neurotransmitter release, and cell death. (McIntosh, Smith et al. 1996, Blumbergs 1997, Davis 2000, Giza and Hovda 2001, Werner and Engelhard 2007, McAllister 2011) Although few treatment options are available for the primary injury, secondary injury pathways are potentially modifiable. (Graham, McIntosh 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 open-head injury models including fluid percussion (FP) and controlled cortical impact (CCI) systems, and closed-head injury (CHI) models that use either gravity or mechanical methods to impact the intact skull. (reviewed in (Namjoshi, Good et al. 2013)) 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 typical human injuries, which result from impact and/or acceleration on an intact skull. In contrast, closed-head injury (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 more accurately represent 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, and response of the animal’s head to mechanical loading) are often poorly controlled, which can contribute to the considerable experimental variation across cognitive, histological, and biochemical outcome measures. (reviewed in (Namjoshi, Good et al. 2013))

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 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 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.
2.2 Materials and methods

2.2.1 CHIMERA impactor

The CHIMERA impactor consists of an aluminum frame that supports an animal holding platform above a pneumatic impactor system (Fig 2.9). 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 is projected to contact 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 upon the head plate. 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 lie flat over the hole in the head plate, thus delivering impact to the dorsal cortical region.

The pneumatic impactor system includes an accumulator air tank, pressure regulator, digital pressure gauge, two-way solenoid valve, and trigger button. The pressure regulator and digital pressure gauge allow precise adjustment of air pressure to 0.1 psi (0.69 kPa), enabling accurate delivery of piston velocity and impact energy. Impact is induced with a 50 g free-floating chrome-coated steel impact piston whose trajectory is constrained to linear motion by a steel barrel. An array of holes drilled through the piston barrel near the muzzle end allows the air to vent and equalize the pressure as the piston moves past them towards the impact. The piston is accelerated by a controlled pulse of compressed air along the length of the barrel until it clears the venting holes.

CHIMERA was calibrated by measuring the exit velocity of the piston at various air pressures (0.5, 1, 1.5, 2, 3, 5, 7, and 10 psi) to determine the relationship between air pressure and piston velocity. Three measurements were taken at each pressure value. Each impact event was recorded by a high-speed video camera at 10,000 fps and tracked by video motion analysis.
software (TEMA Motion, Image Systems AB, Sweden). A 2nd order polynomial curve was used to fit the data. The $r^2$ value was 0.9996 (Fig 2.9D). Using this curve, the desired impact velocity or energy can be independently interpolated. By choosing the appropriate air pressure, impacts of input energy ranging from 0.01 J to 1 J can be precisely generated.

2.2.2 CHIMERA TBI procedure

All animal procedures were approved by the University of British Columbia Committee on Animal Care (protocol # A11-0225) and were carried in strict accordance with the Canadian Council on Animal Care guidelines. Male C57Bl/6 mice (mean body weight 33.9 ± 4.6 g) at 4 mo of age were housed with a reversed 12 h light-12 h dark cycle for at least 10 days before TBI. Animals were anaesthetized with isoflurane (induction: 4.5%, maintenance: 2.5%) in oxygen (0.8 L/min). 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. Animals were placed supine in the holding bed such that the top of the animal’s head lay flat over a hole in the head plate, aligned using crosshairs such that the piston strikes the vertex of the head covering a 5 mm area surrounding the bregma (Fig 2.9B and C). Impact was induced by pressing a trigger button that simultaneously fires the piston and activates a high-speed camera to record the resulting head trajectory. Isoflurane delivery was immediately stopped and the animal was continuously monitored until fully ambulatory. Twenty-four hours after the first impact, a second identical impact was delivered. Sham animals underwent all of these procedures and except for the impact. Approximately 3% of animals did not regain consciousness for > 45 min or displayed severe motor dysfunction after TBI, and were euthanized.

2.2.3 High-speed videography and kinematic analysis

For kinematic analysis, an independent 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. Videos were analyzed using ProAnalyst (Xcitex Inc., Woburn, MA). 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. Velocities and accelerations were determined by discrete differentiation of the position data. Resultant linear velocity and acceleration were calculated as the magnitude of their respective X and Y components. Linear kinematic parameters were assessed from trajectories obtained by tracking the paint mark. 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. After video capture, the dental floss was removed. Energy transferred from the piston to the head was determined by \( KE = 0.5 \times M_e \times \Delta V^2 \), where \( M_e \) is the effective mass and approximated by head mass (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.

### 2.2.4 Behavioral analyses

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 NSS (Flierl, Stahel et al. 2009) determined at 1 h and at 1, 2, and 7 d following the second TBI. NSS is a composite of ten different tasks, including tasks of motor function, alertness, and physiological behavior (Supplementary Table S6). One point is awarded for the lack of a tested reflex or for the inability to perform the tasks and no point for succeeding the task. A maximal NSS of 10 points thus indicates severe neurological dysfunction, with failure of all tasks. Motor performance was evaluated at 1, 2, 7, and 14 d after the second TBI using an accelerating rotarod as previously described. (Namjoshi, Martin et al. 2013) 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. The animal was placed in the center of the box and spontaneous activity was recorded for 10 min, 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 and spatial reference memories were assessed from days 7-13 after the second TBI using the passive avoidance task (days 7-10) and the Barnes maze (days 8-13), respectively. 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 shocker (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. Barnes maze testing was conducted on a grey circular platform (91 cm diameter) with 20 circular holes (5 cm diameter; Stoelting Co) 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. A digital camera was placed 4 m above the center of the maze to record trials for the ANY-maze tracking system. For motivational purposes, mice were food restricted to 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) that hung 10 cm above the maze. On day 8 after the second TBI 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 after the second TBI mice were tested for memory retention. Mice were given four trials per day (15-min inter-trial interval) for five days. For each trial, mice were placed in a black plastic
start tube (7 cm diameter, 12 cm height) on 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.

### 2.2.5 Tissue collection

For histological analyses, mice were anesthetized with an intraperitoneal injection of 150 mg/kg ketamine (Zoetis) and 20 mg/kg xylazine (Bayer) at 2, 7, or 14 d after the second TBI, and brains were collected from perfused animals as described (Namjoshi, Martin et al. 2013) except that 4% paraformaldehyde rather than neutral buffered formalin was used to post-fix hemisected brain tissue for histology. For biochemical analyses, brains were harvested as above at 6h, 12h, 2d, 7d, and 14d post-rTBI, longitudinally hemisected and rapidly frozen over dry ice and stored at -80°C until analysis.

### 2.2.6 Microglial activation and Silver staining

Silver staining and Iba-1 histology was performed as described. (Namjoshi, Martin et al. 2013) Microglial morphology was quantified using fractal analysis using ImageJ (NIH). Three to four microglia were randomly chosen from 40X-magnified Iba-1 stained images of olfactory nerve layer, corpus callosum, optic tract and brachium of superior colliculus, 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. Silver staining intensity was quantified using ImageJ (version 1.48, NIH) on 40X-magnified images of olfactory nerve layer, corpus callosum and optic tract. 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. The number of microglia in each white matter regions was quantified by scanning the entire area using Olympus BX61 microscope at 10X with a ProScan motorized XY stage (Prior Scientific Inc, Rockland, MA). The individual images were combined as a scan image using ImagePro. The number of Iba1-positive cells were counted manually in the
entire region. The area of ROI was measured by ImageJ as pixels and scaled to \( \mu \text{m}^2 \). Cell density was finally expressed as number of cells per mm\(^2\).

2.2.7 Biochemical analyses

**Tissue Processing.** For protein determination, half-brains were homogenized in RIPA lysis buffer as described. (Namjoshi, Martin et al. 2013)

**Cytokine ELISA.** Endogenous TNF\(\alpha\) and IL-1\(\beta\) 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.

**Quantitative Assessment of Phosphorylated and Total Tau.** Phosphorylated and total tau were assessed using an automated capillary electrophoresis-sized-based blot-free Western-like system called WES (ProteinSimple, San Jose, CA). (O'Neill, Bhamidipati et al. 2006) All procedures were performed with manufacturer’s reagents according to the user manual. Briefly, 5 \( \mu \)l of RIPA lysate (2 \( \mu \)g of protein) was mixed with 1.2 \( \mu \)l 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, separation and immunodetection were performed automatically using default settings. Data were analyzed with Compass software (ProteinSimple). Samples were immunodetected using following monoclonal antibodies (all kind gifts from Dr. Peter Davies, Albert Einstein College of Medicine, Manhasset, NY, USA): RZ3 is directed against tau protein phosphorylated at Thr231 (1:25), PHF1 is directed against tau protein phosphorylated at Ser396 and Ser404 (1:25), CP13 is directed against tau protein phosphorylated at Ser202 and Thr404 (1:25) and DA9 is directed against phosphorylation-independent (total) tau (1:5000). GAPDH (clone 6C5, 1:5000, Chemicon) was used as a loading control. Levels of phosphorylated 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.

The rTBI protocol and post-rTBI end points are summarized in Fig 2.12.
2.2.8 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-positive cell morphology and cell density 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 for 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).

2.3 Results

2.3.1 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 an impact energy of 0.5 J (Fig 2.1; peak kinematic parameters depicted in Fig 2.1H). Trajectories of the mouse head in the sagittal plane during peak acceleration following two impacts are depicted in Fig 2.1A. Following vertical impact, the head followed a looped trajectory in the sagittal plane (Fig 2.10). The average head trajectories following two repetitive TBIs in 8 mice were highly consistent (Fig 2.1A). The head traveled a peak linear displacement of 49.6 ± 3.5 mm in 15.7 ± 2.4 ms (Fig 2.1B) and exhibited a peak angular deflection of 2.6 ± 0.28 rad in 24.8 ± 3.1 ms (Fig 2.1C). Peak linear velocity was 6.6 ± 0.8 m/s at 3.4 ± 1.0 ms (Fig 2.1D), and peak angular velocity was 305.8 ± 73.7 rad/s at 2.8 ± 1.9 ms following initial impactor contact (Fig 2.1F). The head experienced large linear and angular accelerations following impact, achieving peak linear acceleration of
385.3 ± 52 g at 1.5 ± 0.3 ms (Fig 2.1E), whereas the peak angular acceleration of 253.6 ± 69.0 krad/s² was observed at 0.8 ± 1.1 ms (Fig 2.1G). As the head was stationary before impact, the change in head velocity (ΔV) 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.

Using the equal stress/equal velocity approach (Holbourn 1943, Gutierrez, Huang et al. 2001, Viano, Hamberger 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 (Supplementary Table S5).

2.3.2 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, Perez-Polo et al. 2013) Mice subjected to CHIMERA rTBI showed significantly increased LRR duration compared to sham animals (Fig 2.2A, p<0.001). LRR duration was consistent between the first and second impacts (Fig 2.2A). We further assessed injury severity using the Neurological Severity Score (NSS), which is a composite of ten tasks that assess motor reflexes, alertness, and physiological behavior. (Flierl, Stahel et al. 2009) The NSS of injured animals was significantly higher than sham mice at 1h to 7d post-procedure (Fig 2.2B, p<0.0001). In injured mice, the NSS score showed maximum deficits at 1h post-procedure followed by steady spontaneous improvement over the next 48h, albeit remaining significantly higher than sham animals at each post-rTBI time point (Fig 2.2B). 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 (Fig 2.2C). Fall latencies showed both time (p<0.001) and injury (p<0.01) effects. Injured mice showed anxiety-like behavior as indicated by significantly increased thigmotaxis in an open field test (Fig 2.2D, p<0.001). 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 mice (Fig 2.11). Repeated TBI induced working memory impairment as indicated by decreased latencies to enter the darkened compartment on the passive avoidance task (Fig 2.2E, \( p = 0.041 \)). For all mice, a main effect of Day indicated that mouse behavior changed during the training and testing sessions evaluated in this study \(( p < 0.001)\). For the main effect of Day, pairwise comparisons indicated that mice entered the darkened compartment significantly faster on 7d \(( p < 0.001)\) than on 8-10d \(( p > 0.05)\) signifying that all mice remembered the shock to some degree (Fig 2.2E). Injured mice also showed spatial reference memory impairment as indicated by increased latencies to locate the escape hole on the Barnes maze (Fig 2.2F, \( p = 0.018 \)).

2.3.3 CHIMERA rTBI induces widespread persistent diffuse axonal injury
Silver staining was used to assess post-rTBI axonal damage at 2, 7, and 14d post-rTBI (Figures 3 and 4). rTBI brains revealed widespread axonal injury, as indicated by intense punctate and fiber-associated argyrophilic structures in several white matter tracts including the olfactory nerve layer of the olfactory bulb, corpus callosum, and optic tracts (Fig 2.3A and B, Fig 2.4). 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 (Fig 2.3C), which is a characteristic histological feature of human axonal pathology after TBI. (Johnson, Stewart et al. 2013) Quantitative analysis of silver staining revealed that in the olfactory nerve layer, following a spike at 2 d, silver uptake returned to sham levels over 7-14d (Fig 2.4A). On the other hand, silver stain uptake continued to increase from 2 through 14d post-rTBI in the corpus callosum (Fig 2.4B, \( p<0.05 \)) and optic tract (Fig 2.4C, \( p<0.05 \)) indicating ongoing axonal degeneration in the coup and contrecoup regions, respectively. Silver uptake was most intense in the optic tract and showed both time and injury effects (Figures 3B and 4C, \( p<0.05 \)).

2.3.4 CHIMERA rTBI induces widespread microglial activation and increases pro-inflammatory cytokine levels
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 5 and 6). 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 (Fig 2.5C, upper row, Fig 2.6A-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 (Fig 2.5C, lower row, Fig 2.6A-D, black bars). In the injured olfactory nerve layer and optic tract, microglial morphological changes were significant at 2d \( (p < 0.05) \) and returned to sham levels by 7d post-rTBI (Fig 2.6A and D). Microglial morphology was significantly different from sham controls from 2-7d in the injured corpus callosum and brachium of superior colliculus \( (p < 0.05) \), and returned to sham levels by 14d post-rTBI (Fig 2.6B and C).

In addition to changes in microglial morphology, we observed significant increases in the number of microglia in the same white matter regions (Fig 2.6E-H, \( p < 0.001 \)) up to 14d post-rTBI, indicating that injury induced proliferation or recruitment of immune cells. In the olfactory nerve layer (Fig 2.6E, \( p < 0.01 \)) and optic tract (Fig 2.6G, \( p < 0.05 \)), microglia cell number increased significantly at 2d. The increase resolves by 7d and 14d in olfactory bulb, but persists up to 14d in optic tract (Fig 2.6G, \( p < 0.001 \)). In corpus callosum (Fig 2.6F, \( p < 0.05 \)) and brachium of superior colliculus (Fig 2.6H, \( p < 0.01 \)), a delayed but persistent increase in cell number is observed from 7d to 14d. In sham animals, microglia density did not significantly change over time. However, in injured animals, microglia density significantly increased from 2d to 7d and 14d post-rTBI in optic tract (Fig 2.6G, \( p = 0.0007 \)) and brachium of superior colliculus (Fig 2.6H, \( p = 0.0005 \)), but not olfactory bulb (Fig 2.6E, \( p=0.078 \)) or corpus callosum (Fig 2.6F, \( p=0.058 \)). In summary, rTBI induced microglia activation, as indicated by both altered cell morphology and cell number, in multiple white matter tracts.

In addition to the microglial response, we also measured protein levels of the pro-inflammatory cytokines TNF\( \alpha \) and IL-1\( \beta \) in half brain homogenates. Protein levels of TNF\( \alpha \) (Fig 2.7 A) and
IL-1β (Fig 2.7B) were significantly higher at 2d post-TBI compared to the respective sham levels ($p<0.01$).

2.3.5 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). Total murine tau levels were determined by the antibody DA9. WES analysis (a Western-like system) showed significantly increased phosphorylation of all the probed epitopes in rTBI brain lysates at 6h, 12h, and 2d compared to the respective sham brain lysates (Fig 2.8A-C and 8G-I, $p<0.01$). The change in tau phosphorylation reflected a significant increase in the ratio of phosphorylated tau:total tau, but not a change in total tau levels (Fig 2.8D-F and 8G-I, $p<0.01$).
2.4 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 existing rodent TBI models. 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 exposure to multiple anesthetic agents including opioid analgesics (i.e., buprenorphine) and sedatives (i.e., xylazine) that can interfere with rapid neurological assessment. Surgical models are also low throughput, require extensive operator training, and have limited suitability for studies involving repetitive TBI or long-term TBI outcomes. By contrast, CHIMERA produces injury using a simple, reliable and semi-automated procedure that requires <10 min per animal to produce defined injury (Fig 2.9). As the biomechanical input parameters are highly adjustable across impact energy, velocity, and directional parameters, CHIMERA offers a wide dynamic range of precisely controllable inputs to reproduce specific 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, which potentially enables greatly improved translational relevance to human TBI. CHIMERA produces diffuse injury characterized by activation of inflammatory reactions, axonal damage, and tau phosphorylation, replicating many aspects of the neuropathology 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.

In this study, a 50 g piston was used to deliver 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 murine TBI is 0.1 J to 1 J) in minimum steps of 0.01 J, with highly reproducible performance (Fig 2.9D). Adjusting the 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.

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, Huang et al. 2001, Viano, Hamberger et al. 2009) Thus, 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, Huang et al. 2001, Viano, Hamberger et al. 2009), and has been applied to a rat model (Viano, Hamberger 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. Nevertheless, 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, Viano et al. 2003, Pellman, Viano et al. 2003) 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, Foda et al. 1994, Flierl, Stahel et al. 2009) have poor control over biomechanical input parameters, including friction and air resistance inside the guide tube that may contribute to variable outcomes. High incidence of skull fractures and limited dynamic range pose additional challenges in comparing results using weight-drop TBI models across different laboratories. (Namjoshi, Good et al. 2013) 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 various types of 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, Angoa-Perez 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, Cassidy 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, Lucas et al. 2011, Putukian 2011), as well as mood changes such as irritability or anxiety (Blume, Lucas et al. 2011, Daneshvar, Riley et al. 2011). Though general locomotor activities were not severely affected (Fig 2.11), CHIMERA TBI resulted in deficits in fine motor coordination and neurological performance. Similar changes have also been reported by other groups. (Tsenter, Beni-Adani et al. 2008, Mouzon, Chaytow et al. 2012) CHIMERA also increased thigmotaxis in TBI animals, suggesting an anxiety-like behavior. (Simon, Dupuis et al. 1994) Our model also revealed deficits in both working and spatial reference memory as assessed by the passive avoidance test and Barnes maze, respectively. Intriguingly, recovery of motor performance was faster than cognitive performance under the conditions of our study.

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, Toulmond et al. 1993, Shohami, Gallily et al. 1997, Rooker, Jander et al. 2006, Shein, Doron et al. 2007, Shitaka, Tran et al. 2011, Perez-Polo, Rea et al. 2013) Under our conditions, rTBI led to elevated IL-1β and TNF-α levels at 2d after injury, which was accompanied by histological evidence of microglial activation and microgliosis. 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, Toulmond et al. 1993, Shohami, Gallily et al.
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 was significant at 2 d in all white matter tracts analyzed. While microglial activation in the corpus callosum and brachium of superior colliculus was persistent until 7d, it was resolved in the olfactory nerve layer and optic tract within the same time period. In all areas, microglia morphology returned to resting state by 14d. Intriguingly, the change in microglia cell density showed a similar but not identical pattern. An increase in microglia cell density at 2d was only observed in the olfactory bulb and optic tract. The increase in microglia cell numbers in the optic tract was persistent until 14d, but the olfactory bulb was resolved by 7d. In contrast, the corpus callosum and brachium of superior colliculus showed a delayed but persistent increase of microglia cell numbers at 7d and 14d. These findings suggest that rTBI-induced changes in microglia morphology and cell number may not necessarily be parallel. The quicker resolution of microglia morphology is comparable to the trend observed in cytokine expression, whereas the persistent increase in microglia cell density is more comparable to the trend of persistent axonal injury as shown by silver staining results. Since 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, recruitment of immune cells from the periphery, or both.

Diffuse axonal injury (DAI) is one of the characteristic pathologies of TBI. (Adams, Doyle et al. 1989, Johnson, Stewart 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, Stewart 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 that both coup and contra-coup injuries are present in our model. Affected white matter areas, including the corpus callosum, optic tract, and olfactory system, have been reported in other CHI models that induce impact at the superior side of skull. (Foda and Marmarou 1994, Creeley, Wozniak et al. 2004, Shitaka, Tran et al. 2011, Namjoshi, Martin et al. 2013) Several white matter areas showed increased argyrophilic staining and microglial
activation as well as microgliosis following rTBI, suggesting a possible relationship between axonal damage and neuroinflammation, in agreement with previous reports. (Engelhardt 1996, Shohami, Gallily et al. 1997, Namjoshi, Martin et al. 2013) Interestingly, axonal injury in the corpus callosum and optic tract continued to increase from 2 to 14d suggesting that ongoing secondary injury processes overwhelmed endogenous repair mechanisms in the time frame examined in this study. Moreover, the optic tract, which is contrecoup to the impact site, showed the most intense silver uptake, in agreement with the common clinical observation that contrecoup injuries are more severe than the coup injuries. (Drew and Drew 2004) On the other hand, axonal injury was resolved in the olfactory neuronal layer within 7d, suggesting efficient neural repair with active/neuroregeneration in this region. Future studies will be designed to assess changes in cognitive functions and the dynamics of axonal injury across several brain regions in our model over a longer-term (up to 6 mo) post-rTBI follow up.

Hyperphosphorylation of the cytoskeletal protein tau is a pathological event observed in many neurodegenerative diseases including Alzheimer’s Disease (Ballatore, Lee et al. 2007) and chronic traumatic encephalopathy (McKee, Stern et al. 2013). In our model, we demonstrate that endogenous tau hyperphosphorylation is an early and dynamic event after rTBI in wild-type mice, again in agreement with other models of CHI. (Genis, Chen et al. 2000, Goldstein, Fisher 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.

In summary, here we report a novel, surgery-free CHI model that fully integrates biomechanical, functional and neuropathological characteristics of TBI. CHIMERA allows precise control over mechanical inputs allowing reproducible head kinematics. Our study also shows that CHIMERA-TBI reliably replicates several key behavioral, biochemical and neuropathological characteristics of human TBI including axonal injury, neuroinflammation, and functional deficits. Future studies will be conducted to characterize in more detail 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.
Figure 2.1  Head kinematics during rTBI

Head kinematic parameters during impacts were assessed in 8 mice subjected to rTBI. Data are represented as the means for each impact. (A) Head trajectory during the maximum acceleration phase 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. Data in (A) are represented as mean ± 95% CI in both X- and Y- direction, respectively. Data in B-G
are represented as mean ± 95% CI. (H) Summary of peak values of kinematic parameters averaged across all 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.
Figure 2.2  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 1h, 1d, 2d, and 7d post-rTBI. Cohort size: Sham (1 h: N = 34, 1d: N = 31, 2d: N = 35, 7d: N = 21); rTBI (1 h, 1d and 2d: N = 42, 7d: N = 25). (C) Motor performance was assessed on an accelerating rotarod at 1d, 2d, 7d and 14d 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 1d, 7d and 14d post-rTBI. Cohort size: Sham (1d: N = 23, 7d: N = 16, 14d: N = 15); rTBI (1d: N = 24, 7d: N = 10, 14d: N = 15). Data in A-D were analyzed by repeated measures two-way ANOVA followed by the Holm-Sidak post-hoc test. (E) Working memory was assessed by the passive avoidance test from 7d to 10d post-rTBI. (F) Spatial reference memory was assessed by Barnes maze from 9d to 13d post-rTBI. Data at each time point represent the mean of four trials. Data in E and F (Cohort size: N = 15/group) were
analyzed by repeated measures two-way ANOVA. For all graphs, data are presented as mean ± SEM. For all graphs, * indicates a significant rTBI effect within a particular time point and # indicates a significant time effect within each group. ***: p < 0.001. ###: p < 0.001.
Figure 2.3   CHIMERA rTBI induces diffuse axonal injury
Axonal degeneration was assessed by silver staining at 2, 7, and 14d post-rTBI. (A) Coronal sections showing white matter areas including the olfactory nerve layer, corpus callosum, and optic tract with regions of prominent silver staining indicated by black rectangles. (B) Representative 40X-magnified images of the same brain regions in sham-operated (upper row) or rTBI-induced (lower three rows) animals at the indicated time points. (C) 100X-magnified images of the same brain regions in rTBI-induced animals. Axonal varicosities are indicated by arrows.
Figure 2.4  Quantitative analysis of silver stain images

Silver stained images were quantified by calculating the % of region of interest (ROI) in the white matter tract area that was stained with silver. Bars indicate mean ± SD 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 by a Tukey post-hoc test. Cohort size: olfactory nerve layer: Sham (2d: N = 4, 7d: N = 5, 14d: N = 6); rTBI (2d: N = 8, 7d and 14 d: N = 5); corpus callosum: Sham (2d and 7d: N = 5, 14d: N = 6); rTBI (2d: N = 16, 7d: N = 5, and 14 d: N = 6); optic tract: Sham (2d: N = 5, and 7d: N = 4, 14d: N = 6); rTBI (2d: N = 17, 7d and 14d: N = 5). For all graphs, * indicates a significant rTBI effect within a particular time point and # indicates a significant time effect within rTBI group. **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. ###: p < 0.001, ####: p < 0.0001.
Figure 2.5  CHIMERA rTBI induces widespread microglial activation
Microglial activation was assessed using Iba-1 immunohistochemistry at 2, 7, and 14d 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 the same white matter tract regions showing resting microglia in sham brains (upper row) and activated microglia in injured brains (lower three rows) at the indicated time points. (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 2.6  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.
Figure 2.7  CHIMERA rTBI increases proinflammatory cytokine levels

Bar graphs represent mean ± SD % fold change in TNFα (A) and IL-1β (B) levels in rTBI brain lysates compared to the levels in the sham brain lysates at the respective time points. For both graphs, ** indicates p < 0.01 in comparison of rTBI vs sham, using multiple t-tests with Bonferroni corrections for multiple comparisons (p = 0.05/5 = 0.01).
Tau phosphorylation was analyzed using the Simple Western system (ProteinSimple). 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, Panel A), RZ3 (pThr231, Panel B) and PHF1 (pSer396 and pSer404, Panel C), respectively. Graphs in the middle column (D-F) depict quantitation of phosphorylated tau as a
proportion of total tau (DA9). Representative digital immunoblots of phosphorylated and corresponding total tau are depicted in the right column (G-I). Arrows on the left of the blots indicate molecular weight marker at 66 kDa. Data are presented as the mean ± SD 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 for multiple comparison (p = 0.05/5 = 0.01).
Figure 2.9  CHIMERA device and mouse head positioning

(A) The picture depicts the CHIMERA device. Various parts are labeled with numbers 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) Close-up view of animal strapped on the holding platform. (C) Location of impact relative to the mouse head and brain. P: impact piston. (D) 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 three measurements for each air pressure value.
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. 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.
Figure 2.11 CHIMERA rTBI does not affect general mobility
General mobility was tested by the open field test at 1, 7 and 14d post-injury. No significant differences were observed between sham and rTBI mice in total distance travelled (A), number of lines crossed (B), or time spent immobile (C). Data are presented as the mean ± SEM and analyzed by repeated measures two-way ANOVA followed by Holm-Sidak post-hoc test.
Figure 2.12  Experimental plan

The figure indicates the timeline for rTBI/sham procedure and behavioral, biochemical and histological end points at various post-rTBI time points used in this study. BM: Barnes maze, Iba-1: Iba-1 immunohistochemistry, NSS: neurological severity score, OF: open field behavior, PA: passive avoidance, RR: rotarod, SS: silver stain.
### Table 2.1 Comparison of kinematic parameters between TBI models

The table compares four 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 (\( \lambda \)), angular acceleration is scaled by \( \lambda^2 \). \( \lambda \) 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 likelihood of head injury. HIC15 for the CHIMERA was calculated based on the definition:

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

, where linear acceleration \( (a) \) is in g and scaled by \( \lambda \), time 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.

<table>
<thead>
<tr>
<th>Study</th>
<th>Injury Type</th>
<th>Species</th>
<th>( \Delta V ) (m/s)</th>
<th>Angular Velocity (rad/s)</th>
<th>Linear Acceleration (g)</th>
<th>Angular Acceleration (krad/s(^2))</th>
<th>HIC15</th>
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<td>14.2</td>
<td>16.9</td>
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<tr>
<td>Study</td>
<td>Injury Type</td>
<td>Species</td>
<td>ΔV (m/s)</td>
<td>Angular Velocity (rad/s)</td>
<td>Linear Acceleration (g)</td>
<td>Angular Acceleration (krad/s²)</td>
<td>HIC15</td>
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<td>-</td>
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<td>1.1</td>
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<td>-</td>
<td>1.1</td>
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<tr>
<td>Davidsson et al (2011)</td>
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<td>Human (Hybrid III dummy)</td>
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<td>Olympic Boxing – Uppercut</td>
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Chapter 3: Defining the biomechanical and biological threshold of murine mild TBI using CHIMERA

3.1 Introduction

Traumatic brain injury (TBI) is a leading worldwide cause of death and disability in persons under 45 yr of age with an annual cost of over $80B. (Faul and Coronado 2015) Over 2 million TBIs occur every year in the United States, mostly from falls, motor vehicle accidents and sports. (Faul and Coronado 2015) The Glasgow Coma Scale (GCS), developed in 1974 by Graham Teasdale and Brain Jennet (Teasdale and Jennett 1974), is widely used to assess a patient’s level of consciousness using eye, verbal and motor responses to classify TBI into categories of mild (GCS 13-15), moderate (GCS 8-12) and severe (GCS 3-7). (United Sates Departments of Defense and Veterans Affairs 2008) Current data from the Centers for Disease Control and Prevention show that approximately 75% of TBI cases are mild, and most occur in two major age groups including children-young adults and the elderly. (Faul and Coronado 2015)

Although the term “concussion” is often used interchangeably with mild TBI (mTBI) (Levin and Diaz-Arrastia 2015), it is important to realize that human concussion is defined by nonspecific clinical criteria such as confusion, amnesia, disorientation, or headache. It has long been recognized that clinically observable features, such as presence and duration of unconsciousness, duration of post-traumatic amnesia, and GCS on presentation are poor predictors of outcome and correlate weakly with neurodegeneration. In recent years, several schemes have been developed to sub-categorize mTBI. (Kamins and Giza 2016) Currently, mTBI is defined as GCS 13-15 within 24h of impact, and complicated mTBI is defined as mTBI combined with intracranial imaging findings. Concussion is defined as a “clinical syndrome in which a biomechanical force, via acceleration-deceleration or rotational forces, transiently disturbs normal brain function, causing neurological-cognitive-behavioral signs and symptoms”. (Kamins and Giza 2016) Subconcussion is a “proposed construct of biomechanical force causing subclinical injury in the absence of overt acute signs and symptoms”. (Kamins and Giza 2016) These definitions are in
flux, and many expect the current clinical classification scheme will eventually be replaced by one based on biomarkers that more closely reflects the underlying pathophysiology. (Saatman, Duhaime et al. 2008, Diaz-Arrastia, Kochanek et al. 2014)

Although historically considered benign, mTBI can result in significant disability. Most mTBI patients recover without sequelae, yet approximately 10% have intracranial lesions on computed tomography scanning (complicated mTBI) and a minority of these can develop a potential life-threatening condition that requires neurosurgical intervention. (Haydel, Preston et al. 2000) Additionally, up to 30% of mTBI subjects, despite having normal neuroimaging, can suffer persistent impairment of physical, cognitive, and psychosocial functioning known as post-concussive syndrome. (Carroll, Cassidy et al. 2014) The recognized incidence of mTBI complications and poor outcomes is rising, owing to greater awareness and reporting especially for sports-related concussion. Although the monetary cost of TBI varies by severity, the high incidence of mTBI leads to a total cost that is nearly three times that for moderate to severe TBI. (Te Ao, Brown et al. 2014) The prevalence and socioeconomic burden of mTBI are driving the demand to develop objective diagnostic, prognostic and therapeutic tools to address this large unmet medical need.

A major gap in clinical TBI research, especially for mTBI, is the lack of human brain specimens to study, as human brain tissues are only available under extreme life-threatening circumstances, or at postmortem where the time between injury and autopsy can be highly variable. Cerebrospinal fluid (CSF), which has been invaluable for the development of biomarkers of neurodegenerative disease, is rarely obtained after TBI. Blood specimens are being collected in several clinical TBI studies and several promising blood TBI biomarkers are emerging. (Bogoslovsky, Gill et al. 2016, Oresic, Posti et al. 2016, Shahim, Gren et al. 2016, Zetterberg and Blennow 2016) Still, how blood biomarkers may reflect brain changes after TBI is not yet clear.

Experimental TBI animal models from which pre-injury and longitudinal data can be obtained and from which brain biospecimens can be collected under controlled conditions therefore have
considerable potential to address a multitude of questions about the tissue level mechanisms of mTBI and the factors that modulate mTBI recovery and repair. Popular rodent TBI models include open-head injury models including fluid percussion (FP) and controlled cortical impact (CCI) systems, and closed-head injury (CHI) models that use either gravity or mechanical methods to impact the intact skull [reviewed in (Namjoshi, Good et al. 2013)]. Although FP and CCI models employ highly reproducible mechanical inputs and can mimic many pathological features of human TBI, their predominately focal tissue destruction and lack of head movement decreases their resemblance to the majority of human impact acceleration injuries, which most often occur without skull fracture and are chiefly characterized by diffuse axonal injury (DAI). The importance of understanding the relationship between biomechanical inputs and pathological responses in TBI is increasingly being recognized. (Namjoshi, Cheng et al. 2014, Courtney and Courtney 2015, Cullen, Harris et al. 2016, Coats, Binenbaum et al. 2017)

We recently developed a neurotrauma animal model called CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) to address the absence of a simple, nonsurgical impact acceleration model of CHI that reliably produces DAI and white matter inflammation. We previously showed that mice subjected to two mild, closed-head TBIs with an impact energy of 0.5 J using CHIMERA show human-like head kinematics and faithfully reproduce behavioral features and diffuse white matter tract pathology similar to human TBI. (Namjoshi, Cheng et al. 2014) However, much remains to be learned about the parameters of the nascent CHIMERA technology to substantiate it as a useful platform for the TBI research community. This study was therefore defined to establish the threshold energy for single impact mTBI in the sagittal plane and to test the hypothesis that CHIMERA mechanical inputs predict behavioral and neuropathological outcomes in mice.
3.2 Materials and methods

3.2.1 CHIMERA TBI procedure and high speed videography:
All animal procedures were approved by the University of British Columbia Committee on Animal Care and were carried in strict accordance with the Canadian Council on Animal Care guidelines. Male C57Bl/6 mice (mean ± SD body weight: 33.05 ± 3.63 g) at 4-5 months of age (mean ± SD age: 4.44 ± 0.56 mo) were housed with a reversed 12 h light-12 h dark cycle. Animals were prepared for single TBI in the sagittal plane using CHIMERA as described. Briefly, mice received isofluorane anesthesia (mean ± SD total isoflurane exposure duration: 278.67 ± 60.77 s) and were positioned on the CHIMERA impactor. TBI animals received a single closed-head impact at 0.1, 0.3, 0.4, 0.5, 0.6 or 0.7 J of energy using a 50-g stainless steel piston. Sham animals (0 J) received an equivalent isoflurane exposure as TBI animals but experienced no impact. Five to six animals were included per energy group per post-TBI time point (described below). Head kinematics at each impact energy were assessed using high-speed (9,000 fps) videography with two reference points including a snout and a cheek marker, and were analyzed by ProAnalyst motion analysis software (Xcitex Inc., Woburn, MA) as described. (Namjoshi, Cheng et al. 2014) Pre-TBI body weight, age at TBI and isoflurane exposure duration were comparable across all impact energy groups (Fig 3.10).

The Head Injury Criterion (HIC) was calculated using Eq. (1). The HIC quantifies head impact severity by incorporating the acceleration magnitude and duration of acceleration exposure. The resultant linear head acceleration (a), measured in g, was used to calculate HIC with a time interval (t2 - t1) chosen to maximize HIC over a maximum duration of 15 ms.

\[
HIC_{15} = \left\{ \left[ \frac{1}{t_2 - t_1} \int_{t_1}^{t_2} a(t) dt \right]^{2.5} (t_2 - t_1) \right\}_{max}
\]

Scaling mouse head kinematic insults to equivalent relations in humans used a single scale factor of 13.8 (\lambda) and followed the equal stress equal velocity relationship as described by Ommaya (Ommaya, Yarnell et al. 1967) and summarized by Panzer et al. (Panzer, Wood et al. 2014)
3.2.2 Behavioral analyses

Behavioral analyses were performed as described. (Namjoshi, Martin et al. 2013, Namjoshi, Cheng et al. 2014) Briefly, duration of loss of righting reflex (LRR) was recorded immediately following head impact. Neurological impairment was assessed using the neurological severity score (NSS) at 1 h. Motor function was assessed using the accelerating rotarod test at 1, 2, 7 and 14 d post-TBI. Anxiety-like thigmotactic behavior was assessed in an open field at 1 and 7d after TBI. Post-TBI anxiety behavior was further tested with the elevated plus maze (EPM) at 2d post-TBI. For EPM testing, the animal was placed in the central zone of a custom-built EPM and spontaneous activity was recorded using AnyMaze (Stoelting Co., Wood Dale, IL) for 5 min to assess time spent in the open and closed arms.

3.2.3 Tissue collection

Brains were collected at 6 h and at 1, 2, 7 and 14 d post-TBI as described. (Namjoshi, Martin et al. 2013, Namjoshi, Cheng et al. 2014) Briefly, animals were anesthetized with ketamine and xylazine and perfused with ice-cold heparinized phosphate-buffered saline (PBS). Brains were rapidly collected, longitudinally bisected and hemibrains were processed for histology or biochemistry as described. (Namjoshi, Martin et al. 2013, Namjoshi, Cheng et al. 2014)

3.2.4 Histological analyses:

Hemibrains were cryoprotected with 30% sucrose and 40 µm coronal sections were prepared using a cryostat. Three to five coronal sections per brain spanning the olfactory bulb to the posterior hippocampus were assessed for histology as described below. Microglial activation and axonal damage were assessed using Iba1 immunohistochemistry and silver staining, respectively, as described. (Namjoshi, Martin et al. 2013, Namjoshi, Cheng et al. 2014) Astrocyte reactivity was assessed with glial acidic fibrillary protein (GFAP) immunohistochemistry. Briefly, coronal brain sections were treated with 0.3% hydrogen peroxide for 10 min and blocked with 5% normal goat serum (NGS) in PBS for 1 h. Sections were incubated overnight at 4°C with rabbit anti-mouse GFAP antibody (Abcam, AB_305808, 1:500) in 5% NGS. After washing in PBS, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody (1:1000 in PBS) for 1 h at room temperature. Sections were stained with horseradish peroxidase (Vectastain Elite
ABC Kit PK-6120, Vector Laboratories (Canada) Inc, Burlington, ON) and DAB substrate, mounted and dehydrated.

All stained sections were digitally imaged at a commercial histology service (Wax-it Histology Services Inc., Vancouver, BC) with a ScanScope CS-R scanner (Aperio Technologies) using a 20X magnification objective lens. From the whole-mount images, 20X magnified images containing regions of interest were extracted using the Aperio ImageScope Viewer (v. 12.2.2.2015, Aperio Technologies) software.

3.2.5 Image analyses:
Image quantification was carried out with ImageJ (1.51f, NIH). The microglial response was quantified by counting the number of Iba-1 positive cells in white matter regions as described. (Namjoshi, Cheng et al. 2014) The astrocyte response was quantified by counting GFAP-positive cells in the corpus callosum. Silver staining was assessed by quantifying percent stained area of the region of interest of white matter as described. (Namjoshi, Cheng et al. 2014)

3.2.6 Biochemical analyses:
Half-brains were homogenized in 1.5 ml of ice-cold RIPA lysis buffer containing 0.1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor (Roche Applied Science), and PhosSTOP tablets (Roche Applied Science) in a Tissuemiser homogenizer at full speed for 20 s and then sonicated at 20% output for 10 s. After incubating on ice for 10 min, lysates were clarified by centrifugation at 9000 rpm for 10 min at 4°C and the supernatant was taken for analysis. Levels of IL-6, IL-1β and TNFα were measured on lysates diluted 1:2 using a customized version of the V-PLEX Proinflammatory Panel 1 (mouse) (K152A0H-2, Meso Scale Discovery, Rockville, MD) following the manufactures protocol, with the exception that sample incubation was increased to overnight at 4°C. Levels of total and phosphorylated (Thr231) tau were measured on samples diluted 1:50 using a MSD® MULTI-SPOT assay (K15121D-2, Meso Scale Discovery, Rockville, MD) following the manufacturers protocol. Plates were read on a
Sector S 600 (Meso Scale Discovery, Rockville, MD) and concentrations were normalized to total protein content determine by bicinchoninic acid assay (Pierce).

3.2.7 Statistical analyses
All statistical analyses were performed using GraphPad Prism (v. 6.07) unless otherwise stated. Normality of data was checked to determine if parametric or non-parametric tests were used. Peak head kinematic parameters data were analyzed with one-way ANOVA followed by Tukey post-hoc tests. LRR and 1h NSS data were analyzed with Kruskal-Wallis test. Rotarod latencies and thigmotaxis index data were analyzed with a linear mixed model using SPSS Statistics (v.23, IBM Corporation). Histological quantification and EPM data were analyzed with two-way ANOVA followed by Tukey post-hoc tests. Correlation analyses were employed to assess the relationships between head kinematic data and post-TBI outcomes including LRR, 1h NSS, silver stain quantification, and Iba-1 positive cell count using Spearman’s rank correlation coefficient. All data are presented as mean ± SD unless otherwise stated.
3.3 Results

3.3.1 Data binning
One of the objectives of the current study was to determine the lower limit of impact energy required to induce significant alteration(s) in one or more of our post-TBI outcomes (kinematics, behavior and neurological deficits, neuropathology and biochemical changes) compared to sham controls. Initial analysis of each energy group showed that impact energies from 0.1 – 0.4 J caused no significant changes in any post-TBI behavioral and neuropathological outcome compared to the uninjured group. Significant changes in at least one measure of behavior and neuropathology were observed at impact energy of 0.5 J, and impact energies of 0.6 and 0.7 J produced significantly more injury than 0.5 J but were not significantly different from each other (not shown). Accordingly, we binned data from the seven energy groups into four categories: Sham (0 J), sub-threshold (0.1, 0.3 and 0.4 J), threshold (0.5 J) and mild TBI (mTBI, 0.6 and 0.7 J). The analyses reported hereafter represent binned data obtained from a total of N=31 mice in the sham category, N=94 mice in the subthreshold category, N=36 mice in the threshold category, and N=65 mice in the mTBI category. Experiments were designed with the target sample size of 5-6/energy group/time point for biochemical and histological analyses. We achieved the target N for all assays except for the quantitative analysis of tau where for the sham group we used N = 2/time point/energy group. The limited space on the assay plates precluded us from running all samples from all time points at the same time. The details of sample size for each analysis are presented in Table 3.2

3.3.2 Single impact CHIMERA induces energy dose-dependent changes in head kinematics
We assessed changes in head kinematics by analyzing peak linear (head displacement, velocity and acceleration) and angular (angle, velocity and acceleration) kinematic parameters over increasing impact energies. Each parameter showed an impact energy dose-dependent increase (Fig 3.1). Except for head angle, we observed significant impact energy dose effects for all other head kinematic parameters [Displacement: $F(2, 53) = 15.21, p < 0.0001$; Linear Velocity: $F(2, 53) = 24.62, p < 0.0001$; Linear Acceleration: $F(2, 53) = 19.58, p < 0.0001$; Angle: $F(2, 52) = 2.445, p = 0.0966$; Angular Velocity: $F(2, 53) = 31.37, p < 0.0001$; Angular Acceleration: $F(2,
52) = 15.71, \( p < 0.0001 \); One-way ANOVA followed by Tukey’s post-hoc test. Post-hoc analyses revealed that increasing impact energies caused a significant increase in all head kinematic values except head angle compared to the sub-threshold TBI group (Fig 3.1).

The mean ± SD HIC15 values scaled to human for the sub-threshold, threshold and mTBI groups were 43.01 ± 51.33, 91.94 ± 39.55, and 108.4 ± 57.05, respectively.

3.3.2 Single impact CHIMERA induces energy dose-dependent increases in righting time, neurological severity, motor deficits and thigmotactic behavior

We recorded LRR duration immediately following closed head impact, which showed a robust injury dose effect (Fig 3.2A, \( p < 0.0001 \), Kruskal-Wallis test). Post-hoc analysis showed that LRR durations of the threshold and mTBI groups were significantly longer compared to the sham and sub-threshold TBI groups (Fig 3.2A). Mice in the mTBI group exhibited the longest post-TBI LRR duration compared all other groups (Fig 3.2A). Similarly, the NSS determined at 1 h post-TBI also showed an overall injury dose effect (Fig 3.2B, \( p < 0.0001 \), Kruskal-Wallis test). Mice in the threshold and mTBI groups exhibited significantly higher NSS values compared to sham controls (Fig 3.2B). Post-TBI anxiety behaviors were assessed with the EPM and open-field thigmotactic behavior. For EPM we compared the number of entries by the animal in the open and closed arms of the maze and found no significant differences across any energy group (Fig 3.2C, \( p > 0.05 \), two-way ANOVA). By contrast, thigmotactic behavior showed significant impact energy dose (\( p = 0.016 \)) and time (\( p < 0.0001 \)) effects (Fig 3.2D, linear mixed model). Post-hoc analysis showed significantly increased thigmotactic behavior of mice in the mTBI group compared to sham and sub-threshold TBI groups regardless of time. However, thigmotactic behavior did not show a significant impact energy x time interaction (\( p = 0.559 \), linear mixed model). We assessed post-TBI motor deficits using the accelerating rotarod task. Analysis of rotarod latencies revealed significant effects of impact energy dose (\( p < 0.0001 \)) and time (\( p < 0.0001 \)), and revealed a significant interaction of impact energy dose x time (\( p < 0.001 \)) (Fig 3.2E, linear mixed model). Post-hoc analysis of impact energy dose revealed significantly reduced rotarod latencies of mice in the mTBI group, while fall latencies of mice in sham, sub-threshold and threshold TBI groups were comparable over all post-TBI time points.
tested. Mice in the mTBI group exhibited sustained motor deficits compared to the sham group over all post-TBI time points except 7 d (Fig 3.2E).

3.3.3 Single impact CHIMERA induces energy dose-dependent increases in microgliosis, astrogliosis and pro-inflammatory cytokines

The post-TBI microglial response was assessed with Iba1 immunohistochemistry at 6 h and 1, 2, 7 and 14 d post-TBI. Compared to sham brains, injured brains showed significant increases in the number of Iba1-positive microglia in several white matter regions including the optic tract (Fig 3.3), olfactory nerve layer (Fig 3.11), corpus callosum (Fig 3.12), and brachium of superior colliculus (Fig 3.13), indicating that CHIMERA injury induced proliferation or recruitment of immune cells. In addition to white matter, we also noticed significant increases in Iba1-positive microglia in the lateral geniculate nucleus, which may suggest secondary transsynaptic degeneration (Fig 3.14). Quantitative analysis of Iba1 images revealed an impact energy dose-dependent increase in the number of Iba1-positive microglia in the olfactory nerve layer (Fig 3.4A, energy dose effect: $F(3, 125) = 23.9, p < 0.0001$), corpus callosum (Fig 3.4B, energy dose effect: $F(3, 130) = 26.58, p < 0.0001$), brachium of superior colliculus (Fig 3.4C, energy dose effect: $F(3, 132) = 25.4, p < 0.0001$) and optic tract (Fig 3.4D, energy dose effect: $F(3, 135) = 27.06, p < 0.0001$). Microgliosis was significant in all white matter regions in the mTBI group. Microglia number showed persistent increases over 1-14 d in the olfactory nerve layer (Fig 3.4A, Time effect: $F(4, 125) = 5.499, p = 0.0004$) and over 2-14 d in the corpus callosum (Fig 3.4B, Time effect: $F(4, 130) = 16.84, p < 0.0001$), brachium of superior colliculus (Fig 3.4C, Time effect: $F(4, 132) = 17.86, p < 0.0001$) and optic tract (Fig 3.4D, Time effect: $F(4, 135) = 5.499, p < 0.0001$). Finally, we observed a significant impact energy x time interaction in microglia number in the corpus callosum (Fig 3.4B, $F(12, 130) = 16.84, p = 0.0044$), brachium of superior colliculus (Fig 3.4C, $F(12, 132) = 1.833, p = 0.0489$) and optic tract (Fig 3.4D, Time effect: $F(4, 135) = 4.026, p < 0.0001$), indicating that increased impact energy doses led to persistently increased microglial proliferation or recruitment over time. The temporal profile of post-TBI microgliosis also showed region-specificity. Thus, microgliosis in the olfactory nerve layer and corpus callosum showed an early peak at 2 d post-TBI, while that in the brachium of superior colliculus and optic tract peaked later at 7 d (Fig 3.17A)
We also assessed astrocytic reaction at the same post-TBI time points by GFAP immunohistochemistry, and found a significant increase in the number of GFAP-positive astrocytes exclusively in the corpus callosum of injured animals (Fig 3.5A). Quantitative analysis of GFAP images showed significant effects for impact energy dose (Fig 3.5B, $F(3, 101) = 16.55$, $p < 0.0001$) and time ($F(4, 101) = 11.17$, $p < 0.0001$), and revealed a significant energy dose X time interaction ($F(12, 101) = 4.407$, $p < 0.0001$). Astrogliosis peaked in the threshold and mTBI groups at 2 d, and showed persistent increase in mTBI group up to 7 d followed by return to baseline in all groups by day 14 (Fig 3.5B).

In addition to microglial and astrocyte response, we also measured protein levels of pro-inflammatory cytokines IL-6, IL-1β and TNFα in half-brain homogenates at 6 h. Protein levels of all the three pro-inflammatory cytokines were significantly higher than sham levels (Fig 3.6, $p < 0.05$, one-way ANOVA).

### 3.3.4 Single impact CHIMERA induces energy dose-dependent increases in axonal damage

We used silver staining to assess axonal pathology as a function of energy level and time after single impact CHIMERA. Compared to the sham group, injured brains showed widespread axonal injury, as indicated by intense punctate and fiber-associated argyrophilic structures in the optic tract (Fig 3.7), olfactory nerve layer (Fig 3.15), and corpus callosum (Fig 3.16).

Quantification of silver stained images showed an impact energy dose effect in the olfactory nerve layer (Fig 3.8A, $F(3, 126) = 11.75$, $p < 0.0001$), corpus callosum (Fig 3.8B, $F(3, 141) = 15.68$, $p < 0.0001$) and optic tract (Fig 3.8C, $F(3, 125) = 33.26$, $p < 0.0001$). Post-hoc testing revealed significantly higher silver uptake in all white matter regions by 2 d post-injury in the mTBI group compared to sham (Fig 3.8).
We also noticed region-specific changes in the temporal profile of silver uptake (Fig 8 and Supplementary S9B). Thus, in mTBI group silver uptake was prominent in the olfactory nerve layer at 1 and 2 d followed by return to sham levels by day 7 (time effect: $F(4, 126) = 11.39$, $p < 0.0001$; impact energy dose x time interaction: $F(12, 126) = 6.393$, $p < 0.0001$). On the other hand, silver uptake in the mTBI group showed a delayed but persistent increase over 2-14 d in corpus callosum (time effect: $F(4, 141) = 0.7414$, $p = 0.5653$; energy dose x time interaction: $F(12, 141) = 2.044$, $p = 0.0245$) and optic tract (time effect: $F(4, 125) = 12.93$, $p < 0.0001$; energy dose x time interaction: $F(12, 125) = 3.308$, $p = 0.0003$), indicating axonal injury continued to evolve over time with distinct patterns in different white matter tracts.

3.3.5 Total and phosphorylated tau are not altered after single impact CHIMERA TBI up to 0.7 J

We assessed endogenous levels of total and phosphorylated (Thr231) tau in half brain homogenates using a MSD® MULTI-SPOT assay (K15121D-2, Meso Scale Discovery). No significant changes were observed for total tau, phosphorylated tau, or the ratio of phosphorylated:total tau for any injury group at any time point tested (Fig 3.9).

3.3.6 Neurological and neuropathological outcomes following single CHIMERA TBI correlate significantly with linear head kinematics

We also tested for correlations between changes in the biomechanical response of the head following single impact CHIMERA injury in the sagittal plane with neurological and neuropathological outcomes at 2 d post-injury (Table 1). We observed a strong positive correlation between linear head kinematic parameters with several post-TBI outcomes. Among all post-TBI outcomes, LRR showed the strongest positive correlation with both linear (displacement, velocity and acceleration) and angular (velocity and acceleration) head kinematic parameters ($p < 0.0001$). NSS at 1 h was positively correlated with head displacement ($p = 0.0146$), linear velocity ($p = 0.006$), linear acceleration ($p = 0.0168$) and angular velocity ($p = 0.0011$). The microglial response in all white matter regions showed a significant positive correlation with all three linear kinematic parameters but not with any angular kinematic parameters. Silver stain uptake in the olfactory nerve layer and corpus callosum showed a
significant positive correlation with head displacement and linear velocity only, whereas, silver
stain uptake in the optic tract showed a strong positive correlation with all three linear kinematic
parameters.
3.4 Discussion

The neuropathology of TBI has historically been investigated using brain tissues from patients who succumbed within hours to weeks after severe TBI. (Blennow, Hardy et al. 2012, Johnson, Stewart et al. 2013) More recently, the neuropathological hallmarks of chronic traumatic encephalopathy (CTE) have been defined using brain tissues obtained from athletes in high contact sports, often years after their peak of exposure to repetitive mTBI. (McKee, Cairns et al. 2016) These various streams continue to provide tremendous value in understanding the neuropathology of severe and repetitive mTBI, yet, the degree to which they are representative of a single mTBI remains unknown. Additionally, although impact tolerance thresholds (i.e., the applied force at which injury occurs) for skull fracture have been determined using cadaver studies (Hodgson and Thomas 1971, Yoganandan, Pintar et al. 1995), the corresponding acceleration-deceleration force tolerances of living brain tissue for structural damage (brain contusion, DAI etc.) and functional disturbances (sensory, cognitive, and motor deficits) are not well understood. Current knowledge relies primarily on injury reconstructions using anthropometric test devices, finite element models of the brain and head or helmet-mounted acceleration measurements in athletes. (Pellman, Viano et al. 2003, Zhang, Yang et al. 2004, Kleiven 2007) As single mTBI represents the largest proportion of head injuries that bring people to medical attention, it is increasingly important to understand what severity of impact the living brain can experience without triggering pathological responses that can have long-term consequences. Further, once brain tissue injury tolerance levels are exceeded, it is equally important to define which the most sensitive potential diagnostic and prognostic markers are, and to understand how the temporal profiles of these markers change as a function of acceleration-deceleration force and time.

This study was, therefore, designed to leverage the advantages of the CHIMERA platform to define the lower limit of mTBI in mice upon a single impact in the sagittal plane. Here we show that impact energies up to 0.4 J, which lead to average peak linear and angular accelerations of 305 g and 86.9 krad/s² respectively, do not produce any significant phenotype except for Iba-I reactivity in the optic tract and brachium of superior colliculus at 14d, and can thus be considered sub-threshold. An impact energy of 0.5 J, which produced average peak linear and
angular accelerations of 510 g and 149 krad/s² respectively, resulted in significant changes in behavioral (LRR and NSS), and histopathological (Iba-1 reactivity in corpus callosum, brachium of superior colliculus and optic tract, and GFAP reactivity in corpus callosum) outcomes, and is defined as the threshold of acute injury for murine CHIMERA. Impact energies of 0.6 and 0.7 J resulted in average peak linear and angular accelerations of 539 g and 183 krad/s², respectively, and led to robust and significant changes for many behavioral (LRR, NSS, thigmotaxis, rotarod), and histopathological (Iba-1 reactivity and silver uptake in all white matter tracts examined, and GFAP reactivity in corpus callosum), and can be considered in the mTBI range. Using the current CHIMERA piston design, impact energies greater than 0.7 J resulted in skull fracture and posed an ethical upper limit to our studies. These results demonstrate that single impact CHIMERA in the sagittal plane effectively models both behavioral and histopathological phenotypes of diffuse axonal injury.

Our results define critical experimental parameters that can now be used to determine how well our categories of subthreshold, threshold and mTBI groups using murine CHIMERA reflect human subconcussive, concussive and mTBI categories. Importantly, current definitions that categorize human mTBI are thus far based on clinical and neuroimaging approaches. Our study employed several behavioral measures reminiscent of clinical measures, including LRR as a proxy of loss of consciousness, NSS as a proxy of neurological outcomes, EPM and open field analyses as proxies for anxiety, and rotarod latency as a proxy of motor function and coordination. We observed that LRR and NSS are the most sensitive behavioral assessments in our current battery, as they distinguish both threshold and mTBI categories from sham and demonstrate significant differences between the threshold and mTBI groups. By contrast, significant changes in open field thigmotactic behavior and motor competency were observed only in the mTBI category. Although considerable future studies will be needed to refine the animal model behavioral assessments to more closely resemble the clinical criteria used for mTBI classification, the data presented here serve as a starting point for this effort.

Although neuroimaging outcomes were beyond the scope of the present study, we anticipate observing white matter changes using neuroimaging based on our observation of robust white
matter histopathological changes induced by CHIMERA. Consistent with our previous report of axonal damage and white matter inflammation after double TBI at 0.5 J (Namjoshi, Cheng et al. 2014), here we observed impact energy-dependent increases in silver uptake in several white matter regions including the olfactory nerve layer, corpus callosum, and optic tract, with significantly increased argyrophilic axons in all regions examined for the mTBI category. Several white matter regions also displayed significant Iba-I reactivity. Our analyses also revealed that both axonal injury and white matter inflammation continued to evolve over 14 d post-injury. Interestingly, we observed transient but significant increases in the number of GFAP-positive astrocytes only in the corpus callosum within 2-7 days post-TBI, suggesting GFAP may be a less sensitive marker of axonal damage than either Iba-I or silver uptake. Several additional histological markers of axonal injury, including neurofilament, amyloid precursor protein, and tau, will be examined in future studies and related to neuroimaging changes. Future studies will also address post-TBI outcomes over chronic periods (> 14 d), and assess how age at injury and sex influence post-TBI outcomes.

The response of tau to single vs repetitive mTBI now becomes an important question. Perivascular tau deposits, particularly in sulcal depths, is the pathognomonic feature of CTE. (McKee, Cairns et al. 2016) Biomarker studies, most commonly performed in studies in athletes who participate in high impact sports, also report that blood tau levels can be used as a diagnostic biomarker for mTBI. (Bogoslovsky, Gill et al. 2016, Oresic, Posti et al. 2016, Shahim, Gren et al. 2016, Zetterberg and Blennow 2016) Using Western blotting, we previously observed transient phosphorylation of tau in a study that used 2 x 0.5 J impacts, which we now define as the threshold for injury. (Namjoshi, Cheng et al. 2014) Using a more quantitative ELISA assay, here we observed that single impact TBI up to 0.7 J does not lead to significant increases in total or phosphorylated (Thr231) tau, at least in the acute 14 d period studied here, despite robust silver uptake. This observation raises the important question of whether tau changes may become more robust as a function of cumulative injury, which can be rigorously tested using the CHIMERA platform.
Our data suggest that not all affected white matter regions recover at the same rate (Fig 3.17). The most striking example of this is in the olfactory nerve layer, which displays both a larger dynamic range of histopathologic changes and a faster recovery than any other white matter region studied. On the other end of the spectrum is the corpus callosum, which appears to develop a modest but relatively stable phenotype. In our study, the later bloomer of white matter pathology is the optic tract, which has not yet peaked with respect to silver uptake at the 14 d time point used here, and is consistent with our previous report using double 0.5 J impacts. Future studies that refine the dynamic range and temporal profiles of additional markers in white matter regions, and relate them to neuroimaging findings, will be an important future avenue of investigation.

Our study is limited to describing the associations of impact energy with acute behavioral, neuropathological and biochemical outcomes using single impact CHIMERA in the sagittal plane. Under these conditions, we observed that linear kinematic values were better predictors of biological outcomes than angular kinematic values. Future studies will leverage the ability of CHIMERA to precisely deliver impact in any desired plane to investigate the relationships between lateral impact, which will induce head acceleration-deceleration in the coronal plane, and mTBI outcomes. The threshold of injury for mice is 0.5 J, which produced an average peak linear head velocity of 6.8 m/s, peak linear acceleration of 524 g, peak angular velocity of 326 rad/s, and peak angular acceleration of 149 krad/s². Scaling these mechanical insults based on an equal stress equal velocity relationship and single scale factor of 13.8 (λ) (Panzer, Wood et al. 2014), results in the following equivalent human head kinematics values: peak linear acceleration of 38 g, peak angular velocity of 23.6 rad/s and peak angular acceleration of 782 rad/s². Peak linear head acceleration of 38 g is below most reported human concussion tolerances generated through instrumented helmet data (Sinha, Gupta et al. 1968, Guskiewicz, Mihalik et al. 2007, Rowson, Duma et al. 2012) but above measured head accelerations in volunteer soccer heading (23.5 g) (Shewchenko, Withnall et al. 2005) and low speed (16 km/h) rear impact vehicle crashes (17.2 g) (Szabo and Welcher 1996). Scaled peak angular velocity was in the range reported to result in human concussion, however, peak angular acceleration was well below threshold levels developed from analysis of sports collisions. The average sub-concussive
impact in an American football study had a rotational acceleration of 1230 rad/s² and a rotational velocity of 5.5 rad/s, while the average concussive impact had a rotational acceleration of 5022 rad/s² and a rotational velocity of 22.3 rad/s. (Rowson, Duma et al. 2012) Based on reconstructed head impacts in Australian football, a 50% likelihood for concussion was proposed for peak linear acceleration of 65.1 g, peak angular velocity of 22.2 rad/s and peak angular acceleration of 3958 rad/s². (McIntosh, Patton et al. 2014) One possible interpretation of the present results, given that linear kinematic values were better predictors of biological outcomes than angular kinematics, is that impact in the sagittal plane generates limited rotational motion. As CHIMERA can generate precise impact in additional planes of motion, future studies will be able to address how injury tolerance may vary by anatomical plane of movement. Additional studies will also address current limitations in scaling animal model mechanical insults to humans. Biomechanical scaling techniques, while remarkably successful in some applications, have yet to be experimentally validated across a range of species. (Panzer, Wood et al. 2014)

The initiation of skull fractures at impact energies greater than 0.7 J posed an upper ethical limit to our current experimental protocol. In order to produce pathology indicative of more severe brain injury, it will be necessary to induce larger head accelerations while simultaneously decreasing skull fracture risk. Increased skull stiffness and failure strength of murine cranial bones is expected by distribution of impact forces over a greater area of the mouse skull, as has been demonstrated in human cadaver cranium impact tests. (Allsop, Perl et al. 1991) Our data support CHIMERA as a useful model of several mTBI subcategories, and confirm DAI as a key neuropathology associated with mTBI. By defining the impact energy parameters at the threshold of mild injury, we are now poised to launch several additional studies to further refine our understanding of the tissue level mechanisms of mTBI and repair.
Figure 3.1  Head kinematics during different levels of CHIMERA TBI

Head kinematics during single impact for was assessed in mice subjected to single TBI of varying impact energies from 0.1 to 0.7 J (N = 8–10/impact energy group), that were binned into categories reflecting subthreshold, threshold and mTBI groups. The graphs depict peak values of linear (A–C) and angular (D–F) head kinematic parameters over different impact energies. Data are presented as mean ± SD. Data were analyzed by one-way ANOVA followed by a Tukey post-hoc test. Asterisks (*) denote significant difference compared to the lowest energy group (i.e., sub-threshold). Hash (#) denote significant differences between energy groups other than 0.1 J. For each graph, * and #: p < 0.05, ** and ##: p < 0.01, *** and ###: p < 0.001, **** and ####: p < 0.0001.
Figure 3.2  Behavior changes after single impact CHIMERA TBI

In all graphs, 0 J indicates the sham group. (A) Loss of righting reflex (LRR) duration was assessed immediately after single impact. The graph depicts LRR duration over increasing impact energies. (B) Neurological severity score (NSS) was assessed at 1 h following single TBI. The graph depicts total NSS value (out of 10) over increasing impact energies. (C,D) Anxiety-like behavior was assessed with elevated plus maze (EPM) at 2 d post-TBI (C) and open field thigmotaxis at 1 and 7 d post-TBI (D). Graphs in (C) and (D) show the time spent by mice from different injury categories in the open and closed arms of the EPM (C), or near the edge of the open field box (D), respectively. Graphs represent the thigmotaxis index of injury categories at 1 and 7 d post-TBI. (E) Post-TBI motor performance was assessed with the accelerating rotarod task up to 14 d. The graph depicts fall latencies of injury categories at baseline and at different post-TBI time points. LRR and 1 h NSS data are presented as mean ± SEM. All other data are presented as mean ± SD. LRR and NSS data were analyzed by one-way ANOVA on ranks. Rotarod and thigmotaxis data were analyzed by Linear Mixed Model. EPM data were analyzed by one-way ANOVA. For graphs A and B, Asterisks (*) denote significant difference compared...
to the sham. Hash (#) marks denote significant differences among the groups. For graphs in (A) and (B), * and #: p < 0.05, ** and ##: p < 0.01, ###: p < 0.001, **** and ####: p < 0.0001.
Figure 3.3  Microglial response in the optic tract after single impact CHIMERA TBI

Microglia were assessed using Iba1 immunohistochemistry in tissue sections prepared from 6 h to 14 d after single CHIMERA TBI, presented as representative 20 ×-magnified images. The
region of interest in the optic tract is outlined by dotted black lines in the image in the sham 6 h panel (top left corner). Injury categories are arranged in rows and time points are arranged in columns.
Figure 3.4  Quantification of the post-TBI microglial response to single impact

**CHIMERA TBI**

The post-TBI microglial response was quantified by counting the number of Iba1-positive cells in white matter regions of interest. Graphs depict the number of Iba1-positive cells over time in the olfactory nerve layer (A), corpus callosum (B), brachium of superior colliculus (C) and optic tract (D). For all graphs, data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey post-hoc test. * and #: p < 0.05, ** and ##: p < 0.01, *** and ###: p < 0.001, **** and ####: p < 0.0001.
Figure 3.5  Astrocytic reaction in the corpus callosum after single impact CHIMERA TBI
The post-TBI astrocyte response was assessed using GFAP immunohistochemistry on tissues prepared from 6 h to 14 d after single CHIMERA TBI. (A) Representative 20X-magnified images of the corpus callosum showing GFAP-positive astrocytes. Injury categories are arranged in rows and time points are arranged in columns. (B) The post-TBI astrocyte response was quantified by counting the number of GFAP-positive cells in the corpus callosum and depicted graphically over time. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey post-hoc test. * and #: p < 0.05, ** and ##: p < 0.01, *** and ###: p < 0.001, **** and ####: p < 0.0001.
Figure 3.6  Quantification of levels of endogenous proinflammatory cytokines after single impact CHIMERA TBI

Post-TBI levels of endogenous proinflammatory cytokines were quantified in half-brain lysates at 6 h post-TBI. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test. * and #: p < 0.05, **: p < 0.01.
Figure 3.7 Axonal damage in the optic tract after single impact CHIMERA TBI

Post-TBI axonal pathology was assessed using silver staining on tissues prepared from 6 h to 14 d after single CHIMERA TBI, presented as representative 20X-magnified images of the optic
tract showing silver uptake. Injury categories are arranged in rows while time points are arranged in columns.
Figure 3.8  Quantitative analysis of axonal pathology after single impact CHIMERA TBI

Silver stain images were quantified by calculating the % region of interest (ROI) in white matter areas that displayed silver uptake, including the olfactory nerve layer (A), corpus callosum (B) and optic tract (C). Graphs depict mean ± SD % of the ROI showing in sham and injury categories over time. Data were analyzed by two-way ANOVA followed by Tukey post-hoc test. * and #: p < 0.05, ** and ##: p < 0.01, *** and ###: p < 0.001, **** and ####: p < 0.0001.
Figure 3.9  Quantification of endogenous tau levels

Endogenous and phospho(Thr231) tau levels in half-brain homogenates were assessed with a MSD® MULTI-SPOT assay kit. Levels of total tau (A), phospho (Thr231) tau (B) quantification and ratio of phospho:total tau (C) are presented as Mean ± SD. Data were analyzed by two-way ANOVA.
Figure 3.10  Animal morphological data and isoflurane exposure time

(A) The graph shows age (in days) of animals at the time of TBI across all energy groups. (B) Pre-TBI body weight (in g) of animals in all energy groups. Both age and body weight were comparable across all energy groups. (C) Total isoflurane exposure (in s) during the CHIMERA TBI procedure, which was recorded as the time started with anesthesia induction until head impact when isoflurane delivery was stopped. Numbers within bars indicate sample size.
Figure 3.11  Microglial response in the olfactory nerve layer after single impact
CHIMERA TBI
Post-TBI microglial response was assessed using Iba1 immunohistochemistry, presented as representative 20 ×-magnified images showing the region of interest in the olfactory nerve layer outlined by dotted black lines in the top left corner panel. Energy groups are arranged in rows and time points are arranged in columns.
Figure 3.12  Microglial response in the olfactory nerve layer after single impact
CHIMERA TBI
Post-TBI microglial response was assessed using Iba1 immunohistochemistry, presented as representative 20 ×–magnified images showing the region of interest in the olfactory nerve layer outlined by dotted black lines in the top left corner panel. Energy groups are arranged in rows and time points are arranged in columns.
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Figure 3.13  Microglial response in the brachium of superior colliculus after single impact CHIMERA TBI
Post-TBI microglial response was assessed using Iba1 immunohistochemistry, presented as representative 20 ×-magnified images showing the region of interest in the superior colliculus outlined by dotted black lines in the top left corner panel. Energy groups are arranged in rows and time points are arranged in columns.
Figure 3.14  Microglial response in the lateral geniculate nucleus after single impact
CHIMERA TBI
Post-TBI microglial response was assessed using Iba1 immunohistochemistry, presented as representative 10×-magnified images showing the region of interest in the lateral geniculate nucleus outlined by dotted black lines in the top left corner panel. Energy groups are arranged in rows and time points are arranged in columns.
Figure 3.15  Post-TBI axonal pathology in the olfactory nerve layer
Post-TBI axonal pathology in the olfactory nerve layer was assessed using silver staining, presented as representative 20×-magnified images. Energy groups are arranged in rows and time points are arranged in columns.
Figure 3.16  Post-TBI axonal pathology in the corpus callosum
Post-TBI axonal pathology in the corpus callosum was assessed using silver staining, presented as representative 20 ×-magnified images. Energy groups are arranged in rows and time points are arranged in columns.
Figure 3.17  Temporal profile of microgliosis and axonal injury following single mTBI

The graphs depict the temporal profiles of microgliosis (A) and axonal injury (B) in various white matter regions of the mTBI group over 6 h to 14 d post-TBI. Data are represented as percentage of sham values at the respective time points. BSC: Brachium of superior colliculus, CC: Corpus callosum, ON: Olfactory nerve layer, OT: Optic tract.
### Table 3.1  **Summary of correlation analyses.** The table summarizes the results of correlation analyses between linear / angular head kinematic parameters and post-TBI outcomes. For each analysis, the coefficient of correlation is presented in the upper row, and the $p$ value is indicated in lower row. Significant correlations ($p < 0.05$) are highlighted in bold.

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Table 3.2 **Sample size.** The table details sample number used for the statistical analyses of each post-TBI parameter at the respective post-TBI time point assessed in this study.

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Chapter 4: Age at injury and genotype modify acute inflammatory and neurofilament-light responses to mild CHIMERA traumatic brain injury in wild-type and APPPS1 mice

4.1 Introduction

Traumatic brain injury (TBI) occurs when the head experiences a mechanical force that leads to brain dysfunction. Most commonly, this occurs when the brain experiences rapid acceleration and deceleration events, such as in a fall, motor vehicle accident, or impact with an object. The Glasgow Coma Scale (GCS) is used to classify TBI severity into mild (GCS 13-15), moderate (GCS 8-12) and severe (GCS 3-7) categories. (Teasdale and Jennett 1974) Of the more than 2 million TBIs that occur each year in the USA, approximately 75-90% are mild. ((NHDS) 2010, Faul and Coronado 2015) TBI can lead to persistent changes in cognitive and neurological function (Kraus, Susmaras et al. 2007, Hayes, Miller et al. 2015, Miller, Hayes et al. 2016), and can increase risk for neurodegenerative diseases such as Alzheimer’s Disease (AD), chronic traumatic encephalopathy (CTE), and Parkinson Disease (PD). (Mortimer, van Duijn et al. 1991, Fleminger, Oliver et al. 2003, May 2011, Gardner, Burke et al. 2015, Gardner and Yaffe 2015, Crane, Gibbons et al. 2016, Hayes, Logue et al. 2017) Moderate-severe TBI is reported to increase risk of dementia, including AD, by approximately 1.5-1.8 fold (Mortimer, van Duijn et al. 1991, Fleminger, Oliver et al. 2003, Gardner, Burke et al. 2014), however, the relationship of mild TBI (mTBI) to dementia risk is less clear. (reviewed in (Gardner and Yaffe 2015))

TBI incidence peaks in two major age groups, namely in youth-young adults (~15-24 years) and in persons over 65 years. (Faul 2010) While older age is associated with increased hospitalization rate, poorer functional outcomes and poorer recovery(Mosenthal, Lavery et al. 2002, Mosenthal, Livingston et al. 2004, Willemse-van Son, Ribbers et al. 2007, Faul 2010), how age alters the brain’s response to injury to affect dementia risk and age of onset is not fully understood. In a study of 1,283 TBI cases who were 40 years of age or older, Nemetz et. al. (Nemetz, Leibson et al. 1999) found that injured subjects had significantly earlier onset of AD (median 10 years) than uninjured subjects (median 18 years), particularly if the injury occurred prior to 65 years of age.
With respect to dementia risk, a retrospective study by Gardner et al. (Gardner, Burke et al. 2014) involving 51,799 TBI patients reported that moderate-severe TBI increased dementia risk for patients of all ages, but mTBI significantly increased dementia risk only if the injury occurred in persons over >65 years of age. A recent study of 984 community-dwelling non-demented adults aged 51 years and older found that a history of TBI with loss of consciousness (LOC) increased the likelihood of reporting subjective memory impairment compared to those without TBI even though no difference in objective cognitive function were observed. (Gardner, Langa et al. 2017) In a study of 1M adult Taiwanese patients, mTBI increased dementia risk by >3-fold. (Lee, Hou et al. 2013) In a cohort of >800,000 male military conscriptees, mTBI led to a 70% increased risk of non-AD type dementia developing before age 65 years. (Nordstrom, Michaelsson et al. 2014) Hayes et. al. studied 160 Afghanistan and Iraq war veterans between the ages of 19 and 58 years of age, and found that a history of mTBI exacerbated thinning of the posterior cingulate cortex and indirectly influenced episodic memory performance in subjects with high genetic risk for AD. (Hayes, Logue et al. 2017)

The neuropathological hallmarks of AD include amyloid plaques composed of aggregated β-amyloid (Aβ) peptides and neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein. (Castellani 2010, Querfurth and LaFerla 2010) Although both neuropathologies can be observed in TBI autopsy brain tissue, there are noteworthy differences. TBI primarily leads to tau deposition, which is especially prominent after highly repetitive TBI where tau in the perivascular regions and sulcal depths form the pathognomonic features of CTE. (McKee, Cantu et al. 2009, McKee, Stern et al. 2013) A recent study involving 114 CTE samples (mean age 60 years) found that diffuse Aβ deposits are observed in approximately 52% of cases, with an unknown time between injury and death. (Stein, Montenigro et al. 2015) In severe TBI, the time between TBI and death/subsequent histological analysis appears to define the appearance of these aggregates. Diffuse Aβ deposits are present in 30% of short term TBI survivors (mean survival time hours to weeks) (Roberts, Gentleman et al. 1994, Gentleman, Greenberg et al. 1997) but are largely absent in long term survivors (mean survival time 245 days) (Chen, Johnson et al. 2009), suggesting that altered Aβ deposition kinetics may be a transient response to TBI. Intriguingly, a study involving 39 long-term survivors (1-47 years after injury) of a single moderate-severe TBI showed that neurofibrillary tangles were more common in TBI survivors than controls, particularly in patients
<60 years of age. The frequency of Aβ plaques was not different between TBI survivors and controls, but the Aβ plaques in TBI survivors had higher density and were predominantly of the mature fibrillar form (Johnson, Stewart et al. 2012), suggesting that TBI may affect both tau and Aβ pathologies in the long term. Recently, combined data from three prospective cohort studies demonstrated that TBI with LOC was associated with increased risk for Lewy body accumulation, progression of parkinsonism and PD, but not for amyloid plaques, neurofibrillary tangles, AD or dementia (Crane, Gibbons et al. 2016), raising additional questions about the effect of TBI on the neuropathological hallmarks of common neurodegenerative diseases.

Diffuse axonal injury (DAI) is a common pathology of mTBI. Its neuropathological features include increased uptake of silver ions in white matter tracts, inflammatory changes in white matter, and axonal swelling and accumulation of amyloid precursor protein (APP), which is indicative of disrupted fast axonal transport, and neurofilament, which results from excitotoxicity and calcium-dependent protease activity. (McKenzie, Gentleman et al. 1994, Blumbergs, Scott et al. 1995, Graham, McIntosh et al. 2000) Increased APP in the post-TBI brain has been hypothesized to be accompanied by a burst of Aβ production, which can theoretically form deposits. (Roberts, Allsop et al. 1990, Horsburgh, Cole et al. 2000, Gavett, Stern et al. 2011) However, the effect of TBI on Aβ dynamics is far from simple. (Magnoni and Brody 2010) Microdialysis experiments in living human subjects have shown that the levels of Aβ in interstitial fluid (ISF) may be different depending on the type of TBI. (Marklund, Blennow et al. 2009) In addition, ISF Aβ levels correlates with the patient’s GCS, and they increase as neurological status improves, remain unchanged in clinically stable patients, and decline as neurological status worsens. (Brody, Magnoni et al. 2008) These observations suggest that Aβ release into ISF depends on injury type and is associated with recovery of synaptic function, a conclusion supported by experiments in animal models. (Cirrito, Yamada et al. 2005) An important question is how the presence of pre-existing amyloid deposits or APP-immunoreactive neurites may influence Aβ dynamics after TBI, as microdialysis measurements of Aβ half-life in experimental animal models show that the presence of pre-existing amyloid plaques significantly slows the rate of Aβ decay. (Cirrito, May et al. 2003)

Given the complex association between TBI and AD, many groups have turned to animal model
experiments to elucidate causal relationships. These studies largely use controlled cortical impact (CCI), fluid percussion (FP), or a variety of closed head models that use either gravity or mechanical methods to impact the intact skull. (reviewed in (Namjoshi, Good et al. 2013)) Although FP and CCI models employ highly reproducible mechanical inputs, they typically involve impact onto the brain parenchyma and produce focal rather than diffuse injury, and are generated with no or minimal head motion. To address the absence of a simple, nonsurgical impact acceleration model of TBI that reliably produces DAI, we developed a neurotrauma animal model called CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration), which uses precise and quantifiable biomechanical inputs to reliably induce head motion, behavioural changes, and DAI characterized by white matter inflammation and axonal damage. (Namjoshi, Cheng et al. 2014, Namjoshi, Cheng et al. 2016, Namjoshi, Cheng et al. 2017) Here we applied the advantages of CHIMERA to test how age at injury affects acute behavioural, neuropathological and biochemical changes in APP/PS1 mice and wildtype (WT) littermates that were subjected to two mild concussive-like TBIs. An outline of the study is illustrated in Fig 4.13. Two age groups of mice were used in this study, namely 6 months and 13 months of age, as the seeding phase of Aβ deposition in these APP/PS1 mice starts around 6 months. By 13 months, Aβ deposition is established but not yet maximal. Although age at injury did not alter acute behavioural responses to mTBI in either strain, age at injury transiently modified the pattern of Aβ deposits in APP/PS1 mice. Age at injury also modified microglial and cytokine responses, where neuroinflammation is increased by aging in WT mice but attenuated in old APP/PS1 mice. In both strains, the formation of neurofilament-immunoreactive axonal swellings was robust in young animals but almost entirely attenuated in older mice, despite equivalent axonal injury defined by silver staining. In plasma, neurofilament-light levels were elevated only in young APP/PS1 mice. Our study shows that age and genotype each contribute to altered inflammatory and neurofilament responses after mild impact-acceleration TBI, and that age at injury affects how acute Aβ dynamics are transiently modified after mTBI. An illustrative summary of these complex interactions is provided at Fig 4.14.
4.2 Materials and methods

4.2.1 Animals

All experiments were approved by the University of British Columbia Committee on Animal Care and are compliant with the Canadian Council of Animal Care (A15-0096). Male APP/PS1 transgenic mice (B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax) and WT non-transgenic littermate controls were used in this study (total N=367). APP/PS1 mice co-express two transgenes from the murine prion promoter: a chimeric mouse/human APP650 cDNA containing the Swedish (K670M/N671L) mutations and the human PS1 gene deleted for exon 9, and exhibit emergent Aβ deposits by approximately 6 months of age. The colony was maintained on an F1 hybrid C57Bl/6 × C3H background to control for genetic admixture between APP/PS1 and WT littermates. After weaning, mice were housed with environmental enrichment on a 12 h/12 h reverse light cycle, and received the 2918 Teklad Global 18% Protein rodent diet (Harlan) and autoclaved reverse osmosis water ad libitum. Two age groups of mice were used. “Young” animals had TBI or sham procedures at 183 ± 21 days (mean ± s.d.; approximately 6.1 months) when amyloid deposition in APP/PS1 mice is emerging. “Old” mice had TBI or sham procedures at 404 ± 20 days (approximately 13.5 months) when amyloid deposits in APP/PS1 mice are more established. The study design is summarized in Fig 4.13.

4.2.2 CHIMERA procedure

The CHIMERA model (Namjoshi, Cheng et al. 2014) was used to induce mTBI as described. Procedures were as reported, except that the strap design was adapted to accommodate the larger body size of the hybrid mice. Immediately prior to TBI, mice received 0.5 ml NaCl for fluid supplementation and 1 mg/kg meloxicam for analgesia. Anesthesia was induced with 5% isoflurane in 0.8 L/min oxygen and maintained at 4.0-4.5%. Anesthetized mice were restrained by two abdominal straps in the supine position on the CHIMERA device, such that their heads were free to move and rested at an angle of 145° relative to the body. Two mild head impacts (0.5 J impact energy) were given 24 h apart, each delivered using a pneumatically-driven 50 g piston with a 5 mm tip enclosed by a rubber cap. The piston impacted the midline parietal region, perpendicular to the long axis of the head, driving the head to rotate towards the body along the sagittal plane. Sham-operated mice, which experienced restraint, anaesthesia, NaCl and meloxicam, but no impact, served as uninjured controls.
4.2.3 High speed videography and head kinematic analyses
High speed videography was performed as described (Namjoshi, Cheng et al. 2014) on a subset of 20 mice. Two reference points were marked on the mouse head using paint on the cheek and dental floss wrapped around the snout, which was removed immediately after filming. The entire TBI event was captured at an angle perpendicular to the sagittal plane using a high-speed camera (Q-PRI, AOS Technologies) at 9,000 frames per second. Head kinematics were analyzed using ProAnalyst software (Xcitex Inc., Woburn, MA). Linear kinematic parameters were reported based on the motion of the cheek marker, and rotational kinematic parameters were reported based on the angle between the snout marker, the cheek marker and the horizon.

4.2.4 Behaviour
Behavioural analyses, including loss of righting reflex (LRR), neurological severity score (NSS), RotaRod (RR), open field (OF), passive avoidance (PA), and Barnes mazes (BM), were performed as described. (Namjoshi, Cheng et al. 2014) For LRR, mice were tested immediately after each TBI procedure. For NSS, mice were tested 2 days before the CHIMERA procedure to obtain pre-injury data, and at 1 h, day 1 (D1), D2, and D7 after the second injury. For RR, mice received training 1 week before the experiment, and were evaluated at D1, D2, D7, and D14 after the second injury. For OF, mice were tested on D1 and D6 after the second injury. For BM, mice were trained for acquisition trials from D8 to D12 after the second injury. Probe tests and reverse trials were conducted on D13. For PA, mice were given a foot shock on D7, and tested on D8, D9, and D10 after the second injury. Mice were tested on the elevated plus maze (EPM) for anxiety-like behaviours on D7 and D11 after the second injury. The EPM consisted of two open arms (66 x 5 x 15 cm) and two enclosed arms (66 x 5 x 15 cm) with an open roof, arranged such that the two open arms were opposite to each other. The maze was elevated to a height of 140 cm. During EPM testing, the animal was placed in the center of the maze and spontaneous activity was recorded for 5 min, Animal activity in OF, EPM, and BM was recorded using AnyMaze (Stoelting Co., Wood Dale, IL).
4.2.5 Tissue collection, histology and image analyses

Animals were euthanized with 150 mg/kg ketamine (Zoetis) and 20 mg/kg xylazine (Bayer) at 6 h, 12 h, D2, D7 and D14 post-injury and perfused with 50 ml ice-cold heparinized PBS (5 USP unit/ml). Hemibrains were fixed in 4% PFA for 2 days and cryoprotected with 30% sucrose for 2 days, after which 40 µm-thick coronal sections were cut using a cryotome (Leica). Immunohistochemistry for the microglial marker Iba1 was performed as described. (Namjoshi, Cheng et al. 2014) Briefly, sections were quenched with hydrogen peroxide for 10 min, blocked with 5% BSA, and incubated with primary antibodies overnight at 4°C. Sections were then incubated with biotin-conjugated secondary antibodies (1:1000), then with ABC reagent (Vector, 1:400) before color development with 3,3’ Diaminobenzidine (DAB) (Sigma). SMI312 immunostaining of phosphorylated neurofilament was performed using the M.O.M. kit (Vector) following the manufacturer’s instructions, before applying the ABC kit and DAB. Aβ was immunodetected with 6E10 using procedures similar to SMI312, but with an extra initial step of incubation in 88% formic acid. The dilution of antibodies used were: Iba1 (Wako 019-19741, 1:1000), SMI312 (BioLegend 837904, 1:1000), 6E10 (Biolegend 803015 1:1000). Injured axons were stained using the Neurosilver Staining Kit (FD Neurotechnologies) following the manufacturer’s instructions, and fibrillar amyloid was stained using 1% Thioflavin S (Thio-S) (Sigma), as described. (Namjoshi, Cheng et al. 2014, Robert, Stukas et al. 2016)

Entire coronal sections stained for Iba1, 6E10, and Neurosilver were imaged at a commercial histology service (Wax-it) using a ScanScope CS-R scanner (Aperio) at 20X magnification. Regions of interest were extracted using Aperio ImageScope Viewer (Aperio). ThioS and SMI312 stained sections were imaged using Axio Microscope (Zeiss) with an Olympus Axiocam 503 mono camera at 10x and an Axiocam MRC camera at 20x magnification, respectively. ThioS images were captured using a FITC filter and subsequently tiled to reconstruct the entire sections. Image analyses of 6e10 and ThioS included the entire cortex present in the coronal section. The dorsal/anterior hippocampus was used as anatomical landmark for consistent coronal section selection (roughly 2 mm posterior to bregma). Image quantification was performed using ImageJ (NIH). For Iba1 staining, images were quantified by thresholding and reporting the number of microglia per area (Iba1+ve signals) of the optic tract, after filtering background noise of particles less than 27 µm². This size threshold is empirically determined as it is able to distinguish between
microglia cell bodies and cell processes. Coronal sections at the plane of the dorsal hippocampus were selected, and a region of interest covering approximately two-thirds of the optic tract or more was cropped for analysis. For each sample, two close sections were selected for analysis and the average result for microglial density or size was used. SMI312 stained images were quantified by thresholding and reporting the number of axonal swellings per area of the optic tract, after filtering background noise of particles less than 21 µm² or with circularity <0.2. 6E10 and ThioS images were quantified by thresholding and reporting the percentage area containing signal relative to the total cortical area. Silver staining images were quantified by thresholding and reporting the percentage area containing signal relative to the white matter area.

4.2.6 Biochemistry

Unfixed hemibrains were homogenized as described. (Robert, Stukas et al. 2016) Tissues were extracted using 1.5 ml of ice-cold carbonate buffer (100 mM Na₂CO₃, 50 mM NaCl, pH 11.5) containing cOmplete protease inhibitor (Roche), 1 mM PMSF and PhosSTOP (Roche) in a Tissuemite homogenizer at full speed for 20 s and then sonicated at 20% output for 10 s. After incubating on ice for 10 min, lysates were separated by centrifugation at 16,600 g for 45 min at 4 °C. The carbonate-soluble fraction was extracted and neutralized with 1.5 volumes of 1 M Tris-HCl pH 6.8, to achieve a final pH of approximately 7.4. The insoluble fraction was then resuspended in 1.5 ml of 5 M guanidine hydrochloride (GuHCl) in 50 mM Tris-HCl pH 8.0, at room temperature for 3 h with continuous rotation. All samples were frozen at -80 °C until use. Protein concentration was determined using a Lowry Protein Assay (Biorad).

Human Aβ40 and Aβ42 levels in carbonate and GuHCl fractions were assayed using commercial ELISA kits (Invitrogen KMB3482, KMB3442) at dilutions: 1:2-1:5 for young carbonate soluble; 1:4-1:10 for old carbonate soluble; 1:50-1:100 for young GuHCl soluble; 1:250-1:1000 for old GuHCl soluble. Interleukin-6 (IL-6) and Interleukin-1β (IL-1β) levels were assayed using a customized murine V-PLEX Proinflammatory Panel 1 (Mesoscale Discovery K152A0H-2) at 1:2 dilution and overnight incubation. Total tau and phosphorylated (Thr231) tau were assayed using the MULTI-SPOT assay (Mesoscale Discovery K15121D-2) at 1:50 dilution. The signals were read on a Sector S600 plate reader (Mesoscale Discovery), and concentrations were normalized to
total protein concentration where applicable. VCAM-1 and ICAM-1 levels were measured in carbonate fractions using commercial ELISA kits (Abcam, ab100750, ab100688) at 2000:1 and 20:1 respectively.

Plasma NF-L levels were measured using the NF-L Advantage Assay (cat no. 102258, Quanterix Corporation) on the Simoa HD-1 Analyzer according to the manufacture’s protocol. Samples were run in singlicate and manually diluted 4x offboard. All samples were analyzed within the same run. The lower limit of detection (LLOD) was 0.1 pg/ml, and the lower limit of quantification (LLOQ) was 0.696 pg/ml, after correction for the dilution factor. The mean coefficient of variation (CV) and % error (difference between calculated and theoretical concentration) was 3.0% and 4.0%, and 9.7% and 22%, for the standard curve and internal calibrator controls, respectively.

4.2.7 Immunoblotting
Immunoblotting was performed by resolving 50 μg of carbonate-soluble lysate through denaturing gels (NuPAGE 4-12% Bis-Tris, Thermo), which was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and probed for soluble APP (22c11, Millipore MAB348, 1:4000) and APP C-terminal fragment (Biolegend 802801, 1:1000). HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (ECL) (GE) were used to develop light signals, which were captured by a ChemiDoc MP imaging system (Biorad) and subsequently compared to GAPDH (Millipore MAB374, 1:20000) expression and analyzed by Image J (NIH).

4.2.8 Statistics
All statistical analyses were performed using SPSS (IBM) and graphs were plotted using GraphPad. Analyses of pre-injury body weight and head kinematics were performed on the 4 genotype-age combinations (APP/PS1-Young, APP/PS1-Old, WT-Young, and WT-Old), to compare if these animals received different biomechanical inputs during impact. Subsequent analyses were performed separately for the APP/PS1 cohort and the WT cohort. For any given assay, the same procedures were used to analyse each cohort. All behaviour data (except LRR and BM-probe) were analyzed using mixed linear model, with fixed factors including “Group” (4 age-injury combinations per genotype cohort: APP/PS1-Young-Sham/TBI and APP/PS1-Old-Sham/TBI, or WT-Young-Sham/TBI and WT-Old-Sham/TBI) and “Time” (various testing days depending on
experiments, which was absent in BM-reverse as this test was conducted on a single day). For mixed linear model analyses, each mouse was considered a subject and accounted for random effects. In BM-acquisition, BM-reverse and RR, there was an extra fixed factor, “Trial”, as multiple separate observations were made on the same day. LRR and head kinematic data were analyzed using Repeated Measure General Linear Model, with the independent variable of “Group”, and “Time” was considered the repeated measure variable. For cytokine assays, samples below the detection limit were assigned a value of “zero”, and data were analyzed using Kruskal Wallis test, with post-hoc analyses using the Mann-Whitney U test and Bonferroni correction for multiple comparisons. All other histological, biochemical and BM-probe data were analyzed by univariate ANOVA, with independent variables including “Group” and “Time” (if applicable). For 6E10 and Thio-S histology, analyses were performed on fold change over respective average sham level in each experiment. In all analyses, post-hoc tests were performed between pairs of the groups, if the “Group” factor or “Group*Time” interaction reached statistical significance (p<0.05). A summary of statistically significant results is presented in Table 4.1.
4.3 Results

4.3.1 Head kinematics are not affected by the greater body weight of APP/PS1-Old mice
APP/PS1 mice and WT littermates were subjected to two successive CHIMERA mTBI 24 h apart at either 6 (young) or 13.5 (old) months of age. Analysis of pre-injury body weight across the 367 mice used in this study revealed that the APP/PS1-Old mice were significantly heavier than the other groups (post-hoc analysis: APP/PS1-Old vs. WT-Old, p=0.001; APP/PS1-Old vs. APP/PS1-Young or WT-Young, p<0.001) such that the average APP/PS1-Old mouse was 4 g (8%) heavier than the average mouse from other groups. All mTBI mice received the same biomechanical input of 2 x 0.5 J of kinetic energy in the sagittal plane. High speed videography was then performed for a subset of 4-6 animals per group to determine if the greater body weight of APP/PS1-Old mice affected their biomechanical response to impact (Table 4.2). Linear velocity, linear acceleration, rotational velocity and rotational acceleration were not significantly different in any experimental group.

4.3.2 Age at injury does not affect acute behavioural responses to very mild CHIMERA TBI
A battery of behavioral tests was performed to assess acute neurological impairment after CHIMERA mTBI. Loss of righting reflex (LRR) was measured in all experimental animals as an analogue to human LOC. Sham controls were exposed to the same procedures as mTBI animals, including time under anaesthesia, restraint in the CHIMERA apparatus, saline supplementation and meloxicam (analgesic) application, but without impact. For LRR, significant injury effects (p<0.001) were observed in all groups except for APP/PS1-Old mice (p=0.075), although the APP/PS1-Old mice showed a similar trend of increased LRR after injury (Fig 4.1 a,b).

Neurological performance was evaluated using the composite neurological severity score (NSS), at pre-injury baseline and post-injury time points (1 h, D1, D2, and D7). Unlike our previous observations in C57Bl/6 mice, 2 x 0.5J impacts did not lead to significant increases in post-injury NSS scores in hybrid C57Bl/6-C3H mice except for the APP/PS1-Young group (p=0.009), suggestive of very mild injury conditions (Fig. 4.1c,d).
Motor performance was evaluated by recording the time animals could remain on an accelerating rotarod at pre-injury baseline or post-injury time points (D1, D2, D7, D14). Consistent with very mild injury, APP/PS1-Old-TBI mice were the only group to show significant injury effects (p=0.041). Post-hoc analysis showed that these mice performed significantly worse than their sham counterparts on D1 (p=0.026). At D14 the post-hoc test was not statistically significant (p=0.108), but the data show a clear trend of poor motor recovery (Fig 4.2a,b). This contrasts with the injured APP/PS1-Young-TBI group, which had similar motor performance as the APP/PS1-Old-TBI group at D1 and D2, but showed full recovery by D14. General mobility evaluated by open field activity on D2 and D7 post-injury showed no significant injury effects either in distance traveled or time spent immobile for any group (Fig 4.8 a,b,c,d).

Spatial memory performance was measured using the Barnes maze (BM). Spatial learning was assessed using acquisition reference trials, which measures the time required to locate the BM hideout. Three acquisition trials per day were performed on 5 consecutive days after either CHIMERA mTBI or sham procedures, from D8-D12. Although WT-Young-TBI and APP/PS1-Young-TBI groups showed no deficit in spatial learning after mTBI, WT-Old-TBI and APP/PS1-Old-TBI groups each showed significant injury effects (p<0.001 and p=0.035, respectively) (Fig 4.2 c,d). It should be noted, however, that the modestly significant APP/PS1-Old-TBI result appears to be at least partly driven by the better-than-expected performance of the APP/PS1-Old-Sham group, and therefore may be less biologically meaningful. Probe trials, which assess spatial memory recall, were performed on D13 by removing the hideout and recording the amount of time the mice spent in the target quadrant or the previous hideout area. This task revealed no significant injury effects for either the target quadrant or hideout analysis (Fig 4.2). Reverse trials, which assess unlearning and relearning, were also performed on D13 by moving the hideout to the opposite side of the maze and evaluating the latency to find the new hideout using 3 trials. No significant differences were observed between groups with sham injury or mTBI. In summary, mTBI did not induce deficits in recall of spatial memory, but led to subtly slower spatial learning in old mice.

Neither fear memory tested with the passive avoidance (PA) task nor anxiety tested with the elevated plus maze (EPM) task showed significant injury effects in any of the groups (Fig 4.8).
Together, these very subtle behavioural observations suggest that hybrid C57Bl/6-C3H mice may be more resilient than C57Bl/6 mice to CHIMERA conditions of 2 x 0.5J impacts, suggesting that the cohorts in this study were likely within the sub-concussive to concussive range of TBI severity.

4.3.3 Age at injury transiently modifies diffusivity of Aβ deposits after mTBI

Immunohistochemical (IHC) staining using 6E10 was performed to characterize how CHIMERA mTBI affected Aβ deposits in APP/PS1-Young-TBI and APP/PS1-Old-TBI mice up to 7 days post-injury. As Aβ and amyloid levels vary dramatically by age in APP/PS1 mice, young and old groups were analysed separately for these measures. After injury, APP/PS1-Young-TBI mice showed significantly increased Aβ deposits (p=0.043). Specifically at D2, we observed an increased area of 6E10-positive cortical deposits in APP/PS1-Young-TBI animals (240% increase at D2, post-hoc analysis p=0.002), which was not observed either at 6 h or 7D (Fig 4.3 a,c). In APP/PS1-Old mice, which had a well-established baseline amyloid load, no significant changes in 6E10-positive cortical deposits were observed (p=0.213) (Fig 4.3 b,d).

Intriguingly, a different effect of age at injury was observed when we used Thio-S staining to examine fibrillary amyloid plaques. Although CHIMERA mTBI had no significant effects on cortical fibrillary amyloid load in APP/PS1-Young mice at any time point examined (Fig 4.3e-h), a significant injury*time effect was observed in APP/PS1-Old mice (p<0.001). Specifically, post-hoc analyses revealed a significant 30% increase in Thio-S-positive plaques in APP/PS1-Old-TBI mice compared to APP/PS1-Old-Sham mice at 6 h post-injury (p<0.001), whereas at D2 and D7, Thio-S-positive plaque burden was significantly decreased by 23% and 27%, respectively, in APP/PS1-Old-TBI mice (p=0.002 and 0.017, respectively) (Fig 4.3e-h). By D14, there was no longer any significant difference of Thio-S signals between APP/PS1-Old-TBI and APP/PS1-Old-Sham mice (not shown). Two examples of Thio-S stained amyloid, one from APP/PS1-Old-Sham and one from APP/PS1-Old-TBI, are shown at higher resolution in (Fig 4.3i), where the fibrillar structure of the plaques is clearly visible.

We also quantified carbonate soluble and guanidine-HCl soluble pools of Aβ40 and Aβ42 using ELISA to determine whether whole brain Aβ levels correlated with the deposition patterns
observed above. APP/PS1 mice displayed the expected age-associated increases in Aβ40 and Aβ42 levels in both pools (Fig 4.9). However, CHIMERA mTBI had no significant effect on whole brain Aβ levels, with one exception, where D14 samples from APP/PS1-Young-TBI mice had an Aβ40 level significantly lower than APP/PS1-Young-Sham mice (p=0.031), albeit this is unlikely to be biologically significant as the sham level at D14 is higher than sham levels on other days. Whole brain carbonate-soluble Aβ42 levels trended toward increased levels in APP/PS1-Young-TBI mice, however, this was not statistically significant (p=0.057). No significant differences were observed between sham and mTBI mice in any other fraction, including carbonate-soluble Aβ40 levels in APP/PS1-Old mice (p=0.609), carbonate-soluble Aβ42 levels in APP/PS1-Old mice (p=0.491), or guanidine-HCl-soluble Aβ40 and Aβ42 levels in either APP/PS1-Young (p=0.683 and p=0.520, respectively) or APP/PS1-Old (p=0.365 and p=0.326, respectively) mice (Fig 4.9). Western blot analysis showed no significant change in the levels of carbonate soluble APP and APP-C-terminal fragment after mTBI (Fig 4.12) suggesting that the very mild injury paradigm in this study does not cause a significant acute change in APP processing or net Aβ levels in the whole brain despite a transient and subtle change in Aβ deposits defined by structural configuration and brain region (cortex).

Together, these observations suggest that age at injury can modify the acute response of deposited Aβ to mTBI without affecting total APP processing or whole brain Aβ levels. APP/PS1-Young-TBI mice showed a transient increase in cortical diffuse Aβ deposits that resolved as the animal recovered from very mild injury, without affecting the infrequent fibrillary deposits at this age. In contrast, APP/PS1-Old-TBI mice had no change in diffuse Aβ deposits but display a sinusoidal change in fibrillary Aβ deposits, with a transient increase followed by a transient decrease during the acute recovery period.

4.4.4 Age and APP/PS1 genotype can modify the inflammatory response after CHIMERA mTBI

White matter tracts are highly vulnerable to inflammation after TBI (Johnson, Stewart et al. 2013), and we previously demonstrated that CHIMERA TBI increases both microglial density and morphological changes consistent with microglial activation in several white matter regions. (Namjoshi, Cheng et al. 2014, Namjoshi, Cheng et al. 2016, Namjoshi, Cheng et al. 2017) Here,
we also observed a microglial response in various white matter areas including the optic tract, brachium of superior colliculus, and olfactory bulb, and selected the optic tract as the most robustly affected area for quantification purposes. Intriguingly, we observed a striking effect of age at injury on white matter inflammation, as mTBI significantly increased optic tract microglial density only in the WT-Old-TBI (p=0.028) and APP/PS1-Young-TBI (p<0.001) mice. Post-hoc tests showed that although no significant changes were observed at D2, microglial density was significantly increased at D7 specifically in the WT-Old-TBI (post-hoc analysis at D7 p=0.003) and APP/PS1-Young-TBI (p<0.001) groups (Fig 4.4). We also examined the mean microglial size in the optic tract after mTBI and found a significant injury effect in the APP/PS1-Young-TBI group (44% increase, p<0.001) (Fig 4.10), but no change in the other groups.

Mesoscale ELISA assays were used to profile the inflammatory response to mTBI in brain homogenates. As the concentrations of IL-6, IL-1β, TNF-α in some of these brain samples were below the lower limit of detection and assigned value of zero, data are presented using scatter plots and analyzed using non-parametric tests. No changes were observed for any of the groups except for APP/PS1-Young-TBI mice, which showed a significant increase in IL-1β (p=0.020), and IL-6 (p=0.043) and TNF-α (p=0.076) showed a similar trend toward increased levels in APP/PS1-Young-TBI mice but did not reach significance after Bonferroni correction (threshold p value=0.05/2=0.025) (Fig 4.5).

Together, these data suggest that CHIMERA mTBI induces an inflammatory response that is modified by age at injury as well as by genotype. In WT mice, our results support the conclusion that older animals show a heightened microglial response (Kumar, Stoica et al. 2013) in affected areas compared to younger animals inflicted with the same impact severity. By contrast, for APP/PS1 mice, an exaggerated microglial and cytokine response is observed in young mice when Aβ deposits are just emerging, perhaps reflecting a hyper-sensitivity to a further insult. However, when Aβ and amyloid loads in APP/PS1-Old mice are established, the brain may be desensitized by a chronic stage of inflammation, and become less able to mount an acute inflammatory response after an additional mild insult.
4.4.5 Age at injury modifies axonal neurofilament pathology after mTBI

DAI is a cardinal neuropathological feature of mTBI that is associated with complex changes in a number of axonal cytoskeletal markers that accumulate as swellings or varicosities along the length of the axon and in terminal axonal bulbs. (Siedler, Chuah et al. 2014) We previously demonstrated that CHIMERA reliably induces DAI in several white matter tracts, including the optic tract, brachium of superior colliculus, and corpus callosum. (Namjoshi, Cheng et al. 2014) Using NeuroSilver staining, we observed similar patterns of injury in this study and focused on the optic tract for quantification purposes. Significantly increased silver uptake was observed at D14 post-injury in all groups; WT-Young-TBI (p=0.001), WT-Old-TBI (p<0.001), APP/PS1-Young-TBI (p<0.001), and APP/PS1-Old-TBI (p=0.017). (Fig 4.6)

As APP immunoreactive axonal bulbs are a hallmark of human DAI, we attempted to examine these in APP/PS1 mice. However, due to the high expression level of the APP transgene in these animals, the baseline signal overwhelmed our ability to clearly define APP-stained axonal bulbs (not shown). Though we did not assay tau level in detergent-soluble fraction of brain homogenates, we measured total tau and p-tau levels in the carbonate-soluble fraction using Mesoscale assays. No significant injury effects for tau, p-tau, or p-tau/tau levels were observed, consistent with very mild injury (Fig 4.11).

As neurofilament-light is a promising biomarker of axonal damage for human TBI (Shahim, Gren et al. 2016, Thelin, Zeiler et al. 2017), we used SMI312 to label phosphorylated neurofilament medium and heavy chains using IHC to quantify the density of axonal swellings in the optic tract. (Ljungqvist, Zetterberg et al. 2016, Shahim, Gren et al. 2016, Shibahashi, Doi et al. 2016) Strikingly, significant neurofilament-positive axonal swellings were observed specifically at D2 in the WT-Young-TBI (p=0.020, 180% increase) and APP/PS1-Young-TBI (p<0.001, 330% increase) groups, but not in either WT-Old-TBI (p=1.000) or APP/PS1-Old-TBI (p=0.995) groups (Fig 4.6), despite clear evidence of equivalent axonal injury by silver uptake in all mTBI groups. We also observed that the occurrence of neurofilament-positive axonal swellings in both WT-Young-TBI and APP/PS1-Young-TBI groups were transient, as the number of axonal swellings returned to sham levels by D7 (p=0.991 and p=0.876, respectively).
We then performed an ultrasensitive Simoa ELISA assay for plasma neurofilament-light chain levels, and observed that at D2, APP/PS1-Young-TBI mice showed significantly elevated levels compared to APP/PS1-Young-Sham mice (p=0.030, 233% increase). Plasma neurofilament-light levels in WT-Young-TBI mice at D2 were slightly elevated but not statistically significant (p=0.087, 74% increase). (Fig 4.7)

In summary, IHC for axonal neurofilament phosphorylated heavy and medium chains revealed an increased number of axonal bulbs in APP/PS1-Young-TBI mice, and, to a lesser extent, WT-Young-TBI mice. However, this neuropathological response was completely attenuated in old mice for both genotypes. Using the same neurofilament-light assay used in clinical TBI blood biomarker studies, we observed significantly elevated plasma neurofilament-light levels only in APP/PS1-Young-TBI mice, perhaps reflecting their increased susceptibility to axonal injury around the time that Aβ begins to be deposited. As plasma neurofilament reflects clearance of brain neurofilament, the less pronounced neurofilament effect in brains of WT-Young-TBI mice became insignificant in the plasma samples. The fact that both the brain IHC and plasma ELISA assays showed that the neurofilament response is highly transient and peaks around D2 post-TBI is consistent with its transient response in human data. This finding also adds additional translational value to the CHIMERA model of TBI.
4.4 Discussion

Some of the tremendous heterogeneity in TBI outcomes may due to age at injury, as older subjects are at higher risk for poor or partial recovery (McIntyre, Mehta et al. 2013, Sherer, Sander et al. 2015). The odds for a poor outcome following TBI is estimated to increase by 40–50% for every 10 years of age, with patients 60 years or older being at four times greater risk for incomplete recovery compared to a 30-year-old TBI patient (Hukkelhoven, Steyerberg et al. 2003). Notably, being over 60 years of age was reported to be the only independent predictor of insufficient recovery after mTBI (Tierney, Nayak et al. 2016). Although further research is needed, the aged brain is believed to be less able to mount effective recovery pathways than the young brain in response to a similar injury severity or type of injury (Hukkelhoven, Steyerberg et al. 2003). Our study was therefore designed to test the effects of age at injury on acute neurological and cognitive behaviours, neuropathological markers of DAI, and the inflammatory response to mTBI in cohorts of WT and APP/PS1 animals, controlled for genetic admixture, that were subjected to two very mTBI using CHIMERA.

The most striking effect of age at injury was to modify the brain neurofilament IHC response, which was robust in both WT-Young-TBI and APP/PS1-Young-TBI animals but markedly attenuated in WT-Old-TBI and APP/PS1-Old-TBI mice. Notably, as neurofilament-light is of tremendous interest as a potential blood biomarker (Blennow, Hardy et al. 2012, Zetterberg, Smith et al. 2013), our results also suggest that neurofilament-light may be a more useful biomarker of mTBI in young individuals, if further research reveals that the neurofilament response to TBI is also attenuated by age at injury in humans. In addition to silver staining and neurofilament IHC, we also performed APP staining in some of our samples. However, we did not observe significant and consistent APP immunopositive axonal bulbs. (Data not shown) Part of the reason could be due to the different mechanisms leading to APP or neurofilament axonal pathologies, as APP axonal bulb is often considered a hallmark of disrupted fast axonal transport, where neurofilament bulbs can be resulted from excitotoxicity and activation of calcium-dependent proteases. (Siedler, Chuah et al. 2014, Hill, Coleman et al. 2016) In addition, other studies have suggested that the type and temporal profile of injury may lead to different axonal pathologies. For example, following a FPI, c-terminal APP-immunopositive axonal bulbs were found to be present at the proximal axonal segment, whereas axonal varicosities that do not label APP were mainly found at
the distal segment, suggesting Wallerian degeneration. (Hanell, Greer et al. 2015) Another possible explanation is that due to highly overexpressed amount of APP in this APP/PS1 mouse model, there is huge increase in the baseline noise level, rendering the comparison of the post-injury pathology difficult.

The post-injury inflammatory response was also affected both by age at injury and by genotype, such that white matter inflammation was more pronounced in WT-Old-TBI than WT-Young-TBI mice, whereas the opposite pattern was observed in APP/PS1 animals. These results suggest that APP/PS1 mice may be sensitized to mount an exacerbated inflammatory response at an age when amyloid deposition is beginning, and that this response may be attenuated at an older age when chronic inflammation may be established. Surprisingly, the only noteworthy differences between APP/PS1 and WT cohorts after CHIMERA mTBI related to the inflammatory response, suggesting that the presence or absence of human Aβ may have a considerably more minor role in TBI outcomes than previously hypothesized. Our results are consistent with age-related differences in the neuroinflammatory process in other preclinical TBI studies (Kumar, Stoica et al. 2013, Webster, Van Eldik et al. 2015, Gupta and Prasad 2016), although the many differences in TBI models and experimental designs make direct comparisons challenging.

IHC results showed that in APP/PS1-Young mice, mTBI causes a transient increase in non-fibrillar Aβ deposits, although the mechanisms underlying this phenomenon are not yet known. Though we did not directly measure the levels of APP or CTF in membrane fractions, there was no change of their levels in the carbonate-soluble fraction, and so it is unlikely that the mTBI conditions used in this study altered Aβ production. We hypothesize the increased incidence of diffuse Aβ deposits in APP/PS1-Young-TBI mice may reflect a transient slowing of Aβ clearance pathways that subsequently recover before the deposited Aβ is converted to fibrillar forms. Conversely, in APP/PS1-Old-TBI mice, total Aβ deposits did not change significantly, perhaps due to an approaching ceiling effect, but fibrillar amyloid transiently increased and then decreased to baseline, suggesting a dynamic remodeling of amyloid fibrillar structure in the acute phase after injury. Whether this represents true clearance or breakdown of fibrils will require future studies including microdialysis or neuroimaging that can better examine the dynamic nature of Aβ species after injury. Our observations are consistent with neuropathological studies in human TBI,
where diffuse Aβ deposits can be observed in young-middle aged subjects who would not be expected to have a significant age-related Aβ load (Roberts, Allsop et al. 1990, Graham, Gentleman et al. 1996, Gentleman, Greenberg et al. 1997, Ikonomovic, Uryu et al. 2004). Interestingly, increased Pittsburgh compound B neuroimaging shows increased amyloid deposition in subjects imaged up to 1 year post-TBI (Hong, Veenith et al. 2014). Reduced cerebrospinal fluid levels of Aβ42 can be observed months after severe TBI (Bagnato, Andriolo et al. 2017), and cerebral microdialysis of a cohort of 7 patients aged 18-76 years shows consistently higher Aβ40 and Aβ42 levels at 113-116h post-injury in subjects with predominate DAI, regardless of age, level of consciousness, or interstitial energy metabolism (Marklund, Farrokhnia et al. 2014), demonstrating that TBI can also markedly affect Aβ dynamics in humans.

Many studies in animal models have been performed to investigate how TBI influences Aβ dynamics in genetically engineered models of amyloidosis, with a wide variation in outcomes (Bird, Sohrabi et al. 2016) that are likely due to the different animal models, TBI mechanisms, injury severities and ages at injury used in these studies. For example, increased Aβ levels and deposits were observed in Tg2576 mice up to 16 weeks after repetitive mTBI using CCI at 9 months of age, prior to the onset of Aβ deposition (Hsiao, Chapman et al. 1996, Uryu, Laurer et al. 2002). PDAPP mice subjected to CCI at 4 months of age, again prior to onset of deposition (Games, Adams et al. 1995), led to acute transient increased Aβ levels (Smith, Nakamura et al. 1998), whereas severe CCI injury in 24-month old PDAPP mice with extensive plaque burden led to decreased deposited Aβ as measured by IHC (Nakagawa, Reed et al. 2000). Moderate-severe CCI in 3 month old PSAPP mice, when Aβ deposition is beginning (Holcomb, Gordon et al. 1998), increased plaque burden 6 weeks post injury (Tajiri, Kellogg et al. 2013). Severe CCI in 5-7 month old 3xTg mice that express human Aβ and human tau (Oddo, Caccamo et al. 2003) caused axonal accumulation of both Aβ and tau by independent mechanisms (Tran, LaFerla et al. 2011), whereas CCI prior to plaque onset led to acute increases in both insoluble and soluble Aβ (Washington, Morffy et al. 2014). CCI in a human Aβ knock-in mouse model (hAβ KI mice) (Abrahamson, Ikonomovic et al. 2009) increased both levels and deposits of Aβ and phosphorylated tau (Abrahamson, Ikonomovic et al. 2006, Abrahamson, Foley et al. 2013, Webster, Van Eldik et al. 2015). FP in aged guinea pigs led to diffuse Aβ deposits and age-dependent increases in immunoreactivity to APP and tau (Bates, Vink et al. 2014). In pigs, rotational acceleration injury
increased Aβ accumulation in injured axons and led to plaque-like structures (Smith, Chen et al. 1999).

Although our study was specifically designed to mimic mild, concussive TBI, one limitation is that the CHIMERA injury was milder than expected in hybrid C56Bl/6-C3H animals compared to our previous results in C57Bl/6 mice. Although LRR clearly demonstrated an injury response, no other behavioural measure showed consistent injury effects. This surprising result suggests that contributions of the C3H genetic background may provide resilience toward mTBI-induced behavioural responses. The Mouse Phenome Database (Jackson Laboratory) notes several differences between these two strains, including increased growth rate, body weight, skull and whole body mineral density in C56Bl/6-C3H mice compared to pure C57/B6 mice, as well as differential susceptibility to bacterial infection that may reflect genetically-determined differences in immune responses. Nevertheless, silver staining clearly indicated robust DAI effects across all groups. An additional limitation of the current study is that our cohort consisted entirely of male animals examined at only two ages. Future studies will be required to provide additional evidence of how age, and possibly sex, may modify acute and chronic effects of TBI as a function of injury severity. Despite these limitations, our study reveals that even under very mild impact-acceleration TBI conditions, age at injury can significantly modify the acute inflammatory and neurofilament responses in both wild-type and APP/PS1 mice, and transiently alter Aβ metabolism in APP/PS1 mice.
Figure 4.1  Neurological behaviour after mTBI.

(a,b) Loss of righting reflex (LRR) was recorded immediately after sham or TBI procedures on both days. (c,d) Neurological severity score (NSS) was assessed at baseline and at 1 h, Day 1 (D1), D2, and D7 after the second injury. Panels in the left column represent the wildtype “WT” cohort and those in the right column represent the transgenic APP/PS1 cohort. For all graphs, dark blue solid lines, light blue dotted lines, dark red solid lines, and light red dotted lines represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green solid lines, light green dotted lines, dark orange solid lines,
and light orange dotted lines represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. LRR was analyzed by Repeated Measure ANOVA, and NSS was analyzed by mixed linear model. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons between the corresponding Sham/TBI pairs at each time point shown in the figure panels. For LRR, N=33-49 per injury per genotype per age per day (average N=41). For NSS, N=14-52 per injury per genotype per age per time point (average N=27).
Figure 4.2  Motor and spatial cognitive behaviour after mTBI
(a,b). RotaRod (RR) latency to fall was assessed at baseline and on D1, D2, D7, and D14 after second injury. (c,d) Barnes maze (BM) reference acquisition training was performed for 5 days after injury (D8 to D12), and the duration of time the mice took to enter a fixed hideout was measured. On each acquisition day three trials were performed, and the daily average performance is plotted. (e,g) On D13 after injury, the hideout was removed and a probe trial was performed. The amount of time the mice spent in the north quadrant, where the hideout was previously located, was measured. (f,h) On D13 after injury, three reverse trials were conducted on the same day. The average value of the day is shown. For the graphs, dark blue solid lines/bars, light blue dotted lines/bars, dark red solid lines/bars, and light red dotted lines/bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green solid lines/bars, light green dotted lines/bars, dark orange solid lines/bars, and light orange dotted lines/bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. RR was analyzed by mixed linear model. BM acquisition and reverse trials were analyzed by mixed linear modeling, and probe trials were analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons between the corresponding Sham/TBI pairs at each time point shown in the figure panels. For RR, N=10-32 per injury per genotype per age per time point per trial (average N=22). For BM reference acquisition, N=10-14 per injury per genotype per age per day per trial (average N=12). For BM probe and reverse, N=8-12 per injury per genotype per age per trial (average N=10).
Figure 4.3  Brain Aβ and amyloid deposition after mTBI

(a,b) Aβ deposits in APP/PS1 cortex were detected by 6E10 immunohistochemistry at 6 h, D2, and D7 post-injury. (c,d) Quantification of the percentage area of 6E10-stained cortex stained is shown. (e,f) Amyloid burden in APP/PS1 brain cortex was detected by Thioflavin-S (Thio-S) histochemistry at 6h, D2, and D7 post-injury. (g,h) Quantification of the percentage area of Thio-S stained cortex is shown. Two boxed area of (f) are shown at higher resolution in (i), where the core and fibrillary structure of the Thio-S stained plaques are clearly visible. The left image is from an APP/PS1-Old-Sham mouse, and the right image from an APP/PS1-Old-TBI mouse at D2. Figures in (a,c,e,g) represent the APP/PS1-Young-Sham (AYS) and APP/PS1-Young-TBI (AYT) groups, and those in (b,d,f,h) represent the APP/PS1-Old-Sham (AOS) and APP/PS1-Old-TBI groups.
(AOT) groups. In (a,c,e,f), a sham-operated mouse at D2 is shown as a representative sham. In the quantification graphs, dark green solid lines/bars, light green dotted lines/bars, dark orange solid lines/bars, and light orange dotted lines/bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Aβ and Thio-S data are expressed as fold changes over the respective sham groups, and analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons between the corresponding Sham/TBI pairs at each time point shown on the figure panels. For Thio-S, N=3-8 per injury per age per time point (average N=5). Scale = 500 µm.
Figure 4.4  White matter microglial changes after mTBI

(a,b,e,f) The microglial response in the optic tract was visualized using Iba-1 immunohistochemistry (IHC) on D2 and D7 post-injury. (d,h) The density of microglia in the optic tract was quantified and plotted. Panels (a,b,c,d) represent the WT cohort and (e,f,g,h) represent the APP/PS1 cohort. In figures (a,b,e,f), a sham-operated mouse at D2 is shown as a representative sham. In the quantification graphs, dark blue bars, light blue bars, dark red bars, and light red bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green bars, light green bars, dark orange bars, and light orange bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Microglial density was analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons between the corresponding Sham/TBI pairs at each time.
point shown in the figure panels. N=3-8 per injury per genotype per age per time point (average N=5). Scale = 250 μm.
Figure 4.5  Cytokine changes after mTBI
(a,b,c,d,e,f) Cytokine levels in carbonate soluble half-brain homogenates at 6 h post-injury were analyzed using Mesoscale assays for IL-6 (a,c), IL-1β (b,d), and TNF-α (e,f). Panels on the left column (a,b,e) represent the WT cohort and those on the right column (c,d,f) represent the APP/PS1 cohort. In the graphs, dark blue dots, light blue dots, dark red dots, and light red dots represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green dots, light green dots, dark orange dots, and light orange dots represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. IL-6, IL-1β, and TNF-α levels are analyzed by Kruskal-Wallis test followed by post-hoc comparison using Mann-Whitney U test with Bonferroni correction of multiple comparison. All samples are represented as individual dots on the graphs, with median value indicated, and significant post-
hoc comparisons between the corresponding Sham/TBI pairs at each time point are shown in the figure panels. Samples below detection limit were considered “zero”. N=4-5 per injury per genotype per age per time point (average N=4).
Figure 4.6  Axonal injury after mTBI.
Degenerative axonal injury in the optic tract, was visualized by Neurosilver staining on samples at D2, D7, and D14 post-injury. The percentage of stained area was quantified and shown in (c) and (f). (g,h,k,l) Neurofilament immunohistochemistry was performed using SMI312, and axonal bulbs were examined in the optic tract at D2 and D7 post-injury. The density of the SMI312-positive axonal bulbs was quantified and shown in (j) and (n). Panels in (a,b,c,g,h,j) represent the WT cohort and those in (d,e,f,k,l,n) represent the APP/PS1 cohort. In (a,b,d,e,g,h,k,l), a sham-operated mouse at D2 is shown as a representative sham. In the quantification graphs, dark blue bars, light blue bars, dark red bars, and light red bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green bars, light green bars, dark orange bars, and light orange bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Neurosilver and neurofilament data were analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant variables and interactions stated for each figure. For Neurosilver, N=3-9 per injury per genotype per age per time point (average N=5). Scale = 500 µm. For neurofilament IHC, N=3-7 per injury per genotype per age per time point (average N=5). Scale = 500 µm.
Figure 4.7  **Plasma neurofilament-light after mTBI.** (a,b) Plasma neurofilament-light level was measured using Simoa ELISA at 6h, D2, and D7 post-injury. Panel on the left (a) represents the WT cohort and that on the right (b) represents the APP/PS1 cohort. In the graphs, dark blue bars, light blue bars, dark red bars, and light red bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green bars, light green bars, dark orange bars, and light orange bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Plasma neurofilament was analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons between the corresponding Sham/TBI pairs at each time point shown in the figure panels. N=4-5 per injury per genotype per age per time point (average N=5).
Figure 4.8  Passive Avoidance, Elevated Plus Maze, and Open Field performance after mTBI
(a,b,c,d) Open field test (OF) was performed on D1 and D6. (e,f) Passive avoidance (PA) was performed on D6 (shock) and D7 to D9 (test) after TBI. (g,h) Elevated Plus Maze (EPM) was performed on D7 and D10 after TBI. Panels in the left column represent the WT cohort and those in the right column represent APP/PS1 cohort. In the graphs, dark blue dots, light blue dots, dark red dots, and light red dots represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green dots, light green dots, dark orange dots, and light orange dots represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. The total distance travelled and time spent immobile during OF were analyzed by mixed linear model, PA was analyzed by Repeated Measure ANOVA, and EPM was analyzed by mixed linear model. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons of each Sham/TBI pair shown in each panel. For OF, N=12-21 per injury per genotype per age per time point (average N=17). For PA, N=10-18 per injury per genotype per age per day (average N=14). For EPM, N=6-16 per injury per genotype per age per time point (average N=11).
**Figure 4.9  Brain Aβ levels after mTBI**

Half-brains of APP/PS1 mice at 6 h, D2, D7, and D14 were homogenized, and Aβ40 and Aβ42 levels were assayed in carbonate- (a, b, c, d) and guanidine HCl- (e, f, g, h) soluble fractions by ELISA. Panels on the left (a, b, e, f) represent the 6 month “APP/PS1-Young” cohort and those on the right (c, d, g, h) represent the 13 month “APP/PS1-Old” cohort. Dark red bars and light red bars represent the APP/PS1 TBI group and the APP/PS1 Sham group, respectively. Aβ data were analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons of each Sham/TBI pair shown in the figure panels. For Aβ ELISA, N=3-5 per injury per genotype per age per time point (average N=4).
Figure 4.10  White matter microglial size after mTBI

(a,b) The average (mean) microglial size in the optic tract was quantified from Iba-1 immunohistochemistry (IHC) on D2 and D7 post-injury. Panels (a) represent the WT cohort and (b) represent the APP/PS1 cohort. Dark blue bars, light blue bars, dark red bars, and light red bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green bars, light green bars, dark orange bars, and light orange bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Average microglial density was analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant post-hoc comparisons between the corresponding Sham/TBI pairs at each time point shown in the figure panels. N=3-8 per injury per genotype per age per time point (average N=5). Scale = 500 μm.
Figure 4.11  Tau levels after mTBI
(a,b,c,d,e,f) Levels of total tau, phosphorylated tau (Thr231), and phosphorylated:total tau ratio in carbonate-soluble fractions at 6h and D2 post-injury were measured using Mesoscale assays. Panels in the left column represent the WT cohort and those in the right column represent APP/PS1 cohort. In the graphs, dark blue bars, light blue bars, dark red bars, and light red bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green bars, light green bars, dark orange bars, and light orange bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Data were analyzed by ANOVA and represented in all graphs as mean ± standard error, with significant post-hoc comparisons of each Sham/TBI pair shown in the figure panels. For total tau and p-tau, N=3-6 per injury per genotype per age per time point (average N=4).
Figure 4.12  sAPP and APP-CTF levels after mTBI

(a,b,c,d,e,f) Levels of sAPP and carboxyl terminal fragment (CTF) APP in carbonate-soluble fractions at D2 post-injury were assayed using Western blot. In the quantification graphs, dark blue bars, light blue bars, dark red bars, and light red bars represent the groups WT-Young-TBI (WYT), WT-Young-Sham (WYS), WT-Old-TBI (WOT), and WT-Old-Sham (WOS), respectively; dark green bars, light green bars, dark orange bars, and light orange bars represent the groups APP/PS1-Young-TBI (AYT), APP/PS1-Young-Sham (AYS), APP/PS1-Old-TBI (AOT), and APP/PS1-Old-Sham (AOS), respectively. Data were analyzed by mixed linear model and represented in all graphs as mean ± standard error. For sAPP and APP-CTF, N=3-5 per injury per genotype per age (average N=4).
Figure 4.13 Outline of experimental design.

An outline of the experimental plan is shown in the diagram.
Figure 4.14  A graphical summary of the complex interactions of Aβ, ageing, and TBI.
Table 4.1  Summary of significance table.

The significance values of all effects and interactions for each assay is show below.

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<th>Kinematics (TBI mice only) - Linear Acceleration</th>
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<td>IL-1β</td>
<td>TNF-α</td>
<td>Mg-density</td>
<td>Mg-size</td>
<td>Silver</td>
<td>NF (IHC)</td>
<td>NFL (ELISA)</td>
<td>CTF</td>
<td>App</td>
<td>Tau (Total)</td>
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<td>-</td>
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<td><strong>0.001</strong></td>
<td><strong>0.004</strong></td>
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<td>-</td>
<td>-</td>
<td>0.053</td>
<td>0.680</td>
<td>0.309</td>
<td>0.058</td>
<td>0.921</td>
<td>-</td>
<td>-</td>
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<td>0.086</td>
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<td>0.389</td>
<td>0.083</td>
<td><strong>0.001</strong></td>
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<td>0.264</td>
<td>0.488</td>
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<tr>
<td><strong>WOS vs WOT</strong></td>
<td>0.221</td>
<td>0.623</td>
<td>0.618</td>
<td><strong>0.028</strong></td>
<td>0.373</td>
<td>&lt;0.001</td>
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<td>0.524</td>
<td>1.000</td>
<td>1.000</td>
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<td>-</td>
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<td><strong>0.022</strong></td>
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<td>-</td>
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<td>0.054</td>
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<td><strong>0.020</strong></td>
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<td><strong>0.029</strong></td>
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<tr>
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<td>0.221</td>
<td>0.737</td>
<td>0.989</td>
<td>0.976</td>
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<td>0.997</td>
<td>0.888</td>
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Table 4.1 (cont’d)

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<th>Aβ40-old (IHC)</th>
<th>ThioS-young</th>
<th>ThioS-old</th>
<th>Aβ40-young (carb)</th>
<th>Aβ40-old (carb)</th>
<th>Aβ42-young (carb)</th>
<th>Aβ42-old (carb)</th>
<th>Aβ40-young (GuHCl)</th>
<th>Aβ40-old (GuHCl)</th>
<th>Aβ42-young (GuHCl)</th>
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</thead>
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<td><strong>APP/PS1</strong></td>
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<tr>
<td>Group</td>
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<td>0.213</td>
<td>0.568</td>
<td>0.609</td>
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<td>0.491</td>
<td>0.683</td>
<td>0.365</td>
<td>0.365</td>
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<td>Time</td>
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<td>0.278</td>
<td>0.620</td>
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<td>0.965</td>
<td>0.325</td>
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<td>0.357</td>
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<td>0.808</td>
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<tr>
<td>Group*Time</td>
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<td>0.278</td>
<td>0.428</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>0.045</strong></td>
<td>0.411</td>
<td>0.527</td>
<td>0.490</td>
<td>0.119</td>
<td>0.778</td>
<td>0.483</td>
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Table 4.2  Head kinematic analysis of CHIMERA mTBI.
Head motion during the two 0.5 J impacts was tracked using high-speed videography and used to compute linear and rotational acceleration and velocity parameters. Body weights of each group of animals were shown as reference. The peak values of each parameter and body weight were analyzed by repeated measure ANOVA, and the average value (± s.e.) across the two days of TBI are shown. Reference head kinematic values from a previous study using C57Bl/6 mice (mean ± s.e.m.) are also given. For kinematic analyses, N=4-6 per genotype per age (average N=5).

<table>
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<tr>
<th></th>
<th>Linear Velocity (m/s)</th>
<th>Linear Acceleration (g)</th>
<th>Rotational Velocity (rad/s)</th>
<th>Rotational Acceleration (krad/s²)</th>
<th>Body Weight (g)</th>
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<td>WT- Young</td>
<td>5.9 ± 0.5</td>
<td>359.6 ± 35.1</td>
<td>352.1 ± 54.1</td>
<td>360.4 ± 68.0</td>
<td>46.7 ± 0.6</td>
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<tr>
<td>WT-Old</td>
<td>6.6 ± 0.4</td>
<td>400.4 ± 28.7</td>
<td>376.3 ± 44.1</td>
<td>327.5 ± 55.5</td>
<td>47.4 ± 0.6</td>
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<tr>
<td>APP/PS1-Young</td>
<td>6.8 ± 0.5</td>
<td>462.2 ± 35.1</td>
<td>449.5 ± 54.1</td>
<td>462.9 ± 68.0</td>
<td>46.9 ± 0.6</td>
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<tr>
<td>APP/PS1-Old</td>
<td>6.1 ± 0.4</td>
<td>372.9 ± 28.7</td>
<td>332.9 ± 44.1</td>
<td>323.0 ± 55.5</td>
<td>50.8 ± 0.7</td>
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<tr>
<td>Reference C57/B6 (Namjoshi, Cheng et al. 2014)</td>
<td>6.6 ± 0.1</td>
<td>385.3 ± 17.7</td>
<td>305.8 ± 21.6</td>
<td>253.6 ± 18.8</td>
<td>33.9 ± 0.4</td>
</tr>
<tr>
<td>Significant Difference</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>APP/PS1-Old vs other groups (p=0.001)</td>
</tr>
</tbody>
</table>
Chapter 5: CHIMERA repetitive mild traumatic brain injury induces long-term pathological and behavioral changes in APP/PS1 mice

5.1 Introduction

Traumatic brain injury (TBI) is defined as “an alteration in brain function, or other evidence of brain pathology, caused by an external force”. (Menon, Schwab et al. 2010) In United States, the annual incidence of TBI is over 2.5 million (Centers for Disease Control and Prevention 2016), and over 3-5 million are living with residual problems from TBI (Thurman, Alverson et al. 1999, Corrigan, Selassie et al. 2010), showing that TBI is an important public health concern. In the previous Chapters, we used our innovative CHIMERA mouse model to show that repetitive mild TBI (mTBI) may induce acute behavioral, neuroinflammatory and axonal changes. The focus of this Chapter is to investigate the long-term effects of repetitive mTBI.

One common clinical classification of TBI is based on injury severity. In this system TBI is categorized based on the Glasgow Coma Scale (GCS) (mTBI: GCS 13-15; moderate TBI: GCS 8-12, severe TBI: GCS 3-7), and the presence or severity of symptoms such as loss of consciousness and post-trauma amnesia. (Teasdale and Jennett 1974, Centers for Disease Control and Prevention 2014) Moderate and severe TBI can result in high rates of disability or death within 1 yr of injury (Jiang, Gao et al. 2002) and also significantly increases risk of dementia, including Alzheimer’s disease (AD), by approximately 1.5 fold (Mortimer, van Duijn et al. 1991, Fleminger, Oliver et al. 2003, Gardner, Burke et al. 2014). In addition to disability and dementia, long-term survivors of moderate and severe TBI often show psychiatric symptoms, such as anxiety and depression (Jourdan, Bayen et al. 2017) and cognitive impairments, such as in memory and processing speed. These symptoms may persist both at the recovery plateau (6-18 mo post-injury) and after. (Ruttan, Martin et al. 2008) At the other end of the injury severity spectrum, mTBI, which comprises at least 70% of all TBI cases (Kraus and Nourjah 1988, Cassidy, Carroll et al. 2004), may also have dire long-term consequences. Research has shown
that 10%–55% of mTBI patients may show persistent symptoms at 3 mo to 1 yr after injury. (National Center for Injury Prevention and Control 2003, McInnes, Friesen et al. 2017)

Clinically, the term “post-concussion syndrome” describes a complex of cognitive, somatic, sleep or other changes that occur as a consequence of TBI and persist for at least 3 mo. (Barlow 2016) Examples of these symptoms include irritability, mood disturbances, concentration difficulties, depression, and anxiety disorders. (Jorge and Robinson 2011, Barlow 2016) Post-traumatic stress disorder (PTSD), which is characterized by intrusive thoughts and deficient fear memory extinction (Kennedy, Jaffee et al. 2007, Wicking, Steiger et al. 2016), is another frequent occurrence among TBI patients, particularly in the military setting. Overall, mild TBI subjects have an elevated risk for any psychiatric illness in 6 mo post-injury (OR = 2.8 or 1.6, for subjects without/with a prior history of psychiatric illness). (Fann, Burington et al. 2004) In addition, repetitive exposure to mTBI is also linked to the development of a neurodegenerative condition known as Chronic Traumatic Encephalopathy (CTE). This is of particularly high relevance to athletes of contact sports and military personnel (Roberts, Allsop et al. 1990, McKee, Cantu et al. 2009), as they may be exposed to a large number of mTBI during their careers. Behaviorally, CTE is associated with symptoms including irritability, impulsivity, aggression and suicidality, and memory loss. (McKee, Cantu et al. 2009) These evidences show that TBI of any severity may induce long-term behavioral changes.

In addition to behavioral changes, neuroinflammatory changes are also common long-term features of TBI. Immunohistological examination (using CR3/43 and CD68) and imaging studies (using [11C](R)PK11195) of brains from moderate or severe TBI patients have shown that chronic neuroinflammatory changes may persist for months up to over 10 yr after TBI. (Ramlackhansingh, Brooks et al. 2011, Johnson, Stewart et al. 2013) More importantly, chronic inflammatory changes of the white matter and thalamus are associated with degeneration of white matter (Johnson, Stewart et al. 2013) and cognitive impairment (Ramlackhansingh, Brooks et al. 2011), respectively. In a clinical study involving 66 deceased CTE football athletes, the number of years playing the sport was associated with microglia activation, which was further associated with dementia status. (Cherry, Tripodis et al. 2016)

Neurodegenerative changes are another common feature in TBI brains in long-term. In 30% of patients that die from severe TBI, diffuse Aβ depositions were found during the acute phase.
Recent imaging studies using position emission tomography-computer tomography have confirmed increased $[^{11}\text{C}]$ Pittsburgh Compound B ($[^{11}\text{C}-\text{PiB}]$) binding in the living brain during the acute phase after moderate or severe TBI. (Hong, Veenith et al. 2014) The long-term Aβ changes after a single moderate or severe TBI, however, are less conclusive, with studies showing no change in amyloid (Chen, Johnson et al. 2009), showing region-specific changes in amyloid (Gatson, Stebbins et al. 2016), or showing changes in the structure but not the density of amyloid (Johnson, Stewart et al. 2012). In CTE brains, amyloid deposits are only observed in up to 50% of all cases. (Tierney, Nayak et al. 2016) On the other hand, CTE is defined by progressive tauopathy, particularly the presence of perivascular neurofibrillary tangles at sulcal depths, as well as axonal loss/axonal varicosities. (McKee, Stern et al. 2013) Other common CTE neuropathologies include TAR DNA-binding protein 43 (TDP-43) inclusions (over 80%). (McKee, Stern et al. 2013) These neuropathological presentations of CTE partly overlaps, but is distinct from, those of AD, which is characterized by both amyloid plaques and neurofibrillary tangles. Notably, recent advances in the development of Positron Emission Tomography (PET) tracers of tau have opened the possibility to perform longitudinal study of tau pathologies in vivo. (Mitsis, Riggio et al. 2014, Barrio, Small et al. 2015, Dickstein, Pullman et al. 2016) Up-to-date, only a handful of case studies or small reports have been published. However, it is expected these novel PET tracers will greatly advance our understanding of the long term consequences of TBI across the severity spectrum.

Regarding pre-clinical TBI studies, many different TBI models have been used. Based on injury mechanisms, TBI can be classified as impact, penetrating, or blast. TBI models that involve impacts can be further classified as open head injury (e.g. fluid percussion injury (FPI), controlled cortical impact (CCI)) or closed head injury (e.g. weight drop (WD)). (Xiong, Mahmood et al. 2013) A summary of chronic outcomes of experimental TBI has been reviewed by Osier. (Osier, Korpon et al. 2015) In short, after open head injury in rodents, reported chronic changes (1 mo to 1 yr post injury) include: shrinkage of gray matter and neuronal loss (e.g. cortex and hippocampus)(Bramlett, Kraydieh et al. 1997, Smith, Chen et al. 1997, Carbonell, Maris et al. 1998, Dixon, Kochanek et al. 1999, Kamper, Pop et al. 2013), enlargement of

Though there are a plethora of publications on preclinical TBI research, reports that include long-term outcomes are less common. A review in 2013 reported that among rodent TBI research with functional tests, only 32% include post-injury time-points of 1 mo or beyond, and only 2.5% include 6 mo post-injury. (Gold, Su et al. 2013) This relative lack of research on chronic post-TBI outcomes reveals a misalignment with the clinical need, as evidence suggests that TBI may cause a number of long-term consequences. In addition, most of the historical literature of preclinical TBI uses the CCI or FPI models, which is not a representative model of the vast majority of clinical TBI. (Namjoshi, Good et al. 2013) Given the current interest in understanding how repetitive concussion leads to CTE, many current preclinical studies are using a wide variety of closed-head injury models to explore relationships between repetitive impact and cerebrovascular flow, persistent white matter astrogliosis and microgliosis, and changes in tau levels. (Ojo, Mouzon et al. 2016, Winston, Noel et al. 2016) These studies have tremendous variations in experimental design with respect to biomechanical inputs, resultant injury severity, plane of head motion (if any), number of consecutive impacts, and interval between impacts, all of which could influence the overall results. Furthermore, as many CHI
models are not yet standardized across different laboratories, identifying the most salient and clinically relevant results from pre-clinical literature is challenging.

To enable reliable investigation of chronic effects of TBI in an animal model that uses mechanically validated methods to produce injury, we developed the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) as a biomechanically and clinically relevant model of mTBI. (Namjoshi, Cheng et al. 2014, Namjoshi, Cheng et al. 2017) CHIMERA induces TBI by delivering highly reproducible impacts to head. The CHIMERA design allows unrestrained head motion and thus enables integrated analysis of head kinematics with biological outcomes. CHIMERA also has the precision to differentiate between concussive and subconcussive injuries, which offers improved tenability for mTBI research. In Chapter 4, we have discussed the acute outcomes (6h – 14d) of CHIMERA-induced repetitive mTBI at 2x0.5J in male APP/PS1 mice, a well-established model of Alzheimer’s disease beta amyloidosis. We observed that age at injury, in addition to genetic predisposition to AD, modulates several post-TBI outcomes including Aβ, neuroinflammation, and axonal injury responses. In the current Chapter, we aim to characterize the long-term behavioral, histological, and biochemical outcomes of repetitive mTBI (2x0.5J) up to 8 mo post-injury, and to test if such outcomes are affected by WT or APP/PS1 genotype. We find that chronically, mTBI reduced anxiety-like behavior and induced prolonged microgliosis and axonal injury, which are not significantly affected by genotype. However, mTBI chronically intensified long-term fear memory and impaired reversal spatial learning only in APP/PS1 mice.

These results suggest that TBI in APP/PS1 mice may chronically impair updates of existing memory, or unlearning / relearning of formed memory. The neurocircuitry involved in fear learning in one-trial inhibitory avoidance tasks, such as PA, has been widely studied. It has been shown that the acquisition, consolidation, and extinction of fear memory critically depends on brain regions including the hippocampus, amygdala, and prefrontal cortex, with additional contributions from structures including the entorhinal cortex, perirhinal cortex, and posterior parietal cortex. (Reviewed in (Izquierdo, Furini et al. 2016)) The processing of PA task and association of conditioned stimulus (entering the dark chamber) and unconditioned stimulus
(foot shock) mainly takes place through the trisynaptic circuit of the hippocampus (dentate gyrus > CA3 > CA1), whereas the emotional component involved in PA consolidation takes place in the basolateral amygdala. The processing of PA is then relayed to the central amygdala, which is the output nucleus of the amygdala, and is responsible for the fear response. The retrieval and extinction of conditioned fear, on the other hand, requires the function of prelimbic and infralimbic ventromedial prefrontal cortex (vmPFC), respectively. Specifically, during extinction of conditioned fear, the infralimbic vmPFC stimulates intercalated cells in the amygdala, which in turn leads to increased inhibitory output from central amygdala. Conversely, on the other end of the unlearning/relearning spectrum, reconsolidation of fear learning heavily depends on the hippocampus. (Izquierdo, Furini et al. 2016)

The clinical features of PTSD include involuntary and recurrent fear, in reaction to mood or cognition alteration, or mere elements or components of a previous traumatic experience, even without a stimulus resembling the conditioned stimulus. (Shalev, Liberzon et al. 2017) While PTSD in human is a very complicated disorder that can hardly be studied in animal models using one or two simplified behavioral tasks, many studies have suggested that PTSD may be caused by a deficit in fear learning and extinguishing (Wicking, Steiger et al. 2016). In this study, we used PA, the most widely studied form of fear learning, to probe for deficits in fear behavior after TBI. We find that TBI in APP/PS1 mice, but not WT mice, may chronically enhance fear response for an extended period of time, suggesting TBI may exacerbate fear consolidation and extinction in animals predisposed to Alzheimer’s amyloidosis. Our finding resonates with clinical findings. For example, one study followed 181,093 war veterans (53,155 developing PTSD) for a median period of seven years. (Yaffe, Vittinghoff et al. 2010) They found that war veterans with PTSD were more than twice as likely to develop dementia (OR for dementia in general: 2.31; OR for AD: 1.71; OR for FTD: 2.19) than those without PTSD. Our experimental data supports the idea that there is a strong overlap between the development of PTSD and AD pathogenesis.
5.2 Materials and methods

5.2.1 Western blotting
The Western blotting procedures in this Chapter was exactly the same as in Chapter 4.2.6. Notably, there were additional antibodies used in this Chapter, including anti-ZO1 (Life Technology 339100, 1:2000), SRB1 (Novus NB400-113, 1:2000), and TDP-43 (ProteinTech 12892-1-AP, 1:2000).

5.2.2 Barnes Maze Weibull learning curve analysis
The daily average duration to locate the hideout during the BM acquisition trials were used to fit the Weibull function commonly used in modelling psychometric learning curves:

\[ 90 - y = A \left(1 - e^{-\left(\frac{x}{L}\right)^S}\right), \]

where \(x, y, A, L, S, e\) represents the acquisition day, duration to find the hideout, curve asymptote (learning plateau), onset latency (the number of trials before observable learning response), abruptness of onset, and the natural exponential constant, respectively. (Gallistel, Fairhurst et al. 2004)

All other materials and methods used in this Chapter was the same as in the previous Chapter. See Section 4.2 for details.
5.3 Results

5.3.1 Two successive very mild impacts produce no significant acute neurological or motor deficits in hybrid C57Bl/6-C3H mice

In Chapter 2, we demonstrated that 2x0.5J impacts produce phenotypes consistent with mTBI in C57Bl/6 mice. In Chapter 3, we subsequently titrated impact energies for single impact CHIMERA to produce no phenotype at 0.1-0.4J mimicking sub-concussive impact, injury threshold at 0.5J mimicking concussive impact, and 0.6-0.7J mimicking mTBI. In Chapter 4, we demonstrated that 2x0.5J impacts produce milder than expected injury resembling concussive-concussive type injury in hybrid C57Bl/6-C3H mice (including APP/PS1 animals), suggesting that hybrid vigor may provide resilience to concussive impacts. To maintain consistency with the work described in Chapter 4, in this Chapter we delivered 2x0.5J impacts to hybrid C57Bl/6-C3H WT and APP/PS1 mice at 6 months of age and assessed outcomes up to 8 months post-injury, or 14 months of age, the same chronological age at which “old” animals were assessed in Chapter 4. A graphic of the study designs for Chapters 4 and 5 is provided in Fig 4.13 and Fig 5.9.

As required by our animal ethics protocol, acute neurological behaviors including the duration of loss of righting reflex (LRR), which is analogous to loss of consciousness in human, and the neurological severity score (NSS), which is analogous to the GCS in humans, were assessed immediately after CHIMERA injury. In this study with average N=12 per group, post-procedure LRR did not show a significant effect of injury (p=0.361), genotype (p=0.851) or injury*genotype (p=0.917). (Fig 5.1A) There was a significant time effect (p=0.018) for LRR, indicating that both sham and TBI mice recovered from anaesthesia earlier on the second day. In NSS (baseline to D7 post-TBI), there was no significant injury effect (p=0.173), but there was a significant genotype effect (p=0.003), indicating that APP/PS1 mice generally had a higher NSS compared to WT, regardless of injury status. (Fig 5.1B). We also used rotarod to assess motor performance in these mice and found a significant genotype effect (p=0.004), indicating APP/PS1 mice had worse motor performance than WT in general. (Fig 5.1C) However, there was no injury effect (p=0.100) or genotype*injury interaction (p=0.374). In summary, in the
current cohort, we did not observe neurological or motor deficits induced by TBI with this sample size, consistent with very mild TBI, likely in the subconcussive-concussive range.

5.3.2 mTBI intensifies long-term fear memory
Long-term fear memory was investigated using the Passive avoidance (PA) task from D6 up to 8 mo post-TBI. (Fig 5.2A) The first foot shock (Shock 1) was given on D6 post-TBI, and PA performance was followed from D7 up to 3 mo post-TBI. After a 3 mo interval to minimize task acclimatization, a second foot shock (Shock 2) was given at 6 mo post-TBI and PA performance was followed from 6 mo to 8 mo post-TBI. The PA performance after Shock1 and Shock2 shared a similar pattern.

Overall, we observed a significant time effect (p<0.001), a significant injury effect (p<0.001) and a significant genotype*injury interaction (p<0.001). The two prominent peaks of PA duration after the two shock days showed that all groups of mice were able to learn the fear during both shock events. All groups showed a gradual reduction in post-shock PA duration over time, indicating extinction of fear memory. Post-hoc comparison of WT-TBI and WT-Sham did not show significant difference between these two groups (p=0.225), suggesting both WT-Sham and WT-TBI mice had comparable fear learning and extinction. In contrast, the APP/PS1-Sham mice showed the lowest post-shock duration to enter light chamber of all four groups, indicative of poorest fear memory in these non-TBI mice (p<0.001 compared to WT-sham, p<0.001 compared to APP/PS1-TBI). In fact, previous studies have reported deficiency in various types of fear learning in this strain of APP/PS1 mice or similar models. (Knafo, Venero et al. 2009, Zhang, Hao et al. 2011) However, most strikingly, APP/PS1-TBI mice greatly increased the duration to enter light chamber compared to APP/PS1-Sham (p<0.001), and even greater than that of WT-TBI (p=0.004), suggesting that TBI in APP/PS1 mice greatly intensified fear memory and/or reduced extinction.

When we analyzed the temporal profile of PA duration among the four groups, the APP/PS1-TBI group showed the most long-lasting fear response. Comparing to the respective PA duration on Shock 1, WT-Sham and WT-TBI had a significant increase in post-shock duration for only 3 days, and APP/PS1-Sham had a significant increase for only 1 day post-shock. In contrast, the APP/PS1-TBI had a significant increase for 1 mo post-shock. When we perform a day-by-day
post-hoc analysis, at D7 (1\textsuperscript{st} post-shock day after Shock 1) the APP/PS1-TBI group was not significantly different from the WT-TBI group (p=0.507). However, at 1 mo post-shock, the APP/PS1-TBI group was significantly higher than the WT-TBI group (p=0.040), suggesting the greater fear response was largely due to an impairment of extinction.

In summary, TBI in APP/PS1, but not WT mice, induced more intense fear memory and impaired its long-term extinction. These results suggest that very mild TBI can induce a chronic PTSD-like behavior in APP/PS1 mice.

\textbf{5.3.3 mTBI reduces long-term anxiety-like behavior}

We performed Elevated plus maze (EPM) from D7 to 8 mo post-TBI to test whether mTBI altered long-term anxiety-like behavior in these mice. (Fig 5.2B) In this experiment, we measured the amount of time the animal spent in the open arms and the closed arms, and expressed the difference of the two values as a percentage of total time. We observed a significant injury effect (p<0.001) but no significant genotype*injury interaction (p=0.404) such that injured mice of both genotypes demonstrated a preference for the open arms over the closed arms. Particularly noteworthy is that even though the injury*time (p=0.957) or genotype*injury*time (p=0.952) interactions were not significant, the distinction between the sham and TBI groups became more and more pronounced over time, especially at 6 mo and 8 mo post-TBI, suggesting that decreased anxiety after mTBI can lead to increased chronic risk-taking behavior.

\textbf{5.3.4 mTBI impairs spatial learning, spatial memory, and unlearning and relearning}

We used the Barnes maze to study spatial learning and spatial memory. Mice were trained for acquisition trials from D14 to D18 post-TBI, and probe trials and revere trials were performed from 1 mo to 8 mo. In acquisition trials (D14 – D18 post-injury), we observed a significant injury effect (p<0.001) and a significant genotype effect (p<0.001), indicating that generally APP/PS1 mice learn more slowly than WT mice, and that TBI mice learn more slowly than sham mice. (Fig 5.3A) The genotype*injury interaction was not significant (p=0.164), and post-hoc analysis showed that both APP/PS1-Sham and APP/PS1-TBI mice took longer to learn than WT-
Sham and WT-TBI mice, respectively (p<0.001 and p=0.033). These results suggest that in this cohort, APP/PS1 genotype and TBI had additive, rather than synergistic, effects on spatial learning impairment. Analysis by Weibull function curve fitting showed no significant injury effects in learning latency, learning plateau or learning slope (p=0.175, p=0.735, and p=0.507, respectively). (Fig 5.3B,C,D,E)

We then used probe trials to test their spatial memory in long-term (D19 – 8 mo post injury). When analyzing the amount of time spent near the previous target location, we observed a significant genotype effect (p=0.019) but not an injury effect (p=0.575), indicating that the WT mice had significantly better spatial memory than APP/PS1 mice. (Fig 5.3G) When we analyzed the amount of time spent in the quadrant containing the previous target, we observed a significant genotype*injury interaction (p=0.027) but not genotype or injury effect (p=0.175 and 0.762, respectively). (Fig 5.3F) Post-hoc analysis showed that WT-sham performed significantly better than APP/PS1-Sham (p=0.013), indicating uninjured APP/PS1 mice had poorer spatial memory. On the other hand, APP/PS1-TBI was not significantly different from APP/PS1-Sham (p=0.204) or WT-TBI (p=0.532). WT-Sham showed a trend of performing better than WT-TBI, but this did not reach statistical significance (p=0.081).

In short, while there were considerable variations in mouse performance during probe trials (particularly APP/PS1 mice), both the target area analysis (a more precise spatial location) and the quadrant analysis (a broader spatial location) showed that the genotype separation became more pronounced at later time points. However, there was no significant separation between sham and injured mice.

During reverse trials (D20 – 8 mo post-injury), we observed a significant genotype effect (p<0.001) and a significant genotype*injury interaction (p=0.002) but no injury effect (p=0.862). (Fig 5.3H) Post-hoc analyses showed that sham mice of both genotypes performed similarly (p=0.724), whereas APP/PS1-TBI mice performed worse than WT-TBI mice (p<0.001). Comparing the effects of TBI in APP/PS1 mice, we observe that APP/PS1-TBI mice performed significantly worse than APP/PS1-sham mice (p=0.032), and that the difference became larger
towards the end of the study, suggesting that TBI may be associated with progressively poorer unlearning and relearning in APP/PS1 mice in the long-term. Potentially of concern, WT-TBI mice performed significantly better than WT-sham mice (p=0.025), which is opposite to the expected results and may indicate our sample size is underpowered to reliably detect biologically meaningful results for classical tests of spatial cognitive performance,

In summary, this study suggests that acute spatial learning can be additively impaired by APP/PS1 genotype status and TBI. In long-term, TBI can impair unlearning and relearning in APP/PS1 mice, but not in WT mice.

5.3.5 mTBI induces chronic white matter inflammation and axonal injury,

We used Iba1 immunohistochemistry to analyze microglial activation in the optic tract, a white matter area we have previously shown to be robustly affected after CHIMERA mTBI. Intriguingly, we observed persistent microgliosis at 8 mo post TBI, as a significant injury effect was observed for both microglia density (p<0.001) and microglia size (p<0.001). Neither genotype nor genotype*injury interactions were significant. (Fig 5.4D,E,F) We also assessed axonal damage in the optic tract using NeuroSilver staining, and found a significant injury effect (p=0.005) but no genotype effect (p=0.264), again suggesting persistent axonal damage up to at least 8 mo post-TBI. (Fig 5.5A,B) independent of genotype. Neurofilament IHC showed no significant difference between the sham and TBI mice in both genotypes, which is consistent with our previous observation that neurofilament-positive axonal bulbs were only found in 2d post-TBI but not after. (See Section 4.4.5)

Although the histological assays to assess chronic white matter injury and inflammation led to robust results, cytokine ELISA of IL1β, IL6 and TNFα using brain carbonate-soluble homogenates did not show significant differences between groups genotype (p=0.618, p=0.207, p=0.152, respectively). Note that for IL6 and TNFα, some samples did not reach lowest detection limit of the assay and were assigned “zero” in analysis. For this reason, non-parametric tests were used to analyze these two cytokines. (Fig 5.4A,B,C) In summary, these observations are
consistent with our previous findings in acute CHIMERA, where the cytokine response is transient and returns to baseline levels within 48 h of injury.

5.3.6 mTBI does not affect long-term Aβ deposition, APP production or cell adhesion molecules
Aβ40 and Aβ42 ELISA performed on brain homogenates of 13 mo APP/PS1 mice, 8 mo post-TBI, revealed no significant injury effect in either the carbonate-soluble (p=0.504 and p=0.854, respectively) or guanidine HCl-soluble (p=0.662 and p=0.593, respectively) fractions. (Fig 5.6A,B), consistent with our previous observations for Aβ levels at acute time points after CHIMERA injury. Cortical Aβ deposition or fibrillary amyloid, as revealed by 6e10 immunohistochemistry or Thioflavin S staining, also showed no significant injury effects at 8 mo post-TBI (p=0.056 and p=0.621, respectively) (Fig 5.6C,D,E,F), again consistent with our previous observation of transient and subtle changes in plaque morphology at acute time points after CHIMERA injury.

Western blotting was performed on the carbonate-soluble fraction to analyze sAPP and APP-CTF levels. As expected, APP/PS1 mice had significantly higher levels of sAPP and APP-CTF (p=0.003 and p<0.001, respectively) compared to WT mice. However, in this study, TBI had no long-term effect (p=0.746 and p=0.485) compared to sham animals. TBI also had no long-term effect on other markers related to endothelial cell function (SR-BI p=0.747), neurodegeneration (TDP-43 p=0.389), or blood-brain-barrier integrity (ZO1 p=0.547). (Fig 5.7)

Mesoscale analyses were performed on the carbonate soluble fraction to quantify total tau and p-T231 tau levels. As expected from our previous CHIMERA studies, mTBI did not have any significant effect on total tau, p-tau, or the p-tau:total tau ratio. However, we observed a significant genotype effect in all three analyses (p=0.024, p<0.001, p=0.001, respectively), as APP/PS1 mice showed a lower level of total tau, p-tau, and p-tau:total tau ratio compared to WT animals. (Fig 5.5C,D,E)

We assessed VCAM1 and ICAM1 levels using ELISA. As expected from our previous results, there was a significant genotype effect (p<0.001 and p<0.001) with APP/PS1 having higher adhesion molecule levels than WT mice, indicating endothelial activation due to overexpression
of Aβ. There was no significant injury effect (p=0.097 and p=0.056, respectively). Interestingly, though genotype*injury interactions were not significant (p=0.319 and p=0.089), we observed that VCAM1 and ICAM1 level decreased by 16% or 20% respectively after TBI in APP/PS1 mice (post-hoc test p=0.055 and p=0.010, respectively.) (Fig 5.8A,B)
5.4 Discussion

In Chapter 4, we described acute outcomes of two mTBI to APP/PS1 mice and WT littermates, where age-of-injury was at either 6 mo or 13 mo. In this study, two mTBI were delivered using the identical impact energy to the same strains of mice at 6 mo of age, and outcomes were followed longitudinally up to 8 mo post-injury (14 mo old). This study was specifically designed to address a major knowledge gap of chronic consequences to injury, as most pre-clinical studies focus on acute TBI outcomes and the long-term consequences of TBI remain poorly characterized in animal models. (Gold, Su et al. 2013)

Consistent with the results in Chapter 4, 2x0.5J injuries produced very mild injury phenotypes as assessed by acute behavioural responses in the hybrid C57Bl/6-C3H mice used in this study. Despite these very mild injury conditions, striking chronic effects were observed for both behavioural and white matter inflammatory outcomes.

Behaviorally, we observed that repetitive mTBI induced PTSD-like behavior in APP/PS1 mice. When given a foot-shock at 6d post-injury, APP/PS1-TBI mice showed an intensified fear memory that was maintained for 2 mo and extinguished by 3 mo. A second foot-shock given at 6 mo post-TBI again triggered intensified fear memory for another 2 mo post-shock that persisted up to 8 mo post-injury. This behavior strongly suggests that mTBI can induce chronic post-traumatic stress disorder (PTSD)-like changes in APP/PS1 mice. In fact, enhanced fear conditioning and amygdala abnormalities have been reported in some previous mTBI models (Reger, Poulos et al. 2012) but not others. (Genovese, Simmons et al. 2013) Furthermore, a clinical study conducted by Yaffe et al. (Yaffe, Vittinghoff et al. 2010) (n = 53,155 and 127,938, for veterans with or without PTSD, respectively) showed that military veterans diagnosed with PTSD are twice more likely to develop dementia. This suggest that TBI-induced PTSD may be at least partly contributed by neurodegenerative changes in the brain.

Consistent with the clinical observations that cognitive dysfunctions after TBI are more variable, we observed less robust effects in spatial memory performance in this study, although TBI induced chronic impairment in reverse spatial learning specifically in APP/PS1-TBI mice. Taken together with impairment in fear memory extinction, these data suggest that TBI may induce deficits in synaptic plasticity in APP/PS1 brains, and raises the hypothesis of amygdala involvement in addition to the hippocampus. Such changes may result in impairment of the
ability of the mice to rewire their existing brain network. Supporting evidence for this hypothesis includes the results from the BM task, which revealed a reduced ability to unlearn the previous location of the hideout, and relearn its new location, as well as in the PA task, which revealed an inability to dissociate the dark chamber from the fear memory of the foot shocks for months after shock exposure, thereby leading to a PTSD-like phenotype. It will be of great interest in future to investigate synaptic changes in hippocampus and amygdala in these APP/PS1-TBI mice, using electrophysiological or immunohistochemical techniques.

Chronic neuroinflammation, such as microgliosis, has also been shown to persist for months and years after TBI in clinical studies. (Ramlackhansingh, Brooks et al. 2011, Johnson, Stewart et al. 2013, Cherry, Tripodis et al. 2016) It should be noted that these clinical reports generally focused on patients with a single moderate or severe TBI or with CTE. Pre-clinical studies using open head injuries also showed persistent microglial response in grey matter and white matter up to 2 mo post injury (Cao, Thomas et al. 2012, Acosta, Tajiri et al. 2013) In addition, pre-clinical studies have shown that CHI in rodents may induce argyrophilic responses in white matter up to at least 21d post-injury (Lindner, Plone et al. 1998, Shitaka, Tran et al. 2011), but evidence of prolonged axonal pathology at 6 mo post-injury or beyond is lacking (Mannix, Meehan et al. 2013). Our current findings show that as few as two mTBI using CHIMERA is sufficient to trigger microgliosis and axonal damage, up to at least 8 mo post injury. Prolonged neuroinflammation has long been hypothesized as one of the triggers for neurodegeneration, and recent interest has surged after genome-wide association studies have identified several genes involved in inflammation as strong genetic risk factors of AD, such as complement receptor 1 and inositol polyphosphate-5-phosphatase. (Harold, Abraham et al. 2009, Lambert, Ibrahim-Verbaas et al. 2013)

Previous studies have targeted post-TBI neuroinflammatory responses in rodents with therapeutic or genetic manipulations. For example, one report (Thau-Zuchman, Shohami et al. 2012) showed that carprofen (a specific COX-2 inhibitor) treatment for 7d reduced pro-inflammatory cytokine levels and improved neurological behavior during the acute phase after CHI in mice. However another report (Browne, Iwata et al. 2006) showed that ibuprofen (a COX inhibitor) treatment for 4 mo in rats worsened post-TBI spatial learning. In another study, Febinger (Febinger, Thomasy et al. 2015) et al. reported that CX3CR1-/- mice had less motor
deficits and neuronal loss than WT during the acute phase (24h to 15 d post-injury), but more
cognitive deficits and neuronal death at 30d post-injury. Antagonism of the IL-1 receptor has
been shown to acutely reduce contusion volume (Toulmond and Rothwell 1995, Jones, Prior et
al. 2005) and improve cognitive deficits (Sanderson, Raghupathi et al. 1999, Clausen, Hanell et
al. 2009). In addition, genetic deletion and pharmacological studies show that TNF-α has a dual
function in TBI, being functionally and histopathologically detrimental in the acute phase
(Shohami, Bass et al. 1996, Bermpohl, You et al. 2007, Chio, Lin et al. 2010), but required for
subsequent regeneration and healing (Scherbel, Raghupathi et al. 1999, Shohami, Ginis et al.
1999, Sullivan, Bruce-Keller et al. 1999). Taken together, these observations suggest that
neuroinflammation is a highly prevalent and equally complicated process after TBI. In summary,
it may appear that on one hand, an excessive neuroinflammatory response is detrimental during
the acute recovery phase, yet suppression of neuroinflammatory cues may also inhibit proper
healing. Our study provides evidence that chronic neuroinflammation may be associated with
long-term axonal damage, though we are unable to delineate any cause-and-effect relationship in
the present study.

Our previous work, summarized in Chapters 2-4, shows that mild CHIMERA injury induces an
acute neuroinflammatory response that is modified by both age at injury and genetic
predisposition to amyloidosis. In Chapter 4 we showed that acute neuroinflammation is
exacerbated in 6 mo APP/PS1 mice and 13 mo WT littermates, and muted in 13 mo APP/PS1
mice when assayed up to 14d post-injury. (See Chapter 4) In this chronic study, no genotype
differences remained when neuroinflammation is assayed 8 mo post-injury by both microglial
count and cytokine levels. It will be interesting in the future to further characterize these Iba1-
immunoreactive microglia, using markers of activation and phagocytosis (e.g CD68), pro-
inflammation or anti-inflammation (e.g. iNOS, TGFβ, arginase 1).

Chronic induction of Aβ deposition (1.5 mo to 4 mo post-injury) has been reported previously in
some models of AD, up to 4 mo post-CCI or CHI. (Uryu, Laurer et al. 2002, Tajiri, Kellogg et al.
2013, Webster, Van Eldik et al. 2015) By contrast, a few other studies (Nakagawa, Nakamura et
al. 1999, Nakagawa, Reed et al. 2000, Miszczuk, Debski et al. 2016), which used different
models of AD assessed up to 4 to 8 mo post injury, found that TBI actually lowered Aβ levels in
the long-term. In our study, we observed no significant change in Aβ levels, Aβ deposits, or
It is very likely that Aβ dynamics depend on many factors including AD model, rate of deposition and clearance, TBI severity, extent of pre-existing amyloid, chronological age, and age-at-injury. Although some studies have shown that TBI in rats may lead to accumulation of TDP-43 or α-synuclein at 60d post-injury (Acosta, Tajiri et al. 2015, Wang, Lee et al. 2015), we did not observe significant change of TDP-43 levels in the soluble fraction of brain homogenates.

In Chapter 4, we found that TBI acutely reduces the levels of cell adhesion molecules VCAM-1 and ICAM-1 in APP/PS1 mice, which were elevated at baseline compared to WT control. In this study, we did not observe any significant TBI effects at 8 mo post-injury, although there is an interesting trend toward reduced cell adhesion molecules in APP/PS1-TBI compared to APP/PS1-sham controls. It will be of interest to investigate if such changes in are specific to a particular brain region.

One limitation of our study is the smaller cohort used here (total N=46 mice) compared to our previous acute study of WT and APP/PS1 mice (N=367 mice over 2 age cohorts) such that we did not fully replicate all of the immediate behavioural data between the two studies. For example, both the acute study (Chapter 4) and the chronic study (Chapter 5) used hybrid C57Bl/6-C3H WT or APP/PS1 mice that received 2x0.5J CHIMERA impacts at 6 mo of age. In the previous acute study, we observed a significant injury effect for both LRR and NSS that was not observed in the present study. We believe this is likely due to differences in sample size and sampling error, as an average N=47 per group (LRR) and 37 per group per time point (NSS) were used in the previous study compare to an average N=12 for either assay in the current study. Regarding Barnes maze results, in the previous report, we did not observe any significant genotypic difference in spatial learning when assayed up to 12d post-injury. However, in the current study, a significant genotype effect emerged when analyzing spatial learning (up to 18d post-injury). Specifically, the WT-sham mice in this study achieved a better learning plateau than the previous study. One explanation for the differences of spatial learning is that, due to constraints in study design, the Barnes Maze task was performed between D8 and D12 in the acute study and overlapping with other behavioral tests such as the testing phase of passive
avoidance, elevated plus maze, and rotarod. These overlapping behavioral tests may have incurred stress in these animals and compromised the optimal condition for their spatial learning. By contrast, in this longitudinal study, the Barnes Maze task was performed from D14 to D18, with minimal overlap with other behavioral tests, which may have allowed the WT-sham to achieve a better learning plateau.

In conclusion, this current study shows that two very mild CHIMERA impacts that are within the subconcussive-concussive range of severity are sufficient to produce chronic white matter neuroinflammation in both WT and APP/PS1 mice and lead to chronic PTSD-like behaviour in APP/PS1 mice.
Figure 5.1  TBI did not impair long-term motor performance

(A) Loss of righting reflex (LRR) was recorded immediately after sham or TBI procedures on both days of operation. (B) Neurological severity score (NSS) was assessed at baseline and at 1
h, D1, D2, and D7 post-TBI. (C). Rotarod (RR) latency to fall was assessed at baseline and on post-TBI days including D1, D2, D7, D14, and 2 consecutive days in 1 mo, 2 mo, 3 mo, 6 mo, 7 mo, and 8 mo post-TBI, respectively. Dark blue solid lines, light blue dotted lines, dark red solid lines, and light red dotted lines represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. LRR was analyzed by Repeated Measure ANOVA, and NSS and RR were analyzed by mixed linear model. Data in all graphs are represented as mean ± standard error, with significant effects and interactions stated for each figure. For LRR, N=10-13 per injury per genotype per day (average N=12). For NSS, N=10-13 per injury per genotype per time point (average N=12). For RR, N=3-12 per injury per genotype per time point per trial (average N=10).
Figure 5.2  TBI induced long-term PTSD-like behavior and risk-taking behavior

(A) During passive avoidance (PA) task, the mice received the first foot shock on D6 post-TBI and were tested for avoidance duration from D7 to D9, 1 mo, 2 mo, and 3 mo. As the avoidance duration returned to pre-shock level, the mice received a second foot shock at 6 mo post-TBI, and were tested for 3 consecutive days afterwards, as well as at 7 mo and 8 mo. (B) Elevated
Plus Maze (EPM) was performed on the post-TBI time points D7, D10, 1 mo, 2 mo, 3 mo, 6 mo, 7 mo, and 8 mo. Dark blue solid lines, light blue dotted lines, dark red solid lines, and light red dotted lines represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. PA and EPM were analyzed by mixed linear model. Data in all graphs are represented as mean ± standard error, with significant effects and interactions stated for each figure. For PA, N per injury per genotype per day = 10-13 for first half of study and 7-11 for second half (average N=11). For EPM, N=5-12 per injury per genotype per time point (average N=9).
Figure 5.3  TBI resulted in poor spatial unlearning and relearning in APP/PS1 mice
(A,B,C,D,E) Barnes maze (BM) reference acquisition trainings were performed for 5 consecutive days after injury (D14 to D19), and the duration of time the mice took to enter a fixed hideout was measured. On each acquisition day three trials were performed, and the daily average performance is plotted in (A). The daily average acquisition data of each mouse were fitted to a Weibull function learning curve and plotted in (B). The latency, plateau, and slope of learning were extracted from the Weibull curves and plotted. (C,D,E) On D19, 2 mo, 6 mo, 8 mo after injury, the hideout was removed and a probe trial was performed. The amount of time the mice spent in the entire north quadrant (where the hideout was previously located) (F), or the time spent around the previous hideout location (G), was measured. On D20, 2 mo, 6 mo, 8 mo after injury, three reverse trials were conducted on the same day. The average value of the day is shown. (H) Dark blue solid lines/bars, light blue dotted lines/bars, dark red solid lines/bars, and light red dotted lines/bars represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. BM acquisition and reverse trials are analyzed by mixed linear modeling. Weibull latency of learning is analyzed by Repeated Measure ANOVA, and probe trial by ANOVA. Data in all graphs are represented as mean ± standard error, with significant effects and interactions stated for each figure. For BM reference acquisition, N=10-13 per injury per genotype per day per trial (average N=12). For BM probe and reverse, N=6-13 per injury per genotype per trial (average N=10).
Figure 5.4  TBI induced long-term white matter microgliosis
Cytokine levels in carbonate soluble half-brain homogenates at 8 mo post-injury were analyzed using Mesoscale assays for IL-1β (A), IL-6 (B), and TNF-α (C). The microglial response in the optic tract was visualized using Iba-1 immunohistochemistry (IHC) at 8 mo post-injury (D). The density (E) and average size of microglia (F) in the optic tract were quantified and plotted. Dark blue bars, light blue bars, dark red bars, and light red bars represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. IL1-β was analyzed by ANOVA, and IL6 and TNF-α were analyzed by Kruskal-Wallis-test due to data lower than detection limit and being assigned as zero. Iba-1 staining was analyzed by ANOVA. Individual data points were plotted for IL1-β, IL6 and TNF-α, with mean (IL1-β) or median (IL6 and TNF-α) represented by a horizontal line. Data in Iba-1 are represented as mean ± standard error, with significant effects and interactions stated. For cytokines, N per injury = 6-7 (average N=7). For Iba-1, N=6-7 per injury per genotype (average N=6).
Figure 5.5  TBI induced long-term axonal injury

Degenerative axonal injury in the optic tract, was stained by Neurosilver staining on samples at 8 mo post-injury. (A) The percentage of stained area was quantified and shown in (B). Total tau, phosphorylated tau (Thr231), and ratio of phosphorylated tau to total tau in carbonate-soluble fractions of brain samples at 8 mo post-injury were measured using Mesoscale assays. (C,D,E) In
the graphs, dark blue bars, light blue bars, dark red bars, and light red bars represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. Neurosilver and tau data were analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant variables and interactions stated for each figure. For Neurosilver, N=4-8 per injury per genotype (average N=7). For tau, N=4-7 per injury per genotype (average N=5).
Figure 5.6  TBI did not change long-term Aβ level in APP/PS1 mice
Half-brains of APP/PS1 mice at 8 mo post-TBI were homogenized, and Aβ40 and Aβ42 levels were assayed in carbonate- (A) and guanidine HCl- (B) soluble fractions by ELISA. Fibrillar amyloid burden in APP/PS1 brain cortex was detected by Thioflavin S (Thio-S) histochemistry at 9 mo post-TBI (C). Quantification of the percentage area of cortex stained is shown in (E). Aβ deposits in APP/PS1 cortex were detected by 6E10 immunohistochemistry at 8 mo post-TBI (D). Quantification of the percentage area of cortex stained is shown in (F). In the graphs, dark red bars and light red bars represent the APP/PS1 TBI group and the APP/PS1 Sham group, respectively. Aβ, Thio-S and 6E10 data were analyzed by t-test. Data in all graphs are represented as mean ± standard error, with significant variables and interactions stated for each figure. For Aβ, N=6-7 per injury. For Thio-S, N=6-7 per injury. For 6E10, N=4-6 per injury. Scale = 500 µm.
Figure 5.7  TBI did not change long-term brain Aβ metabolism, neurodegeneration proteins, or cerebrovascular proteins.
Levels of sAPP, carboxyl terminal fragment (CTF) APP, ZO1, SRB1, TDP-43 in carbonate-soluble fractions of TBI brains at 8 mo post-injury were assayed using Western blot, and normalized to the level of GAPDH. Dark blue bars, light blue bars, dark red bars, and light red bars represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. Data were analyzed by ANOVA and represented in all graphs as mean ± standard error, with significant variables and interactions stated for each figure. For all protein targets, N=4.
Figure 5.8  TBI did not affect long-term brain VCAM1 and ICAM1 levels.

VCAM1 (A) and ICAM1 (B) levels in carbonate-soluble fractions of half-brain homogenates at 8 mo post-injury were assayed by ELISA. Dark blue bars, light blue bars, dark red bars, and light red bars represent the WT TBI group, the WT Sham group, the APP/PS1 TBI group, and the APP/PS1 Sham group, respectively. VCAM1 and ICAM1 data were analyzed by ANOVA. Data in all graphs are represented as mean ± standard error, with significant variables and interactions stated for each figure. For VCAM1 and ICAM1 ELISA, N=6-7 per injury per genotype (average N=7).
Figure 5.9 Outline of experimental design and most significant findings

An outline of the experimental plan is shown in the diagram.
Chapter 6: Conclusion

6.1 Summary of findings

In Chapter 2, we detailed the development of CHIMERA, a novel impact-acceleration TBI model that can perform surgery-free TBI procedures without restricting head motion. CHIMERA distinguishes itself from many existing models in that it incorporates rigorous biomechanical considerations in its design. It induces reproducible head motions that are relevant to real-world TBI in terms of injury mechanism and head kinematics. We also characterized the acute pathologies of CHIMERA repetitive mTBI, and showed that they are highly clinically-relevant, including behavioural deficits (neurological, motor, cognitive), diffuse axonal injury (silver staining), and neuroinflammation (white matter microgliosis and brain cytokine upregulation). These results demonstrate that CHIMERA produces clinically-relevant head kinematics and TBI pathologies.

In Chapter 3, we characterized single CHIMERA TBI ranging from sub-threshold to mTBI, which comprises the vast majority of TBI cases in real world. We showed that by increasing impact energy, CHIMERA induced greater extent of head motion. Furthermore, the acute pathological outcomes of CHIMERA TBI (e.g. neurological deficits, microgliosis, and axonal injury) correlated with the head kinematic parameters experienced during TBI. These results show that CHIMERA is a valuable research tool and can induce an adjustable range of injury by varying its mechanical input.

In Chapter 4, we characterized the acute response of CHIMERA repetitive mTBI in young/old mice of WT or APP/PS1 genotype. We showed that age-at-injury is a significant modifier of TBI outcomes, including neuroinflammation, neurofilament changes, and the structure of deposited Aβ deposits. In most analyses, WT and APP/PS1 mice performed similarly with a few important exceptions where old WT mice were more prone to have an increased inflammatory response than young WT mice whereas young APP/PS1 mice had an exacerbated neuroinflammatory
response that was attenuated in old APP/PS1 mice. These results show that both chronological ageing and genetics are important modifiers of acute TBI outcomes.

In Chapter 5, we characterized the chronic response of CHIMERA repetitive mTBI in WT and APP/PS1 mice. We showed that two mTBI is sufficient to result in prolonged white matter microgliosis and axonal injury up to at least 8 mo post-injury. More importantly, TBI chronically induced prolonged intensification of fear memory in APP/PS1 mice, suggesting a PTSD-like phenotypic change in these mice.

6.2 Significance of findings

CHIMERA is a novel TBI model that induces injuries that are relevant to real-world TBI with respect to both biomechanics (injury mechanism and head kinematics) and pathologies (behavior, neuroinflammation, diffuse axonal injury, etc). We have demonstrated that CHIMERA TBI outcomes are scalable and can be adjusted by varying the input mechanical parameters. We define age-at-injury and APP/PS1 genotype are both significant modifiers of acute TBI outcomes. Specifically, age-at-injury modifies cognitive deficits, TBI-induced neuroinflammation, and Aβ deposit changes. We further showed that even very mild CHIMERA TBI induced chronic pathologies including microgliosis and axonal injury, and results in PTSD-like behaviour in APP/PS1 mice in the long-term. In a collaborated study, we have shown that CHIMERA-induced DAI is detectable using clinical imaging method such as diffusion tensor MRI. (Haber, Hutchinson et al. 2017) The fact that CHIMERA has a high relevance to real-world TBI biomechanics and is able to induce clinically relevant TBI pathologies highlights its potential in future research of TBI biomarkers and therapeutics. Accordingly, in consultation with UBC’s University Industry Liaison Office, we have elected not to patent the CHIMERA technology, which could potentially stall research progress for this critically important unmet medical need. Rather, we are disseminating CHIMERA systems to academic laboratories through licensing agreements and have completed 11 agreements to date, and these laboratories are starting to publish their own research findings using CHIMERA (Chen, Desai et al. 2017), demonstrating successful transfer of the CHIMERA technology developed through this thesis.
In summary, we have verified our research hypotheses. By developing a reproducible TBI model with clinically relevant injury mechanism, we have improved our understanding on TBI pathophysiology. Using our novel model we have demonstrated the interactive effects of factors such as age-at-injury and APP/PS1 genotype in determining acute and long-term TBI outcomes.

6.3 Limitations of these studies

6.3.1 Tau pathologies

From Chapter 2 to 5, we have shown robust evidence that CHIMERA TBI may induce behavioural changes and pathological changes (including inflammatory, glial, axonal, and amyloid changes, etc). However, strong evidences of two important aspects of TBI, namely tau and cerebrovascular changes, have not been thoroughly investigated thus far.

The defining pathology of CTE is “an accumulation of abnormal hyperphosphorylated tau in neurons and astroglia distributed around small blood vessels at the depths of cortical sulci and in an irregular pattern”. (McKee, Cairns et al. 2016) In Chapter 2, by using immunoassays with antibodies targeting various phospho-tau epitopes, we showed that repetitive mTBI in C57B/6 mice resulted in an acute and transient elevation of phosphorylated endogenous tau. In Chapter 4 and 5, by using ELISA against one phospho-tau species, we showed that repetitive mTBI in C3HxB/6 mice (of either WT or APP/PS1 genotype) did not significantly change phospho-tau levels. Thus, with two CHIMREA mTBI, we did not observe strong evidences of increase in phospho-tau. In fact, it is a known issue that there is a lack of consistent and prolonged tau changes in TBI studies involving mice that express endogenous mouse tau. (Ojo, Mouzon et al. 2016)

Several hypotheses have been proposed to explain such discrepancies based on the biological and biomechanical differences between mouse and human tau. For example, unlike human tau, the mouse homologue of tau is less prone to tangle formation in vivo (Adams, Crook et al. 2009, Ando, Leroy et al. 2011), possibly partly due to differences in the amino acid sequence at the N-terminus (Alonso, Zaidi et al. 2001, Ojo, Mouzon et al. 2016), which has been implicated in conformational stability in physiological and pathological settings (Jeganathan, von Bergen et al. 2006, Jeganathan, Hascher et al. 2008). In addition, the tau tangles in CTE contain both 3R and
4R isoforms. (McKee, Stern et al. 2013) However, while both isoforms are expressed in adult human brains, only the 4R form is found in adult mouse brains. (McMillan, Korvatska et al. 2008, Abisambra and Scheff 2014) Anatomically, the human brain and the mouse brain are also quite different. The human brain is gyrencephalic whereas the mouse brain is lissencephalic. When the gyrencephalic brains experience acceleration, mechanic stress concentration occurs at tissue folds and boundaries (e.g. gyri and blood vessels). Therefore, even when exposed to the same extent of head acceleration, the gyrencephalic brain will experience greater tissue deformation and damage. Also, the human brain has a much greater proportion of white matter, compared to rodents. (Ojo, Mouzon et al. 2016) Therefore, in order to better model the pathological tau changes in CTE cases, we may need to use animal models that are biologically or biomechanically closer to humans. For example, we can employ the use of “humanized” animals in which their endogenous tau gene is replaced by the human homologue, or animal models that have bigger gyrencephalic brains, such as ferrets or pigs. In ferret we have engineered a prototype ferret CHIMERA, which is currently undergoing characterisation.

Another possible explanation for the lack of consistent tau pathologies in animal models of TBI is that, in clinical CTE cases, most athletes have been exposed to thousands of concussions or sub-concussions over years of the professional career. In contrast, in the research settings, most TBI paradigms only involve several, or at most dozens, of TBI due to the shorter life-span of animal models or budget/resources reasons. Thus the use of animal models that have an accelerated development of tauopathy (e.g. animals harbouring transgenes of disease-causing mutations, such as P301S or P301L tau mice) may be useful, as they may hasten the experimental time frame of concussion-related tauopathy to a practical duration. However, the use of these transgenic mice may impose additional complications regarding translatability to human cases, as most human subjects that experience TBI do not harbour genetic mutations predisposed to neurodegeneration. Therefore, due to biological, biochemical and biomechanical differences between small animal models and human, there may be intrinsic limitations in “perfectly” modelling the temporal profile and clinical signs of CTE. While larger animal models may be helpful in addressing some of these aspects, we may have to balance with practical considerations, and aim at designing multiple studies that aim at answering different questions regarding the development of CTE.
6.3.2 Cerebrovascular changes

Cerebrovascular changes are another clinical feature of TBI. (Salehi, Zhang et al. 2017) However, at the current mTBI paradigm, we have not observed significant changes of BBB integrity or extravasation of plasma proteins (data not shown). As discussed in the previous section, the absence of such pathologies may be partly explained by anatomical differences between the gyrencephalic human brain and the lissencephalic mouse brain. In addition, the smaller size of the mouse brain renders them more physically resilient to acceleration-induced mechanical stress. (Ommaya 1967) Even though mouse-to-human scaling factors have been incorporated in our analysis of head kinematic parameters, it is likely that such simplistic scaling functions (which assumed the mouse and the human brains are homogeneous materials and are geometrically similar objects) did not fully account for the physical and anatomical differences. In other words, the blood vessels in the mouse brains in our experiment may have experienced smaller mechanical stress than those in clinical TBI cases. Therefore, future studies that seek to investigate TBI-induced cerebrovascular injuries should aim at inducing a greater extent of tissue deformation – either by increasing input mechanical parameters and thus increasing the extent of head motion, or by using animal models that have bigger gyrencephalic brain and a greater density of cerebrovasculature. In addition to the current approaches, i.e. immuno-assays that target particular cerebrovascular markers (such as proteins involved in BBB integrity/breach or in endothelial activation), a more integral approach, such as functional assays that measure in vivo cerebrovascular flow or functional/capnic reactivity, may provide more meaningful insights regarding the cerebrovascular changes induced by TBI.

6.3.3 Other Limitations

As studies described in this thesis used cohorts of entirely male mice, future studies will be required to reveal possible sex differences in acute or chronic TBI outcomes.

Our data demonstrate that CHIMERA reliably mimics the mild end of the human TBI severity spectrum. However, upon increasing impact energy in an attempt to produce moderate-severe
TBI, energies >0.7J led to skull fracture in over 40% of animals, thus reaching the humane limit of mortality in our animal protocol, and necessitating their immediate euthanasia. During the course of this thesis, we continued our engineering work to enable reproducible moderate-severe injuries without inducing skull fracture, by designing and evaluating 8 impactor interfaces designed to disperse high impact forces across the skull, thereby protecting from skull fracture, whilst increasing linear and rotational velocity and acceleration of the head. Performance criteria for each device included skull protection ability, consistency of head kinematics, ease of use, durability and production factors. The best results were achieved using a 3D-printed, polylactic acid, silicone-lined interface that enables impact energies up to 2.5J to be applied with an acceptable incidence of skull fracture (0%) and mortality (25%), and produced phenotypes consistent with moderate-severe human TBI in a preliminary cohort of 55 C57Bl/6 mice (37 male, 25 female) at 4-6 months of age. These very new results now enable CHIMERA to be used to study moderate-severe TBI with the same precision as for mild TBI.

6.4 Future directions

6.4.1 Synaptic changes

We have shown that CHIMERA TBI may induce behavioral deficits in the acute phase, e.g. spatial learning, and the chronic phase, e.g. fear memory. Since we have not observed overt TBI-induced neuronal loss, it would be of interest to investigate if such behavioural deficits are reflected by changes at the synaptic level (e.g. synaptic density or plasticity), or neurotransmitter or receptor levels, particularly in areas related to spatial learning and fear memory, such as the hippocampus (dentate gyrus, CA1 and CA3), parahippocampal regions (entorhinal cortex), amygdala (basal lateral and central), and prefrontal cortex (prelimbic and infralimbic). (van Strien, Cappaert et al. 2009, Izquierdo, Furini et al. 2016)

In fact, clinical studies have shown that TBI-patients with PTSD symptoms may show altered emotional processing. In one study that involved 852 military personnel, patients exposed to multiple TBI had increased fear acquisition during fear-potentiated tasks. (Glenn, Acheson et al. 2017) In another study that involved 74 military personnel, patients with strong PTSD symptoms displayed altered cortical neural activity during a facial emotion processing task. (Zuj, Felmingham et al. 2017) Similar observations have been made in animal studies as well. For
example, FPI-induced mTBI has been shown to enhance fear learning and increase expression of NMDA receptor subunit in amygdala. (Reger, Poulos et al. 2012) Thus it will be of interest to use biochemical and electrophysiological techniques to study the neuronal pathways and molecular mechanisms behind the behavioural deficits induced by CHIMERA TBI.

### 6.4.2 Connectivity changes and other imaging analysis

Since we have shown that CHIMERA TBI results in injury at multiple white matter areas and leads to complex chronic behavioural changes, it is of great interest to investigate if these histological and functional observations are paralleled by deficits in brain connectivity, e.g. by assessing the correlation of neural activity (through measurement of blood oxygenation signals) of different brain regions during resting state or task-based functional magnetic resonance imaging. In fact, a few studies have suggested that connectivity deficits may play a significant role in TBI pathologies. One hypothesis is that the healthy brain performs analogously to “a small world”, in which neighbouring neurons form densely connected clusters or hubs, and such clusters communicate with each other through long distance connections. (Han, Mac Donald et al. 2014, Zhou 2016) In TBI brains, however, it is suggested that global long distance connectivity is disrupted due to the multifocal axonal injuries, whereas local connectivity is increased for compensation. Therefore, it would be of interest to use optical, ultrasonic, or magnetic resonance imaging techniques to verify if connectivity changes are correlated to the histological and behavioral changes observed after CHIMERA TBI. Notably, we have recently published a collaborative study demonstrating that CHIMERA TBI leads to ex vivo changes in diffusion tensor imaging, which correlate with histologically confirmed axonal pathology. (Haber, Hutchinson et al. 2017) Future in vivo imaging studies may provide more information regarding the functional significance of these changes.

We have shown that the optic system (optic tract and brachium of superior colliculus) are among the most robustly affect areas of TBI, in terms of microgliosis and axonal injury. These findings echoes clinical cases, as deficits of the optic system (e.g. eye tracking and convergence) are increasingly recognized as common features of TBI. (Alhilali, Yaeger et al. 2014, Radomski, Finkelstein et al. 2014) Therefore, it would be of interest to examine the connectivity and function of the visual system after TBI (e.g. by inducing visual stimulation and simultaneously
measuring activity of the visual cortex), potentially enabling non-invasive measures to assess recovery from CHIMERA using eye tracking methods.

6.4.3 Longitudinal imaging
Current advances in imaging techniques has provided new opportunities in longitudinal tracing of biological changes in vivo. For example, by coupling with different radioisotope labelled ligands, Positron Emission Tomography (PET) have been used to detect changes in amyloid (Hong, Veenith et al. 2014, Yang, Hsiao et al. 2015), tau (Mitsis, Riggio et al. 2014, Barrio, Small et al. 2015, Dickstein, Pullman et al. 2016, Wooten, Guehl et al. 2017), inflammation (Coughlin, Wang et al. 2015, Velazquez, Ortega et al. 2015), and cerebrovascular or neural activity (Buchsbaum, Simmons et al. 2015) in clinical cases of neurodegeneration or TBI, as well as in animal models (Wang, Yue et al. 2014, Israel, Ohsiek et al. 2016, Brabazon, Wilson et al. 2017). While it is more challenging to perform such experiments in small animal models due to resolution limitations, it would be of invaluable interest as they provide information over changes at multiple time points of the same subject, rather than just providing a snapshot at one particular time point (as in regular histological or biochemical assays). This approach will overcome some of the limitations of the current studies, as we will be able to compare the post-TBI status of every subject to its own baseline value, which will greatly improve the variability and accuracy of the findings. In addition, we will be able to trace the temporal profile over a long period of time, instead of investigating at only specific arbitrary time points.


Burgess, S., R. B. Abu-Laban, R. S. Slavik, E. N. Vu and P. J. Zed (2016). "A Systematic Review of Randomized Controlled Trials Comparing Hypertonic Sodium Solutions and


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Taupin, V., S. Toulmond, A. Serrano, J. Benavides and F. Zavala (1993). "Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. Influence of pre- and post-traumatic
treatment with Ro5 4864, a peripheral-type (p site) benzodiazepine ligand." J Neuroimmunol 42(2): 177-185.


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Appendices

Appendix A  Neurological Severity Score (NSS)

The NSS test used in this study is composed of 10 separate tasks. One point will be assigned for each failed task, render a minimum possible score of 0 (no neurological deficits) to a maximum possible score of 10 (maximum neurological deficits). The list of tasks include

- Exploration
- Straight line walking
- Exit circle
- Startle reflex
- 3 cm beam walk
- 2 cm beam walk
- 1 cm beam walk
- Beam balance
- Stick balance
- Monoparesis/Hemiparesis (Grapping reflex)
Appendix B  List of Publications

All publications that were produced during the course of this PhD study are listed below:


