CHARACTERIZATION AND VALIDATION OF TWO ZEBRAFISH MODELS OF TRAUMATIC BRAIN INJURY (TBI)

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Collaborative Program in Neuroscience
University of Toronto

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ABSTRACT

Characterization and Validation of Two Zebrafish Models of TBI

Victoria McCutcheon, Master of Science, 2015

Institute of Medical Science
University of Toronto

Traumatic brain injury (TBI) is a leading cause of death and morbidity in industrialized countries. The cost and time associated with pre-clinical development of TBI therapeutics is lengthy and expensive with a poor track record of successful translation to the clinic. The zebrafish is an emerging model organism with unique advantages in amenability to high-throughput automated pre-clinical drug evaluation. We developed a chemical model of TBI in zebrafish larvae as a reliable surrogate for TBI with quantifiable outcome measures for use in high-throughput drug screening. The aim is then for results to be confirmed in our adult model of TBI. We used a targeted high intensity focused ultrasound to produce the first non-penetrating brain injury model in adult zebrafish. Results indicate that the zebrafish exhibits responses to injury and intervention similar to mammalian pathophysiology after TBI and suggest the possibility of using the model to efficiently screen for potential therapeutic candidates.
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CONTRIBUTIONS

The author performed all experiments and analyses described in this thesis. Dr. Jahan Tavakkoli and his student Pooya SobheBidari were responsible for calibration and execution of the pHIFU system at Ryerson University. Dr. Xiao-Yan Wen and his team at the Keenan Research Centre at St. Michael's Hospital provided zebrafish breeding and maintenance.
# TABLE OF CONTENTS

*Title Page* .............................................................................................................................................. i
Abstract .................................................................................................................................................. ii
*Acknowledgements* .............................................................................................................................. iii
*Contributions* ......................................................................................................................................... iv
*Table of Contents* ................................................................................................................................... v
*List of Tables* ......................................................................................................................................... vii
*List of Figures* ........................................................................................................................................ viii
*Abbreviations* ...................................................................................................................................... ix

## 1.0 Introduction ........................................................................................................................................ 1
  1.1 Traumatic Brain Injury ................................................................................................................... 1
    1.1.1 Definition .................................................................................................................................. 1
    1.1.2 Classification ............................................................................................................................ 1
    1.1.3 Epidemiology .......................................................................................................................... 3
    1.1.4 Primary and Secondary Injury ................................................................................................... 7
    1.1.4.1 Glutamate Excitotoxicity and Calcium Overload ................................................................. 11
    1.1.4.2 Oxidative Stress and Mitochondrial Dysfunction ................................................................. 13
    1.1.4.3 Caspase and Calpain ........................................................................................................... 14
    1.1.4.4 Apoptosis ............................................................................................................................ 16
    1.1.4.5 The Blood-Brain Barrier and Inflammation ...................................................................... 17
    1.1.4.6 Summary ............................................................................................................................. 18
    1.1.5 TBI and Chronic Neurodegeneration ....................................................................................... 18

## 1.2 TBI Modelling .................................................................................................................................. 20
  1.2.1 3 Types of Validity .................................................................................................................... 20
  1.2.2 Animal Models .......................................................................................................................... 21
  1.2.3 Animal Modelling to Clinical Trials ......................................................................................... 26
    1.2.3.1 Hypothermia ....................................................................................................................... 27
    1.2.3.2 Hypoxia preconditioning .................................................................................................... 29

## 1.3 Zebrafish as a Model Organism .................................................................................................... 30
  1.3.1 Anatomy .................................................................................................................................... 30
  1.3.2 Advantages ............................................................................................................................... 33
  1.3.3 Disadvantages .......................................................................................................................... 38
  1.3.4 History as (neuro) model organism ........................................................................................... 39
  1.3.5 TBI/trauma modelling in zebrafish ............................................................................................ 42

## 2.0 Rationale and Hypothesis .............................................................................................................. 44
  2.1 Rationale ....................................................................................................................................... 44
  2.2 Hypothesis ..................................................................................................................................... 44
  2.3 Specific Aims ................................................................................................................................... 45

## 3.0 Methodology ...................................................................................................................................... 46
  3.1 Animals ......................................................................................................................................... 46
  3.2 Injury Model ................................................................................................................................... 48
    3.2.1 Larval Chemical Injury .......................................................................................................... 48
LIST OF TABLES

Table 1: Common Animal Models of TBI
Table 2: Conservation of mammalian CNS features in zebrafish
Table 3: Selected advantages and limitations of zebrafish models for neuroscience research
LIST OF FIGURES

Figure 1: TBI secondary injury mechanisms
Figure 2: Zebrafish brain anatomy
Figure 3: Zebrafish life cycle/development
Figure 4: Zebrafish prevalence in research
Figure 5: SecA5-YFP transgenic
Figure 6: Ultrasound set-up
Figure 7: Novel tank test (NTT)
Figure 8: Hypoxic preconditioning set-up
Figure 9: Glutamate survival
Figure 10: Glutamate and behaviour
Figure 11: Inhibitors and behaviour
Figure 12: Confocal imaging
Figure 13: Recovery times
Figure 14: NTT: Locomotor activity
Figure 15: NTT: Locomotor activity (cont’d)
Figure 16: NTT: Vertical exploration
Figure 17: Shoaling Test
Figure 18: Protein Analysis
Figure 19: Hypothermia: Effect on survival following 11 MPa pHIFU
Figure 20: Hypothermia: NTT
Figure 21: Hypothermia: NTT (cont’d)
Figure 22: Hypothermia: NTT vertical exploration
Figure 23: Hypoxic preconditioning: NTT
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A5</td>
<td>Annexin V</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis activating protein-1</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B-III Tub</td>
<td>Beta-III Tubulin</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-secretase</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaN</td>
<td>Calcineurin</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled cortical impact</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin-A</td>
</tr>
<tr>
<td>CTE</td>
<td>Chronic traumatic encephalopathy</td>
</tr>
<tr>
<td>DAI</td>
<td>Diffuse axonal injury</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post-fertilization (zebrafish age)</td>
</tr>
<tr>
<td>E3</td>
<td>Zebrafish embryo medium</td>
</tr>
<tr>
<td>ED</td>
<td>Emergency department</td>
</tr>
<tr>
<td>FPI</td>
<td>Fluid percussion injury</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCS</td>
<td>Glasgow coma scale</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>LOC</td>
<td>Loss of consciousness</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascals (ultrasound intensity)</td>
</tr>
<tr>
<td>MPT</td>
<td>Membrane permeability transition (pores)</td>
</tr>
<tr>
<td>mTBI</td>
<td>Mild traumatic brain injury</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NF160</td>
<td>Neurofilament 160</td>
</tr>
<tr>
<td>NF200</td>
<td>Neurofilament 200</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NTT</td>
<td>Novel tank test</td>
</tr>
<tr>
<td>PBBI</td>
<td>Penetrating ballistic-like brain injury</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>pHIFU</td>
<td>Pulsed high-intensity focused ultrasound</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PS-1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PTSD</td>
<td>Post-traumatic stress disorder</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TAI</td>
<td>Traumatic axonal injury</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline Tween-20</td>
</tr>
<tr>
<td>TNFa-1</td>
<td>Tumor necrosis factor a receptor 1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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1.0 INTRODUCTION

1.1 Traumatic Brain Injury

1.1.1 Definition

Traumatic brain injury (TBI) is defined as damage to the brain resulting from an external mechanical force (Centers for Disease Control and Prevention (CDC), 2013; Defense and Veterans Brain Injury Center, February 2013 update; Faul, Xu, Wald, & Coronado, 2010). Injury can be caused by blunt or penetrating forces, rapid acceleration or deceleration, blast waves, impact or penetration by a projectile (Maas, Stocchetti, & Bullock, 2008). Injuries cause confusion, dizziness, loss of consciousness, seizures, coma, and death (Algattas & Huang, 2013; Kolias, Guilfoyle, Helmy, Allanson, & Hutchinson, 2013). Accompanying impairment of cognitive, physical, and psychosocial functions can be temporary or persist long-term (Masel & DeWitt, 2010).

1.1.2 Classification

Mild TBI (mTBI) is often used interchangeably with the term concussion. Concussion, defined as a traumatically induced transient disturbance of brain function, is a subset of mTBI which is generally self-limited and at the least severe end of the brain injury spectrum (Harmon, Drezner et al., 2013a). Both involve a complex pathophysiological process. As a whole, mTBI is typically characterized by short-term memory and attention impairments (H. S. Levin & Robertson, 2013; Walker & Tesco, 2013). Up to 15% of mTBI patients develop post-concussion
syndrome, resulting in persistent cognitive impairments as well as neurobehavioural and psychosocial difficulties (H. S. Levin & Robertson, 2013). Headaches, dizziness, and memory and attention deficits are common examples of persisting symptoms, as are emotional distress and personality changes (H. S. Levin & Robertson, 2013). Currently, there is no known biomechanical threshold for a clinical mTBI. Clinical diagnosis of mTBI and classification of injury severity is difficult.

Traditionally, TBI severity classification is based on the hospital admission Glasgow Coma Scale (GCS) score. Although exact ranges are often debated, generally injury is classified into mild (GCS 13–15), moderate (GCS 9–12), and severe (GCS <8) (Teasdale & Jennett, 1974). The GCS covaries with the risk of dying from TBI, which is low after mild (~1 %), intermediate after moderate (up to 15 %) and high (up to 40 %) after severe TBI (Teasdale & Jennett, 1974). However, the underlying pathology cannot be inferred from the GCS, and different structural abnormalities may result in a similar clinical presentation (Andriessen, Jacobs, & Vos, 2010). Therefore, at present, research efforts are focused on the pathological features of injury such as the moment of onset (primary or secondary) and distribution of structural damage (focal or diffuse).

Intracranial damage can also be classified as focal or diffuse. Focal injury is when a direct impact to the brain results in contusion and/or epidural or subdural haematomas. Diffuse brain injury, for example traumatic axonal injury (TAI), results from closed-head, usually rapid acceleration/deceleration injury (Andriessen et al., 2010; Bayly et al., 2005; Gennarelli, 1994; Le & Gean, 2009;

Axonal injury is the most common consequence of diffuse brain trauma. It was first described in 1956 by the pathologist Strich as a devastating clinicopathological syndrome with extensive damage to the white matter (STRICH, 1956). Later, the term diffuse axonal injury (DAI) was suggested by Adams and colleagues, referring to prolonged coma (more than 6 hrs) and widespread injuries to white matter regions that they classified into three stages (Adams et al., 1989). The more recent term of traumatic axonal injury, which is better suited to describe the whole spectrum of axonal injury severity in both human beings and animal models (Geddes, Whitwell, & Graham, 2000), will be used in this report. TAI is characterized by the delayed and progressive deterioration of white matter following trauma and typically presents histologically with microscopic axonal damage accompanied by myelin loss, axonal degeneration, and/or axonal swellings (Dashnaw, Petraglia, & Bailes, 2012; E. Park, Velumian, & Fehlings, 2004).

1.1.3 Epidemiology

TBI is a leading cause of death and morbidity in industrialized countries, with considerable associated healthcare costs. Specifically in this developed world, TBI is a leading cause of death and disability in young people (Sharp & Ham, 2011). Recent data show as many as 2% of the US population or 5.3 million citizens are disabled as a result of TBI (Centers for Disease Control and Prevention (CDC), 2013).
The economic costs of such disability in 2010 were estimated at US$76.5 billion, including $11.5 billion in direct medical costs and $64.8 billion in indirect costs (e.g., lost wages, nonmedical expenditures) (Centers for Disease Control and Prevention (CDC), 2013).

Over one million TBI’s occur annually and 75-85% of all such injuries suffered in civilian (Faul et al., 2010) and military populations (Defense and Veterans Brain Injury Center., February 2013 update) are categorized as mild. These numbers may be gross underestimates due to the undefined classification of mTBI and its occult presentation. Even more skewing to incidence data, is the lack of mTBI reporting. As recently as 1986, mTBI grading criteria relied heavily on loss of consciousness (LOC). Because fewer than 10% of mTBI’s involve LOC (Broglio, Surma, & Ashton-Miller, 2012; Lovell et al., 2006), reported numbers could be significantly decreased. Sufferers may also be reluctant to seek medical attention without clear evidence of sufficient injury. Also, typical methodological limitations in most studies of mTBI and concussion have included variability in definition and reporting methods (eg, self-report, discharge diagnosis on retrospective review, and survey).

These issues limit the strength of any conclusions regarding the epidemiology of mTBI (Carroll et al., 2004; Laker, 2011). In a meta-analysis, Cassidy et al reported that the incidence of mTBI in the United States was between 100-300 per 100,000 (Cassidy et al., 2004). However, because many of these patients did not present for care, estimates were as high as 600 per 100,000(Cassidy et al., 2004). Furthermore, because most patients with mTBI do not lose consciousness or
experience persisting symptoms, reported data likely underestimate the true incidence of mTBI, and conclusions as to which mechanisms of injury are most common cannot be drawn accurately.

mTBI also asserts a major military concern and has become increasingly recognized as a major cause of mortality and morbidity in the wars in Iraq and Afghanistan. It is estimated that 10–20 % of returning veterans have sustained a TBI (Elder et al., 2012). In American soldiers returning from Iraq, reports of mTBI, especially with LOC, were associated with higher rates of PTSD, depression, and physical health symptoms than were other injuries (Hoge et al., 2008). mTBI’s in military personnel vastly outnumber more severe brain injuries. Our lab has demonstrated that blast-related mTBI contributes analogous biological outcomes as other conventional closed-head traumas, including cytoskeletal degradation, apoptosis and behavioural deficits (E. Park, Bell, Siddiq, & Baker, 2009; E. Park, Gottlieb, Cheung, Shek, & Baker, 2011; E. Park, Eisen, Kinio, & Baker, 2013).

Contact and non-contact sports represent a large proportion of TBI in children and young adults. The US Centers for Disease Control and Prevention (CDC) estimates that 135,000 patients with mTBI from sports and recreation present to emergency departments each year (Centers for Disease Control and Prevention (CDC), 2013). Concussions represent 5.8 % of all collegiate sports injuries and upwards of 8.9 % of high school athletic injuries (Gessel et al. 2007). As of 2011, at least 46.5 million American children and adolescents participated in team sports annually, including 11.6 million basketball players, 8.0 million soccer players, 5.8 million baseball players, 5.3 million tackle football players, and 3.7
million softball players, many of whom participated in multiple sports (Noble & Hesdorffer, 2013).

Several large multiyear studies have been conducted on the epidemiology of mTBI in sport, predominantly based on age group or athletic level (Lincoln et al., 2011; Schulz et al., 2004). Football consistently causes the highest numbers and percentage of injury at the high school and college levels, ranging as high as nearly 75% (Frommer et al., 2011; Lincoln et al., 2011). Soccer is the most common cause of concussion in female athletes and is implicated in nearly 50% of sport-related cases (Frommer et al., 2011; Lincoln et al., 2011). More than 75% of the concussions reported were attributed to player-to-player contact, followed by 15.5% from contact with the playing surface, and nearly 78.5% occurred during competition rather than practice (Meehan, d'Hemecourt, & Comstock, 2010).

Gender differences have long been discussed in mTBI epidemiology. When comparing similar sports (eg, boy’s baseball and girl’s softball), investigators have found that girls have nearly twice the rate of concussion than their male counterparts (Gessel, Fields, Collins, Dick, & Comstock, 2007; Lincoln et al., 2011). Interpreting epidemiological differences becomes difficult when similar sports are not prevalent (eg, boy’s football and girl’s volleyball), therefore gender differences are difficult to translate into common underlying causes. Sociological factors may also influence the observed gender differences, including divergent societal pressures placed on male and female athletes, perceived stigma, and fear of removal from play associated with “concussion” in males, the latter contributing to decreased reporting. Potential biomechanical issues such as head to ball ratio,
smaller head/neck mass ratio, weaker neck muscles, and greater angular velocity in girls compared with boys have been cited as contributors to gender differences (Dick, 2009). High school boys and girls also self-report different symptoms: girls report more drowsiness and sensitivity to noise and boys report more confusion/disorientation and amnesia (Frommer et al., 2011).

Outside sport, men suffer more TBI’s than women. In an Ontario-based, cross-sectional retrospective chart review, men made up 57.8 % of work-place occurrences. The highest incidence of work-place TBI was found in the transportation and storage industry (81.5 per 100,000) (Colantonio et al., 2010). Based on CDC estimates, in 2013 men were 1.4 times as likely as women to sustain a work-place TBI resulting in emergency department (ED) visits or hospitalization (Algattas & Huang, 2013; Carroll et al., 2004; Faul et al., 2010). This is in opposition to the gender trend reported in sports in high school and young adults, although it is more prevalent in overall TBI reporting (Lovell et al., 2006; Stern et al., 2011).

1.1.4 Primary and Secondary Injury

TBI is not a single pathophysiological event but a complex disease causing structural damage and functional deficits due to both acute and long-term injury mechanisms (Maas et al., 2008; Xiong, Mahmood, & Chopp, 2013). The pathological processes underlying damage of neurons and their axons can be separated temporally into primary and secondary injury mechanisms. The primary injury is the result of the immediate mechanical disruption of brain tissue that occurs at the time of exposure to the external force. Primary injury includes contusion, damage to
blood vessels (haemorrhage) and axonal shearing, in which the axons of neurons are stretched and torn (Cernak, 2005; Geddes et al., 2000; Kolas et al., 2013). At the moment of impact, primary injury results from the translation of kinetic energy and force vectors linearly and/or rotationally (Dashnaw et al., 2012). During this movement, certain segments of the brain move at slower rates than others due to varying degrees of stabilization as well as sizes and weights, causing shear, tensile, and compressive forces within brain tissue (Dashnaw et al., 2012; Farkas, Lifshitz, & Povlishock, 2006; Povlishock & Jenkins, 1995). Secondary injury evolves over minutes to months after the primary injury and is the result of cascades of metabolic, cellular, and molecular events. These ultimately lead to axonal degeneration, cell death, tissue damage, and atrophy (Barkhoudarian, Hovda, & Giza, 2011; Giza & Hovda, 2001; Marklund & Hillered, 2011; Shrey, Griesbach, & Giza, 2011).

Secondary injury is classically described as the indirect result of the initial trauma and its subsequent pathophysiological processes. These pathophysiological processes involve both immediate and delayed cellular events, including ultrastructural damage, ionic shifts, and neurotransmitter release (Xiong et al., 2013; Yi & Hazell, 2006). There are also remarkable effects on cerebral blood flow dynamics and the blood-brain barrier (BBB) (Dashnaw et al., 2012; Korn, Golan, Melamed, Pascual-Marqui, & Friedman, 2005). Secondary injuries are multiple, parallel, interacting, and interdependent cascades of biological reactions (Johnson, Stewart, & Smith, 2013; Walker & Tesco, 2013) (Fig 1). Early sequelae is caused by prompt failure of neuronal energy, glial injury and dysfunction (swelling of astrocytic foot processes, reversal of neurotransmitter re-uptake and reactive
astrocytosis), inflammation (invasion of the injury site by microglia and release of proinflammatory cytokines and chemokines), destruction and stenosis of microvasculature, glutamate excitotoxicity, calcium overload, mitochondrial dysfunction, free radical generation, and progressive white matter deterioration (X. H. Chen et al., 2004; Giza & Hovda, 2001; Kolias et al., 2013; Masel & DeWitt, 2010; McKee et al., 2013). These cellular injury processes result in neuronal cell loss, axonal degeneration, cell death, and synaptic dysfunction (E. Park et al., 2008; Tavazzi et al., 2007).
Fig. 1. TBI secondary injury mechanisms.
The major pathways associated with secondary injury after TBI:
(i) Microvascular stenosis and death; (ii) Astrocyte foot process swelling and BBB breakdown; (iii) Astrogliosis (proliferation of astrocytes); (iv) Reversal of glutamate uptake (dysfunction of astrocytes); (v) Inflammatory cells, microglial infiltration – release of cytokines and chemokines that contribute to activation of programmed cell death. (1) Neuronal depolarization (via excitotoxicity due to excess glutamate); (2) Release of excitatory neurotransmitters (glutamate) and activation of NMDA/AMPA receptors leading to intracellular calcium overload; (3) Massive efflux of potassium; (4) Increased activity of (ATP-dependent) membrane ionic pumps to restore homeostasis; (5) Hyperglycolysis to generate more ATP; (6) Oxidative phosphorylation releases reactive oxygen species (ROS); (7) Calcium influx and sequestration in mitochondria; (8) Decreased energy (ATP) production; (9) Calpain activation and initiation of apoptosis. (A) Calcium influx and intra-axonal accumulation; (B) Protease activation and microtubule disassembly (neurofilament compaction via phosphorylation or sidearm cleavage, microtubule disassembly and accumulation of axonally transported organelles); (C) Axonal swelling and disconnection (secondary axotomy).
Adapted from (Andriessen et al., 2010; Giza & Hovda, 2001; E. Park et al., 2008)
White matter exhibits different patterns of injury and deterioration compared with grey matter (E. Park et al., 2008). Importantly, the extent of axonal injury closely correlates with outcome (Adams et al., 1989; Bailes, Petraglia, Omalu, Nauman, & Talavage, 2013; Johnson et al., 2013; Kou & VandeVord, 2014; Sharp & Ham, 2011) and many of the mechanisms leading to delayed white matter injury have been identified as occurring beyond the initial axonal shearing from primary injury. It has also been argued that most axons do not undergo axotomy as a result of primary shearing stress (Maxwell, Watt, Graham, & Gennarelli, 1993). In vitro studies have shown that forces generated under TBI are not sufficient for axonal disconnection and most axotomy is necessarily secondary, namely a result of the secondary injury cascade (Smith, Wolf, Lusardi, Lee, & Meaney, 1999). Nonetheless, progressive and delayed degeneration of white matter occurs in mTBI as well as severe injuries and its main pathways begin with excess extracellular glutamate and abnormal calcium homeostasis (Farkas et al., 2006; E. Park et al., 2008).

1.1.4.1 Glutamate Excitotoxicity and Calcium Overload

The primary excitatory neurotransmitter in the brain is glutamate. Extracellular concentrations of glutamate become significantly increased after injury in multiple ways (Faden, Demediuk, Panter, & Vink, 1989; Katayama, Becker, Tamura, & Hovda, 1990; Yi & Hazell, 2006). The potency of glutamate as neurotoxin has been identified and studied for several decades. In vitro studies have suggested a dose–response relationship (Choi, Maulucci-Gedde, & Kriegstein, 1987). Glutamate is released by pre-synaptic vesicles after depolarization due to ionic
imbalances but also leaks through damaged cell membranes after primary injury (Katayama et al., 1990; Yoshino, Hovda, Kawamata, Katayama, & Becker, 1991). In addition, the normal glutamate re-uptake by astrocytes, via an adenosine triphosphate (ATP)-dependent sodium-cotransport system, decreases when neighbouring astrocytes are destroyed and energy is depleted (Giza & Hovda, 2001; Yi & Hazell, 2006). Excessive extracellular glutamate initiates a massive influx of Ca\(^{2+}\) and Na\(^{+}\) influx into neurons and glial cells due to receptor overactivation (Kou & VandeVord, 2014). Glutamate binds the N-methyl-D-aspartic acid (NMDA) and \(\alpha\)-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, resulting in an overactivation of the ion channels responsible for Na\(^{+}\) and Ca\(^{2+}\) influxes (Faden et al., 1989). Additional Ca\(^{2+}\) is sequentially released from intracellular stores, like the endoplasmic reticulum, further raising intracellular Ca\(^{2+}\) levels. Rising Ca\(^{2+}\) levels cause depolarization of the neuronal membrane which activate voltage-dependent Ca\(^{2+}\) channels (VGCC) which further results in additional Ca\(^{2+}\) influx (Faden et al., 1989; Johnson et al., 2013; E. Park et al., 2008). ATP-dependent pumps then struggle to restore ionic homeostasis, which results in a hypermetabolic state. This energy crisis can begin as early as 30 minutes after injury (Kawamata, Katayama, Hovda, Yoshino, & Becker, 1992; Yoshino et al., 1991) and results in an imbalance of energy supply and demand (Barkhoudarian et al., 2011; Harmon, Drezner et al., 2013b; Prins & Giza, 2012).

In neuronal cells, calcium overload is associated with excitotoxic cell death, initiation of programmed cell death, and post-synaptic receptor modifications (E. Park et al., 2009; Stern et al., 2011). Intracellular calcium levels initiate cellular
pathways, including the activation of phospholipases such as calcineurin (CaN), proteases, including calpains and caspases, transcription factors, nitric oxide synthase (NOS), and DNA-degrading endonucleases, culminating in programmed cell death (Cassidy et al., 2004; Walker & Tesco, 2013). In axonal injury, calcium initiates cytoskeletal breakdown and subsequent axonal disconnection (Buki & Povlishock, 2006; Maxwell, Domleo, McColl, Jafari, & Graham, 2003) by activating protein degradation cascades that cause the accumulation of transported proteins. This leads to axonal swelling and blebbing, and ultimately axotomy (Algattas & Huang, 2013; Andriessen et al., 2010; Vagnozzi et al., 2007). Axonal bulbs and grossly swollen axons are a pathological hallmark of TAI (Angoa-Perez et al., 2014; Bailes et al., 2013; Le & Gean, 2009). Initial suggestions that these axonal bulbs are the result of immediate mechanical tearing of the axon, followed by axonal retraction and axoplasmic leakage (Adams et al., 1989), have now been refuted. This conclusion is especially evident in the axonal injury post-mTBI where primary axonal shearing is minimal or nonexistent. Rather, even mild trauma evokes a cascade of changes to the axon that may ultimately result in secondary disconnection (Buki & Povlishock, 2006; E. Park et al., 2008; Sharp & Ham, 2011).

1.1.4.2 Oxidative Stress and Mitochondrial Dysfunction

Glutamate-mediated accumulation of intracellular calcium ions also results in mitochondrial oxidative dysfunction (Lifshitz, Sullivan, Hovda, Wieloch, & McIntosh, 2004; A. M. Robertson, Bird, Waddell, & Currie, 1978; Verweij, Amelink, & Muizelaar, 2007; Xiong et al., 2013). Calcium overload is directly linked to mitochondrial
membrane depolarization, opening of membrane permeability transition (MPT) pores, and subsequent mitochondrial dysfunction and energy depletion (E. Park et al., 2008; A. M. Robertson et al., 1978; Schouten, 2007). To maintain cytoplasmic calcium homeostasis, the raised intracellular calcium concentration results in calcium sequestration within mitochondria (Lifshitz et al., 2004). However, mitochondrial calcium overload directly impairs oxidative phosphorylation processes (Mustafa, Singh, Wang, Carrico, & Hall, 2010). This causes membrane depolarization and the formation and opening of MPT pores (Lifshitz et al., 2004; C. L. Robertson, Saraswati, & Fiskum, 2007). Subsequently, passive water entry into the mitochondria results in osmotic swelling (A. Singh, Subhashini, Sharma, & Mallick, 2013) and eventually loss of mitochondrial function (Tavazzi et al., 2007; Vagnozzi et al., 2010). Following the increased membrane permeability, oxygen radicals (reactive oxygen species (ROS)), a by-product of the regular oxidative phosphorylation process, and the pro-apoptotic protein cytochrome-c, located between the inner and outer membranes of the mitochondria, are released into the cytoplasm (I. N. Singh, Sullivan, Deng, Mbye, & Hall, 2006). The result is widespread oxidative stress.

1.1.4.3 Caspase and Calpain

Both the caspase and the calpain proteins belong to the cysteine protease family and are key regulatory enzymes in the molecular processes of delayed cell death following TBI (Clark et al., 1999; Q. Liu et al., 2004; K. K. Wang, 2000). Caspases play a central role in apoptotic cell death, especially after mTBI (Q. Liu et al., 2004).
Cytochrome-c (part of the mitochondrial dysfunction discussed above) binds to the apoptosis activating protein-1 (Apaf-1), forming the apoptosome. This quarternery protein structure subsequently initiates the caspase cascade: activation of caspase-9, followed by caspase-3, resulting in apoptotic cell death (Clark et al., 1999; Hengartner, 2000; J. F. Kerr, Wyllie, & Currie, 1972; K. K. Wang, 2000). This is considered the ‘intrinsic’ caspase pathway. The ‘extrinsic’ pathway is mediated through cell surface death receptors such as tumour necrosis factor a receptor 1 (TNFa-1) and CD95 (Apo-1/Fas). Caspase-8 and caspase-10 are activated by interacting with the intracellular domains of such factors, culminating in similar programmed apoptotic death (Kruman & Mattson, 1999; K. K. Wang, 2000).

Calpain activity is calcium-mediated and also results in programmed cell death (Pike et al., 2000). After TBI, specific calpains are activated. Calpain-1 (calpain-µ, referring to the micromolar concentration of calcium required for its activation) is activated under normal homeostatic function. When calcium levels are increased to micromolar levels as they are following TBI, calpain-2 (calpain-m) is activated (K. K. Wang, 2000). These activated calpains cleave several enzymes and structural proteins (Q. Liu et al., 2004). Calpain’s exact physiological role is not entirely clear, although it is known to function in mechanisms of cell death and axonal injury (Huang & Wang, 2001; K. K. Wang et al., 1998). The elevation of spectrin breakdown products, for example those products resulting from cleavage of the cytoskeletal protein all-spectrin by calpain (E. Park et al., 2009; E. Park et al., 2011) and to a lesser extent by caspase (Pike et al., 2000), has been demonstrated following experimental TBI. Other targets of calpain with respect to TBI include
microtubule-associated protein (MAP2) (Fischer, Romano-Clarke, & Grynspan, 1991) and light and heavy neurofilaments (Kampfl et al., 1997; Posmantur, Hayes, Dixon, & Taft, 1994; Posmantur et al., 1997). Calpain can be inhibited by a number of pharmacological compounds or by the endogenous inhibitor, calpastatin (Shiraha, Glading, Chou, Jia, & Wells, 2002). There is evidence to indicate that calpastatin, is also cleaved by caspase-3 (K. K. Wang et al., 1998). This, along with the finding that pro-caspase-3 can also be activated by calpain (McGinnis, Gnegy, Park, Mukerjee, & Wang, 1999) underscores the complexity of molecular signaling cascades and the looped interactions that can make isolating the actual sequence of events difficult.

1.1.4.4 Apoptosis

Both necrosis and apoptosis can occur simultaneously in traumatically injured brain tissues (Rink et al., 1995; Walker & Tesco, 2013). Whereas necrosis in neuronal tissue is energy independent, apoptosis occurs only in the presence of ATP, i.e. in the presence of functional mitochondria as opposed to the total cell loss that is associated with severe TBI (Hengartner, 2000). Hence, apoptosis plays a large role in mTBI and its associated secondary cellular cascade (Johnson et al., 2013). Apoptotic cell death, also known as programmed cell death, is characterized by changes in nuclear morphology, cell shrinkage, blebbing of the plasma membrane, and formation of apoptotic bodies (H. Liu et al., 2013). During apoptosis, the phospholipid phosphatidylserine (PS) is exposed on the outer leaflet of the plasma membrane (Bredesen, Rao, & Mehlen, 2006; Fadok et al., 1992). Exposure of PS on the surface of apoptotic lymphocytes triggers specific recognition and removal by
macrophages (Fadok et al., 1992). The calcium-dependent protein Annexin V (A5) binds PS with high affinity (Martin et al., 1995). Biochemically purified, fluorescently labeled A5 probes have been widely used to detect apoptotic cells in vitro (Koopman et al., 1994; van Genderen et al., 2006).

1.1.4.5 The Blood-Brain Barrier and Inflammation

The BBB is the highly regulated separation between the intravascular and extravascular content of the central nervous system (CNS). BBB breakdown is well-documented in animal models of severe TBI, as well as mild and moderate injury (Shapira, Setton, Artru, & Shohami, 1993). Furthermore, a study of patients with post-concussion syndrome demonstrated that BBB disruption can be observed weeks to months after the original insult (Korn et al., 2005). The shearing forces of primary injury are believed to damage the BBB endothelium, resulting in increased small vessel permeability and dysregulation (Shlosberg, Benifla, Kaufer, & Friedman, 2010). Microvascular stenosis, as well as astrocyte foot process swelling, and inflammation-mediated breakdown during secondary injury, further exacerbate BBB destruction (Giunta et al., 2012; Kou & VandeVord, 2014; Shlosberg et al., 2010).

In brain tissue, microglia play a key role in the initiation of inflammatory events following injury (Dela Pena et al., 2014; Kumar & Loane, 2012; Shlosberg et al., 2010). As microglia become activated, anti- and pro-inflammatory cytokines, chemokines, nitric oxide, prostaglandins, trophic factors, free radicals, lipid peroxidation products, and excitatory molecules are released into the extraneuronal
space (Dashnaw et al., 2012; Giunta et al., 2012; Kumar & Loane, 2012; Marklund & Hillered, 2011). Inflammation is a double-edged sword: it is biologically designed to help the brain but its activity also exacerbates injury.

1.1.4.6 Summary

Collectively, the cascade of secondary injury events culminates in neuronal, endothelial and glial cell death, and white matter degeneration (Morales et al., 2005; Xiong et al., 2013) (Fig 1). Cell death occurs within minutes after injury and extends over a period of days to months (Cernak, 2005; Hengartner, 2000; Yuan & Yankner, 2000). Both acute cell death and delayed apoptosis have important roles in mediating functional deficits after TBI. However, human (Kolias et al., 2013) and rodent studies (Marklund & Hillered, 2011; Morales et al., 2005; Morganti-Kossmann, Yan, & Bye, 2010; Uryu et al., 2007) have indicated that even mTBI without notable cell death can lead to cognitive deficits. These are associated with secondary injury and TAI.

1.1.5 TBI and Chronic Neurodegeneration

There is accumulating evidence that TBI accelerates the onset of cognitive decline leading to dementia (X. H. Chen et al., 2004; Fakhran, Yaeger, & Alhilali, 2013; Q. Liu et al., 2004; M. V. Rao & Nixon, 2003; Sivanandam & Thakur, 2012; Uryu et al., 2007). Furthermore, multiple TBI's, however mild, can exacerbate the cognitive decline (Gavett, Stern, & McKee, 2011; Harmon et al., 2013b; Johnson et al., 2013; Longhi et al., 2005; Lovell et al., 2006; McKee et al., 2009; Omalu et al., 2006;
Pellman, Viano, Tucker, Casson, & Waeckerle, 2003a). Head trauma carries the potential to precipitate conditions such as Parkinson’s disease (PD), Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS), and even the more recently topical chronic traumatic encephalopathy (CTE). TBI has been found to be one of the strongest risk factors for the development of AD (Walker & Tesco, 2013). Recent studies suggest that even a single event can be sufficient to trigger neurodegenerative processes (Goldstein et al., 2012a; Mac Donald et al., 2013; McKee et al., 2013).

Evidence from histopathological and biochemical analysis of biopsy material from TBI patients and sufferers of neurodegenerative disorders indicates that the molecular pathways of TBI-induced neurodegeneration and those of neurodegenerative disease have overlapping characteristics (Walker & Tesco, 2013). Material from both conditions show remarkably similar pathology and accumulation of identical proteins implicated in disease pathogenesis, including amyloid precursor protein (APP), β-secretase (BACE1), presenilin 1 (PS-1), tau, and α-synuclein (X. H. Chen et al., 2004; Sivanandam & Thakur, 2012; Uryu et al., 2007). APP is a precursor to Aβ peptide, a peptide fragment that together with intracellular neurofibrillary tangles (NFT) is a hallmark of neurodegenerative disease. APP is cleaved by β-secretase (BACE1) and γ-secretase enzymes, resulting in Aβ formation and is most commonly found in the early stages of neurodegeneration (McKee et al., 2009; Mez, Stern, & McKee, 2013; Stern et al., 2011). Increased levels of the key components of the amyloidogenic cascade (APP cleaved by BACE1) have been identified in the damaged axons from human TBI patients (X. H. Chen et al., 2004;
Uryu et al., 2007). Enhanced beta APP immunoreactivity is used as a diagnostic marker for axonal damage post-TBI. In addition, chronic stimulation of NMDA receptors due to glutamate excitotoxicity following TBI has been demonstrated to alter APP processing and stimulate Aβ production in neurons (Lesne et al., 2005; Willoughby, Rozovsky, Lo, & Finch, 1995).

1.2 TBI Modelling

1.2.1 3 Types of Validity

Validity is defined as the extent to which a concept, conclusion, or measurement is well-founded and corresponds accurately to the real world (McKinney, 1984). Animal model validity is discussed in terms of the similarity between the model and the human condition it is intended to simulate, but no formal validation criteria of models is applied globally. Influential works such as Willner's (Willner, 1986; Willner, 1991) and McKinney's (McKinney, 1984) have provided the three criteria that are most widely cited in terms of validity: face, predictive, and construct validity. Generally, face validity is the phenomenological analogy with the modeled condition. Predictive validity is how accurately the performance in tests predicts performance in the modeled condition. Construct validity indicates the model has a sound theoretical rationale (Varga, Hansen, Sandoe, & Olsson, 2010).
1.2.2 Animal Models

Valid, sensitive animal models are crucial for understanding the pathobiology of human disorders. TBI is not a single pathophysiological event but a complex disease process (Kolias et al., 2013; E. Park et al., 2008). As such, the construct, face, and predictive validity are of utmost importance when designing and characterizing models that will have objective translational value (A. M. Stewart, Yang, Nguyen, & Kalueff, 2014; van der Staay, 2006). Traditionally, an animal model is considered to be valid, if it “resembles the human condition in aetiology pathophysiology, symptomatology and response to therapeutic interventions” (Van Dam & De Deyn, 2006). The basis for predictive validity, arguably the most important when analyzing animal models, is that basic processes are sufficiently similar across species. If the results of an animal study (pre-clinical research) are intended to be translated into human treatments, the ultimate proof of a model’s value is its predictive validity (McKinney, 1984; Varga et al., 2010; Willner, 1991). That is, the animal model must depict sufficient similarity to the human condition it is intended to simulate. In simple terms, predictive validity can be calculated in terms of reliability and relevance (A. M. Stewart & Kalueff, 2013).

The pathophysiology of TBI observed in humans is extremely heterogeneous and is confounded with a variety of primary injury types and severity and the complexity of interwoven secondary cellular injury pathways. Hence, experimental animal models of TBI that replicate the human condition are invaluable for the study of these mechanisms. Predictably, a single model cannot accurately replicate all processes involved in TBI, but often a single process or symptom can be accurately
mimicked. For an animal model to be viable, it must not only mimic injury characteristics observed in mammalian pathophysiology, but it must possess additional features of reproducibility, low costs, ease of manipulation, and ease of technical performance. Most importantly, in terms of replicating aspects of chronic illness, the model should produce long-lasting behavioural deficits (Morales et al., 2005; A. M. Stewart & Kalueff, 2013).

Aspects of TBI-induced pathology have been successfully modeled using various methods, including weight-drop, fluid percussion injury (FPI), controlled cortical impact (CCI), and blast waves (Marklund & Hillered, 2011; O’Connor, Smyth, & Gilchrist, 2011; E. Park et al., 2009; E. Park et al., 2011; E. Park et al., 2013; Xiong et al., 2013) (Table 1). The majority of research modelling cognitive dysfunction has been carried out in rodents using FPI and CCI models owing to their similarity in histopathological response, but also their amenability to sensitive behavioural tests (Panzer, Wood, & Bass, 2014; Xiong et al., 2013). Much of what we know regarding the pathophysiology of TBI has been gleaned from animal work using models that produce moderate and severe representations of TBI. There has been concern, however, over the effectiveness of prior animal studies modelling mTBI (Angoa-Perez et al., 2014; Gennarelli, 1994; H. S. Levin & Robertson, 2013; Loane & Faden, 2010; Marklund & Hillered, 2011; Panzer et al., 2014; A. M. Stewart & Kalueff, 2013). CCI and FPI injury models involve surgical procedures in order to apply trauma upon the exposed brain or dura (Dashnaw et al., 2012; Marklund & Hillered, 2011; Morganti-Kossmann et al., 2010). Milder models, producing lower cell death, and closed-head injuries, are currently being characterized but a variety of methods of
generating injury are still being used (Table 1). This is due in part to the inability of a single animal model to adequately mimic all aspects of human TBI, but also owes to the heterogeneity of clinical TBI. Also, models that fall under the category of mild struggle to demonstrate sensitivity to routine outcome measures such as histology and behavioural analysis.
<table>
<thead>
<tr>
<th>MODEL</th>
<th>MAIN TYPE OF INJURY</th>
<th>SPECIES</th>
<th>HIGHLIGHTS</th>
<th>LIMITATIONS</th>
<th>REFS</th>
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</thead>
<tbody>
<tr>
<td>Weight-drop</td>
<td>Focal or diffuse</td>
<td>Rat, mouse</td>
<td>Varying devices; similar human biomechanics of injury</td>
<td>Craniotomy; high mortality rates; varying models; variability in skull fracture response</td>
<td>(Albert-Weissenberger &amp; Siren, 2010; Cernak, 2005; Y. Chen, Constantini, Tremblovler, Weinstock, &amp; Shohami, 1996; Feeney, Boyeson, Linn, Murray, &amp; Dail, 1981; Kilbourne et al., 2009; Marmarou et al., 1994; Shohami, Shapira, &amp; Cotev, 1988)</td>
</tr>
<tr>
<td>FPI</td>
<td>Mixed</td>
<td>Rat, mouse, swine, rabbit, dog, sheep,</td>
<td>Middle or lateral models; highly reproducible; fine tuning of injury</td>
<td>Craniotomy; variability in injury severity applied</td>
<td>(Dashnaw et al., 2012; Hartl, Medary, Ruge, Arfors, &amp; Ghajar, 1997; Hayes et al., 1987; McIntosh et al., 1989; Millen, Glauser, &amp; Fairman, 1985; E. Park et al., 2009; Pfenninger, Reith, Breitig, Grunert, &amp; Ahnefeld, 1989)</td>
</tr>
<tr>
<td>CCI</td>
<td>Focal</td>
<td>Rat, mouse, swine, monkey</td>
<td>Highly reproducible; low mortality rate</td>
<td>Craniotomy</td>
<td>(Dixon, Clifton, Lighthall, Yaghmai, &amp; Hayes, 1991; King et al., 2010; Lighthall, 1988; Manley et al., 2006; Panzer et al., 2014; Smith et al., 1995)</td>
</tr>
<tr>
<td>Blast</td>
<td>Diffuse</td>
<td>Rat, mouse, swine</td>
<td>Biomechanics of injury similar to</td>
<td>Needs standardization; great variation</td>
<td>(Bauman et al., 2009; Cernak et al., 1996; DePalma, 1995)</td>
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</table>
military TBI  between injury designs  Burris, Champion, & Hodgson, 2005; Garner, Watts, Parry, Bird, & Kirkman, 2009; Goldstein et al., 2012b; Kovacs, Leonessa, & Ling, 2014; Nakagawa et al., 2008; E. Park et al., 2011)

<table>
<thead>
<tr>
<th>Penetrating ballistic-type injury</th>
<th>Focal</th>
<th>Rat, cat</th>
<th>Similar to human biomechanics of penetrating TBI</th>
<th>Needs standardization</th>
<th>(Carey, Sarna, &amp; Farrell, 1990; Williams et al., 2005; Williams, Hartings, Lu, Rolli, &amp; Tortella, 2006)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeated mTBI</td>
<td>Diffuse</td>
<td>Rat, mouse, swine</td>
<td>Similar to human biomechanics of long-term sport TBI</td>
<td>Needs characterization; long-term evaluation; no consensus on the optimal number or frequency of applied injury</td>
<td>(DeRoss et al., 2002; Friess et al., 2009; Gold et al., 2013; Y. Wang et al., 2011)</td>
</tr>
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</table>

Modified from (Albert-Weissenberger & Siren, 2010; Xiong et al., 2013)
1.2.3 *Animal Modelling to Clinical Trials*

Although primary injury comprises the initial tear, shear, or hemorrhage in brain tissue, the mechanisms of secondary injury can dramatically exacerbate the initial injury. The damage from primary injury is immediate and irreversible (Maruta et al., 2010; Walker & Tesco, 2013) and therefore, is only amenable to preventative measures. However, the delayed process of secondary injury suggests the potential for intervention post-injury. Attenuating secondary injury biochemical cascades confers neuroprotection and improves cognitive outcome following experimental TBI (Walker & Tesco, 2013). Unfortunately, results of approximately 30 randomized controlled clinical trials targeting secondary injury following TBI in human beings have been disappointing (Marklund & Hillered, 2011; Schouten, 2007; Tolias & Bullock, 2004) underlining the complex and heterogeneous pathogenesis of TBI.

Drug interventions (such as VGCC blockers, nitric oxide synthetase inhibitors, cyclosporine-A or calcineurin antagonists) successfully applied in experimental models of TBI have not led to effective treatments in humans. In experimental TBI in rats for example, the application of cyclosporin-A (CsA), a known inhibitor of the MPT pore, showed lower levels of intramitochondrial Ca\(^{2+}\) and decreased ROS production compared to untreated animals, implying a neuroprotective property of CsA (Sullivan, Thompson, & Scheff, 1999). A number of further studies have confirmed the dramatic improvement of mitochondrial function by blocking MPT pore formation using CsA as well as cyclosporine analogues such as NIM811.
Glutamate-mediated excitotoxicity is mediated primarily through abnormal activation of NMDA receptors resulting in intracellular calcium overload. As such, many therapeutic interventions have focused on antagonizing NMDA receptors. Pharmacological blockade using MK-801 (Biegon et al., 2004; Han, Hu, Weng, Li, & Huang, 2009; Phillips, Lyeth, Hamm, Reeves, & Povlishock, 1998), CP-89-113 (Okiyama, Smith, White, & McIntosh, 1998), memantine (V. L. Rao, Dogan, Todd, Bowen, & Dempsey, 2001) and telampenal (Belayev et al., 2001) resulted in neuroprotection following experimental TBI. However, due to the important role glutamate plays in normal excitatory signalling this therapy has largely failed in human clinical trials (Feng et al., 2010; Walker & Tesco, 2013). Timing of therapeutic administration is also a factor. Increases in glutamate occur very early after TBI and patients were likely receiving NMDA receptor antagonists too late (Panzer et al., 2014). Two interventions that continue to be subjects of debate in potential neuroprotection trials are discussed below.

1.2.3.1 Hypothermia

Hypothermia is the oldest form of neuroprotection. Lowering core temperature by varying methods has been used clinically to treat TBI patients for over 100 years (Sherman & Wang, 2014). In 1987, Busto and colleagues specifically reported that small changes in temperature can confer protection in experimental models of brain ischemia (Busto et al., 1987). Since then, many experimental
studies have confirmed that moderate hypothermia confers protection against ischemic and non-ischemic brain hypoxia, traumatic brain injury, anoxic injury following resuscitation after cardiac arrest and other neurological insults (Sahuquillo & Vilalta, 2007).

Many post-trauma adverse events that occur in the injured brain at a cellular and molecular level are highly temperature-sensitive and are therefore a good target for induced hypothermia. Therapeutic hypothermia minimizes tissue injury by slowing the secondary cellular injury cascade associated with TBI. The basic mechanisms through which hypothermia protects the brain are multifactorial: reduction in brain metabolic rate, effects on cerebral blood flow, reduction of the critical threshold for oxygen delivery, blockade of excitotoxic mechanisms, calcium antagonism, preservation of protein synthesis, reduction of brain thermopooling, decrease in edema formation, modulation of the inflammatory response, neuroprotection of the white matter, and modulation of apoptotic cell death (Sahuquillo & Vilalta, 2007). Therefore, as a method of neuroprotection, hypothermia has advantages over neuroprotective drugs that typically target a single mechanism and have so far proven ineffective in clinical trials. Nonetheless, the efficacy and ideal level of hypothermia for clinical application remains debated and further critical evaluation is needed to elucidate reasons for previous failures and to warrant further clinical trials.
1.2.3.2 Hypoxia preconditioning

Hypothermia and neuroprotective compounds are aimed at the secondary injury mechanisms following TBI. Increasing attention has also been turned to possible treatments before injury. Preconditioning is a phenomenon in which brief episodes of a sublethal insult induces robust protection against subsequent injuries. Preconditioning has been observed in multiple organisms and can occur in brain and other tissues. Extensive animal studies suggest that the brain can be preconditioned to resist acute injuries, such as ischemic stroke, neonatal hypoxia/ischemia, surgical brain injury, and trauma (Wegener et al., 2004).

Methods of preconditioning are numerous and diverse, ranging from transient ischemia, hypoxia, hyperbaric oxygen, hypothermia and hyperthermia, to exposure to neurotoxins and pharmacological agents (Stetler et al., 2014). In most cases, the phenomenon of ‘cross-tolerance’ results; a sublethal stress of one kind protecting against a different type of acute injury later. Despite research conducted over the past few decades, brain preconditioning is complex and not fully understood. It involves multiple effectors such as metabolic inhibition, activation of extra- and intracellular defense mechanisms, a shift in the neuronal excitatory/inhibitory balance, and a reduction in inflammatory sequelae (Della-Morte et al., 2013; Stetler et al., 2014). Hypoxic preconditioning in particular has been modeled both in vitro and in vivo, effectively conveying neuroprotection against subsequent injury. Typically, cultures or animals are placed in an airtight hypoxia chamber where oxygen is replaced by nitrogen. Most commonly, normobaric hypoxic conditions
(8 % oxygen) are used to induce a tolerant state that lasts approximately 72 h (Bernaudin et al., 2002; Prass et al., 2003; Zhan et al., 2010).

1.3 Zebrasfish as a Model Organism

1.3.1 Anatomy

Native to Southeast Asia, the zebrafish (Danio rerio) has become a popular model organism in biomedical research (Gerlai, 2003). It is a teleost with sufficient physiological complexity and high physiological brain homology to humans, including common conserved cell types, organs, and physiological systems (Ablain & Zon, 2013; Gerlai, 2012) (Table 2, Fig 2). Notably in TBI research, the vertebrate species demonstrates homology in the expression and function of key receptors involved in synaptic neurotransmission, including those responsible for calcium overload following glutamate receptor activation (J. Chen, Patel, Friedman, & Jones, 2010; Hoppmann, Wu, Soviknes, Helvik, & Becker, 2008).
Table 2: Conservation of mammalian CNS features in zebrafish

<table>
<thead>
<tr>
<th>CNS Structures</th>
<th>Mammals</th>
<th>Zebrafish</th>
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<tbody>
<tr>
<td>Forebrain (cerebrum, thalamus, hypothalamus)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Midbrain (tectum, tegmentum)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hindbrain (cerebellum, pons, medulla)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BBB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Meninges</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ventricular system</td>
<td>+</td>
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<thead>
<tr>
<th>CNS cell types</th>
<th>Mammals</th>
<th>Zebrafish</th>
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<tbody>
<tr>
<td>Neurons</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>+</td>
<td>+/-</td>
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<tr>
<td>Microglia</td>
<td>+</td>
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<tr>
<th>Major neurotransmitter systems</th>
<th>Mammals</th>
<th>Zebrafish</th>
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<tbody>
<tr>
<td>Amino acids (glutamate, GABA, glycine)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monoamines (dopamine, serotonin)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Peptides (somatostatin, opioids)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other (acetylcholine)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Adapted from* (Oosterhof, Boddeke, & van Ham, 2014), www.zebrafishbrain.org
Figure 2: Zebrafish brain anatomy. Schematic representations of (A) mouse brain and (B) zebrafish brain morphology. Abbreviations: OB, olfactory bulbs; Tel, telencephalon; OT, optic tectum; CB, cerebellum; FB, forebrain; MB, midbrain; HB, hindbrain.

Adapted from zebrafishbrain.org
1.3.2 Advantages

The potential of the zebrafish for translational neuroscience (Brennan, 2011; Buske & Gerlai, 2011a; Buske & Gerlai, 2011b; Gerlai, 2010; Gerlai, 2012; Kalueff, Stewart, & Gerlai, 2014; Kalueff, Echevarria, & Stewart, 2014; Miller, Greene, Dydinski, & Gerlai, 2013; A. M. Stewart et al., 2014), the methodological benefits of zebrafish for brain imaging, behavioral phenomics and high-throughput screening critical for CNS drug discovery have recently been employed and well-documented (Kalueff et al., 2014). The zebrafish has numerous advantages that are of relevance to neuroscience research (Table 3). These advantages also include addressing the technical and time-dependent hurdles associated with pre-clinical drug validation. Zebrafish are particularly suited to the study of neurological diseases, boasting well-developed nervous systems and brain structures analogous to mammals (Alfaro, Ripoll-Gomez, & Burgos, 2011; Laird & Robberecht, 2011). They also possess significant potential advantages in live imaging and whole-animal documentation of injury progression. The advantages of using this species in biomedicine also include, external fertilization, efficient breeding, rapid development (Fig 3), transparency of embryos and larvae, as well as ease of genetic and other experimental manipulations and cost- and space-effectiveness (Cachat et al., 2010; Collier & Echevarria, 2013; Grossman et al., 2010; Paquet et al., 2009; Paquet, Schmid, & Haass, 2010; Parker et al., 2013; A. Stewart et al., 2012). There are also various strains of zebrafish available with over 100 transgenic and mutant strains (A. M. Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). In both larval and adult stages, their size alone drives zebrafish potential to high-throughput screening as
Table 3: Selected advantages and limitations of zebrafish models for neuroscience research

<table>
<thead>
<tr>
<th>Advantages</th>
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<tr>
<td>High degree of homology to mammals (conserved cell types, organs, and physiological systems)</td>
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<td>Fully sequenced genome</td>
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<td>Rapid development and longer lifespan (compared to mice/rats)</td>
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<td>Transparent, external embryos</td>
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<td>Genetic pliability</td>
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<td>Whole animal imaging and disease progression</td>
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<td>Amenability to high-throughput drug screening</td>
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<td>Cost effective</td>
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<td>Availability of transgenic and mutant strains</td>
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<table>
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<th>Limitations</th>
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<tr>
<td>Duplication of genome (some genes have two copies)</td>
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<td>Certain analogous brain areas are not as developed (eg, neocortex)</td>
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<td>Variety of wild type strains; differences in behavioural phenotypes</td>
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<td>Blood-brain barrier develops at 5-10 days, affects permeability for drug administration via water bath</td>
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<td>Shoaling and other complex behaviours develop later in life cycle</td>
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Adapted from (Kalueff et al., 2014; A. M. Stewart et al., 2014)
Fig. 3: Zebrafish life cycle. Timeline of zebrafish development and life span. Embryo (0-72 hpf); Larvae (3-29 dpf); Juvenile (30-89 dpf); Adult (90 dpf-2 years); Aged (>2 years). Adapted from (Stewart et al., 2014; Bai et al., 2011)
well as whole-mount imaging, histochemistry, and microscopy (Green et al., 2012; Kalueff et al., 2014; Kaslin & Panula, 2001; J. N. Kerr & Denk, 2008; A. M. Stewart et al., 2014; Ullmann, Cowin, Kurniawan, & Collin, 2010).

Furthermore, zebrafish provide numerous cognitive and behavioural outcome measures that can be rapidly quantified through automated image capture and analysis systems to correlate with molecular findings. As behavioural phenotypes are the most complex product of CNS activity, the use of video tracking software and analysis techniques has increased neurobehavioural analyses in zebrafish (Cachat et al., 2013; Green et al., 2012). Automated observations are ideal for measuring locomotor responses (e.g., distance travelled and/or velocity, turn angle, etc.) as well as anxiety and social behaviours that human observation cannot quantify (Ahmad, 2012; A. M. Stewart et al., 2014). Examples of currently available software packages and equipment for zebrafish research include Ethovision (Noldus IT, Netherlands), LocoScan (CleverSys Inc, USA), and ZebraLab (ViewPoint, France).

Locomotor behaviours are the most common behavioural endpoints in zebrafish larvae and have been shown to be effective in neuropsychobehavioural studies (Ahmad, 2012). Adult behaviour is more complex and involves locomotor analysis as well as detailed spacial, anxiolytic, and social interactions. The most commonly used test in neurophenotypical studies is the novel tank test (NTT) (Cachat et al., 2010; Egan et al., 2009). The NTT is the most extensively studied model of anxiety in zebrafish, which has been adapted for pharmacological evaluation. In this standardized test, animals are introduced into a new tank and activity is monitored for 5-10 minutes (R. Blaser & Gerlai, 2006; R. E. Blaser,
Chadwick, & McGinnis, 2010; Gerlai, Fernandes, & Pereira, 2009; Maximino, de Brito, da Silva Batista et al., 2010; Maximino, de Brito, Colmanetti et al., 2010). Typical zebrafish responses to the NTT include diving response, freezing/immobility, erratic movement, and thigmotaxis (staying close to the walls), which all increase during high-anxiety states but can be rescued by anxiolytic treatments (Cachat et al., 2010; Cachat et al., 2011; Grossman et al., 2010). This model exploits the observation that zebrafish tend to spend 70–85% of the first minute in the bottom third of a novel tank, a preference that is reduced to chance levels by the end of a 5 or 10 min test (Egan et al., 2009; Gerlai, Lahav, Guo, & Rosenthal, 2000; E. D. Levin, Bencan, & Cerutti, 2007; Speedie & Gerlai, 2008). This bottom-dwelling behaviour is sensitive to pharmacological intervention as well as anxiety-driven interventions (Cachat et al., 2011; Maximino et al., 2010; Maximino et al., 2010). More recently, advanced tracking software has allowed researchers to track individual fish within a group and measure shoaling (grouping) behaviour. Outcome parameters include distance and interaction between subjects (Buske & Gerlai, 2011b; Maaswinkel, Zhu, & Weng, 2013; Miller & Gerlai, 2007). Shoaling behaviour offers potential relevance to human disorders such as autism spectrum disorder, post-traumatic stress disorder (PTSD), and general anxiety (Bakshi & Kalin, 2002; Bencan, Sledge, & Levin, 2009; R. E. Blaser et al., 2010; Fontaine et al., 2008; Gould & Gottesman, 2006; Kalueff et al., 2013; Maaswinkel et al., 2013; A. M. Stewart, Nguyen, Wong, Poudel, & Kalueff, 2014; Zafeiriou, Ververi, Dafoulis, Kalyva, & Vargiami, 2013).

These systems and numerous published characterizations (Ablain & Zon, 2013; Allison, 2011; Barros, Alderton, Reynolds, Roach, & Berghmans, 2008; Best &
Alderton, 2008; R. Blaser & Gerlai, 2006; Brennan, 2011; Fontaine et al., 2008; Gerlai, 2013; Goldsmith, 2004) have put the zebrafish at the forefront of behavioral neuroscience and psychopharmacology. The zebrafish’s neuropathological feasibility is a novel domain of research. Its utility in trauma pathology explicitly, remains in the early stages. TBI specifically, has yet to be modelled in any closed-head form for the elucidation of secondary injury mechanisms or application for drug discovery.

1.3.3 Disadvantages

Limitations of zebrafish models in biomedical research and relevance to neuroscience have been provided in recent literature (Ablain & Zon, 2013; Kalueff et al., 2014; Maximino et al., 2010; A. M. Stewart et al., 2014) (Table 3). Limited neuro-specific resources (antibodies, protocol, established models) exist, especially in disease areas where no genetic model can be acutely expressed, such as trauma. Also, although major mammalian structures, pathways, and cell types are conserved in zebrafish, certain brain areas (eg, cortex) are not as developed as in mammals (A. M. Stewart et al., 2014). In many cases, analogs exist but mapping them to their mammalian counterparts can complicate the interpretation of behavioural and functional outcomes. In neurobehavioural studies, some complex behaviours in zebrafish (eg, social behaviours) have been shown to develop over time to maturity (Buske & Gerlai, 2011b; Maximino et al., 2010). Although with such rapid development (Fig 3), a 90-day timespan to maturity is relatively fast compared to other species. The duplication of the genome in zebrafish, where some zebrafish
genes have two copies instead of one as in mammals, presents unique challenges in areas of genetic study and manipulation (A. M. Stewart et al., 2014). As a species, zebrafish do not tolerate inbreeding well and rapidly lose fertility (Kalueff et al., 2014). As a result, not as many well-characterized inbred strains of zebrafish exist as they do in mice models. Consequently, strains are widely variable and react differently to interventions, including stress and anxiety (Cachat et al., 2011; Egan et al., 2009; Wright, Rimmer, Pritchard, Krause, & Butlin, 2003). Finally, water solubility can be problematic in drug administration via water immersion, although use of solvents and other routes (such as injections) are available.

1.3.4 History as (neuro) model organism

Mounting evidence indicates that the zebrafish is rapidly becoming one of the main research organisms in translational neuroscience and biopsychiatry research (Fig 4), successfully complementing both rodent and clinical models of almost every major brain disorder (Green et al., 2012; Kalueff & Tuohimaa, 2004; Kalueff et al., 2014; A. M. Stewart et al., 2014). The zebrafish was previously limited to the field of developmental biology (Ablain & Zon, 2013). However, zebrafish research has extended to almost every aspect of human pathology. Models currently exist in many areas of biomedical research, including broad use in neuroscience, ranging from blood disorders and muscular dystrophies to neurodegenerative disease and cancer (Ablain & Zon, 2013; Bai & Burton, 2011; Best & Alderton, 2008; Paquet et al., 2009; Paquet et al., 2010; Santoriello & Zon, 2012). Marked progress has been made recently in neuropharmacology and neurobehavioural research. Zebrafish models
have been developed in neuropsychosocial areas such as autism, sleep disorders, cognitive deficits, depression, psychoses, and addiction (Bakshi & Kalin, 2002; Collier & Echevarria, 2013; Ellis & Soanes, 2012; Griffiths et al., 2012; Kalueff et al., 2014; E. D. Levin et al., 2007; Linney, Upchurch, & Donerly, 2004; Lopez Patino, Yu, Yamamoto, & Zhdanova, 2008; McKinney, 1984; Neelkantan et al., 2013; Ninkovic & Bally-Cuif, 2006; Panula et al., 2010; Rinkwitz, Mourrain, & Becker, 2011; A. Singh et al., 2013; Zafeiriou et al., 2013; Ziv et al., 2013).
Fig. 4. Zebrafish prevalence in research. A: The growing number of published zebrafish models (assessed in PubMed in February 2015, using terms ‘zebrafish’ and ‘model’). B: The number of PubMed publications for various model organisms (assessed in February 2015).
Brain-specific zebrafish protocols and methods are becoming more common. Researchers are capitalizing on the model species’ unique advantages. For example, histochemical markers for certain neurotransmitters (i.e., serotonin and dopamine, known to be involved in affective or neurological disorders) have already been tested in zebrafish (Kaslin & Panula, 2001) and are commercially available. This has enabled systems-level neurotransmitter analyses and mapping of normal and abnormal phenotypes in fish. Recently, Golgi staining in zebrafish shows that fish neuronal ultrastructure is very similar to that of rodents (Kalueff et al., 2014).

Significant progress in direct visualization and imaging of disease models have resulted from the increasing availability of sophisticated optical imaging systems (J. N. Kerr & Denk, 2008) and markedly advancing zebrafish CNS research. Although in vivo optophysiological analyses of neuronal network function (De Marco, Groneberg, Yeh, Castillo Ramirez, & Ryu, 2013; Ljunggren, Haupt, Ausborn, Ampatzis, & El Manira, 2014; Portugues, Severi, Wyart, & Ahrens, 2013; Zhu et al., 2009) have mainly been established in larval zebrafish, they can now also be applied to adult fish. For example, use in the caspr zebrafish strain (Wenner, 2009; White et al., 2008), which remain transparent into adulthood, permits visualization of the CNS even in adults.

1.3.5 TBI/trauma modelling in zebrafish

Despite this progress in modelling neurological disorders and aspects of disease, zebrafish models of trauma are scarce. To date, trauma-induced injury research in zebrafish has been limited to regeneration studies. Adult zebrafish have
the extraordinary ability to regenerate their damaged fins, skin and heart (Antos & Tanaka, 2010; Poss, Wilson, & Keating, 2002). Furthermore, they can regenerate several organs or tissues in the nervous system (spinal cord, photoreceptor, retina, cerebellum and optic nerve) (Cameron, Gentile, Middleton, & Yurco, 2005; Craig, Calinescu, & Hitchcock, 2008; Y. Guo et al., 2011; McCurley & Callard, 2010; Poss et al., 2002; Qin, Barthel, & Raymond, 2009). This feature of the adult zebrafish brain relies on the presence of neural stem cell niches that enable stem cells to continuously proliferate (Kishimoto, Shimizu, & Sawamoto, 2012a). Because the molecular and cellular mechanisms of regeneration remain unclear, the focus of existing TBI models in zebrafish have focused on its capacity for regeneration. These zebrafish models of neurotrauma are still few in number but are characterized by invasive stab injuries to the brain (Kishimoto, Shimizu, & Sawamoto, 2012b; Marz, Schmidt, Rastegar, & Strahle, 2011), or crush or transection injuries to the spinal cord (Fang, Lin, Pan, Shen, & Schachner, 2012; Hui, Dutta, & Ghosh, 2010). A model of closed-head mTBI has yet to be described. Interestingly, Wu and colleagues have described a stab model of zebrafish TBI to study regeneration, and have applied micro-array technology and a ‘zebrafish movement index’, which includes behavioural patterns of total distance, and turn direction as criteria (Wu et al., 2014). These methods capitalize on both the high-throughput screening potential and behavioural analysis capabilities of zebrafish, although the stab injury of the model is not representative of most TBI’s.
2.0  RATIONALE AND HYPOTHESIS

2.1  Rationale

Zebrafish have been demonstrated as effective organisms to model aspects of mammalian pathophysiology and amenable for high throughput drug screening. Larval and adult stages, along with availability of a transgenic line expressing fluorescently-labelled apoptotic cells (van Ham, Mapes, Kokel, & Peterson, 2010), make them a suitable candidate organism for exploration into the secondary injury mechanisms of TBI. The impact of mTBI in industrialized countries is severe and persistent, establishing a grave need for translationally relevant mTBI research and drug discovery.

2.2  Hypothesis

Overarching Hypotheses:

1. Secondary injury mechanisms contribute to the functional deficits following mild brain injury

2. The interruption/modification of secondary injury mechanism will improve functional outcome following mTBI

3. Secondary injury mechanisms following mTBI can be modelled in order to discover modification strategies
Specific Hypothesis: Larvae and adult zebrafish can be employed in a model of TBI in order to suggest candidate strategies that will modify secondary injury mechanisms.

2.3 Specific Aims

Objective: Development, characterization, and validation of a zebrafish model designed to suggest candidate chemotherapeutic agents that will reduce secondary injury in TBI.

Specific Aims:

1: To characterize and validate a dose-dependent chemical model of TBI in zebrafish larvae that can be used in high-throughput screening of compound libraries.

2: To characterize and validate a closed-head model of TBI in adult zebrafish.
3.0 METHODOLOGY

3.1 Animals

All work was conducted in accordance with guidelines established by the Animal Care Committee at St. Michael’s Hospital in accordance with the standards set by the Canadian Council on Animal Care.

Three day post-fertilization (dpf) secA5-YFP transgenic zebrafish larvae were used for all experiments. Embryos and early-stage larvae were incubated at 28-30°C in E3 medium under a 12-hour light/dark cycle until hatched (3dpf). The transgenic secA5-YFP line of zebrafish attaches a fluorescent YFP molecule to Annexin-V, a normally expressed protein that binds the plasma membrane of cells undergoing apoptosis (Fig 5). The secA5-YFP line fluorescently labeling apoptotic cells was previously published (van Ham 2010) and was a gift from the Randall Peterson lab.
Fig. 5. SecA5-YFP transgenic. A: Schematic representation of secA5-YFP fusion construct in transgenic zebrafish (from van Ham et al. 2010), including secretory signal peptide (sec), and human A5 gene fused to yellow fluorescent protein tag (YFP). B: Representation of fluorescently labelled Annexin-V and its ability to bind phosphatidylserine (PS) that is exposed on the outer leaflet of plasma membrane of cells undergoing apoptosis.
Male and female wild-type adult zebrafish (6-10 months) of short-fin wild type phenotype (AB, short fin) from heterogeneous stock were used in all adult experiments (Part 2). Fish were kept in tanks at pH 6.8-7.0 and maintained at 25°C with a light/dark cycle of 12/12 h.

3.2 Injury Model

3.2.1 Larval Chemical Injury

All chemical treatments were performed in 0.5 % dimethylsulfoxide (DMSO) in 96-well plates (one larva per well) and incubated at 28°C. In order to induce neurological insult, glutamic acid was bath-applied at 1, 5, 10, and 20 μM concentrations (Sigma Aldrich, Oakville, Ontario). Control groups were repeated with each experiment in 0.5 % DMSO in identical conditions. Survival studies as well as behavioural tests were performed with the same parameters. For inhibition studies, the NMDA receptor antagonist MK-801 (Sigma Aldrich) was added simultaneously to glutamate treatments to a final concentration of 100, 200, and 400 nM. The calpain inhibitor MDL-28170 (Enzo Life Sciences, Farmingdale, New York) was added to 5, 10, and 20 mM.

3.2.2 Adult Ultrasound Injury

A brain-specific, closed-head injury was created using a targeted 1-MHz pulsed high intensity focused ultrasound (pHIFU) system (Fig 6). Fish were anaesthetized using 100ppm clove oil before being inserted into ultrasound set up.
(Fig 6 inset). Controls were anaesthetized and inserted into setup and allowed to recover in identical conditions.
Fig. 6. Ultrasound set-up. Targeted 1-MHz pulsed high intensity focused ultrasound (pHIFU) system. Inset: Injury set-up, anesthetized adult fish are fixed with brains positioned over target area (hatched box) and positioned in HIFU focal spot.
3.3 Behavioural Monitoring and Analysis

Larvae were monitored every four hours beginning 48 h post-insult. Behavioural measures were recorded in three consecutive 10 min sessions in a temperature-controlled Daniovision (Noldus, Netherlands) unit at 24 h and 48 h post-glutamate insult. EthoVision XT software (Noldus) was used to analyze locomotor activity and used to compute total distance travelled and mean velocity endpoints.

All adult behavioural tests were recorded using a Canon Vixia HF R500 video camera (Canon, Mississauga, Ontario) at 24 Mbps and 1920x1080 resolution, and analyzed using Ethovision XT (Noldus). Adult behavioural studies were performed at 11 MPa and 10,000 cycles (ultrasound duration), unless varying intensities were specified (1, 5, 11 MPa).

3.3.1 Novel Tank Test (NTT)

To evaluate the behaviour of adult zebrafish post-injury, the NTT was performed as previously described (R. Blaser & Gerlai, 2006). Fig 7 depicts representative visualizations of a NTT example. A rectangular tank was opaqued on three sides and virtually divided into two equal horizontal areas (bottom and top) and filled with conditioned water of pH 6.8-7.0 at 25°C. Procedures were executed on a stable surface with all environmental interferences kept to a minimum. After each recovery interval (0, 24, and 48 h post-injury), each fish was individually removed from identical holding tanks, released into the testing tank and recorded for 6 min. The behavioural measures were automatically measured at a rate of 30
frames/sec. Each test was conducted at the same time each day and separate controls were monitored each session. The locomotor activity of the zebrafish was assessed by measuring the total distance travelled, mean velocity, percentage of time immobile, and meandering. Meandering is defined as the absolute turn angle (representative of the variations in direction of the centre point of the animal) divided by the total distance travelled. Latency to the top zone was evaluated to analyze vertical activity and exploration specifically.
Fig. 7. Novel tank test. A: Representative heat map of a control fish in a Novel Tank Test (NTT), where individual fish are introduced into new tank and recorded by side-view camera for 6 min. B: Representative heat map of an injured fish NTT depicts diving, bottom-dwelling, and freezing behaviour. C: Representative track visualization of a control fish NTT. D: Representative track visualization of an injured fish NTT depicts immobility and bottom-dwelling habits of injured fish.
3.3.2 Shoaling Test

The shoaling test measures the average distance between fish in groups, a paradigm that has been used extensively to study anxiety and other psychiatric disorders. Tighter groupings indicate higher anxiety and stress. At 0, 24, and 48 h post-injury, adult zebrafish were placed in a novel test tank in groups of 5 fish. Behaviour was recorded for 10 min (n=5 per group, repeated twice to achieve a final sample size of n=10) and analyzed using EthovisionXT (Noldus, Netherlands). Automatic tracking provides the mean distance between subjects, a shoaling-specific parameter.

3.4 Confocal Imaging

Live imaging of larval zebrafish brains was performed in 0.5 % DMSO water containing clove oil (100 ppm). Larvae were mounted using 1 % low-melting agarose onto glass-bottomed culture dish (Fisher, Toronto, Ontario). Images were captured on an inverted confocal microscope (Zeiss, Toronto, Ontario) using the LSM700 scanning system and a 20/0.6 objective. Points above and below the larvae in the z-plane were defined by driving the microscope to a point just out of focus on both the top and bottom of the zebrafish larvae. Images were collected with a step-size of 10 um. Exposure times of 200 ms were used for all specimens using YFP channel. All images were processed using ImageJ software (National Institutes of Health). The sum intensity projections of z-stacks were used for quantification of fluorescence. Fluorescence was expressed as sum intensity per unit area.
3.5 Western Blotting

Brain tissue samples were collected from adult zebrafish anesthetized in 100 ppm clove oil. Post-injury time points assessed were 6, 12, and 24 h post-injury (n=6 per time point). Brains were placed in oxygenated phosphate buffered saline (PBS) immediately following decapitation and extracted with fine tip forceps and microscissors. Whole brains were homogenized in 300 ul RIPA lysis buffer containing protease inhibitors (50mM Tris-HCl, 1 % NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 lg/mL aprotinin, 1ug/mL leupeptin, and 1 µg/mL pepstatin). Samples were centrifuged for 10 mins at 14,000 rpm and the supernatant extracted. Protein concentration was determined using the modified Lowry method (Peterson, 1977). Samples were normalized for equal loading (40 µg/lane), electrophoresed on 7.5 % or 12 % SDS-PAGE gels, and transferred overnight to nitrocellulose membranes. Blocking of membranes was performed in 5 % non-fat milk blocking solution for 1h at room temperature. Immunoblots were probed for Neurofilament 160 (NF160), B-III tubulin, the activated form of Caspase-3, and APP. In rat studies, Neurofilament 200 (NF200) has been used to show increases in heavy neurofilament proteins following TBI (E. Park et al., 2011; Saljo, Bao, Haglid, & Hansson, 2000), but no zebrafish-specific antibody was available for NF200. Therefore we used NF160, a medium neurofilament protein antibody that was specific to zebrafish. B-III tubulin was used to assess changes in cytoskeletal integrity because it breaks down in axonal injury (Nakamura et al., 1990; Nixon, 1993; Sternberger & Sternberger, 1983). Activated Caspase-3 was chosen as a marker for activated apoptotic pathways. APP was used to assess the activation of
the amyloidogenic cascade following TBI. Primary incubations were performed overnight at 4°C at a dilution of 1:15,000 in blocking solution. Secondary antibody incubation was performed for 1h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:3,000 dilution). Rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich) was used as a loading control (1:15,000 dilution) and visualized with HRP-conjugated goat-anti-rabbit secondary antibodies (1:3,000 dilution). Washes in three changes of TBST were performed between incubations. Bands were visualized using a chemiluminescence kit (Perkin Elmer, Waltham, MA) and images captured on a BioRad gel docking station (BioRad, Mississauga, Ontario).

ImageLab (Mississauga, Ontario) software was used to measure the densitometry of the western blots. This software calculated an integrated optical density of bands based on intensity and size. Protein bands were normalized to the loading control. Total GAPDH expression was chosen as a loading control, as others have demonstrated that total GAPDH expression does not change following brain trauma (Atkins, Falo, Alonso, Bramlett, & Dietrich, 2009; Enomoto, Osugi, Satoh, McIntosh, & Nabeshima, 2005; Otani, Nawashiro, Fukui, Nomura, & Shima, 2002). Western blot samples were run in triplicate to ensure consistency of results.
3.6 Temperature

Temperature NTT experiments were performed as described above, with recovery tanks set 3°C below normal conditions (22°C versus 25°C). Survival proportions post-injury were monitored in recovery tanks at 22, 25, and 28°C.

3.7 Hypoxia

Hypoxia preconditioning preceded ultrasound injury and behavioural analysis as described above. An air-tight chamber was constructed using 1,000mL glass beaker and 500mL of water. A nitrogen (N₂) tank was connected directly to the water while another outlet led from air space to outside the tank (Fig 8). Hypoxia was created by bubbling in pure N₂ at a reading of 2% oxygen. Normoxia was restored at a reading of 100% oxygen. Adult zebrafish were acclimated to the tank (bubbling at 100% O₂) for 5 min before they were subjected to three cycles of 2 min of 1% O₂ and 3 min recovery in 100% O₂. Characteristics of sublethal hypoxia, as outlined by Braga et al., were observed, specifically swimming to the surface and loss of posture(Braga et al., 2013). Subjects were then allowed to recover for 30 min before targeted ultrasound injury. Injury control groups were placed into tank with bubbling O₂ for 20 min before resting in a recovery tank for 30 min before injury. Control subjects were exposed to the same conditions but without ultrasound injury.
Fig. 8. Hypoxic preconditioning set-up. N₂ is bubbled into an airtight chamber to create hypoxic environment. 100% oxygen is added to return to normoxia. Adult fish undergo 3 cycles of 2 min of hypoxia and 3 min normoxia before undergoing ultrasound injury.
3.8 Statistical Analysis

D’Agostino-Pearson omnibus test for normality was performed on all data sets. All data passed normality (p>0.05). Survival curve comparison was evaluated using logrank test and median survivals were computed. Median survival is used in survival analyses for its ability to be completely defined once the survival curve descends to 50 %, whereas mean survival is not defined until every subject dies and no results are censored. These conditions occur in very few studies, thus median survival is computed with its confidence interval. Behavioural endpoints were analyzed by one-way ANOVA with Tukey’s post hoc test for multiple comparisons. Fluorescence quantification was evaluated using repeated measures two-way ANOVA and Tukey’s post hoc test. Data were expressed as mean ± SEM and the significance was set at p<0.05.

Western blot densitometry analysis was performed using one-way ANOVA and Tukey’s post hoc test. Statistical significance was set at p<0.05. Errors are shown in the graphs as standard error of the mean (SEM). Behavioural endpoints were analyzed by one-way ANOVA. Post hoc comparisons were performed using Tukey’s test. Data were expressed as mean ± SEM and statistical significance was set at p<0.05.

3.9 Methodological Limitations

Zebrafish brain-specific antibodies are limited in comparison to other model organisms. Zebrafish protein targets, with established roles in mammalian
neurotrauma models, are limited. Thus, western blotting and staining techniques were limited to commercial availability and specificity.

Brain sectioning and immunohistochemistry were attempted in adult zebrafish. However, given the size of the zebrafish brain (approximately 3 mm long, 2 mm thick, and 2.5 mm wide), it was technically difficult to section in both paraffin and flash-frozen tissue blocks in a manner that achieved consistent slices at comparable angles and levels. Even the slightest change in mounting angle resulted in large morphological and landmark variability when slicing both paraffin and frozen brain samples. Consequently, immunohistochemistry analysis and morphological markers such as hematoxylin and eosin (H&E), commonly used as a histological stain, were difficult to compare between brains. H&E methods were adapted to the zebrafish brain, but in this study their use was of no significant value given that identical anatomical sections could not be compared in both control and injured tissues. In future validation, methods of directly visualizing morphological changes in ultrasound injury (such as high-powered confocal imaging or transgenic lines) could be useful in elucidating both mechanistic elements of injury as well as pathways of pharmacological intervention.

3.10 Bias

Introduction of observer-based methodological bias was minimized in video recording and automated analysis of all behavioural studies. Limitations existed, however, in group selection randomization and observer-based recovery time recording.
4.0 RESULTS AND ANALYSIS

PART 1: LARVAL MODEL

4.1 Study Rationale and Aims

Mild traumatic injury triggers the activation of multiple, interrelated pathways of secondary cellular injury. Glutamate excitotoxicity has been demonstrated as an upstream effector in many of these pathways. While many pathways have been characterized in both general and specific detail, others have yet to be defined. To date, however, results of successful pre-clinical evaluation of candidate neuroprotective pharmacotherapeutics have all gone on to fail in clinical trials. The rationale behind our larval experiments was to provide a cost- and time-effective model to rapidly screen candidate compounds that will act on the secondary injury mechanisms of mTBI. As a simplified model, candidates’ mechanisms, specificity, and dosage would need to be further validated in our adult model, as well as in higher order animal models. This model serves to stand as a high-throughput representation of a portion of secondary injury mechanisms, namely those initiated by glutamate excitotoxicity.

Specific Aims: To characterize and validate a dose-dependent chemical model of TBI in zebrafish larvae that can be used in high-throughput screening of compound libraries.
4.2 Methods

Glutamate injury dose-dependency was established in survival studies of zebrafish larvae. Results were confirmed in behavioural studies before death was induced, thus confirming sensitive endpoints. Brain-specific insult was verified using a transgenic line of zebrafish that fluorescently labels apoptosis (Fig 5) and in vivo confocal imaging of whole animal larvae at time points identical to those validated in behavioural analysis (Fig 12). Detailed descriptions of corresponding methods are provided in section 2.0.

4.3 Results

4.3.1 Glutamate insult results in dose-dependent injury

Increasing concentrations of glutamate resulted in increased rate of zebrafish larvae death (Fig 9 A). Addition of 1, 5, 10, and 20 uM of glutamate provided median survivals of 84, 80, 72, and 64 h respectively. The logrank test indicated survival at 10uM was significantly less than that of the control group (P < 0.0001), which was treated with DMSO only. Treatment at 20 uM resulted in 12 % death within the first 50 h post-injury. The 10 uM concentration provided a sufficient difference in the temporal window of survival from control larvae without significant early death to test inhibitor function. Increasing concentrations of MK-801 resulted in delay of death from glutamate exposure in a dose-dependent manner (Fig 9 B). There was no significant effect on median survival of administration of 100 nM of MK-801 relative to the control insult group, however both 200 and 400 nM concentrations of MK-801 significantly improved compared to glutamate insult (p<0.0001).
Fig. 9. Glutamate Survival. Data represent percent survival for n=96 per condition (logrank test for comparing survival curves) A: Survival proportions of increasing dosages of glutamate (median survivals of 84, 80, 72, and 68 h at 1, 5, 10, and 20 uM, respectively) showed 10 uM to have the greatest effect without inducing early death (logrank p<0.0001 indicates 10 uM curve is significantly different from that of control group). B: Survival proportions of 10 uM of glutamate-injured larvae with increasing concentrations of NMDA antagonist, MK-801, demonstrate delayed death (median survivals of 72, 72, 80, and 84 h at 0 nm, 100 nm, 200 nm, 400 nm inhibitor, respectively). 200 nM curve significantly different compared to control (p<0.0001), whereas 100 nM compared to control was not
4.3.2  *Glutamate challenge in zebrafish larvae results in locomotor deficits*

Daniovisor software was used to analyze video recordings of zebrafish larvae to monitor the effects of glutamate-mediated injury on mobility/activity. Significant reductions in total distance travelled were observed at 5 uM (p=0.001), 10 uM (p<0.0001), and 20 uM (p<0.0001) concentrations of glutamate (Fig 10 A). Distance at 10 uM was also significantly less than at 5 uM (p=0.041). Analysis of distance travelled revealed a statistically significant difference between 5 and 10 uM insults but not between 10 and 20 uM. Mean velocities of the same subjects were significantly decreased in all injury groups relative to controls (1, 5, 10, 20 uM glutamate) (Fig 10 B; p<0.001).
Fig. 10. Glutamate and behaviour. Summary of behavioural data. A: Total distance travelled by larvae decreased with increasing concentrations of glutamate at 24 h post-insult. There were significant decreases at 5 uM (*p=0.001), 10 uM (*p<0.0001) and 20 uM (*p<0.0001) compared to control. Values between 5 and 10 uM were significantly different (**p=0.042). D: Mean velocity of larvae at 24 h post-glutamate insult decreased significantly with increasing concentrations of glutamate 24 h post-injury (*p<0.0001).
4.3.3 **Glutamate receptor antagonist and calpain inhibitor delays glutamate-mediated excitotoxicity**

The use of NMDA receptor antagonist MK-801 and calpain inhibitor MDL-28170 in conjunction with the 10 uM glutamate insult was to evaluate the dose-dependent response to interventions known to be protective in mammalian models of TBI (Ai et al., 2007; Han et al., 2009; Okiyama et al., 1998; V. L. Rao et al., 2001). The 10 uM glutamate level of injury was chosen to evaluate inhibitor treatment because of significant margin between control and treated groups on behavioural outcome measures. MK-801 increased survival an average of 8.2 ± 2.5 hours compared to glutamate treatment groups (Fig 9 B). MK-801 rescued the glutamate-mediated impairment in locomotor activity (Fig 11 A). These improvements in distance travelled were statistically significant at 200 and 400 nM concentrations of MK-801 (p<0.0001). Similar results were found in velocity analysis where MK-801 significantly improved velocity at 200 nM and 400 nM concentrations (Fig 11 C; p=0.041 and p=0.016, respectively).

MDL-28170 had lesser effects in recovery of distance travelled and velocity (Fig 11 B,D). The highest soluble concentration (20 uM) produced significant increases in both outcomes compared to glutamate-only control larvae (p=0.044 and p=0.023).
Fig. 11. Inhibitors and behaviour. Summary of behavioural data with application of inhibitors. A-B: Distance travelled by larvae 24 h after addition of 10 uM of glutamate and increasing concentrations of inhibitors MK-801 and MDL-28170, respectively. There was significant recovery in total distance at 200 and 400 nM MK-801 (*p<0.0001) and 20 uM of MDL-28170 (*p=0.044). The increase in concentration of MK-801 from 100 to 200 nM resulted in a significant increase in distance (**p=0.0075). C-D: Velocity of larvae 24 h after addition of 10 uM of glutamate and increasing concentrations of inhibitors MK-801 and MDL-28170. There was a significant increased at 200 and 400 nM using MK-801 (*p=0.041 and *p=0.016, respectively) as well as recovery of behaviour at 10 uM (*p=0.044) and 20 uM concentrations of MDL-28170 (*p=0.023).
4.3.4 *Glutamate insult is brain specific*

Glutamate excitotoxicity was assessed visually using confocal imaging of live zebrafish larvae. Apoptosis in glutamate-exposed larvae (10 uM) was visualized alongside glutamate-treated specimens that had also been treated with NMDA antagonist MK-801 or calpain inhibitor MDL-28170 (Fig 12 A-H). Glutamate-treated larvae showed significant increase in fluorescence levels at 24 h (Fig 12 B; p<0.0001) and 48 h post-insult (Fig 12 F; p<0.0001). We also observed axonal degeneration evidenced by beading morphology at 24 h and 48 h post-injury (Fig 4 Inset) where no beading was present in control or inhibitor groups. Both MK-801 and MDL-28170 had significantly decreased fluorescence levels at 24 h (Fig 12 C,D,I; p<0.0001) and 48 h post-insult (Fig 12 G,H,I p<0.0001) points. No fluorescence was observed in spinal cord or tail regions (data not shown).
Fig. 12. Confocal imaging. Composite z-stacks of 4 dpf transgenic zebrafish larvae brains. Top row of panels represent 24 h post-insult and bottom row is 48 h post-insult. A: Control brain at 24 h. B: 24 h post-10 uM glutamate. C: 10 uM glutamate with 200 nM MK-801, and D: 10 uM glutamate with 20 uM MDL-28170. E: Control brain at 48 h. F: 48 h post 10 uM glutamate. G: 10 uM glutamate with 200 nM MK-801 and H: 10 uM glutamate with 20 uM MDL-28170. Inset: Arrows indicate axonal swelling and blebbing. I: Data represent mean ± SEM for n=6 larvae/condition. Quantification of sum intensity of YFP-labelled cells/unit area showed significantly more fluorescence in brains of glutamate-injured larvae at both 24 and 48 h compared to controls at identical time points (**p<0.0001). Inhibitor-treated larvae at corresponding time points (24 and 48 h) demonstrated significant reduction in fluorescence levels compared to glutamate exposed larvae using MK-801 and MDL-28170 treatments (all *p<0.0001). There was no significant difference between controls and inhibitor-only treated groups.
PART 2: ADULT MODEL

4.4 Study Rationale and Aims

Adult zebrafish demonstrate high physiological and genetic homology to mammals with respect to neurological function and anatomy. They possess unique capabilities as a model organism, including well-characterized behavioural tests, developmental maturity at 3 months of age, cost-efficient housing, genetic tractability, and a fully sequenced genome. We employed adult zebrafish in a model of closed-head TBI as a means to study mechanisms of secondary injury in a biologically relevant model system amenable to high-throughput, automated capabilities. Following screening of candidate compounds using the larval model of chemical injury, this adult model will be used to validate compounds found to be neuroprotective with a higher degree of predictive validity.

Specific Aim: To characterize and validate a closed-head model of TBI in adult zebrafish.

4.5 Methods

Ultrasound injury was titrated so that induced brain trauma was non-fatal. Behavioural testing was used to establish dose-response endpoints. Protein analysis confirmed secondary cellular injury mechanisms. Hypothermia and hypoxic preconditioning were used as proof-of-concept interventions demonstrating the
model’s responsiveness to known neuroprotective therapeutics for TBI. Detailed descriptions of corresponding methods are described in section 2.0.

4.6 Results

4.6.1 Recovery Times post-ultrasound injury

Adult zebrafish were anesthetized and injured using the pHIFU system. Recovery times were measured from time of injury until fish were upright and swimming independently. As intensity of injury (in MPa) increased, recovery times of injured fish increased (Fig 13 A). At 1,000 cycles, mean recovery times were $4.7 \pm 0.51, 5.5 \pm 0.49$ and $8.7 \pm 1.1$ min at 1, 5, and 11 MPa respectively. Control recovery times remained constant at $1.9 \pm 0.18$ min. Once the maximum deliverable intensity possible on the pHIFU system was attained (11 MPa), this value was kept constant and duration of ultrasound pulse was then increased as a dependent variable. At 5 MPa, recovery times increased to 2,000 cycles ($5.1 \pm 0.42$ min) and continued until 10,000 cycles ($7.8 \pm 0.9$ min) (Fig 13 B). At 11 MPa, recovery times increased until 50,000 cycles where mean recovery times reached $11.0 \pm 1.1$ min (Fig 13 C).
Fig. 13. Recovery times. Data represent means±SEM for n=12 per time point. A: Recovery times following ultrasound injury increase with increasing pHIFU intensity. B-C: Recovery times increase with increasing pHIFU duration (number of cycles) at 5 and 11 MPa, respectively.
4.6.2 Behavioural response to pHIFU injury

4.6.2.1 Novel Tank Test

EthovisionXT software (Noldus) was used to analyze recorded video of zebrafish subjected to ultrasound injury. Locomotion and vertical exploration in the NTT were the outcome variables of analysis (Figs 14-17). Significant reductions in total distance travelled were observed at 11 MPa intensity (p=0.032) at 24 h post-injury (Fig 14 B). Mean velocities of the same subjects were significantly decreased at 5 MPa (p=0.048) and 11 MPa (p=0.027) at 24 h post-insult (Fig 14 E). In both outcomes, activity had recovered by 48 h post-injury (11 MPa distance p=0.84 and velocity p=0.18). Meander, a measure of turn angle divided by distance travelled, indicated a significant decrease immediately after injury as well as at 24 and 48 h. Immediately following injury and 24 h post-injury, 5 MPa and 11 MPa injury levels resulted in significant increases in meander (Fig 15 A,B) (p=0.019 and p<0.0001 respectively at 0 h and p=0.0066 and p<0.0001 at 24 h) while only the higher intensity of 11 MPa resulted in persisting increase by 48 h (p=0.021). The 11 MPa injury level also significantly affected the subjects' overall mobility. The percentage of time spent immobile (freezing) was significantly increased at 11 MPa at all three time points (p=0.033 at 0 h, p=0.00011 at 24 h, and p=0.0031 at 48 h) compared to pre-injury (Fig 15 D-F). The lower intensity (5 MPa) also resulted in significant increases in freezing at both 24 and 48 h post-injury (p=0.0012 and p=0.026) (Fig 15 E,F). Latency to the upper zone of the tank was used as a measure of vertical exploration and indicator for anxiety (Fig 16 A-C). The latency was significantly increased at 11 MPa immediately after injury with a mean of 254.2±47.48 seconds
(p=0.045). Control fish latency was 90.8 ± 43.0 seconds. Both 5 MPa and 11 MPa intensities resulted in significant increases in latency at 24 and 48 h post-injury.
Fig. 14. NTT: Locomotor activity. Summary of behavioural data for n=8. A: Analysis of total distance travelled during NTTs at 0 h, 24 h, and 48 h post-injury, depict a trend of decreasing distances with greater levels of ultrasound injury. Significant decreases in 11 Mpa-injured fish were found at 24 h post-injury (*p=0.032). B: Velocities of NTTs at 0, 24, and 48 h showed similar trends with significant decreases found at 24 h at both 5 MPa and 11 MPa intensities of injury (*p=0.048, *p=0.027).
Fig. 15. NTT: Locomotor activity (cont’d). Summary of behavioural data for n=8. A: Meandering measured at 0, 24, and 48 h post-injury found significant increases at both 0 and 24 h in 5 MPa (*p=0.019, *p<0.0001) and 11 Mpa intensities (*p=0.0066, *p<0.0001) as well as significant enduring changes in 11 MPa 48h-post injury (*p=0.021). B: Subjects’ overall movement depicted significant increases in percentage of time spent immobile at 11 MPa injury level at 0, 24, and 48 h, respectively (*p=0.033, *p=0.00011, *p=0.0031), while also displaying significant increases at the 5 MPa level at both 24 and 48 h post-injury (*p=0.0012, *p=0.026).
Fig. 16. NTT: Vertical exploration. Summary of behavioural data for n=8. Latency to upper zone was significantly increased post 11 MPa injury at 0, 24, and 48 h time points (*p=0.045, *p=0.024, *p=0.024) as well as at the lower injury level (5 MPa) both 24 and 48 h post-injury (*p=0.042, *p=0.018).
4.6.2.2 Shoaling Test

The open tank shoaling test was performed to measure social interaction (grouping) in injured and control (sham) adult zebrafish. Subjects were placed in a novel tank in groups of 5 fish of identical condition (n=5 fish per treatment group) and shoaling distance (mean distance between subjects) was measured following increasing intensities of ultrasound injury at 0, 24, and 48 h post-injury. Effects on shoaling behaviour were observed immediately after injury in all insult groups (1, 5, 11 MPa). Significant decreases in the distance between subjects in each group were observed following injury compared to controls (Fig 17 A). Similar effects were seen at both 24 and 48 h post-injury, although only in 5 and 11 MPa groups (Fig 17 B-C).
Fig. 17. Shoaling Test. Summary of shoaling data for n=10. Mean distances between subjects quantified from open tank Shoaling Test where 5 fish of identical conditions are monitored in an open tank for 10 min immediately after injury as well as 24 and 48 h post-injury. 1, 5, and 11 MPa-level injuries display significant decreases in distances compared to controls (*p<0.01) at 0h post-injury. Mean distances between subjects 24 h post-injury depict significant decreases at both 5 and 11 MPa levels of injury (*p<0.0001) and persist at 48 h post-injury (both *p<0.0001).
4.6.3 Protein Analysis

The expression of several protein markers of relevance to neurotrauma were examined at 6, 12 and 24 h after 11 MPa ultrasound injury. Neurofilament protein NF160, an abundantly expressed protein in myelinated axons, was found to have significantly higher levels of expression 12 h post-injury (Fig 18 B; p=0.029). The same time point revealed a significant increase in the expression of B-III Tubulin, a microtubule protein (Fig 18 C; p=0.047). Activated caspase-3 and beta APP, downstream markers of activated apoptotic pathways, were found to be significantly increased at 24 h post-injury (Fig 18 D, E; p=0.039 and p=0.042).
Fig. 18. Protein analysis. A: Representative western blots for medium neurofilament, neurofilament 160 (NF160), amyloid precursor protein (APP), microtubule protein B-III tubulin, and the activated form of caspase-3 in whole adult zebrafish brain tissue at 6, 12, and 24 h post-11 Mpa injury. B-C: Quantification (n=4 per time point), normalized to GAPDH expression, indicates significant increases in NF160 (*p=0.029) and B-III tubulin (*p=0.047) 12 h post-injury. D-E: Activated caspase-3 and APP quantification showed an increasing trend that was not significant until 24 h post-injury (*p=0.039 and *p=0.042, respectively).
4.6.4  *Hypothermia as an intervention*

Survival post-injury was significantly affected with varying temperature (Fig 19). Recovery carried out in normothermic conditions (25°C) resulted in a median survival of 8 days (n=10). By contrast, injured subjects recovered at 28°C died earlier (median survival of 5 days). When the temperature was decreased to 22°C, all subjects survived past 10 days. Control subjects at the highest temperature (28°C) had less than 1 % total death.

Behavioural testing performed at 0, 24, and 48 h post-injury was used to compare recovery of injured fish at 25°C and 22°C. In the NTT test (n=6), injured fish recovered at 22°C had significantly increased distance (p=0.046, p=0.036) and velocity (p=0.045, p=0.038) both 24 and 48 h post-injury compared to controls (Fig 20). Both meander and percentage of time spent immobile were significantly decreased at 24 (p=0.0073 and p=0.0022) and 48 h (p=0.0098 and p=0.016) in the 22°C groups compared to injured fish recovered at 25°C (Fig 21). Latency to upper zone was also decreased at the same time points in the 22°C group (p=0.017 at 24 h and p=0.016 at 48 h) (Fig 22).
Fig. 19. Hypothermia: Effect on survival following 11 MPa pHIFU. Data represent percent survival for n=10 (logrank test for comparing survival curves). Survival proportions of adult fish injured at 11 MPa and recovering in decreasing temperatures indicate significantly less death at both 25 and 22°C compared to injured fish kept at 28°C (p<0.0001).
Fig. 20. Hypothermia: NTT. Summary of behavioural data for n=6. Effects of decreased temperature on locomotor activity and vertical exploration. Behavioural monitoring and analysis shows improved performance at 22°C compared to standard 25°C zebrafish water. Panels A-C show significantly decreased total distances travelled in NTT tests of 11 MPa-injured fish at 22°C (*p=0.046 as compared to 25°C injured fish) both 24 and 48 h (*p=0.036) post-injury. Panels D-F show similarly significant trends in significantly rescuing velocity at both 24 and 48 h (*p=0.04541 and *p=0.038, respectively).
Fig. 21. Hypothermia: NTT (cont’d). Summary of behavioural data for n=6. (A-C) Meandering (*p=0.0073 at 24 h and *p=0.0098 at 48 h), and (D-F) percentage of time spent immobile (*p=0.0022 at 24 h and *p=0.016 at 48 h) as compared to identically injured fish at 25°C.
4.6.5 *Hypoxia preconditioning*

Zebrafish preconditioned using transient hypoxia before injury (n=5) had significant improvements in locomotor and vertical exploration measured at 24 h post-injury (Fig 23). Distance travelled and velocity both increased significantly compared to injured groups without preconditioning (Fig 23 A; p=0.039 and B; p=0.016). Meander and time spent immobile were significantly decreased with hypoxic preconditioning (Fig 23 C; p=0.038 and D; p=0.039). Latency to the upper zone was also significantly decreased as compared to injured subjects without preconditioning (Fig 23 E; p=0.043). Outcome variables were not significantly different from control values at the same assessment time points and injured values remained significantly different (p<0.05) than controls.
Fig. 22. Hypothermia: NTT vertical exploration. Summary of vertical exploration data for n=6. Effects of decreased temperature on vertical exploration. A-C: latency to upper zone is significantly decreased in injured fish left to recover at 22°C at both 24 and 48 h post-injury (*p=0.017 and *p=0.016, respectively).
Fig. 23. Hypoxic Preconditioning: NTT. Summary of behavioural data for n=5. Effects of hypoxia preconditioning on locomotor activity and vertical exploration 24 h post-injury. A-D: Endpoint analysis of locomotor parameters showing effects on distance travelled, velocity, meandering, mobility/freezing. E: Vertical exploration measured by latency to upper zone (*p<0.05).
5.0 GENERAL DISCUSSION

5.1 Part 1: Larval Model

Animal models have been crucial for understanding the pathobiology of human disorders. TBI is a complex process causing structural damage and functional deficits that are due to both primary and secondary injury mechanisms (Kolias et al., 2013; E. Park et al., 2008). Therefore, a model's value is based on its design and characterization of a system that has objective translational value. The use of animals to model human disease in biomedical research requires that fundamental cellular processes are similar across species to allow translation and extrapolation. Specifically, when the results of an animal study are intended to be translated into human treatments, the ultimate proof of a model's value is its predictive validity (McKinney, 1984; Varga et al., 2010; Willner, 1991). The ability of zebrafish to recapitulate mammalian pathophysiology with rapidly quantifiable outcome measures (e.g., behaviour and cell death) makes them ideal model organisms. The added advantages of time- and cost-efficiency make them a powerful tool for drug discovery. The ease of applicability of the zebrafish larvae to numerous replicates using 96-well plates add to their effectiveness as a high-throughput model organism.

In this study, we first attempted to model the response of zebrafish larvae to chemical injury. Applying glutamate directly in the water, we induced death curves indicative of a dose-dependent response to excitotoxic injury (Fig 9). Behavioural analysis of glutamate excitotoxic injury revealed a more sensitive endpoint. Dose-
dependent outcomes were observed at earlier time points using automated software (Fig 10). The 10 uM concentration of glutamate allowed for significant margins of differences in behavioural outcome measures and avoided the use of early death as a primary endpoint, thereby making the injury more clinically relevant. Increasing concentrations of MK-801 and MDL-28170 produced a dose-dependent relationship between neuroprotection and improved behavioural outcome (Fig 11). Both compounds are known to have successful effects in established mammalian models of TBI (Ai et al., 2007; Faden et al., 1989) and provide evidence that the larval model is responsive to interventions, which are comparable to the mammalian models. Behavioural analysis was performed using automated software that removed potential observer bias thereby providing objective endpoint analysis. Behavioural outcomes provide a measure for milder injury progression, detectable well before death. It is interesting to note that the behavioural analysis was sensitive enough to detect statistically significant differences in both distance and velocity at 5 uM as well as 10 and 20 uM insult.

The 200 nM and 400 nM concentrations of MK-801 produced significant effects in preventing glutamate-induced injury. A second inhibitor, MDL-28170, was also evaluated but provided less effect than MK-801. The less profound effects can be attributed to the role of calpain overactivation in the secondary injury cellular cascade. In contrast to MK-801, which targets the upstream initiator of the excitotoxicity cascade, calpain’s role lies further downstream of calcium-mediated pathways. There are likely other injury pathways being activated, which are not accounted for in the inhibition of calpain. Nonetheless, these data not only
demonstrate a progressive and persistent pathophysiological response to glutamate injury, but also demonstrate the model’s sensitivity to detecting changes in downstream intervention therapy strategies. This has significant implications for identification of novel pharmacological treatments for the secondary injury mechanisms associated with TBI. The larval model of injury is therefore amenable to intervention, and displays predictive validity in its response to known interventions.

Because drug absorption in larvae is mediated through full-body water exposure, the specificity of glutamate excitotoxicity was verified to ensure brain-specific effects. Confocal imaging of fluorescence in the secA5-YFP transgenic strain was used to ensure injury and subsequent behavioural deficits were not due to global cell death or injury to areas other than the larval brain. The genetically encoded secA5-YFP marker allows noninvasive in vivo quantification of apoptosis over time, which we visualized only in larval brains. Morphological changes such as axonal blebbing, and fragmentation of degenerating axons were evident in the in vivo brain of glutamate-injured larvae (Fig 12). Such morphological changes are hallmarks of axonal damage due to secondary injury following TBI (Andriessen et al., 2010; Johnson et al., 2013; K. J. Park, Park, Liu, & Baker, 2014). MK-801 also demonstrated non-specific binding of the fluorescent tag on the surface of the larvae. This non-specific binding was observed in both glutamate-injured larvae as well as controls treated with the inhibitor alone. As such, this fluorescence was not included in quantification analyses.
The zebrafish larvae’s cellular response to excitotoxic injury is consistent with human and animal pathological changes in cell structure (Cernak, 2005; Dashnaw et al., 2012; Giza & Hovda, 2001; Xiong et al., 2013). These cellular changes are associated with behavioural consequences resulting in locomotor deficits. The sensitivity of zebrafish larvae to chemical intervention known to be effective in other animal models further validates the translational potential of zebrafish for drug evaluation and screening.

Glutamate excitotoxic injury was used in the zebrafish larval model as a well-defined initiator of the secondary injury mechanisms of TBI and to provide a clear proof-of-concept of the amenability of the model to intervention and evaluation. Although not a clinically successful target, glutamate receptor antagonism is the most upstream signaling pathway in the injury cascade that follows TBI (H. S. Levin & Robertson, 2013; Walker & Tesco, 2013). The limited effectiveness of the calpain inhibitor, MDL-28170, further demonstrates this concept of upstream targeting having greater impact on outcome. Using the larval injury model, we have reproduced a key aspect of TBI pathophysiology. This model has the potential to be further built upon by adding other chemical modulators of physiological function to model variables associated with TBI, such as vascular injury. Our model is simplified in design and is specifically aimed at high-throughput preliminary evaluation of potential drug discovery and re-purposing. In addition to the advantages of simplicity and reproducibility of injury, using a chemical injury paradigm offers a noninvasive insult that avoids potential confounding aspects of mechanical injury. This allows the isolation of specific secondary mechanisms from
primary insults, which aids in the interpretation and reliability of results obtained from therapeutic drug screening assays. In summary, our model of TBI allows for pathway-specific, well-controlled, noninvasive, reproducible conditions.

Numerous animal models of TBI have been used to elucidate cellular properties of trauma (Elder et al., 2012; Nakagawa et al., 2011; E. Park et al., 2013; Taylor & Ford, 2009). These models include recent developments in primary blast trauma and its contribution to mTBI and CTE sequelae (Goldstein et al., 2012a; McKee et al., 2009; McKee et al., 2013; Mez et al., 2013). The screening of therapeutic compounds, however, remains a laborious and time-consuming process. The lengthy pre-clinical development phase has been a significant hurdle in the identification of novel chemical entities or signalling pathways amenable to treatment of TBI. Zebrafish exhibit methodological advantages for brain imaging and behavioural phenomics, both ideal for high-throughput screening critical for CNS drug discovery and identifying novel candidate targets for intervention (Kalueff et al., 2014). The larvae’s size and ability to live in volumes as small as 100 microliters make them particularly suited to high-throughput and high-content compound screening in 96-well plate format. Larvae also possess an undeveloped BBB up to 10 days post-fertilization (Fleming, Diekmann, & Goldsmith, 2013), allowing direct uptake of neuroprotective compounds from water allowing for greater bioavailability to determine drug effectiveness. Successful chemical screens have already been performed in this way (Barros et al., 2008; S. Guo, 2009; Lockwood, Bjerke, Kobayashi, & Guo, 2004; Maximino et al., 2010; Xiong et al., 2013; Zon & Peterson, 2005). Recent evidence confirms that the zebrafish's ease of
experimental manipulation and observation and overall feasibility as a behavioural-based, whole-organism screening model (Braga et al., 2013; Cachat et al., 2011; Cachat et al., 2013; Maximino et al., 2010; A. M. Stewart et al., 2014; A. M. Stewart et al., 2014).

5.2 Part 2: Adult Model

Does another animal model bring added value to the field of research? This question has been tackled from the standpoint of many symptoms and diseases, including TBI. We propose that the use of zebrafish as a model organism can address the time and cost hurdles associated with pre-clinical drug discovery and therapeutics in TBI. Specifically in closed-head trauma, animal models have already been used extensively to model individual aspects of TBI pathology, including molecular and functional outcomes (Angoa-Perez et al., 2014; Cernak, 2005; Davidson, Lindsey, & Davis, 1987; Garner et al., 2009; Gennarelli, 1994; Marklund & Hillered, 2011; Morganti-Kossmann et al., 2010; O’Connor et al., 2011; Xiong et al., 2013). However, despite an increasing knowledge of secondary injury mechanisms, the advancement of therapeutics to treat TBI has met with limited, if any, clinical success. The zebrafish offers not only key homology to humans, but is uniquely capable of high-throughput screening and automated endpoints.

In terms of validity, the adult model of TBI in zebrafish conforms to requirement for predictive validity, that is, the ability of animal models to accurately and reliably depict the human condition. The adult injury model demonstrates consistency in outcome, as well as ease of replication in larger numbers. Our results
confirm both specificity and reliability across each endpoint. The model’s relevance to human pathophysiology was evaluated using molecular and behavioural outcome measures. Although it is difficult, if not impossible, to model the entire complex process of TBI in an animal, selected processes and symptoms have successfully been modelled in a range of species. In our adult zebrafish model, we created a representation of closed-head injury through ultrasound insult. In contrast to the larval model, the adult model included injury effects from primary and secondary injury elements associated with TBI. The endpoints, both molecular and functional, are representative pieces of this secondary cascade of injury. The larval and adult injury models can potentially be used in conjunction to identify and validate potential therapeutic compounds.

Initially, recovery times following injury were used as an endpoint and were consistent with response to injury level. Post-injury times increased in a dose-dependent manner in response to increases in intensity and duration (Fig 13). Both variables were correlated with titratable outcome measures. However, it should be noted that variability in response was high; recovery times varied between subjects within the same treatment group with large standard errors. Death was not observed at any injury level below 8 MPa and constituted less than 1 % of subjects between 8-11 MPa. In establishing a titratable model, we used increasing intensities (1, 5, and 11 MPa) for behavioural studies and kept duration (cycles of ultrasound per insult) constant. Each cycle duration was 1 microsecond, thus 10,000 cycles constituted a 10 ms injury. This is in line with average closed-head impacts that last on average 10-20 ms, with peak forces at 12-15 ms (Pellman, Viano, Tucker, Casson,
& Waeckerle, 2003b). Our data also correspond with military blast estimates, as cited in Pellman et al., that use 15 ms injury duration averages in research models (Pellman, Viano, Tucker, Casson, & Committee on Mild Traumatic Brain Injury, National Football League, 2003). Impact duration in other TBI research models, however, present very wide margins with no clear consensus; they can range from microseconds to multiple seconds. Shorter durations have been implicated in high impact instances; car crashes involving rails, pillars, and hard structures, for example, last less than 6 ms on average (Pellman et al., 2003b). It is known that accidents involving impact with seatbelts and airbags, however, can last over 40 ms (Pellman et al., 2003b). Our choice of 10 ms insults, therefore, is intended to represent a common intersection between closed-head concussion and military estimates, representative of the greatest body of research available (Pellman et al., 2003; Pellman et al., 2003b; Viano, Casson, & Pellman, 2007).

The adult pHIFU injury model demonstrates changes in white matter protein expression. Expression of axonal cytoskeletal proteins and activated caspase-3 are consistent with the response to trauma in rodent models of non-penetrating TBI. In traumatic axonal injury, the results of complex secondary injury cascades following TBI, ultrastructural alterations of neurofilaments cause axonal swelling and interrupted axonal transport. Phosphorylation and instability of neurofilament protein (Nakamura et al., 1990; Nixon, 1993; Sternberger & Sternberger, 1983) might account for the increase in NF160 (E. Park, Liu, Shek, Park, & Baker, 2007) as well as microtubule B-III tubulin at 12 h post-injury. This process of axonal disconnection (axotomy) has been observed as early as 4 h after injury in animal
models (Maxwell et al., 2003; Povlishock & Christman, 1995; Saatman et al., 2003) and has been reported to persist for days to weeks in human pathobiology (Blumbergs et al., 1994). The delayed increase of caspase-3 and APP, found at 24 h post-injury, can be attributed to their downstream places in secondary injury cascades. Caspase-3 is part of an apoptotic pathway initiated by mitochondrial dysfunction and cytochrome-c, among others. APP is a protein cleaved by β-secretase (BACE-1) and δ-secretase, leading to the generation of Aβ, an amino acid peptide implicated in long-term neurodegeneration and AD. TBI is one of the strongest risk factors for the development of neurodegeneration and AD, and increased levels of APP and BACE-1 have been identified in damaged axons following human TBI (Y. Chen et al., 1996; Uryu et al., 2007). In summary, early alterations in neurofilament and microtubule expression is followed by increased markers of apoptosis and axonal injury. These results suggest that the cytoskeletal response to ultrasound trauma is delayed and attributable to secondary injury mechanisms rather than primary injury due to shearing and stress.

The complex behavioural repertoire of zebrafish has been evaluated in a variety of tests (R. E. Blaser et al., 2010; R. E. Blaser & Rosemberg, 2012; Egan et al., 2009; Maximino et al., 2010). The NTT evaluates locomotor and vertical exploratory activity in a novel environment. Zebrafish initially dive to the bottom and explore horizontally before gradually swimming to the upper levels of the tank (E. D. Levin et al., 2007). Numerous studies have evaluated the NTT pharmacologically using anxiogenic and anxiolytic manipulations as assessment tools to evaluate behavioural changes in zebrafish (Bencan et al., 2009; R. Blaser & Gerlai, 2006;
Cachat et al., 2010; Grossman et al., 2010; Lopez Patino et al., 2008; Rosenberg et al., 2011; Severi et al., 2014; Wong et al., 2010). In our study, adult zebrafish subjected to pHIFU demonstrated locomotor swim deficits, as well as complex behavioural changes in the NTT. Locomotor outcome measures, including total distance travelled and mean velocity, were significantly decreased at 24 h post-injury. Meander, a measure of unspecific swim path, and the percentage of time subjects spent immobile (freezing behaviour), indicated increases at 24 h post-injury as well as immediately after recovery. These effects also persisted until 48 h post-injury. This may indicate that behaviour is a more responsive endpoint to mild injury.

Latency to the upper zone is a measurement widely used to test vertical exploration within the NTT (Cachat et al., 2011). The vertical exploration measure represents the tendency of zebrafish to gradually explore the top areas of a novel environment, which suggests habituation to novel environments and reduced anxiety (R. Blaser & Gerlai, 2006; Rosenberg et al., 2011). We observed latency to the upper half of the tank was maintained from immediately after injury to 48 h post-injury. Notably, this endpoint was also sensitive to 5 MPa injury, as well as 11 MPa at both 24 and 48 h time points. This represents a sensitive measure for complex behaviour deficits after TBI in zebrafish.

Open tank tests involving multiple fish allows for observation of shoaling (grouping) interactions that can be used to evaluate social behaviour. Zebrafish display characteristics of social behaviours that have been well-documented (Grossman et al., 2010; Kalueff et al., 2014; Miller & Gerlai, 2007). Social dysfunction is often apparent in psychiatric disorders and can be influenced by
genetics, chemicals, environment, and disease (Green et al., 2012; Kalueff et al., 2013; A. Stewart et al., 2012). In our study, social interaction was measured by the mean distance found between subjects in a group. This measurement was significantly decreased at all injury levels (1, 5, and 11 MPa) immediately after injury, as well as in both 5 and 11 MPa levels 24 and 48 h post-injury. These results are consistent with documented studies demonstrating increased anxiety in response to predatory stress responses in the grouping activities of zebrafish (Grossman et al., 2010; Miller & Gerlai, 2007). Our results suggest increased anxiety-like responses after trauma, replicating mammalian response following TBI (Elder et al., 2012; Malkesman, Tucker, Ozl, & McCabe, 2013). The two behavioural testing paradigms can be used to evaluate the zebrafish response to injury and potentially intervention post-insult.

There was a distinct delayed temporal profile of the molecular and functional changes in our injury model. We observed delayed activation of cell death pathways, as well as evidence of axonal injury consistent with secondary cellular mechanisms. Behavioural endpoints were most significant at 24 h post-injury, with many outcomes persisting until 48 h. These data not only demonstrate a progressive and persistent pathophysiological response to ultrasound injury, but they may also have significant implications for potential windows of opportunity for the pharmacological treatment of closed-head, non-penetrating TBI pathophysiology.

These implications are further strengthened by evidence that our adult model system is responsive to intervention. First, we plied the concept of hypothermia to treat TBI (Sherman & Wang, 2014) as a possible therapeutic
intervention during recovery. Initial pilot studies have confirmed improved functional outcomes following hypothermia intervention. By lowering water temperature in our model by 2°C, we were able to rescue behavioural deficits post-injury. Immediately after initial recovery from anesthesia (0 h post-injury time point, approximately 30-40 min post-ultrasound injury) subjects at lower temperature depicted small changes in locomotor outcomes as compared to injured fish kept at 25°C. Increases in distance travelled and velocity, as well as decreases in meander and freezing, and decreases in latency to upper zone, became significant at 24 h post-injury. These ameliorations observed in 2°C of hypothermia continued until 48 h. These results indicate our model is responsive to intervention and analogous to results from similar interventions in human trauma care.

A second proof-of-concept intervention has also been previously validated in mammalian trauma and diseases and opens novel treatments for TBI. Hypoxic preconditioning is a physiological phenomenon in which transient episodes of sublethal hypoxia induce neuroprotection against subsequent ischemic injuries. Preconditioning has been observed in multiple organisms and found to confer protection against varying methods of insults, including those that differ from the type of stress induced originally. Its precise mechanisms, however, are largely unknown. Animal studies suggest the brain can be preconditioned to acute injuries, such as ischemic stroke, hypoxia/ischemia insults, surgical brain injury, as well as trauma. In our study, we applied brief episodes of hypoxia to zebrafish 30 minutes before non-penetrating ultrasound injury. In subsequent behavioural testing, preconditioned groups demonstrated significant improvements in each endpoint 24
h post-injury. Our model also allows for further study into the mechanism of unknown possible therapeutics as well as current targets of TBI research. Preconditioning has been postulated to induce neuroprotection through neural, as well as humoral, pathways (Stetler et al., 2014). The possible underlying mechanisms of hypoxic preconditioning remain unclear and the ability to study their intricacies using this zebrafish model strengthens its influence in TBI research.

5.3 Summary of Major Findings

We have developed and characterized a chemical model of TBI in zebrafish larvae with the goal of developing a high throughput, automated system for drug evaluation to treat TBI. We simultaneously developed and characterized an adult model of closed-head TBI using ultrasound technology. In both models we determined dose-dependent response to injury demonstrating a titratable model with responsiveness to intervention. Our results also indicate that zebrafish exhibit responses to injury and pharmacotherapeutic intervention similar to mammalian pathophysiology after TBI. This suggests the possibility of using our zebrafish injury model system to screen compound libraries to identify potential therapeutic candidates for TBI in a manner that increases efficiency over existing rodent-based pre-clinical TBI models.
6.0 LIMITATIONS

The scope of this injury model, namely the larval model of injury, is limited in certain respects. As previously discussed, the use of glutamate itself can be viewed as controversial since methods of neuroprotection involving the suppression of glutamate excitotoxicity have previously failed in clinical applications. The use of glutamate-mediated injury represents a single, albeit significant, arm of the secondary injury mechanisms that follow TBI. Here, we use glutamate as a known mechanism of injury that acts at the top of the secondary injury cascade. Although glutamate receptor antagonists may not be a clinically feasible or effective target, they reveal the concept of responsiveness in the injury model to quantifiable outcome measures, including behaviour and apoptotic cell identification. Presumably other candidate compounds acting further downstream of glutamate receptor overactivation could be used to model specific aspects of the secondary injury cascade. As demonstrated here, calpain, which targets the increased calcium overload and lies downstream of the injury cascade, was also effective in reducing glutamate-mediated excitotoxicity, although less so than direct glutamate receptor antagonism. Antagonists and inhibitors acting in any of the other numerous (and interconnected) pathways within the secondary injury cascade hold neuroprotective potential. These could be known or discovered in screening, thus elucidating potentially unclear mechanisms.

In the adult portion of the model, limitations exist in documenting the molecular and anatomical hallmarks of TBI while maintaining high-throughput capacity. The western blot as means of protein marker analysis is labour-intensive
and thus more of a characterization of the injury model than an endpoint to be employed in future drug screens. Histology and sectioning, as previously discussed, were also found to be too inconsistent to properly represent injury markers in the adult zebrafish.
7.0 CONCLUSIONS

The data presented in this thesis contribute to a novel model system of TBI. The development of treatments for TBI, mTBI, and concussion is an important public health issue given the number of athletes participating in contact sports that involve high risk of brain trauma, military troops frequently exposed to blast events, and the prevalence of motor vehicle accidents and occupational hazards. The use of animal models has been extensively adopted to investigate pathophysiology and treatment of TBI. The time and cost to evaluate chemical entities for therapeutic benefit are long and high. Zebrafish have the potential to address this important pre-clinical hurdle. By capitalizing on the unique properties of zebrafish as a model organism, we have presented an efficient system to address the cost- and time-dependent barriers associated with pre-clinical drug development for TBI.

This thesis characterizes a dose-dependent excitotoxic brain injury in larval zebrafish that is brain-specific, amenable to intervention, and suited for high-throughput screening. Specifically, the larval zebrafish model, consisting of exposure to toxic concentrations of glutamate, exhibited reliable injury that was characterized by readouts of survival proportion and behavioural methods. The readouts demonstrated that they were quantifiable with relatively narrow confidence intervals. With these readouts, we also demonstrated the impairment in a dose-dependent manner, as well as the attenuation of impairment by glutamate receptor antagonist. The larval model was also brain-specific as evidenced by apoptotic cell-surface markers demonstrated in larval brains only. Lastly, the model included downstream pathways (initiated by glutamate but not involving
glutamate) as evidenced by attenuations of behavioural measure impairments using a known antagonist of this downstream pathway. Therefore, our larval zebrafish model of excitotoxic brain injury has been characterized in a manner that will allow its evaluation as a tool to identify compounds that may be subsequently assessed for their ability to attenuate secondary traumatic brain injury.

Secondly, we have developed and characterized a closed-head injury in an adult fish using ultrasound technology with the aim to validate pharmacotherapeutics identified in the larvae screening system. Currently, the only models of brain injury in zebrafish are crude, involving direct stab or penetration into brain tissues with specific focus on regeneration. These models do not adequately characterize subclinical, closed-head trauma or the subsequent injury mechanisms. Here, we report the generation of a first vertebrate animal model of TBI in the zebrafish. Our adult zebrafish model of TBI, consisting of ultrasound-induced trauma, exhibited a reliable injury that was characterized by readouts of behavioural measures. These readouts demonstrated that they were quantifiable by automated means with relatively narrow confidence intervals. These readouts also demonstrated impairment in a dose-dependent manner. Finally, our adult model demonstrated responsiveness to two interventions with previously established lines of evidence of neuroprotection: hypothermia and hypoxic preconditioning. To conclude, our adult zebrafish model of TBI has been characterized in a manner that will allow its evaluation as a tool to assess candidate compounds that may attenuate secondary traumatic brain injury. Together, these two models are an important novel tool for investigating the mechanisms of downstream secondary TBI, and for
the implementation of high-throughput screening of pharmacotherapeutic compounds.
8.0 Future Directions

Our larval zebrafish model is simplified and technically easy to manipulate. Next steps could include the combination of this excitotoxic injury model with other injury mechanisms, including vascular injury and inflammation. These are common results of both primary and secondary injury following TBI. Therefore their inclusion in a larger model to approach a more encompassing representation of injury in larvae is interesting and valuable. Their value, however, must be weighed against the risk of deterioration of high-throughput screening. Mechanical injury and labour-intensive protocols may defeat the advantage of the use of chemical compounds to replicate injury with the key purpose of simplifying the system for screening.

Again, the translational value of this zebrafish injury model lies in its high-throughput, automated capacities. We propose using this two-stage model to screen commercially available compound libraries. Logically, having characterized the model, the next step lies in screening available libraries. Both FDA-approved and novel compound libraries are readily available for screening and automated facilities exist. We are currently in the process of optimizing systems to perform initial screenings in our larval model. This work employs a state-of-the-art automated zebrafish facility that capitalizes on the genetic manipulation techniques of the zebrafish. The technology can screen thousands of compounds for neuroprotective or axonoprotective effects in zebrafish larvae using our excitotoxic injury model. These compounds can then be applied to our adult zebrafish model of
TBI to be validated with a higher degree of similarity to mammalian pathophysiology.

Future possibilities are also present in the adult model’s potential capability to recapitulate aspects of mTBI. Depicting anatomical hallmarks of TBI is a logical future direction in building upon this model. Bleeding, as well as cytoskeletal and axonal damage are commonly identified as markers of secondary injury in animal models of TBI. High-powered confocal imaging, as well as dissection and tractography techniques, can foreseeably be employed to detail the pathological mechanisms of our ultrasound injury and foreseeably identify these hallmarks. Specifically, vibrotome sectioning of adult brains in agar might be employed to stain and image adult zebrafish brains. This technique could allow the precise and consistent sectioning we have lacked in our histology efforts. In this way, demonstrating specific anatomical data following ultrasound insult would further refine our model of adult TBI. Comparing anatomical and histological data to the mechanisms and markers already detailed in both clinical and animal models may provide further detailed insight into TBI itself, as well as confirm our model’s face validity.

Transparent lines of zebrafish, such as the casper line (White et al., 2008) that maintains transparency into adulthood, may also be useful in our adult model. If we can visualize bleeding or other markers of injury and/or secondary injury post-ultrasound insult, we could confirm our behavioural and molecular data in another high-throughput manner. This technology may also be used in conjunction with or in addition to Brainbow methods. As described by Pan et al. in 2013,
zebrafish have been employed with imaging and cell labeling techniques known as Brainbow (thus termed by Pan et al. as Zebrabow), where Cre recombinase-mediated recombination can lead to expression of distinct fluorescent proteins in neighbouring cells. Pan shows that specific Zebrabow lines can be used to “generate broad or tissue-specific expression patterns and facilitate tracing of axonal processes” (Pan et al., 2013). Applying these genetic lines in our adult model of TBI could provide no-touch endpoints that confirm our molecular findings, such as cytoskeletal breakdown or programmed cell death. This would be particularly useful in maintaining the high-throughput screening integrity of our adult model while further validating its effectiveness in recapitulating mammalian aspects of TBI.

Following the evaluation of therapeutic effects of candidate compounds in our adult model, we propose an in depth analysis of the affected molecular pathways as they relate to secondary TBI in higher animal models of mTBI. Our laboratory (the Baker lab) has developed a clinically relevant model of mTBI in rats using an open-ended shock tube device capable of producing a subclinical shockwave. This closed-head injury is similar to our ultrasound delivery characterized here in our adult zebrafish model of TBI. By comparing these validated pathways of secondary cellular injury, the goal would be to avoid redundancies and replication of previously targeted pathways that have met with clinical failure while at the same time identifying novel pathways for intervention. Using our larval model and automated screening, candidate compounds and their specific targets can be identified. Their application in the adult model may then reveal novel targets for neuroprotection.
The mechanistic possibilities also apply to unknown pieces of secondary injury. Parsing out individual pathways in the complicated and interconnected cascade can be completed with this adult zebrafish model of TBI. Because the comparable anatomy of the zebrafish brain to mammals is well-documented and quickly improving in scope and detail, insights into unknown pieces of the secondary mechanisms of TBI may be discovered in future studies. Candidate compounds found in screening may reveal not only new therapeutic targets but also new pathways, or pieces of pathways, themselves. Researchers are already aware that our knowledge of secondary TBI pathways is limited. The targets of candidate compounds found in screening could very well be unknown pieces within the secondary cellular cascade and the specific functions of these targets could then be detailed with further studies.

In order to refine our adult zebrafish model of TBI, a future possibility would be automation of the ultrasound insult itself. As it is characterized here, the adult behavioural endpoints are completely automated and conducive to large-scale high-throughput studies. The ultrasound insult, however, entails anesthetising and immobilizing individual fish and injuring them within our ultrasound set-up one at a time. Next steps could include creating a specialized tray that could hold multiple fish in place and allow sequential injury to each fish. The tray could be automated to position each specimen under the focused ultrasound target, positioning each brain for injury one after another. In this way, the entire adult zebrafish injury process could be automated and adapted to join the behavioural and social endpoints in their application to large-scale screening studies. This would allow faster screens
and validations of more candidate compounds, furthering the goal of bridging translational drug discovery from the bench to the bedside.

The behavioural and social analysis of zebrafish may also be useful in further studies associated with TBI. Our initial characterization of behaviour and social interaction following trauma may apply to study of TBI and mTBI from a social psychology standpoint rather than a purely locomotor and mobility deficit position. The correlation to anxiety and stress may have correlations to PTSD and post-trauma psychological effects that afflict military personnel as well as accident and trauma sufferers. Behavioural endpoints quantified through automated image capture and analysis systems may also extend to memory and cognition studies. Tests such as a T-maze, often used in rodent models of TBI as well as other neural disorders, may be applicable in our adult model of TBI to study memory and conditioning in zebrafish following varying levels of injury.
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