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The KATP -Knockout Mouse: A Model of Susceptibility to Post-Concussion Syndrome

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The K_{ATP} -Knockout Mouse: A Model of Susceptibility to Post-Concussion Syndrome

by

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Abstract

Approximately 1.4 million people in the United States each year sustain a mild traumatic brain injury (mTBI). Unfortunately, approximately 15% of these patients will have post-concussive symptoms that last longer than three months. Knowledge of the pathophysiological processes underlying susceptibility to poor outcomes is limited, which has impaired the development of effective therapeutics for patients with a mTBI. The present study was designed to assess behavioral and molecular changes post-mTBI in an animal model of genetic susceptibility to poor neurological outcomes, the Kir6.2-knockout mouse. We conducted multiple behavioral tests and analyzed the cellular stress response through assessment of heat shock protein (*Hsps*) gene expression. We hypothesized that following mTBI, Kir6.2-knockout mice would have increased behavioral deficits, and aberrant *Hsp* expression. Results from behavioral and molecular analyses demonstrated that outcomes post-mTBI depended on sex and genetic make-up, and that the influence of these factors changed throughout the recovery process.

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This thesis is dedicated to my mom and dad,

Wanda and Peter Farran.

Not only do you both exceed the role as wonderful parents,

But you are also the two best friends anyone could ask for.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ACC	Animal Care Committee
AD	Alzheimer's Disease
ADHD	Attention Deficit/Hyperactivity Disorder
ADP	Adenosine Diphosphate
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
CCAC	Canadian Council on Animal Care
CCI	Controlled Cortical Impact
DAI	Diffuse Axonal Injury
cDNA	Complimentary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
E_2	17β-estradiol
EPM	Elevated Plus Maze
ER	Endoplasmic Reticulum
FPI	Fluid Percussion Injury
FST	Forced Swim Test
GCS	Glasgow Coma Scale
Grp	Glucose-Related Protein
GnRH	Gonadotropin Releasing Hormone
Hsp	Heat Shock Protein

K^+	Potassium Ion
K_{ATP} channel	ATP-Gated Potassium Channel
Kir6.2-KO	Potassium Inward Rectifier 6.2 Channel Knockout
Kir	Potassium Inward Rectifier
LOC	Loss of Consciousness
Mg-ADP	Magnesium-bound ADP
mPFC	Medial Prefrontal Cortex
mTBI	Mild Traumatic Brain Injury
MWT	Morris Water Task
OF	Open Field
P	Post-Natal Day
PD	Parkinson's Disease
PCS	Post-Concussion Syndrome
PFC	Prefrontal Cortex
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SUR	Sulfonylurea Receptor
TBI	Traumatic Brain Injury
WD	Weight Drop

Chapter One: **Introduction**

1.1 Mild Traumatic Brain Injury

1.1.1 Epidemiology

Traumatic brain injury (TBI) is one of the most common causes of death and disability in the United States, and affects over 1.7 million people in the United States each year. Annually, it accounts for over 1.3 million visits to the emergency department, 275 000 hospitalizations, and approximately 52 000 fatalities (Faul, Xu, Wald, & Coronado, 2010). As a result, TBI has become a significant public health concern. Studies have estimated that the direct and indirect cost of care for TBI amount to approximately $60-221 billion per year (Coronado et al., 2012; Finkelstein, Corso, Phaedra, & Miller, 2006; Langlois, Rutland-Brown, & Thomas, 2004). Furthermore, studies assessing the quality of life in patients and their families following TBI indicate that employment status, quality of leisure activities, pain, emotional distress, and social relationships are significantly impaired, and the extent of these disabilities are independent of injury severity (Kersel, Marsh, Havill, & Sleigh, 2001; Kolakowsky-Hayner, Miner, & Kreutzer, 2001; Upadhyay, 2007; van der Naalt, van Zomeren, Sluiter, & Minderhoud, 1999). This places a significant burden on the individual sustaining the injury, their friends and family, as well as society as a whole.

For the vast majority of patients who experience a TBI, approximately 80%, the injury is classified as mild (mTBI) or in clinical populations, a concussion. As this statistic only accounts for those cases that were presented to medical attention, it is possible that the prevalence of

concussion is even higher. A working definition of concussion, as outlined by the WHO Collaborating Centre Task Force on mTBI (Carroll et al., 2004) is that it is:

"An acute brain injury resulting from mechanical energy to the head from external physical forces. Operational criteria for clinical identification include:

(1) One or more of the following:

i. Confusion or disorientation

ii. Loss of consciousness for 30 minutes or less

iii. Post-traumatic amnesia for less than 24 hours

iv. Other transient neurological abnormalities (focal signs, seizure, or intracranial lesion not requiring surgery)

(2) Glasgow Coma Scale score of 13-15 after 30 minutes post-injury or upon presentation to healthcare

These manifestations must not be due to drugs, alcohol, medications, caused by other injuries or treatment for other injuries, caused by other problems, or caused by penetrating craniocerebral injury."

A concussion or mTBI may be caused by a blunt force to the head, but can also result from acceleration/deceleration forces on the brain in the absence of any external trauma. The most common sources of concussion when combining all age groups are falls (35.2%), motor vehicle accidents (17.3%), being struck by an object (16.5%), or assault (10%; (Faul et al., 2010). Following injury, patients typically report a sequelae of symptoms, which may or may not include headaches, motor deficits, learning and memory impairments, anxiety, depression,

attention deficits, and/or changes in sociability (Ryan & Warden, 2003). However, in most cases, symptoms are transient and resolve on their own.

1.1.2 Mild Traumatic Brain Injury Versus Concussion

While the terms mTBI and concussion are often used synonymously in the literature, there are a few important distinctions. The term "concussion" is most often used to describe sports-related injuries and requires the ability to assign a Glasgow Coma Scale (GCS) score to a patient. Because a GCS score involves assessing verbal responses (i.e. comprehendible speech), it is not possible to assign scores to rodents. Therefore, the term "concussion" should only be used with respect to clinical populations. In contrast, the term mTBI, can be applied to both humans and rodents. mTBI also refers to a spectrum of mild head injuries that may or may not include abnormalities on neuroimaging. In contrast, concussion refers to the most "mild" form of mTBI, where there is an absence of brain lesions or bleeding upon brain imaging (McCrory et al., 2009).

1.1.3 Post-Concussion Syndrome

While the majority of patients typically recover from mTBI within 1-3 months without further complications, (Carroll et al., 2004; Gentilini et al., 1985; McCrea et al., 2003) a small but significant subset (approximately 15%) will have symptoms that persist - a disorder known as post-concussion syndrome (PCS; Ruff, 1996). In PCS, the effects of the injury may last anywhere from three months to becoming permanent disabilities. Thus for patients in this category, the term "mild" TBI is perhaps somewhat misleading.

To further complicate this issue, there is a paucity of treatments available for patients with PCS (Kabadi and Faden, 2014). In fact, there have been over 200 clinical drug trials to offset the damage of TBI which have failed (Zhang, Larner, Kobeissy, Hayes, & Wang, 2010). These failures in clinical trials stem, in part, from a failure of basic science to develop drugs that are also efficacious in the clinic. Part of what makes TBI and PCS so difficult to treat is that all aspects of the brain injury (i.e. the mechanism of injury, severity, symptomology, and individual background) are quite diverse (Bigler et al., 2013). As a result, the term PCS refers to a constellation of symptoms in a heterogeneous patient population, making it difficult to study and even more challenging to treat. Despite this varied population, there has been a tendency within both research and the treatment of PCS to lump patients into a homogeneous group, and in turn search for a single target molecule that could be responsible for this disease process (Xiong, Mahmood, & Chopp, 2013). This may, at least partially, explain the lack of success in preclinical drug development treatment options available for patients suffering from PCS. Another possible reason for the failure of the novel therapeutic pipeline is our incomplete understanding of the pathophysiological processes underlying PCS and what makes certain individuals more susceptible to poor outcomes, while others are more resilient. The purpose of this study was to investigate the molecular processes that may underlie variability in individual outcomes using an animal model that displays behavioural characteristics that are consistent with clinical symptoms of PCS.

1.2 Susceptibility Factors

1.2.1 Adenosine Triphosphate (ATP)-sensitive Potassium Channels

In order to improve our understanding of what molecular processes underpin PCS, this study employed a partial adenosine triphosphate - sensitive potassium channel knockout (Kir6.2-KO) mouse as a model of inherent susceptibility to poor outcomes. Originally, these channels were found in the heart, but since then, they have also been characterized in islet cells of the pancreas, muscle tissue, kidneys, and in many regions of the rodent brain (Ashford, Sturgess, Trout, Gardner, & Hales, 1988; Miki et al., 2001; Noma, 1983; Spruce, Standen, & Stanfield, 1985; Suzuki, Fujikura, Inagaki, Seino, & Takata, 1997; Yamada & Inagaki, 2005; Zhou et al., 2008). The K_{ATP}-channel is a hetero-octomer comprised of two main subunits – the sulfonylurea (SUR) receptor and the potassium inward rectifier protein family (Kir6; see figure 1)(Inagaki et al., 1995; Mikhailov et al., 2005). The SUR regulatory subunit is the primary binding site for magnesium-adenosine diphosphate (Mg-ADP), which causes channel opening and results in an efflux of potassium (K^+) ions along the concentration gradient (Nichols et al., 1996). Conversely, Kir6 acts as the pore forming subunit and is responsible for binding ATP, which causes the channel to close (Drain, Li, & Wang, 1998; Tucker, Gribble, Zhao, Trapp, & Ashcroft, 1997). This study was designed using a mouse lacking the Kir6.2 pore-forming subunit and will be discussed in more detail below.

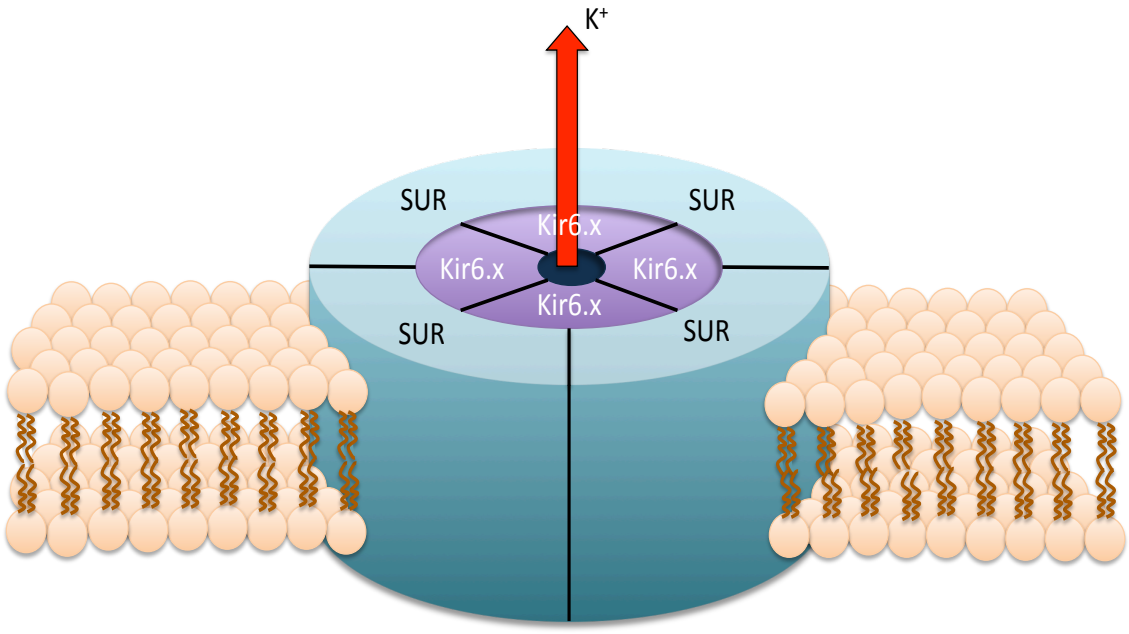


Figure 1.1. Structure of the K_{ATP} channel.

Functionally, K_{ATP}-channels act as an important link between the bioenergetic and excitatory states of a cell. A high intracellular ATP/ADP ratio, such as at normal physiological conditions, causes K_{ATP} channels to remain closed, while during states of high metabolic demand the ATP/ADP ratio is reduced and K_{ATP} channels open (Cook & Hales, 1984; Dunne & Petersen, 1986; Noma, 1983). As a consequence, and due to the high intracellular concentration of K^+ ions, K_{ATP} channel opening leads to an efflux of K^+ ions and hyperpolarization of the cell membrane (see figure 2). In turn, hyperpolarization can cause reduced firing, which decreases metabolic demand and allows for ATP stores to replenish. This ability to link the metabolic state and membrane excitability of a cell may be particularly important during brain injury, as these channels could reduce excitotoxic stress that results from hyperexcitability. Ultimately, this could decrease the likelihood of cell death (Barkhoudarian, Hovda, & Giza, 2011).

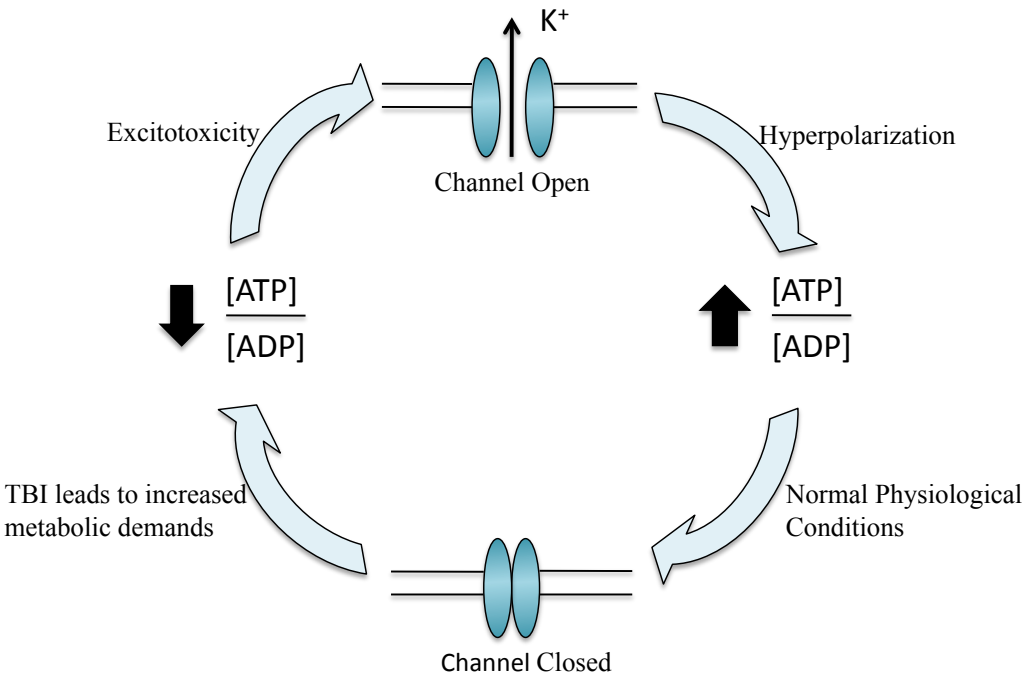


Figure 1.2. K_{ATP} channel configuration during different cellular states.

The neuroprotective importance of effective K_{ATP} channels during seizure or stroke has been well established (Heron-Milhavet et al., 2004; Sun, Feng, Miki, Seino, & French, 2006; Yamada et al., 2001). Overall, these studies have shown that the presence of functional K_{ATP} channels is neuroprotective during stress or injury, at the cellular level. Experiments using Kir6.2-KO mice also demonstrate that the absence of this channel lowers the threshold for induction of generalized seizures, and that opening these channels in wild-types prevents seizure propagation (Yamada et al., 2001). Kir6.2-KO mice have also been used in models of hypoxia/ischemia such as the study by Sun and colleagues (2006) that demonstrated substantially more neuronal injury and increased membrane excitability following *in vitro* middle cerebral artery occlusion in Kir6.2-KO brain slices. They also described an increase in cell death, infarct volume, and neurological deficits *in vivo* after hypoxia/ischemia in these knockout mice.

Together, these studies support the idea that animals lacking the Kir6.2 pore-forming subunit of the K_{ATP} channel may have an increased susceptibility to poor outcomes following brain injury.

In contrast, other results based on using K_{ATP} channel gain-of-function experiments have shown that this channel is sufficient to produce neuroprotective effects following brain injury. Experiments using K_{ATP} channel over-expression demonstrated that following hypoxia/ischemia there was a significant reduction in cell death and damage, as well as a decrease in infarct volume compared to controls (Heron-Milhavet et al., 2004). Thus, in essence, opposite results were observed in over-expression studies compared to knockout experiments.

Together, these gain- and loss-of-function studies provide evidence that K_{ATP} channel opening may play an important neuroprotective role in the brain following stress or injury. The present study was therefore designed to extend the use of Kir6.2-KO mice as a model of susceptibility to poor outcomes in mTBI research in order to study both the behavioral and molecular consequences of adding a susceptibility factor.

1.2.2 Sex differences in mTBI Outcome

While mTBI is a realistic concern at all ages and for both sexes, the prevalence of injury is disproportionately higher among adolescent and young-adult males between the ages of 15-25 years (Carroll et al., 2004; Faul et al., 2010). While the increased risk of injury in males is well established, sex differences related to injury outcome are less straightforward (Bazarian, Blyth, Mookerjee, He, & McDermott, 2010). There are inconsistencies between animal and human literature regarding sex differences in recovery from brain injury, with some results from human populations suggesting that females have a higher propensity for poor outcomes following

mTBI. Bazarian and colleagues (2010) showed that at three months post-injury, females that were in their child-bearing years had higher PCS scores, a trend towards missing more days of work, and had poorer coping skills compared to males. Similarly, other researchers have found greater cognitive impairment, higher PCS symptom reporting, and more severe social impairments in females following mTBI that were all within child-bearing years (Bay, Sikorskii, & Saint-Arnault, 2009; Broshek et al., 2005; Farace & Alves, 2000; Mychasiuk, Hehar, Farran, & Esser, 2014). Researchers from these studies have suggested that the poorer outcomes observed in females might reflect, at least partially, differences in circulating gonadal hormones, such as estrogen or progesterone, due to the appearance of sex differences between puberty and menopausal ages.

Interestingly, animal literature has demonstrated that males tend to fare worse after TBI (O'Connor, Cernak, & Vink, 2003; Roof & Hall, 2000; Wagner et al., 2004). In these and other studies, improved cerebral blood flow, cognitive functioning, and motor performance were observed in females when compared to males in rats and mice. As a result, the animal literature has concluded that female gonadal hormones, estrogen and progesterone specifically, may actually be neuroprotective (Bramlett & Dietrich, 2001; Roof & Hall, 2000).

While the reasons behind these discrepancies in human and animal literature are unclear, there are a few plausible explanations. First, the nature of data collection between human studies and animal experiments differ. Human studies often use self-reported symptoms as a measure of outcome following TBI. Previous literature has indicated that the likelihood of self-reporting symptoms is greater in females than males (Barsky, Peekna, & Borus, 2001). Therefore, sex differences in humans may reflect a difference in the subjective experience of PCS, or coping styles between males and females. However, in the rodent population it is not possible to collect

subjective information about symptoms post-TBI. Therefore, animal research relies on the observation of animals performing certain tasks that are designed to assess specific behaviours. Second, the timeline of assessment between human and animal studies also differs substantially. In human literature, PCS symptoms are often assessed between 3 and 36 months post-injury (Bay et al., 2009; Broshek et al., 2005; Farace & Alves, 2000) whereas the animal literature follows a shorter timeline, usually assessing symptoms between 24h and three weeks post-injury (Mychasiuk, Farran, & Esser, 2014b; O'Connor et al., 2003; Roof & Hall, 2000; Wagner et al., 2004). Lastly, animal models of TBI allow researchers to control for various extraneous factors such as genetics, environment, or mechanism of injury – all of which likely affect final outcome. In studies of human populations, these variables are usually not the same between participants, and despite a researchers best intention, are difficult to control. Thus, extraneous variables in the human population may skew results about how sex affects outcome following mTBI, and in turn, may contribute to differences observed between human and animal literature.

Differences in research methodology, as well as the inherent differences between these two populations, may therefore help explain why sex differentially affects rodents and humans. Because these populations differ on numerous levels, we should not necessarily expect sex differences to be the same between them. Thus, results from animal versus human literature should be considered separately, and research should focus on finding consistency within each population. It is also perhaps more important to note that regardless of the research population, sex differences are apparent.

1.3 Pathophysiology of mTBI

1.3.1 Primary Injury

Primary injury occurs as a direct result of the initial impact. It can be caused by direct mechanical forces applied to the head, or by indirect forces that result in acceleration/ deceleration or rotation of the brain within the cranial vault. Focal injury usually causes direct damage to brain tissue or vasculature at the site of the insult. In contrast, indirect forces that lead to rotation or acceleration/deceleration of the brain tend to cause a more diffuse injury that results from, in part, shearing of axons. Thus, it often called a diffuse axonal injury (DAI) (Barkhoudarian et al., 2011). Because this stage of TBI occurs in a matter of milliseconds, treatment for primary injury is limited to preventative measures.

1.3.2 Secondary Injury Cascade

Following the initial mTBI insult, a secondary cascade of molecular events becomes activated in the brain, which can occur at different time points and on different time-lines (Ryan & Warden, 2003). These molecular processes can be broadly classified into three main groups– bioenergetic dysfunction, excitotoxicity, and neuroinflammation (Barkhoudarian et al., 2011), with each one being negatively associated with outcome after TBI. These themes may therefore be especially important in patients with PCS.

1.3.2.1 Excitotoxicity

Following TBI, axons may become stretched resulting in perturbations of the cell membrane and its ability to maintain homeostatic ion concentration gradients (Farkas, Lifshitz, & Povlishock, 2006). In turn, these changes may result in neuron terminals becoming

persistently depolarized, which can lead to massive glutamate release in a process known as excitotoxicity. Glutamate is one of the main excitatory neurotransmitters in the brain, but when increased to pathologically high levels it can lead to cell death (Olney, 1969). When released into the synaptic cleft, it causes overstimulation of glutamatergic receptors, which ultimately leads to an influx of Na^+ and Ca^{2+} cations into the postsynaptic cell. In turn, calcium overload is responsible for stimulating a series of neurotoxic cascades that can cause mitochondrial uncoupling, apoptosis, and/or necrosis (Schinder, Olson, Spitzer, & Montal, 1996). If the cell becomes necrotic, its intracellular components, such as glutamate, can leak into the extracellular space. Consequentially, glutamate leakage may then initiate the same process in neighboring neurons (for review, see Barkhoudarian et al., 2011).

1.3.2.2 Bioenergetic Dysfunction

Excitotoxicity can also cause calcium overloading of mitochondria, which may lead to uncoupling of the electron transport chain (Khodorov, Pinelis, Storozhevykh, Vergun, & Vinskaya, 1996; Schinder et al., 1996; Xiong, Gu, Peterson, Muizelaar, & Lee, 1997). The consequences of a dysfunctional ETC are twofold. First, it can cause the production of reactive oxygen species, which are ultimately responsible for a process known as oxidative stress that is toxic to the cell (Vagnozzi et al., 1999). Second, it can also lead to a reduction in the amount of ATP that is generated by the mitochondria (Aoyama, Lee, Moro, Hovda, & Sutton, 2008; Khodorov et al., 1996). This is particularly problematic, as ATP is the main source of energy for the cell, and without a functional ETC, ATP is produced through the inefficient process of glycolysis. Ultimately, this can lead to an energy deficit within the cell. Important ATP-dependent cellular processes, such as maintaining concentration gradients via Na^+/K^+ ATPase

pumps, can fail to function. Together, the oxidative stress and energy failures produced by mitochondrial dysfunction can lead to activation of apoptotic and necrotic pathways, which cause cell death (Lewen et al., 2001; Robertson, 2004)

1.3.2.3 Neuroinflammation

Following mTBI, there is an increase in a number of neuroinflammatory markers in the brain such as activated microglia, pro-inflammatory cytokines, chemokines, prostaglandins, and migration of leukocytes due to breakdown of the blood-brain barrier (Ghirnikar, Lee, & Eng, 1998; Koshinaga et al., 2007). However, because of the closed-headed and non-penetrating nature of mTBI, the associated neuroinflammation is not considered to be the typical infection-inflammation reaction, but rather is deemed to be sterile neuroinflammation (O. I. Schmidt, Heyde, Ertel, & Stahel, 2005). Activation of inflammatory pathways in this case is thought to be due to interactions between endogenous molecules within the brain that act as ligands for inflammatory pathways. However, neuroinflammation following mTBI is somewhat of a "double edged sword". While these processes play a vital role in helping remove cellular debris, repairing the blood-brain barrier, or preventing excitotoxicity (van Beek et al., 2001), they can also lead to the activation of cell death pathways (Lenzlinger, Morganti-Kossmann, Laurer, & McIntosh, 2001).

1.4 The Cellular Stress Response and mTBI

1.4.1 Heat shock proteins

While it is unlikely that perturbations of any single molecule are solely responsible for the complex pathophysiology of mTBI, studying a family of molecules or part of a pathway could provide a starting point to understanding how certain pathological processes are interacting. One family of proteins, which are common to all three mechanisms of the secondary injury cascade, are the heat shock proteins (Hsp) (Lai et al., 2006; Lowenstein, Chan, & Miles, 1991; Zhang, Zhang, Wu, & Schluesener, 2012). Hsps were originally discovered in *Drosophila busckii* where experimenters observed that a transient increase in temperature resulted in up-regulated gene transcription, or "puffs" on certain sites of two chromosomes (Ritossa, 1962). It was later discovered that the genes transcribed from these sites were actually quite ubiquitous and could be induced by many different cell stressors (Tissieres, Mitchell, & Tracy, 1974). Thus, they were later more aptly named, "stress proteins" (Lindquist & Craig, 1988).

Heat shock proteins are grouped into 5 main families according to their molecular weight (kDa): Hsp110 (HSPH), Hsp90 (HSPC), Hsp70 (HSPA), Hsp60 (HSPD1), Hsp40 (DNAJ), and the small Hsps (HSPB, 15-30 kDa) (Lindquist and Craig, 1988; Kampinga et al., 2009; Hightower & White, 1981). Within each class there are variations in expression (constitutive (Hsc) versus inducible (Hsp)), function, and subcellular localization. Together, the entire Hsp class spans all the major cellular compartments – the nucleus, cytosol, endoplasmic reticulum, and mitochondria (Snoeckx, Cornelussen, Van Nieuwenhoven, Reneman, & Van Der Vusse, 2001). Some Hscs are constitutively active and act as molecular chaperones that aid in proper

protein folding and creation of functional proteins. Other Hsps are activated during the cellular stress response and help refold damaged proteins, prevent protein aggregation, or target misfolded proteins for degradation. These processes are critical, particularly during stress or injury, as accumulation of misfolded proteins is toxic (Lindquist & Craig, 1988).

1.4.2 Heat Shock Protein Expression in mTBI

Previous literature in animal research has indicated that TBI results in changes in *Hsp* gene expression and protein levels. One of the most extensively studied of these proteins is Hsp70. Multiple research groups have shown that inducing a focal TBI leads to an up-regulation of *Hsp*70 gene expression and protein levels at time points varying from 2 h to one-week post-TBI (M. Chen et al., 1998; Raghupathi, Welsh, Lowenstein, Gennarelli, & McIntosh, 1995; Tanno, Nockels, Pitts, & Noble, 1993; Truettner et al., 2007). There is also some evidence to show that up-regulation of *Hsp*70 could be associated with positive outcomes. For example, mice with either over-expression or pharmacological increases in *Hsp*70 have decreased cell death and lesion volume, improvements in motor coordination, and a reduced volume of hemorrhage between 6 hours to 28 days following TBI or ischemia (Kim, Kim, Zheng, Lee, & Yenari, 2013; Zhao et al., 2013). In contrast, *Hsp*70 knockout mice show increased lesion volumes, and increased hemorrhaging following brain injury (Kim et al., 2013).

However, Hsp70 also has other roles within the cell besides protein folding. For example, Hsp70 is a natural ligand for toll-like receptor 2 and 4 (TLR2 and TLR4), which are key cell surface receptors for two important inflammatory pathways. Thus Hsp70 could act as a mechanism underlying the sterile neuroinflammatory seen in TBI (Asea et al., 2002). While

studies have not been conducted to assess the relationship between TLR2/4 and Hsp70 in TBI, it is possible that persistent activation of this receptor could have negative effects, as its downstream effectors are pro-inflammatory, (Asea et al., 2002) and neuroinflammation is one of the key characteristics of TBI pathophysiology. Therefore, Hsp70 may have important neuroprotective effects, but it also has the potential to play other roles that may vary depending on levels and duration of exposure.

Despite extensive research on the role of Hsp70 following TBI, changes in other Hsps have been less well characterized. However, some insight into the cellular stress response following TBI may be found in ischemia or seizure literature, both of which could also be considered different forms of brain injury. Indeed, many studies have shown an up-regulation of *Hsp*90b1, 60, 40, 27, 10, and 5 gene expression following brain injury and, similar to *Hsp*70, that there are a variety of factors that influence the degree of measured gene expression (Conzlez Villaron, Gonzalez, Gracia-Talavera, & De Castro Del Pozo, 1975; Kawagoe, Abe, Aoki, & Kogure, 1993; Okubo et al., 2000; Paschen, Linden, & Doutheil, 1998; Wagstaff et al., 1996). However, whether up-regulation of these genes leads to positive or negative effects on outcome following injury is still unknown.

Taken together, these data suggests that Hsps likely play a role in the cellular response to brain injury. However, the specific roles of each Hsp remain to be elucidated, as well as how they interact together. Few studies have manipulated *Hsp* expression, or levels, to see how they may affect outcomes after TBI. Results from previous literature therefore beg the question; could the heat shock protein response be perturbed in patients that have an increased susceptibility to PCS?

Most studies assessing the expression of *Hsps* following brain injury have focused on a very limited post-injury timeline, usually no longer than one week post-injury with most only examining the first 24h (Dutcher, Underwood, Walker, Diaz, & Michael, 1998; Fukuda et al., 1996; Raghupathi et al., 1995). However, the deficits observed in patients with PCS often last in the order of months to years. It is therefore important to study the cellular stress response at more long-term time points, as well as the early time points, to establish whether changes in gene expression persist or whether differences may present only at later points. The proposed experiments in this study assessed *Hsp* expression at 24h and 14 days post-injury to compare and contrast levels during periods that correlate to different stages of the injury. Furthermore, we also wanted to determine whether animals with a susceptibility to PCS have persistent changes. Furthermore, we assessed the *Hsp* response in two brain areas to determine if mTBI may affect regions differently. The present study is also novel because it assesses *Hsp* changes following a mild TBI, whereas the majority of studies analyzing *Hsp*s have been conducted in models of moderate to severe TBI.

1.5 Brain Regions of Interest

The present study will look at molecular changes in the prefrontal cortex (PFC) and hippocampus. These two areas are often associated with executive functioning, affective states, and learning and memory, which may become impaired following mTBI. The PFC is responsible for a variety of functions such as working memory, cognitive flexibility, response inhibition, decision-making, attention, and social cognition (Kolb & Whishaw, 2009). It is also a key structure involved in regulating psychological affect, which may become perturbed in disorders

such as anxiety and depression (Davidson, 2002). As PCS is often associated with symptoms such as impaired social behaviour, attention problems, impulsivity, anxiety, and depression (Ryan & Warden, 2003), changes in the PFC may help explain some of these deficits.

The hippocampus was also selected because it plays an important role in memory formation, learning, and spatial navigation (Kolb & Whishaw, 2009). Memory disturbances and learning difficulties are symptoms often reported by patients with mTBI (Ryan & Warden, 2003). The hippocampus is also involved in the body's response to stressful stimuli, as it has a high density of glucocorticoid receptors (Sze et al., 2013) and some studies have shown that deregulation of this brain region may have important implications for the anxiety and depression sometimes seen following mTBI (Boyer, 2000).

1.6 Animal Models of mTBI

When the ultimate goal of basic science research is to translate findings to human populations, a model that adequately reflects the symptomology and pathophysiology observed in the clinic is required. Prior research has capitalized on a variety of TBI animal models that have been developed to better understand the complex disease processes initiated after an injury, and to examine potential therapeutic strategies. Some of the most commonly used models include the controlled cortical impact (CCI), the fluid percussion injury (FPI), and the standard weight drop (WD). More recent modifications have been made to these models in an effort to fine-tune the mechanisms of the injury sustained and increase their clinical translatability (Kane et al., 2012; Mychasiuk, Farran, et al., 2014b).

1.6.1 Controlled Cortical Impact (CCI)

Lighthall originally developed the CCI model for use with the ferret in 1988 (Lighthall, 1988). Briefly, the anesthetized animal is placed into a stereotaxic frame to prevent any head movement, and a craniotomy is typically performed to expose the dura. However, there are closed-head variations of this protocol whereby the researcher only makes an incision to expose the skull. In both situations, a pneumatically operated piston penetrates the brain, or compromises the skull. The resulting injury causes tissue loss, cell death, haemorrhage, and prominent neurobehavioral deficits (Fox, Fan, Levasseur, & Faden, 1998; Gao & Chen, 2011). The major advantages of this technique are the ease at which damage delivered to the brain can be controlled as well as the fact that the velocity and depth of the piston that imparts the brain injury can be regulated, which greatly increases injury reproducibility. However, its use is limited as a model of clinically relevant mTBI for several reasons. First, the animal must be under anesthesia for an extended period of time; an experience that does not reflect mTBI in the human population and that may have an effect on the biochemical cascades in the brain (Stover et al., 2004). Second, the brain or skull must be exposed prior to the injury. This is problematic as the incision or craniotomy itself activates inflammatory pathways in the body, which could confound results. Furthermore, while placing the animal's head in a stereotaxic frame aids in injury reproducibility, it reduces the translatability of the model, as the human head is usually free to move following an impact injury. In a stereotaxic frame, a direct force to the head causes a focal injury that is localized to the specific site of impact. However, when the head is free to move, the brain is subjected to acceleration/deceleration and rotational forces, which cause sheering and stretching of white matter tracts throughout the brain. This is important as the

nature of the forces imparted on the head can lead to different pathophysiological processes and may have different symptom presentation (Andriessen, Jacobs, & Vos, 2010).

1.6.2 Fluid Percussion Injury (FPI)

The FPI is also very frequently used to model TBI (Xiong et al., 2013). It is similar to the CCI in that it requires complete immobilization of the animal's head, as well as a craniotomy/incision to expose the brain or skull. However, in this case, a pendulum swings to hit a pressurized reservoir of saline, which results in rapid injection of the fluid onto the exposed surface. The severity of the injury can be altered by changing the height of the pendulum, and therefore the pressure imparted on the saline solution (Kabadi, Hilton, Stoica, Zapple, & Faden, 2010). Following FPI, there are deficits in learning and memory, motor impairments, and neuronal cell death (Hamm, 2001; Hamm, Pike, O'Dell, Lyeth, & Jenkins, 1994; Raghupathi et al., 2002). However, as with the CCI, the invasive nature of this technique limits is use for translational modeling of clinical mTBI.

1.6.3 Weight Drop

In the weight drop model, a weight is dropped through a Plexiglas® tube onto the animal's head, which is resting below. Changing the height at which the weight is released from and the mass of the weight can alter the severity of the injury. In the original model, a craniotomy was performed so the weight would impact the exposed dura, resulting in significant hemorrhaging and cell death at the site of injury (Feeney, Boyeson, Linn, Murray, & Dail, 1981). Under these circumstances the animal's head was also fixed into a stereotaxic frame to prevent

movement. However, over the years various modifications have been made to this model. One of the most notable changes was the development of a closed headed weight drop injury by the Shohami lab (Y. Chen, Constantini, Trembovler, Weinstock, & Shohami, 1996). In this procedure, a craniotomy was not performed and the weight hit the intact skull producing a milder injury. This small modification significantly increased the translatability of this model to human TBI.

Marmarou et al. (1994) also modified the weight drop in order to allow the animals head to have some degree of movement following impact. In this case, the head of the animal rests on a foam cushion, as opposed to being completely immobile in a stereotaxic frame. The goal behind this modification was to replicate the acceleration/deceleration component of TBI, and to create a model of diffuse axonal injury. However, the Marmarou weight drop still does not allow for complete body rotation, or free movement of the head as it is still somewhat restricted by the cushion. Studies have shown that free, unrestricted movement of the body is an important component in the biomechanics of TBI (Viano, Casson, & Pellman, 2007). Therefore, this model is still limited in its capacity to translate to the clinical TBI population.

1.6.4 Modified Weight Drop

In the present study we chose to use a modified weight drop technique that was originally developed by Kane et al. (2012) and further validated by Mychasiuk et al. (2014). In this model, the animal is placed chest-down on a scored piece of tinfoil, 10 cm above a foam cushion. A weight is then dropped from a predetermined height above the animal's head. The weight

produces a glancing hit to the head, which propels the animal through the tinfoil. The animal rotates 180^{o} during the fall and lands on its back on the cushion below.

There are a few benefits associated with this model for mTBI when compared to the previously used techniques. First, the mechanism of injury more adequately reflects that of a mild injury sustained in humans as it is a closed head injury, which does not penetrate the brain or require an incision to expose the skull, an important characteristic for modeling mTBI. Furthermore, there are rapid acceleration/deceleration and rotational forces applied to the head, which often occurs in humans, and is absent in many of the other models. As previously stated, the type of forces imparted on the brain (i.e. focal or diffuse) leads to different pathophysiological cascades, and can also be associated with different symptoms (Andriessen et al., 2010). Lastly, the behavioural deficits that are created by this injury closely approximate those reported in human mTBI (Mychasiuk, Farran, et al., 2014). This last point is of critical importance when choosing a model where the ultimate goal is to translate findings to a clinical population.

1.7 The Present Study

The general purpose of this thesis was to determine whether Kir6.2-KO mice have an inherent susceptibility to poor outcomes following mTBI, and whether there are any sex differences in this susceptibility. There is currently a limited understanding of what makes certain individuals more susceptible to poor outcomes following mTBI, and the present study was designed to elucidate some possible reasons for susceptibility/resiliency to PCS. In particular, adolescents and young adults between the ages of 15-25 years seem to be at

particularly high risk for concussion. We therefore chose to use the aforementioned modified weight drop model to elicit mTBI at a mouse age of 45 days, which roughly correlates with human adolescence (Semple et al., 2013), in an effort to reliably replicate human symptomology and pathophysiology during this high-risk period. The effects of the Kir6.2-KO and mTBI were explored in the context of behavioural changes as well as the cellular stress response. We hypothesized that Kir6.2-KO mice would have worse behavioral outcomes and differential *Hsp* gene expression following a mTBI, and that these effects would be sex-dependent.

1.7.1 Specific Aim 1 – Behavioural Characterization

To determine the behavioural phenotype of Kir6.2-KO mice following mTBI, animals were subjected to a behavioural test battery over 14 days. Measures of outcomes included loss of consciousness, motor coordination, sociability, anxiety, depression, and learning and memory. These behavioural measures were chosen, as they are consistent with the common symptoms reported in patients with mTBI. It was hypothesized that Kir6.2-KO mice that receive a mTBI would have worse outcomes when compared to control mice, and that some of the outcomes would also be sex-dependent

1.7.2 Specific Aim 2 – Pattern of Cellular Stress Response Following mTBI

To better understand the pathophysiological underpinnings of mTBI, we examined the pattern of changes in mRNA expression of several heat shock proteins as an indicator of the cellular stress response. The effect of Kir6.2-KO was determined through comparison of gene changes between KO animals and controls. Two brain areas, the PFC and the hippocampus, were

examined. It was hypothesized that heat shock proteins would show aberrant expression patterns in Kir6.2-KO mice that received an mTBI relative to controls, and that these changes would be sex dependent.

Chapter Two: **Methods**

2.1 Animals and Husbandry

The Animal Care Committee (ACC) at the University of Calgary approved all procedures in this study under protocol number AC11-0047, and is in accordance with the guidelines set by the Canadian Council on Animal Care (CCAC). The present study used Kir6.2-KO mice that were generated from a C57BL/6J background (n=12 per sex) and C57BL/6J wild-type controls (n=12 per sex), which were randomly assigned to various experimental and control groups as indicated in figure 3. For the duration of testing, mice were housed in a standard colony room in IVC Greenline cages (39 x 20 cm; Techniplast, Italy) with 1-5 mice of the same sex per cage. Due to small litter sizes, Kir6.2-KO mice typically had 1-3 mice per cage, whereas C57BL/6J mice were usually housed in groups of 5. Each cage contained wood chip bedding and a cardboard house for enrichment. The room was maintained on a 12 h light:dark cycle with lights on at 0700 h and a constant temperature (~21^{o}C) and humidity. Food and water was provided to animals *ad libitum*.

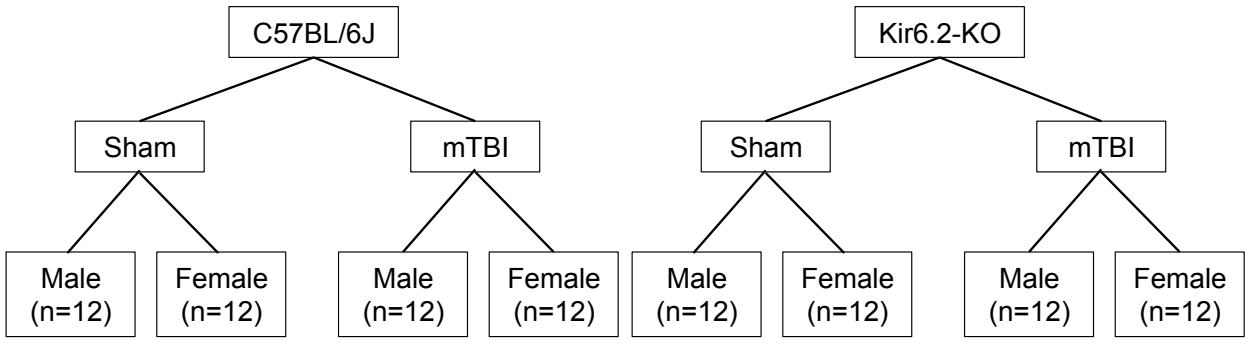


Figure 2.1. Schematic of different experimental groups.

2.2 Mild Traumatic Brain Injury

On post-natal day (PND) 45±5, mice either experienced a mTBI or sham injury using the modified weight drop technique previously validated by Kane et al. (2012) and Mychasiuk et al. (2014). Mice were lightly anaesthetized using Isoflurane (Pharmaceutical Partners of Canada, Canada) until hind-leg withdrawal reflexes were absent. They were then placed chest-down on a sheet of scored tinfoil that is located 10 cm above a foam cushion and 4cm below a Plexiglas© tube (2 cm inner diameter x 1.5 m). A 250 g brass weight was released from a height of 1 m and guided through the tube to hit the head of the mouse. Upon the glancing impact between the weight and head, the mouse breaks through the tin foil and horizontally flips 180^{o} onto the foam below. Mice were treated immediately with topical Xylocaine (AstraZeneca, Canada) and placed on their backs in cages warmed with a heating pad. Sham injured mice underwent the same procedure but failed to receive a mTBI with the brass weight. Animals were monitored until their righting reflexes returned, and time to wake from anesthesia was recorded.

2.3 Behavioral Testing

Behavioral testing occurred between 0900-2100 h. Mice (n=8 per group; see Figures 2.2 and 2.3) were transported from the colony room and allowed to sit for 30 minutes before testing commenced. Behavioral tests were recorded using Noldus Ethovision XT tracking software (Noldus Information Technology, Netherlands) unless otherwise indicated. Each testing apparatus was cleaned between trials with Virkon to remove any olfactory cues. For tests that were scored manually, a second trained observer, blinded to the conditions, was used. Behavioral tests are explained in the same order at which they were conducted.

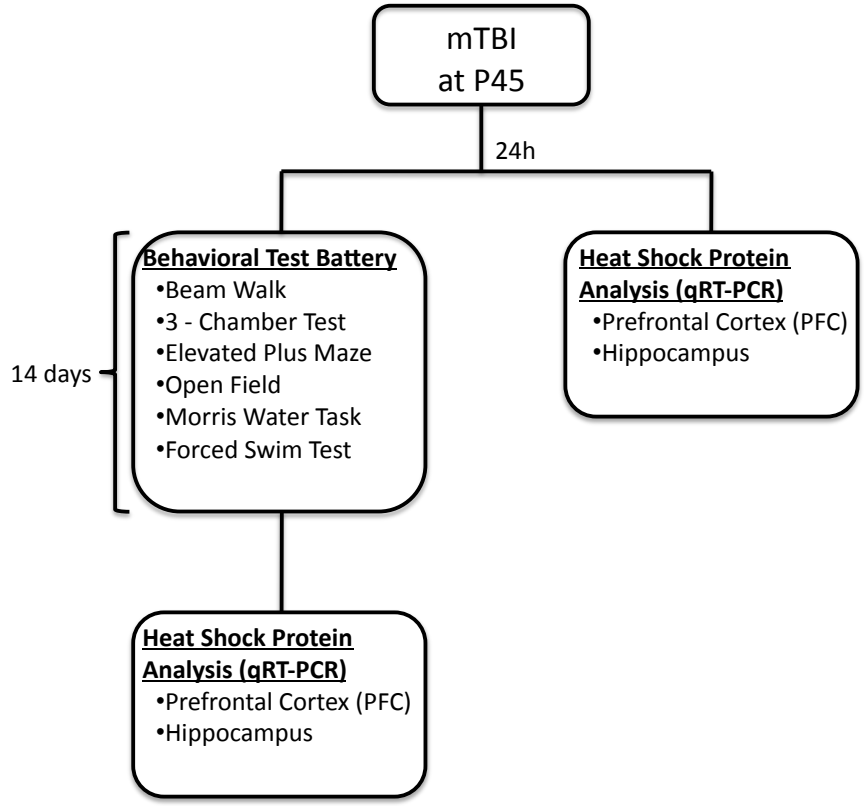


Figure 2.2. Schematic of behavioural tests and molecular analysis.

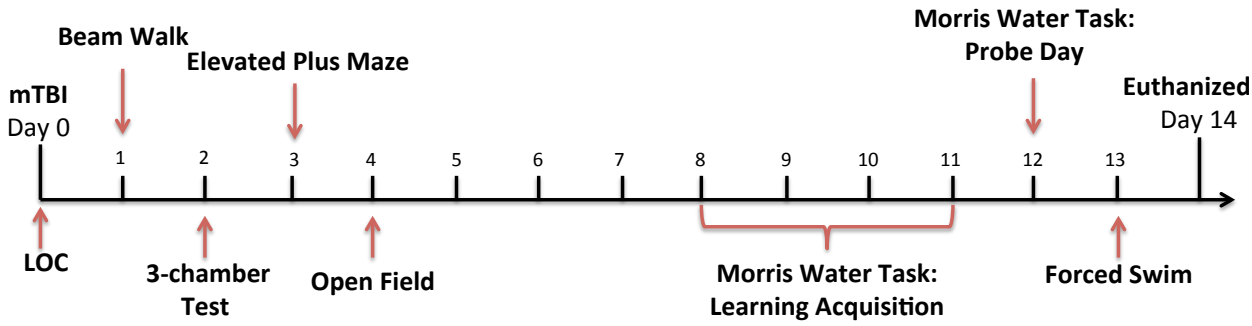


Figure 2.3. Timeline of behavioural Tests.

2.3.1 Beam Walk.

Procedures were conducted in a manner similar to that described by (Schallert, Woodlee, & Fleming, 2002). A wooden plank measuring 1.2 x 4 x 80 cm that was elevated 75 cm above the ground was used to test motor coordination. Mice were placed on one side of the beam and required to cross to the other side. The home cage for each mouse was placed at the opposite end of the beam as an incentive to cross. Each mouse was scored on 4 trials of successful beam crosses, which were recorded using a Sony DCR-SR68 camcorder. Between trials, mice were permitted to sit in their home cage at the end of the beam for 1 minute to reinforce correct behavior. The average time to cross the beam and number of hind-leg foot slips for each mouse was recorded as a measure of motor coordination.

2.3.2 Crawley's Three-Chamber Sociability Test.

The three-chamber test, similar to that described by Moy et al. (2004) was used to assess sociability in mice. The testing apparatus consisted of a clear Plexiglas® box with 3 interconnected compartments (40x23x30 cm). A wire cage was placed within the two side chambers of the apparatus. Following a 2-minute habituation period, an unfamiliar sex-matched mouse was placed in one of the two side-chamber cages. The doors separating each compartment were removed to permit the mouse to move freely between all three chambers. The time spent in each chamber was recorded. Side preference during this experiment was controlled for by systematically alternating which side the stranger mouse was placed in. Lastly, mice that were singly housed were excluded from this test due to a confounding lack of previous social interaction.

2.3.3 Elevated Plus Maze.

The elevated plus maze was used as a measure of anxiety. The maze was comprised of four Plexiglas® arms (27.5 × 5cm), two open and two enclosed by 30cm high walls. The maze was elevated to 50 cm above the floor. Each mouse was placed in the center of the maze facing a closed arm and was allowed to explore for 5 minutes. The time spent in the open, closed, and center was recorded.

2.3.4 Open Field Test.

The open field test was used as a secondary measure of anxiety and to assess overall activity level. Mice were placed in a white, brightly lit, open-aired box (100 x 100 x 50 cm) and allowed to move freely for 5 minutes. The floor was divided into an outer and inner area. The time spent in each area, as well as the total distance moved was recorded.

2.3.5 Morris Water Task.

The pool (1 m diameter) was filled with water (25°C) to a depth of 55 cm, and the hidden platform placed 5 mm below the water's surface. The water was dyed with blue Elmer's© Tempera Paint (USA) so that both white and black mice could be detected with the tracking system. Various cues were placed around the room for the mice to use as reference points when locating the platform. Care will be taken so that all potential cues remain in the same position for the duration of testing. Similar to the protocol described by Vorhees and Williams (2006), mice were given a total of 16 trials over a period of 4 days (4 trials per day) to learn where the platform is. Mice were rotated through the 4 quadrants as start points on each day. On successive days, the order of start locations was altered to force the mouse to use spatial cues instead of

route memorization. A single trial will be comprised of placing the animal in the water, and allowing it to swim for up to 1 minute. If the mouse did not find the platform during this time, they were guided to its location, and allowed to sit on the platform for 20 seconds post-trial to learn the visual cues. Mice completed the trials in groups of four to reduce inter-trial time. The time to reach the platform was recorded for each trial, and an average was calculated for each learning day. On probe day, the platform was removed from the pool, and mice were placed in the water at the starting location farthest from the previous platform location. The mice were permitted to swim for one minute, and the time spent in each quadrant was recorded.

2.3.6 Forced Swim Test.

This procedure was adapted from Porsolt, Le Pichon, and Jalfre (1977) in order to test for depressive- or anxiety-like phenotypes. A clear cylindrical container (12.5 cm diameter) was filled with warm water (~25^{o}C) to a depth of 13cm so that mice could not touch the bottom or escape out the top. Mice were placed in the water and forced to swim for 7 minutes. Time spent immobile versus swimming was recorded. Immobility was defined as no leg movements other than to keep the head and body afloat. Mice were judged as immobile when paw movement was limited to only keeping their head above water.

2.4 RNA Isolation and cDNA synthesis

Mice (n=5 per group) were deeply anaesthetized (i.e. no toe-pinch or righting reflexes) with Isoflurane (Pharmaceutical Partners of Canada, Canada) and decapitated either 24h after mTBI or at 14 days (~P59 ±5, after behavioural testing) as indicated in Figure 4. Brains were

dissected from the skull, and the prefrontal cortex and hippocampus from both hemispheres were collected. Tissue was flash frozen on dry ice and stored in -80^{o}C for later use. RNA and DNA were extracted using the Qiagen Allprep DNA/RNA Mini Kit (Qiagen, The Netherlands) as per the manufacturer's instructions. RNA concentration and integrity was identified using spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, Canada). If samples were contaminated with genomic DNA, they were processed with Deoxyribonuclease I (Invitrogen, USA). RNA was then reverse-transcribed using Superscript III First Strand Synthesis SuperMix (Invitrogen, USA) into cDNA with a final concentration of 10ng/μL.

2.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and purchased from Integrated DNA Technologies (IDT, USA). They were selected to amplify *Hsp*90aa1, *Hsp*90b1, *Hsp*70, *Hsp*60, *Hsp*40, *Hsp*32, *Hsp*27, *Hsp*10, and *Hsp*5. *Ywhaz* and *Cyclin A* were selected as the housekeeping genes, as previously described by Mychasiuk, Muhammad, Ilnytskyy, and Kolb (2013) due to their acceptable stability value and mean M value in brain tissue. See Table 1 for a list of oligonucleotide sequences. Temperature gradients were performed for each primer set to determine the optimal annealing temperature. Primers were used on sample tissue at annealing temperatures between 50-60 ^{o}C in standard PCR with the cycling parameters of 95^{o}C for 2 min, followed by 35 cycles of 95^{o}C for 30, gradient temperature (50-60 ^{o}C) for 30 sec, and 72^{o}C for 30 sec, followed by a final elongation step of 72^{o}C for 10 min. Gradient samples were then run on an ethidium bromide agarose gel to determine which temperature(s) resulted in the most intense band (a measure of amplicon number). Following

annealing temperature optimization, qRT-PCR reactions were run on individual plates for each primer set. Each individual PCR reaction had a total volume of 20μL, which contained 1ng of cDNA, 1x SYBR Green FastMix (Quanta Biosciences, USA), and 10 μM of forward and reverse primers. All samples were tested in duplicate to ensure sample reliability. A standard curve for each plate was created from seven two-fold serial dilutions, with new standards prepared fresh for each PCR reaction. Plates were processed in a BioRad CFX96 Connect Real Time System (Biorad, USA) with the following cycling parameters: 95^{o}C for 3 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 30 sec, with the exception of *Ywhaz* and *Cyclin A* where the 60^{o}C cycle was replaced with an annealing temperature of 56^{o}C. Only those plates with efficiencies between 85-115% were used in the analysis. The $2^{-\Delta\Delta Ct}$ values for each sample were calculated as described by Pfaffl (2001) and used in the statistical analysis.

Table 1.1. Oligonucleotide sequences obtained from Primer3 software and subcellular location for genes of interest.

Gene	Oligonucelotide sequence	Subcellular location
Ywhaz	(+): 5'– TTG AGC AGA AGA CGG AAG GT -3' (–): 5' – GAA GCA TTG GGG ATC AAG AA -3'	----
Cyclin A	(+): 5' – AGC ACT GGG GAG AAA GGA TT -3' (–): 5' – ACG CAC TCA GTC TTG GCA GT -3'	----
Hsp90aa1	(+): 5' – GAT CTG GTC ATC TTG CTG TAC G -3' (–): 5' – TCT TCA GTT ACA GCA GCA CTG G -3'	Nucleus Cytosol
Hsp90b1 (Grp94)	(+): 5' – GTT GTA GGA ATG ACC AGA GAG G -3' (–): 5' – TGG GTA TCA TTG TTG TGT TTC G -3'	Endoplasmic Reticulum
Hsp70	(+): 5' – CTT CGT GGA GGA GTT CAA GAG G -3' (–): 5' – TGG ATG TGT AGA AGT CGA TGC C -3'	Nucleus Cytosol
Hsp60	(+): 5' – TGG TTT ACT GCT GTC ATT GTC C -3' (–): 5' – TGC TTC TGA ACT TCT CAT GTG G -3'	Mitochondria
Hsp40	(+): 5' – GCC TTG TAA CTG TGC TTT TCC C -3' (–): 5' – CTT AAA ACT GCG CAC TGT ACC G -3'	Nucleus Cytosol
Hsp32 (HO-1)	(+): 5' – AGG AAC ACA AAG ACC AGA GTC C -3' (–): 5' – CAA CAG GAA ACT GAG TGT GAG G -3'	Mitochondria
Hsp27	(+): 5' – TGA ACA TGG CTA CAT CTC TCG -3' (–): 5' – CTA CTT GGC TCC AGA CTG TTC C -3'	Nucleus Cytosol
Hsp10	(+): 5' – CCG CTA TTT GAC AGA GTA TTG G -3' (–): 5' – CTC CAA CTT TCA CAC TGA CAG G -3'	Mitochondria
Hsp5 (Grp78)	(+): 5' – AAA GAG AAG CTG GGA GGT AAG C -3' (–): 5' – CCT CCA CTT CCA TAG AGT TTG C -3'	Endoplasmic Reticulum

2.6 Statistical Analysis

All statistical tests will be performed using SPSS version 21.0 (SPSS IBM, USA). A three-way ANOVA with Sex, Genotype, and Injury as factors was used to analyze all main effects and possible interactions. This process was used for both behavioural and molecular data sets. For all tests an alpha level of $p \leq .05$ was deemed statistically significant, and (*) was used to designate significance on graphs. A (*) placed directly above columns was used to compare sham and mTBI groups.

Chapter Three: **Behavioral Changes**

3.1 Results

3.1.1 Body Weight

Statistical analysis revealed that males weighed more than females, and that C67BL/J6 mice were heavier than Kir6.2-KO mice at both 24 h as well as 14 days following mTBI. A three-way ANOVA with sex, strain, and injury as factors demonstrated main effects for sex, F (1, 32) = 154.49, $p < .01$, and for strain, F (1, 32) = 25.64, $p < .01$ at 24 h following mTBI. However, no main effect was revealed for injury, F (1, 32) = 0.05, $p = .82$, nor were any interactions significant, p's > .05. Similar results were obtained at 14 days following injury, whereby main effects of sex, F (1, 58) = 28.98, $p < .01$ and strain, F (1, 58) = 35.97, $p < .01$ were revealed, but not injury, F (1, 58) = 1.70, $p = .20$. Interactions were also not significant at this time point, $p > .05$. See figures 3.1 and 3.2.

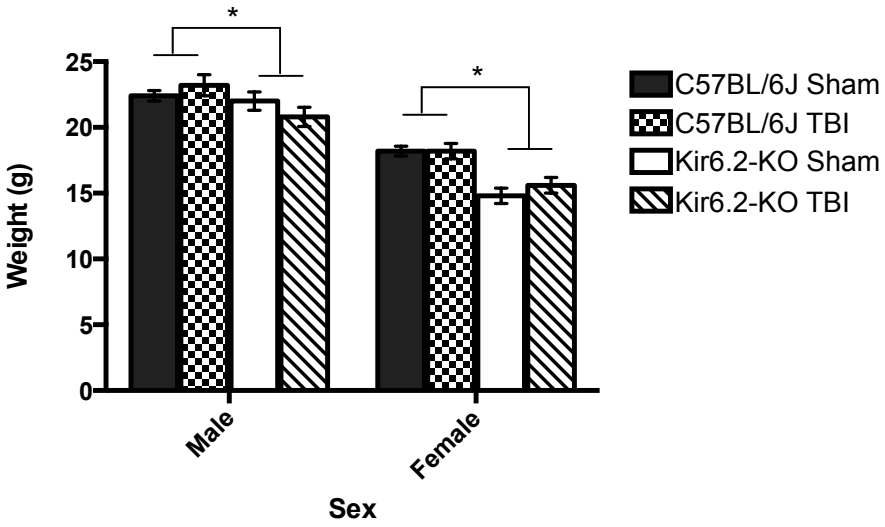


Figure 3.1. Average body weights 24h following mTBI (P46).

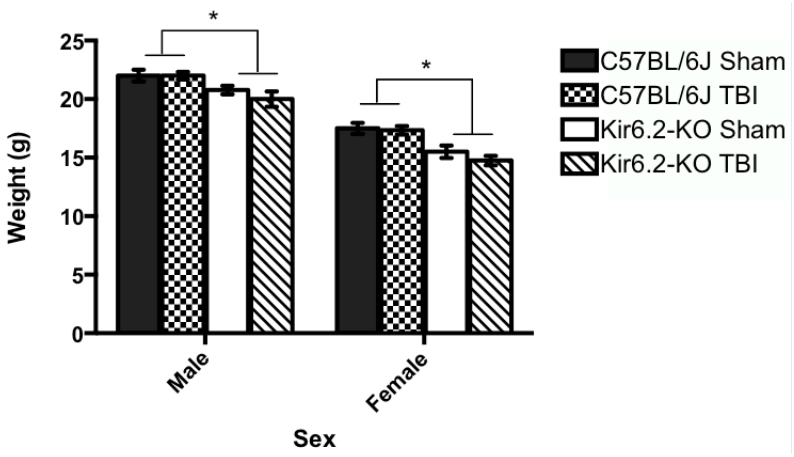


Figure 3.2. Average body weights 14 days following mTBI (P59).

3.1.2 Time to Right

The time required for righting reflexes to return after brief anesthesia was measured. Following mTBI, both males and females took longer to right compared to sham animals that just received anesthesia alone. A three-way ANOVA revealed a main effect of mTBI, F (1, 106) = 57.43, $p < .01$. However, analysis did not indicate main effects of sex, F (1, 106) = .63, $p = .43$ nor genotype, F (1, 106) = 1.00, $p = 0.32$. There were also no significant interactions observed, p's > .05. See figure 3.3.

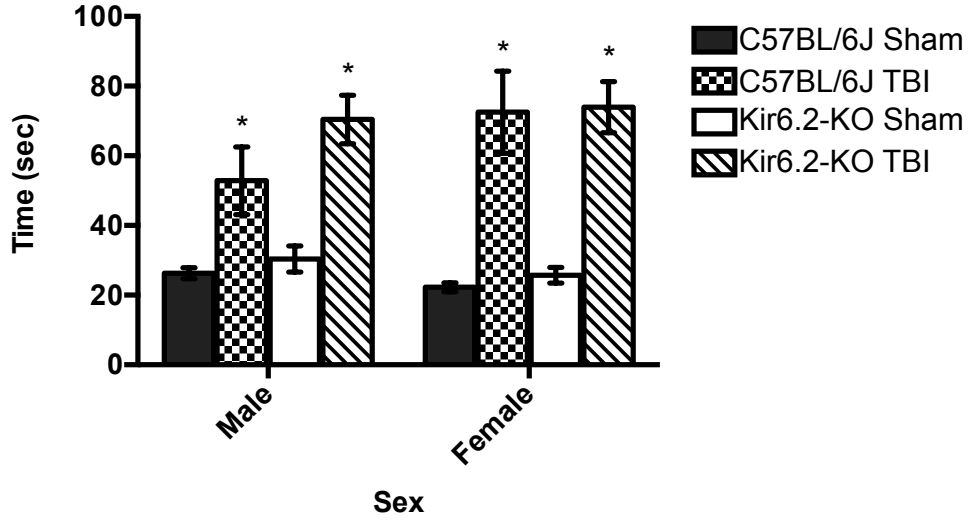


Figure 3.3. Time required for righting reflexes to return following anesthesia.

3.1.3 Beam Walk.

At 24 h post-injury, mTBI led to increased deficits in both hind-leg foots slips as well as the time it took animals to cross the beam when compared to shams. A three-way ANOVA revealed that mice sustaining a mTBI had significantly more hind-leg foot slips, F (1, 56) = 14.79, $p < .01$. However, analysis did not reveal any main effects of sex, F (1, 56) = .004, $p = .95$ nor strain, F (1, 56) = 1.48, $p = .23$. Also, no significant interactions were detected for number of foot slips, $p > .05$. Brain injured animals took significantly longer to cross the beam, F (1, 56) = 4.18, $p < .05$ compared to shams, and a significant main effect of strain, F (1, 56) = 31.51, $p < .01$ was also observed, whereby Kir6.2-KO mice took more time to cross the beam compared to C57BL/6J mice. However, there was no main effect of sex, F (1, 56) = 0.31, $p = .58$, or significant interactions p's > .05. See figures 3.4 and 3.5.

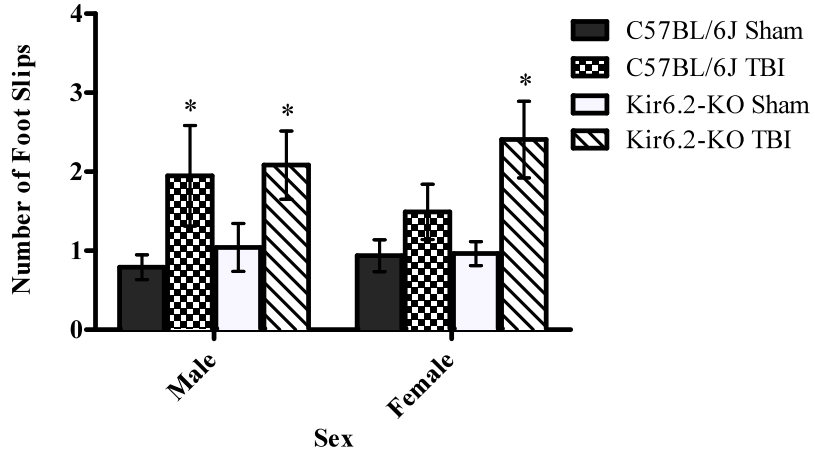


Figure 3.4. Average number of foot slips when crossing the beam.

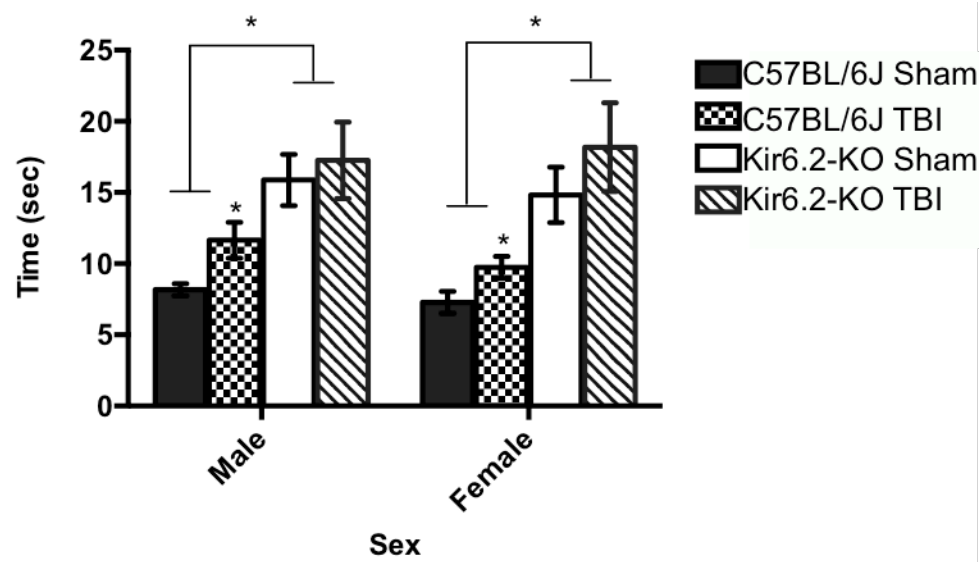


Figure 3.5. Average time required to cross the beam.

3.1.4 Crawley's Three-Chamber Sociability Test.

The effect of mTBI on social behaviour was more profound in Kir6.2-KO mice compared to C57BL/6J controls, and this effect was sexually dimorphic. A three-way ANOVA analyzing the percentage of time spent with the stranger mouse revealed a main effect of strain, F (1, 44) = 56.23, $p < .01$, and a trend towards a sex effect, F (1, 44) = 3.39, $p = .07$, but no main effect of injury, F (1, 44) = .65, $p = .43$. Kir6.2-KO male sham mice spent more time with the stranger mouse than comparative C57 controls. Significant interactions were observed for both sex by strain, F (1, 44) = 4.76, $p < .05$, and sex by injury, F (1, 44) = 8.26, $p < .01$ interactions. A trend, though not significant, was also observed for the strain by injury interaction, F (1, 44) = 3.66, $p = .06$. Lastly, a significant three-way interaction (sex by strain by injury) was revealed, F (1, 44) = 29.65, $p < .01$, indicating that mTBI had opposite effects on social behaviours in male and female Kir6.2-KO mice. Male Kir6.2-KO mice showed a marked reduction in the time they

spent with the stranger mouse following mTBI, yet Kir6.2-KO females increased the time that they spent with the stranger mouse. See figure 3.6.

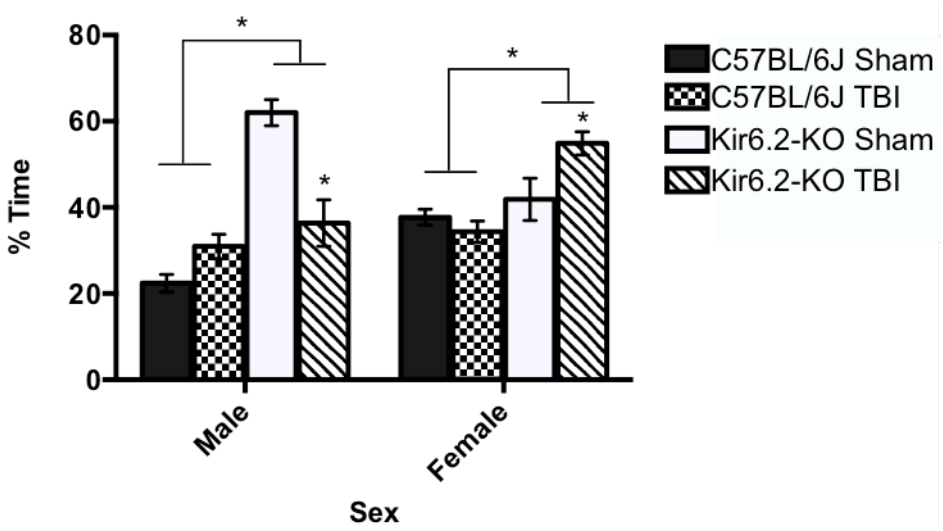


Figure 3.6. Percentage of time mice spent with a stranger mouse in the three-chamber test.

In contrast, opposite results were found for the time animals spent in the chamber containing a novel object. There was a main effect of strain, F (1, 44) = 27.6, $p < .01$ whereby C57BL/6J mice spent more time with the novel object compared to Kir6.2-KO mice. However, no main effects of sex, F (1, 44) = .084, $p = .77$ nor injury, F (1, 44) = 1.02, $p = .93$ were observed. A three-way interaction (sex by strain by injury), F (1, 44) = 7.0, $p < .05$ indicated that male and female Kir6.2-KO mice had opposite responses following mTBI. Females spent significantly less time with the novel object, whereas males more time on this chamber side. Lastly, no significant main effects or interactions were observed for the time animals spent in the middle chamber, $p > .05$. See figure 3.7.

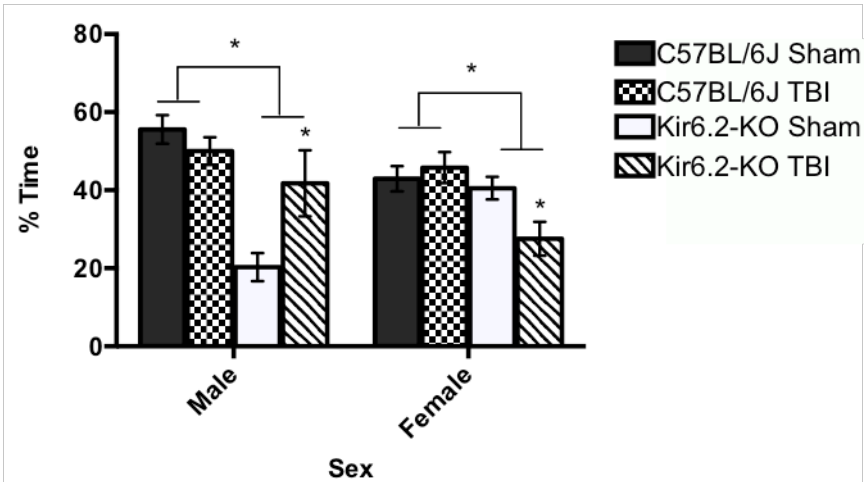


Figure 3.7. Percentage of time mice spent with a novel object in the three-chamber test.

3.1.5 Elevated Plus Maze (EPM).

Overall, males exhibited an increased anxiety-like phenotype on the EPM, as indicated by them spending significantly less time in the open arm compared to females. Anxious behaviours were also higher in C57BL/6J compared to Kir6.2-KO mice. Significant main effects of sex, F (1, 52) = 10.32, $p < .01$, and strain, F (1, 52) = 8.69, $p < .01$ were revealed for the percentage of time spent in the open arm, but not of injury, F (1, 52) = 0.097, $p = .76$. There was also a significant interaction between sex and strain, F (1, 52) = 6.90, $p = .01$. The effect of strain was sex specific with Kir6.2-KO females spending less time in the open arm compared to C57 BL/6J mice. A significant interaction between sex and injury was also detected, F (1, 52) = 4.15, $p < .05$. In females, the time spent in the open arm following injury was greater than controls, whereas in males the time spent in the open arm was shorter. Lastly, no significant three-way interaction (sex by strain by injury) was revealed, F (1, 52) = 0.41, $p = .53$. See figure 3.8.

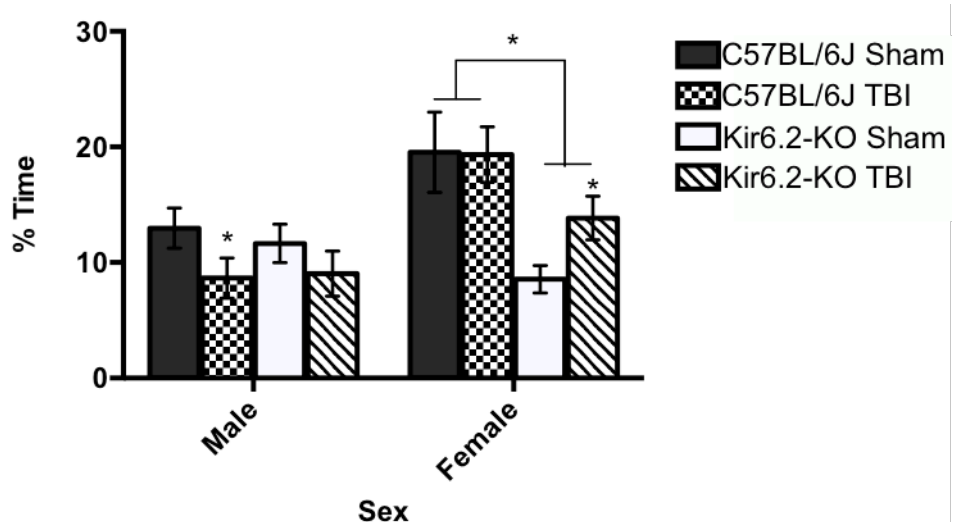


Figure 3.8. Percentage of time mice spent in the open arm of the EPM.

Interestingly, no main effects or significant interactions were observed for the time animals spent in the closed arms or the center of the EPM, *p*'s > .05. However, there was a main effect of strain, F (1, 52) = 3.93, p = .05 for the percentage of time spent in the center of the maze, indicating that Kir6.2-KO mice spent more time in the middle than C57 BL/6J controls. See figure 3.9.

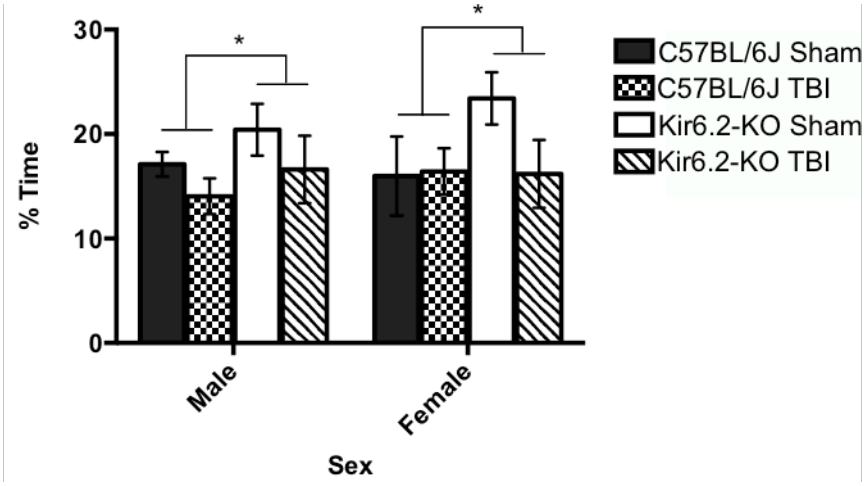


Figure 3.9. Percentage of time mice spent in the center of the EPM.

3.1.6 Open Field Test (OF).

A three-way ANOVA revealed that sex, strain, as well as injury affected the time animals spent in the outer area of the OF. While no main effects were observed for these factors alone, *p's* > 0.05, there were some significant interactions. First, a significant sex by strain interaction, $F(1, 48) = 7.21$, $p = .01$ demonstrated strain effects for the time spent in the outer area was opposite in males versus females. Male Kir6.2-KO mice spent less time in the outer area of the OF compared to C57 BL/6J, whereas female Kir6.2-KO mice spent more time in this area compared to controls. A strain by injury interaction was also observed, $F(1, 48) = 5.03$, $p < .05$. C57 BL/6J mTBI mice spent less time in the outer area compared to their sham injured counterparts. In contrast, Kir6.2-KO mice receiving a mTBI spent more time in the outer area compared to controls. Lastly, a three-way interaction (sex by strain by injury), $F(1, 49) = 6.49$, $p = .01$, indicated that following mTBI, female Kir6.2-KO mice spent the greatest amount of time in the outer area of the OF compared to other groups. See figure 3.10.

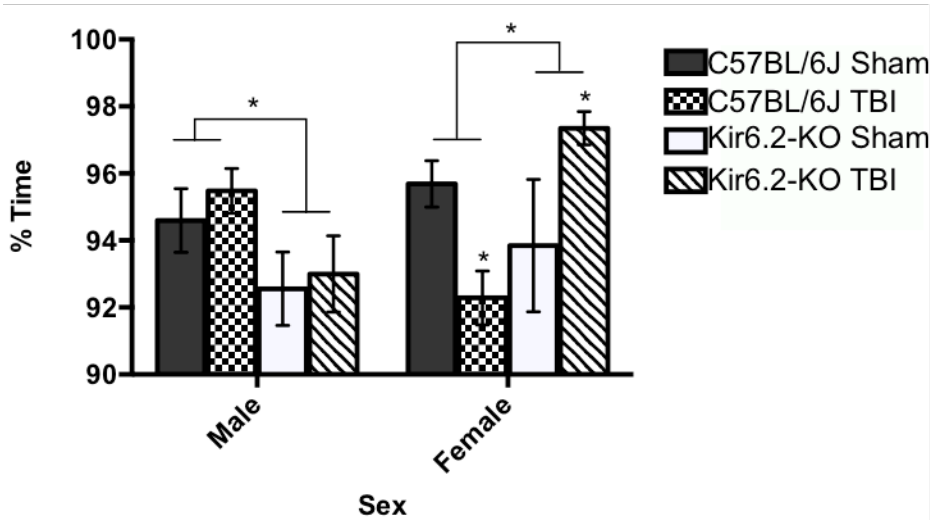


Figure 3.10. Percentage of time animals spent in the outer area of the OF.

The OF test was also used to measure overall activity level of the mice. A three-way ANOVA indicated a main effect of injury, F (1, 48) = 4.66, $p < .05$. However, the main effect was driven by a strain by injury interaction, indicating that the effect of mTBI was specific to Kir6.2-KO mice F (1, 48) = 4.28, $p < .05$. Activity level was also dependent on the interaction between sex and strain, F (1, 48) = 10.70, $p < .01$, where male Kir6.2-KO mice had a greater total distance moved compared to controls, but female Kir6.2-KO sham travelled less distance than C57BL/6J. No main effects of sex or strain were revealed, nor were any other interactions, p's > .05. See figure 3.11.

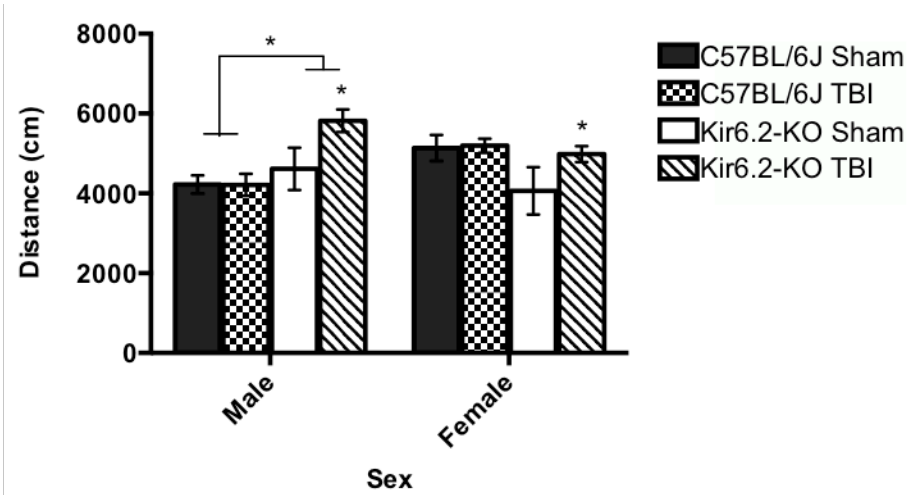
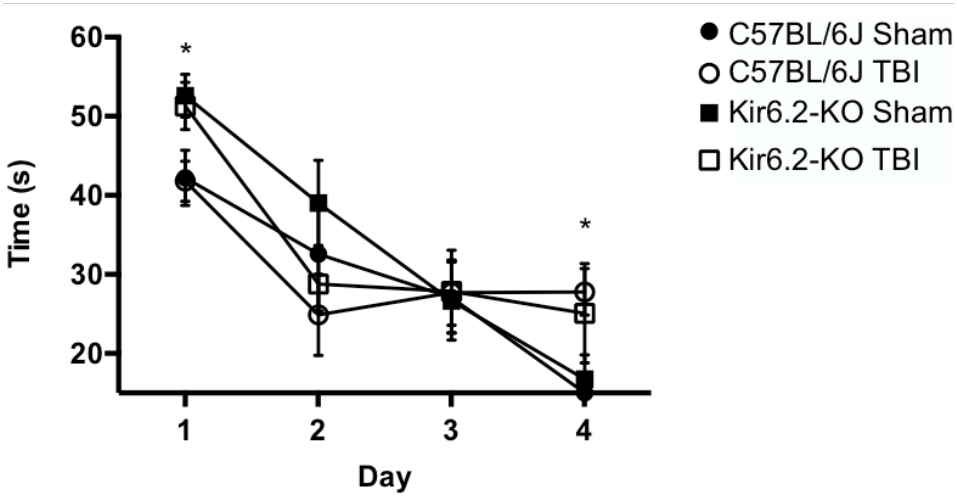


Figure 3.11. Distance travelled in the OF.

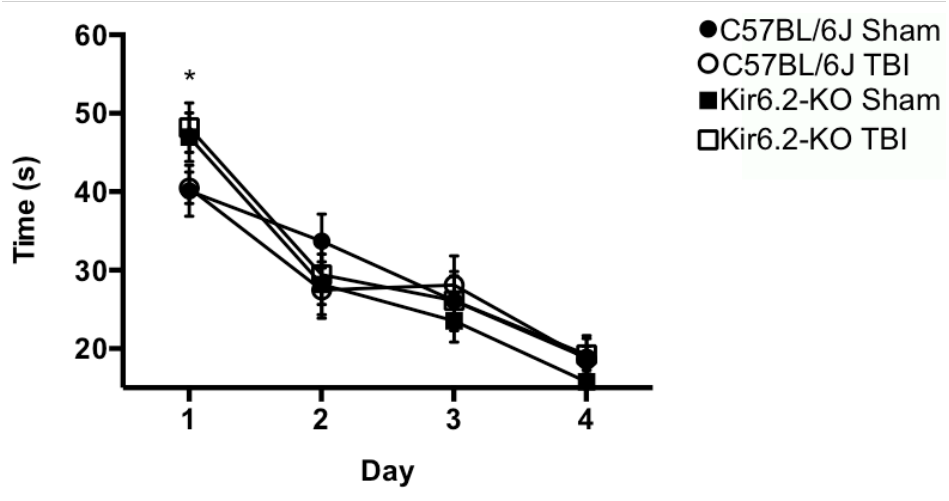
3.1.7 Morris Water Task (MWT)

A three-way repeated measures ANOVA was used to analyze learning acquisition data from the MWT. Sphericity was assumed, as the variance between each day of testing was not statistically different. Analysis revealed a main effect of day, the within subject variable, F (1,

150) = 11.57, $p < .01$, where it took progressively less time for the mice to find the platform between days. A day by strain interaction was also statistically significant, F (1, 150) = 3.61, $p < .05$, and *post-hoc* analysis demonstrated this interaction was only present on day 1. Lastly, a day by injury interaction was revealed, F (1, 150) = 4.35, $p < .01$, with *post-hoc* analysis indicating this interaction occurred only on day 4. Lastly, there was a trend towards a day by sex by injury on day 4, F (1, 50) = 3.56, $p = .07$ demonstrating that the effect of injury was driven by behaviour in females. See figures 3.12 (A) and (B).



(A)



(B)

Figure 3.12. Learning acquisition curves in the MWT for (A) females, and (B) males.

The probe trial indicated that following mTBI, Kir6.2-KO mice spent significantly more time in the target quadrant of the MWT. Significant main effects of strain, F (1, 50) = 4.15, $p < .05$, and injury, F (1, 50) = 7.07, $p = .01$ were detected. However, the main effect of sex was not statistically significant, F (1, 50) = 1.35, $p = .25$, nor were any interactions, p's > .05. See figure 3.13.

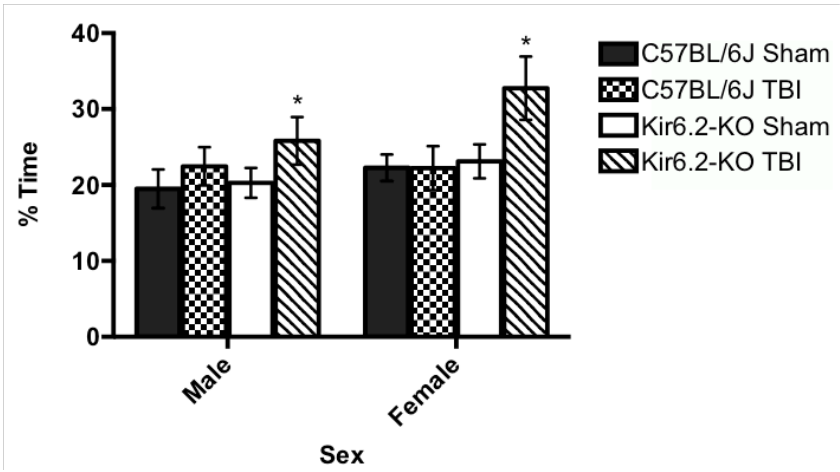


Figure 3.13. Percentage of time mice spent in the platform quadrant on probe day in the MWT.

3.1.8 Forced Swim Test (FST)

Overall, Kir6.2-KO mice that received a mTBI spent less time immobile than their sham counterparts or C57BL/6J mice. A main effect of injury, F (1, 52) = 8.06, $p < .01$, and a trend towards a main effect of strain, F (1, 52) = 3.35, $p = 0.07$ were revealed in the statistical analysis. However, there was no significant main effect of sex, F (1, 52) = .03, $p = .53$. A strain by injury interaction, F (1, 52) = 9.83, $p < .01$, indicated that following mTBI, Kir6.2-KO spent the least amount of time immobile compared to all other groups. See figure 3.14.

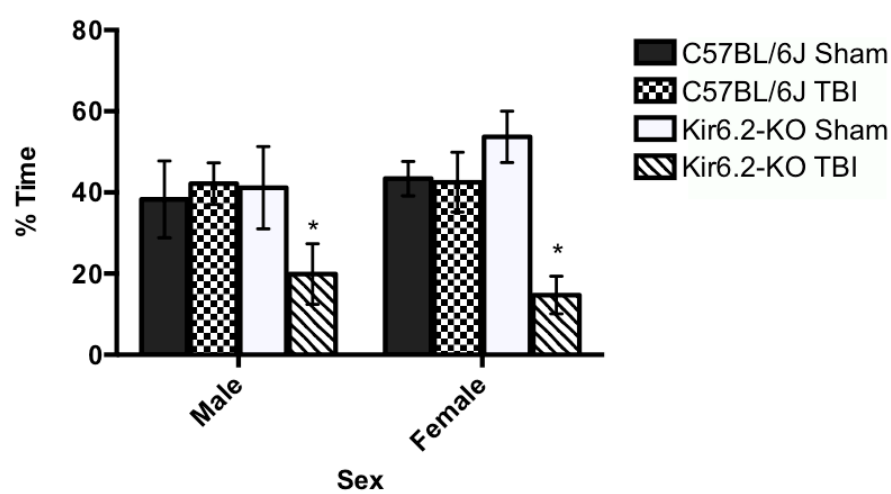


Figure 3.14. Percentage of time mice spent immobile in the FST.

3.2 Discussion

There is a subset of the human population that appears to be predisposed to poor outcomes following mTBI, but what makes these individuals more susceptible to long-term deficits is incompletely understood (Ruff et al., 1996). In its first component, this thesis sought to understand the relative roles that sex, genetics, and mTBI play in determining behavioural outcomes following a brain injury. We employed a novel model of genetic susceptibility, the Kir6.2-KO mouse, to assess behavioural outcomes following mTBI, and hypothesized that genetic difference, as well as sex, would influence outcomes. Tests were chosen in order to assess many of the cognitive and neuropsychological symptoms often reported following mTBI in human populations. Indeed, we found that all three of these factors influenced performance on behavioural tests. However, what is perhaps more interesting, is that the relative influence that sex, genetics, and injury have on outcomes appears to be dependent on the time point following the initial insult and the specific behaviour analyzed (see figure 3.17).

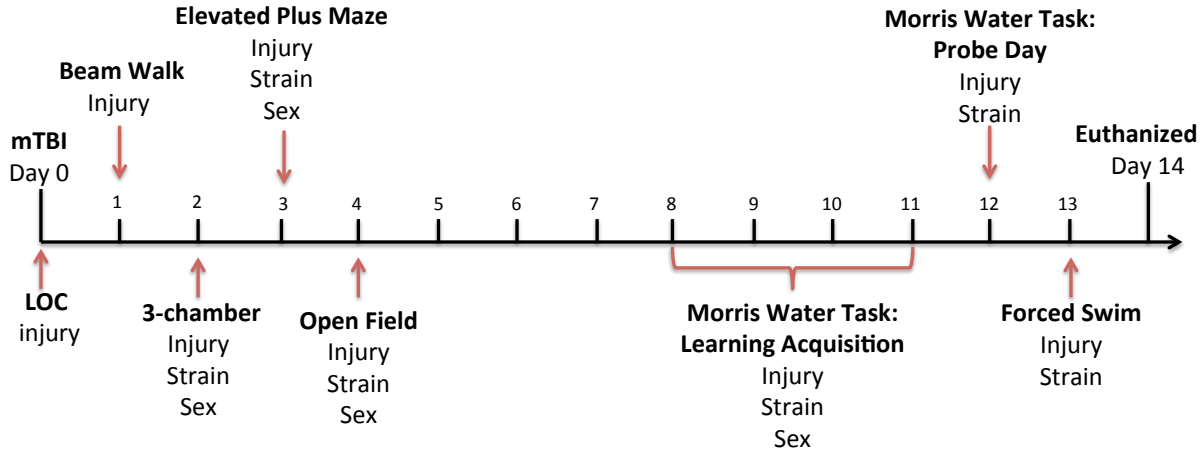


Figure 3.17. Timeline of behavioural tests and factors significantly affecting outcome.

Although loss of consciousness (LOC) is no longer considered a requirement for concussion, some patients sustaining a concussion report a transient LOC following injury (Carroll et al., 2004). This study analyzed the time required for righting reflexes to return following anesthesia as a measure of LOC (Franks, 2008). Although time to right, as used in this study, is not a direct measure of LOC because differences could also be due to an interaction between anesthetic and injury, it does provide some indication of an altered awareness following mTBI. Our results indicated that mice receiving a mTBI took nearly double the time to right themselves compared to sham injured animals. However, neither the sex nor strain of the animal affected this early outcome measure. This is consistent with previous literature that has shown sex does not necessarily affect in the rate or degree of LOC in human studies (Berz et al., 2013; Cantu, Guskiewicz, & Register-Mihalik, 2010; Frommer et al., 2011).

Following concussion, some patients also report a myriad of motor disturbances, such as problems with balance, coordination, or fine motor control (Jang, 2009; Kuhtz-Buschbeck et al., 2003). During post-concussion assessment in the clinic, tests of postural stability are commonly

used to assess motor dysfunction (Guskiewicz, Ross, & Marshall, 2001). We therefore chose to assess vestibulomotor function and motor coordination in mice 24 h following mTBI using the beam-walk test. This task required animals to navigate across a narrow beam in order to reach their home cage on the opposite end. The number of hind-limb foot slips and the time required to traverse the beam were used as measures of motor function. As hypothesized, mice that received a mTBI slipped significantly more often than shams. Multiple studies of mTBI have assessed motor deficits using the beam walk, and have found similar results (Mychasiuk, Farran, & Esser, 2014a; Piot-Grosjean, Wahl, Gobbo, & Stutzmann, 2001; Russell, Kutchko, Fowler, Berman, & Levant, 2011). This data, together with the time to right results, suggest that at least acutely, the effect of injury dominates over other factors such as sex or genetic differences in defining outcomes following mTBI. Unfortunately, this study did not assess motor function at a more distant time point post-injury so we are unable to discern whether motor deficits are also a chronic symptom in these animals. Observationally however, animals were capable of completing subsequent behavioural tasks and did not appear to perform any different than controls on the motor components of the tasks.

While the analysis did not indicate an effect of sex or strain on the number of foot slips, there was an effect of genetics on the time it took to cross the beam with Kir6.2-KO mice taking longer. Interestingly however, there was no effect of injury on this measure in the Kir6.2-KO mice. It is possible that Kir6.2-KO mice were impaired at learning the task and therefore took longer to cross, however data from the Morris water task would suggest that they do not have a learning deficit. Another possibility is that impaired motor coordination leads animals to cross the beam more slowly. Two areas of the brain known to be responsible for coordination of movement are the basal ganglia and cerebellum, both of which also have a particularly high

density of K_{ATP} channels (Dunn-Meynell, Rawson, & Levin, 1998; Thomzig, Laube, Pruss, & Veh, 2005). Increased time to cross the beam in Kir6.2-KO compared to C57BL/6J mice may reflect impaired basal ganglia and/or cerebellar functioning. This idea is supported by similar findings using the rota rod in a study profiling Kir6.2-KO behaviours (Deacon et al., 2006), which demonstrated that without brain injury these mice were impaired at motor functioning when compared to control animals.

Another symptom of PCS that has received significantly less attention in the TBI scientific community is social impairment. In particular, brain injury during childhood or adolescence may be especially detrimental to social development as this time period is critical for the development and consolidation of new social skills (Yeates et al., 2007). Studies of clinical populations have found that following TBI, some patients become more aggressive, antisocial, and have impaired social problem-solving skills (Andrews, Rose, & Johnson, 1998; Janusz, Kirkwood, Yeates, & Taylor, 2002; Yeates, 2010). Unfortunately, only a few studies have extrapolated findings from the clinic to experimental models of TBI (Mychasiuk, Hehar, et al., 2014; Semple, Canchola, & Noble-Haeusslein, 2012). Therefore, this study used Crawley's three-chamber test of sociability two days after the initial mTBI to test for effects of brain injury, and also to determine whether there was an influence of genetic susceptibility, or sex. Brain development of a mouse at this age (P47) roughly correlates with human adolescence (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013), and is therefore within the age range of social skill consolidation (Semple et al., 2012) . Analysis of the time spent socializing with a stranger mouse revealed a three-way interaction of sex, strain, and injury. First, the effect of injury was specific to Kir6.2-KO mice (i.e. no change was observed in C57BL/6J mice) indicating that genetic background played a role in determining social outcomes following

mTBI. Perhaps even more interesting, was that the combined effect of injury and genetics was opposite for males and females.

Following injury, Kir6.2-KO males had a significantly less social preference, whereas female Kir6.2-KO mice showed more sociability. The lower social behaviour observed in Kir6.2-KO males is consistent with another study using the three-chamber test to assess sociability after mTBI (Semple et al., 2012). This is an interesting finding, as in humans following brain injury it is not uncommon for patients to become withdrawn, often due to social rejection (Yeates et al., 2013). The reduction in sociality seen in the male Kir6.2-KO mice following mTBI could be a reflection of these behaviours. In our protocol, following injury, both mTBI and sham injured animals are caged together, it is therefore possible that following brain injury there is a reorganization of the social hierarchy within each cage so that animals receiving a TBI take on a more submissive, less social, role.

It should also be noted that at baseline, male Kir6.2-KO shams had a significantly higher preference for sociability than C57BL/6J mice. This baseline differences could be attributed to an aggressive phenotype in the male Kir6.2-KO's as the amygdala, a region of the brain important in aggression, has a high density of Kir6.2-containing K_{ATP} channels. Knocking out these channels therefore could lead to persistent activation of the amygdala and potentially increase aggressiveness (Dunn-Meynell et al., 1998; Thomzig et al., 2005). Furthermore, the literature has established that male rodents tend to be more aggressive than females (Blanchard, 1984; Edwards, 1969; Nelson & Chiavegatto, 2001) as they have larger amygdaloid volumes, as well as higher levels of circulating testosterone, which can activate this area (Edwards, 1969; Goldstein et al., 2001). This may be a plausible explanation for why male Kir6.2-KO mice, but not females, spend more time with the stranger mouse. Male Kir6.2-KO mice that were caged in

groups also experienced severe barbering and tail bites from their cage-mates, as well as regular fighting within cages, which further supports the idea of an aggressive phenotype. These behaviours were not observed in the female Kir6.2-KO mice, nor were they found to the same severity in male C57BL/6J controls. Future studies therefore could be directed towards analyzing aggressive phenotypes using a task such as the tube dominance test, which may be useful to discern sex differences, as well as the affect that mTBI may have on this behaviour. It would also be useful to extend molecular studies of the cellular stress response to the amygdala in order to determine if differences in male Kir6.2-KO mice are also observed on a molecular level in this area.

In female Kir6.2-KO mice, an increase is sociability was observed following mTBI. Despite these results being opposite to males, the data may indicate an alternative form of social impairment. Some children suffering from TBI become socially withdrawn, while in others, social impairment can manifest as an inability to understand social cues (Dennis, Barnes, Wilkinson, & Humphreys, 1998). The increased sociability observed in female Kir6.2-KO mice could reflect inappropriate interactions with the stranger mouse, or an inability to understand social responses from the stranger mouse. Together, results from the three-chamber test parallel observations in human populations that mTBI can affect sociability, and suggest that both sex and genetic differences can alter behaviour. However, as with all animal experimentation the results can be subject to interpretation and therefore it is important to cross reference behavioural abnormalities in one test with those from other tests.

Patients sustaining a mTBI also frequently report post-traumatic anxiety and depression (Ryan & Warden, 2003). In order to test how sex, genetics, and injury may predispose animals to anxiety or depression, we used three behavioural tests; the elevated plus maze (EPM), open field

test (OF), and forced swim test (FST). Our results demonstrated that outcomes on the EPM were dependent on sex, strain, as well as whether or not animals sustained an injury. Following mTBI, neither C57BL/6J nor male Kir6.2-KO mice showed a significant change in behaviour on the EPM. However, differences were seen in the Kir6.2-KO females. Interestingly, we found that female Kir6.2-KO mice spent more time in the open arms of the EPM after the injury. This may seem paradoxical compared to previous studies analyzing the effects of EPM performance after TBI, which demonstrated less time spent in the open arms, suggestive of increased anxiety (Baratz, Rubovitch, Frenk, & Pick, 2010; Jones et al., 2008; Schwarzbold et al., 2010). However, our study employed a different mechanism of injury, the modified weight drop (Kane et al., 2012; Mychasiuk, Farran, et al., 2014a, 2014b), which induced a diffuse brain injury through rotational and acceleration/deceleration forces. In contrast, previous studies induced a TBI using the fluid percussion injury, or original weight drop method, which most often produces focal injuries and are therefore models of more severe injury. These two types of mTBI- diffuse versus focal, may also result in the induction of different secondary injury cascades, which could account for the discrepancy between our results and previous literature.

There are also three other studies that have reported results similar to ours (Pandey, Yadav, Mahesh, & Rajkumar, 2009; Petraglia et al., 2014; Washington et al., 2012), and have interpreted the increase in time spent in the open arm as an increase in risk-taking behaviour. Furthermore, studies have also reported that adolescent mice, similar to this study, spend significantly more time in the open arms of the elevated plus maze compared to other age groups (Laviola, Macri, Morley-Fletcher, & Adriani, 2003). In addition, other studies have shown that following a stressful event; adolescent female rats were particularly likely to engage in risk-taking behaviours compared to male counterparts (McCormick, Smith, & Mathews, 2008;

Toledo-Rodriguez & Sandi, 2011). This is consistent with our findings, that female Kir6.2-KO mice spent the most time in the open arms of the EPM. Therefore, the age, genetic background, and sex of the mice may have an interactive effect to predispose them to increased risk-taking behaviour following TBI.

The OF was also used to measure anxiety, where the percentage of time animals spent in the outer area of the OF was used as a measure of anxiety. Mice are fearful of exposed areas, therefore when anxious, they tend to spend more time close to the walls of the OF, or what is designated as the "outer area" (Prut & Belzung, 2003). In our study the results suggest marked sex differences in the effect of genetic predisposition as well as injury on outcomes. In male mice, injury did not have an effect. However, there was a prominent difference between C57BL/6J and Kir6.2-KO males at baseline. C57BL/6J males spent significantly more time in the outer area, regardless of injury status, compared to Kir6.2-KO, suggesting that knocking out the Kir6.2 subunit of the K_{ATP} channel in males may lead to reduced anxiety, increased risk-taking behavior, or some other alternate explanation.

In females, there was an effect of injury in both C57BL/6J and Kir6.2-KO mice. However, the effect was opposite between strains, indicating that at least for females, genetic background plays a role in determining outcomes on anxiety measures following mTBI. In female C57BL/6J injury reduced the time they spent in the outer area following injury, which suggests an increase in exploratory or risk-taking behaviours. In contrast, Kir6.2-KO females spent more time in the outer area post-mTBI, indicating an increase in anxiety. Together, this data suggests that genetic background does indeed affect anxiety behaviours following mTBI, but also that there is an effect sex that can modify the affect of genetics, or vice-versa.

Interestingly, results from the OF in Kir6.2-KO females following mTBI contrast with findings from the EPM. There are a few potential reasons why data from these tests do not necessarily coincide. First, the EPM and OF were run on different days; therefore a certain degree of recovery, or acclimation, could have taken place between each subsequent test. This is supported by the fact that different factors namely sex, genetics, and injury; exert different levels of influence over behavioural outcomes depending on the time from initial injury (see figure 3.17). Second, anxiety is a multidimensional and dynamic mental state. There are multiple types of anxiety, and the degree to which an animal or human feels anxious can rapidly change, and be context dependent. Therefore, each test of anxiety may have assessed different anxiety constructs, or levels of emotion within an animal. To this end, multiple studies comparing the results of the open field and elevated plus maze have demonstrated they may not perfectly overlap in assessing anxiety-like phenotypes (Ramos, Pereira, Martins, Wehrmeister, & Izidio, 2008; Trullas & Skolnick, 1993; Vendruscolo, Takahashi, Bruske, & Ramos, 2003). The fact that different results were found on the OF and EPM, two tests that measure similar behaviours, again emphasizes the need to employ multiple behavioural tests when attempting to understand the behavioural phenotype in complex disorders. Assuming that an animal exhibits a certain behaviour based on a single measure of a single time point could lead to incorrect conclusions.

The OF was also used as a measure of general activity in mice. Results indicated that following injury, Kir6.2-KO mice travelled a greater distance than C57BL/6J mice, and that this effect was more pronounced in males. One possible reason that we observed an increase in activity level following mTBI in Kir6.2-KO but not C57BL/6J mice may have to do with genetic susceptibility. It is possible that the weight we used to induce injury was only enough to unmask behavioural deficits in animals with a genetic predisposition to behavioural deficits. The

hyperactivity that was observed in Kir6.2-KO mice post-mTBI is consistent with other literature demonstrating increased spontaneous activity in the OF after brain injury (Budinich, Tucker, Lowe, Rosenberger, & McCabe, 2013; Kimbler, Shields, Yanasak, Vender, & Dhandapani, 2012; Li et al., 2006; Pullela et al., 2006; Schwarzbold et al., 2010). Lastly, hyperactivity is one of the hallmark traits of attention deficit/hyperactivity disorder (ADHD), which can occur in a subset of the human population secondary to TBI, particularly in pediatric populations (Konrad, Gauggel, Manz, & Scholl, 2000), although this association is still under debate. While our results only demonstrate one of the many symptoms of ADHD, it does suggest an important area for future research; namely can experimental models of traumatic brain injury induce an ADHD-like phenotype following injury? Research in our lab is exploring this question.

To determine whether our model also had an effect on other affective behaviours (i.e. depression or anhedonia) we used the Porsolt's forced swim test. Mice were placed in a container of water and the amount of time spent immobile was used as a surrogate measure of depression (Porsolt et al., 1977). Interestingly, Kir6.2-KO mice that received a mTBI spent significantly less time immobile when compared to other groups; an effect that was not sex-dependent. It is difficult to draw any parallels with other studies of TBI using the FST, as the results have been largely inconclusive. Some studies show increased immobility (Milman, Rosenberg, Weizman, & Pick, 2005), while others find no difference between mTBI and sham groups (Petraglia et al., 2014). Due to the inconsistencies in experimental design between studies of TBI, such as the mechanism of injury, time of testing, or age of animals, it is perhaps not surprising that results differ significantly within the literature. Furthermore, as mTBI is a very heterogeneous injury, the symptom profile between different cases will likely vary between individuals and even within individuals over time.

While most studies interpret immobility as a marker of depression, there are other interpretations that could be drawn (West, 1990). Owing to the hyperactivity found in Kir6.2-KO mice after injury in the OF, less time spent immobile in the FST could just be interpreted as a marker of hyperactivity in the Kir6.2-KO mice that is unmasked by mTBI. Another possibility is that the C57BL/6J mice and Kir6.2-KO shams have adapted to the inescapability of the FST and become immobile as an effective coping strategy to conserve energy. In this case, immobility would not be a measure of depression but instead indicative of learning. In this context, reduced immobility in the Kir6.2-KO mice receiving a brain injury may actually suggest a learning deficit. Nonetheless, regardless of the interpretation, results from the FST indicate that following mTBI, different genetic backgrounds alter outcomes on neurobehavioral tests. Further cross-correlative tests will however be needed to try to discern the significance of these findings.

One of the most common tests used to assess cognitive deficits in experimental models of TBI is the Morris water task (MWT). This test assesses the spatial learning and memory abilities of animals by forcing them to learn the location of a hidden platform in a pool by using visual cues (Morris, 1984). In our study, at the onset of learning, C57BL/6J mice were faster at finding the platform than Kir6.2-KO mice. However, this difference in performance between the strains was not evident after day 1. While mice receiving a mTBI improved over the 4-day learning acquisition period, they did not perform as well as shams on the final day of learning, suggesting the possibility of a persistent spatial learning deficit. However, this effect tended to be specific to females ($p = .06$). It has long been suggested that males tend to be superior at performing spatial memory tasks compared to females (Kolb & Whishaw, 2009). Therefore, one possible reason we observed a mTBI effect only in females is that they are predisposed to poorer performance on spatial tasks, and the mTBI unmasks this susceptibility. The MWT is a hippocampus-dependent

task (Morris, Garrud, Rawlins, & O'Keefe, 1982), and therefore deficits in learning acquisition may reflect changes to the hippocampus following mTBI. Results from the molecular analysis (see below) are consistent with this and demonstrate sex- as well as injury- dependent changes to this brain region. Previous studies assessing spatial learning following mTBI have found similar results (Creed, DiLeonardi, Fox, Tessler, & Raghupathi, 2011; Petraglia et al., 2014; Zohar, Rubovitch, Milman, Schreiber, & Pick, 2011). However, these studies showed more severe impairments in spatial learning when compared to our results, possibly owing to different and more severe mechanisms of injury.

Another useful component of the MWT is the probe trail where the platform is removed and the animal's ability to adapt and task switch is measured. Interestingly, the Kir6.2-KO mTBI mice spent significantly more of their time in the platform quadrant compared to other groups. These results are consistent with a previous paper from our lab (Mychasiuk, Farran, et al., 2014), using the same injury model in young rats. In shams, both C57BL/6J and Kir6.2-KO mice initially swam to the initial platform location, but quickly adapted to the platform absence and began to explore the other quadrants until they found the platform. However, Kir6.2-KO mice that received a brain injury perseverated in the initial platform location, and did not as efficiently disengage their attention to search other areas. One important function of the frontal lobe, an area that is thought to be affected in concussion, is cognitive flexibility – the ability to switch attention between different tasks (Brooks, Fos, Greve, & Hammond, 1999). Multiple studies have shown that in human studies patients with TBI have difficulty disengaging from certain tasks to focus their attention elsewhere (Brooks et al., 1999; Howell, Osternig, Van Donkelaar, Mayr, & Chou, 2013), which is consistent with our findings on the probe day of the MWT. Lastly, and similar to results on the FST, OF, and three-chamber test, deficits following mTBI

were specific to Kir6.2-KO indicating that they might be more susceptible to poor outcomes compared to C57BL/6J mice.

3.3 Conclusion

Taken together, results from neurobehavioral testing further support the idea that outcomes following mTBI are incredibly complex and heterogeneous. These results stress that sex, genetics, as well as mechanism of injury should be taken into account when predicting how a patient may recover from brain injury. Furthermore, the degree to which each of these factors influences outcome depends both on the time following injury, and the specific behaviour being assessed. This is particularly important as it indicates a need to tailor diagnostic choices as well as treatment plans to the individual as oppose to searching for global solutions.

Chapter Four: **Molecular Changes**

4.1 Results

4.1.1 Prefrontal Cortex at 24 h post-mTBI

Results for the following section are summarized in table 4.1, and graphs are available in Appendix A.

4.1.1.1 Hsp90aa1

A three-way ANOVA with sex, strain, and injury as factors revealed main effects of strain, F (1, 29) = 5.27, $p < .05$, and of injury, F (1, 29) = 9.44, $p < .01$. *Hsp*90aa1 expression was higher in Kir6.2-KO mice compared to C57BL/6J controls, and following mTBI, expression levels were lower than in shams. A strain by injury interaction was also observed, F (1, 29) = 14.24, $p = .01$, where C57BL/6J mice that received a mTBI had the lowest levels of *Hsp*90aa1 expression when compared to other groups. A main effect of sex, and the other interactions did not reach significance, p*'s* $> .05$.

4.1.1.2 Hsp90b1

A three-way ANOVA revealed a main effect of sex, F (1, 29) = 5.26, $p < .05$, strain, F (1, 29) = 6.89, $p = .01$, and injury, F (1, 29) = 13.6, $p < .01$. Furthermore, an interaction between sex and strain, F (1, 29) = 5.42, $p < .05$, and strain and injury, F (1, 29) = 5.13, $p < .05$ were observed. Lastly, a significant sex by strain by injury interaction was demonstrated, F (1, 29) = 4.78, $p < .05$ where female C57BL/6J mice that received an mTBI had the lowest level of *Hsp*90b1 expression.

4.1.1.3 Hsp70

Statistical analysis revealed a main effect of strain, F (1, 25) = 15.59, p < .01, and injury, indicating that C57BL/6J mice had higher expression than Kir6.2-KO mice. A main effect of injury was also detected, F (1, 25) = 41.87, p < .01, which showed that mTBI increased *Hsp*70 expression following mTBI in the prefrontal cortex 24 h post-injury. There was also a sex by injury interaction, F (1, 25) = 6.67, p < .05, demonstrating that injury resulted in greater expression changes in males compared to females. Lastly, a strain by injury interaction was found, F (1, 25) = 20.13, p < .01, which indicated that the effect of injury was greater in C57BL/6J mice compared to Kir6.2-KO mice. A main effect of sex was not statistically significant, nor were any other the interactions, p's > .05.

4.1.1.4 Hsp60

*Hsp*60 expression was higher in Kir6.2-KO mice compared to C57BL/6J controls, and was reflected by a main effect of strain, F (1, 26) = 9.59, p < .01. Main effects of sex, F (1, 26) = 2.95, p = .10, and injury, F (1, 26) = 2.97, p = .10 were not statistically significant, nor were any of the interactions, p's > .05.

4.1.1.5 Hsp40

A three-way ANOVA revealed a main effect of sex, F (1, 29) = 4.90, p < .05, and strain, F (1, 29) = 5.44, p < .05, as well as a strain by injury interaction, F (1, 29) = 4.81,

$p < .05$. Results indicated that in C57BL/6J mice receiving a mTBI, had lower levels of gene expression, while other groups did not significantly differ. There was no significant main effect of injury, F (1, 29) = 2.89, $p = .10$, nor any other interactions, p's > .05.

4.1.1.6 Hsp32

*Hsp*32 expression was lower in animals that received a mTBI when compared to sham injured controls, and this main effect of injury was statistically significant, F (1, 29) = 5.56, $p < .05$. Furthermore, a sex by strain interaction was also revealed, F (1, 29) = 6.66, $p < .05$, indicating that *Hsp*32 expression was lower in Kir6.2-KO compared to C57BL/6J in males but higher in Kir6.2-KO females. There were no main effects of sex or strain, nor were the other interactions significant, p's > .05.

4.1.1.7 Hsp27

Statistical analysis revealed a significant sex by strain interaction, F (1, 29) = 8.52, $p < .01$. In males, *Hsp*27 expression was lower in Kir6.2-KO mice compared to C57BL/6J, whereas for females, the opposite trend was revealed; Kir6.2-KO mice had higher expression. A significant sex by injury interaction was also revealed, F (1, 29) = 7.51, $p = .01$, where the effect of mTBI on gene expression was opposite for males versus females. mTBI resulted in higher levels of *Hsp*27 expression in males compared to their respective shams, but lower levels of expression in females. Main effects of sex, strain, and injury were not statistically significant, nor were the other interactions, p's > .05.

4.1.1.8 Hsp10

A three-way ANOVA revealed a main effect of sex F (1, 28) = 6.21, $p < .05$, but not of strain, F (1, 28) = 0.03, $p = .87$, or injury F (1, 28) = 1.77, $p = .19$. Males had higher *Hsp*10 expression compared to females. A sex by strain interaction was also revealed, F (1, 28) = 10.30, $p < .01$, indicating that strain had an opposite effect in males versus females. Male Kir6.2-KO mice had lower expression compared to C57BL/6J controls, whereas the opposite pattern was true for females. Furthermore, a strain by injury interaction was also observed, F (1, 28) = 12.78, $p < .01$. C57BL/6J mice had lower expression following injury, whereas Kir6.2-KO mice either had higher expression (males) or no change (females). Main effects of strain and injury were not statistically significant, nor were the other interactions, p's > .05.

4.1.1.9 Hsp5

Statistical analysis revealed a trend towards a main effect of sex, F (1, 29) = 3.95, $p = .06$, indicating that males may have higher Hsp5 expression compared to females. A main effect of injury, F (1, 29) = 6.85, $p < .05$ was also observed. Following mTBI, mice had lower levels of *Hsp*5 expression compared to shams. A strain by injury interaction was F (1, 29) = 7.71, $p = .01$ was also observed. The effect of mTBI was specific to C57BL/6J, resulting in lower levels of *Hsp*5 expression, but did not result in marked changes in Kir6.2-KO mice. Lastly, our results did not indicate a significant main effect of strain, or any other interactions, p's > .05.

Table 4.1. Hsp Expression in the Prefrontal Cortex at 24 h Post-mTBI

		Male		Female	
Brain Region	**Gene**	**C57BL/6J**	**KATP-KO**	**C57BL/6J**	**KATP-KO**
Nucleus / Cytosol	Hsp90aa1	↓1,2,6	Ø1,2	↓1,2,6	Ø1,2
	Hsp70	↑2,3,5,6	↑2,3,5,6	↑2,3,5,6	↓2,3,5,6
	Hsp40	↓1,2,6	Ø1,2	↓1,2,6	↓1,2,6
	Hsp27	Ø4,5	↑4,5	Ø4,5	↓4,5
ER	Hsp90b1	↓1,2,3,4,6	Ø1,2,4	↓1,2,3,4,6,7	↑1,2,4
	Hsp5	↓1,3,6	Ø1	↓1,3,6	Ø1
Mitochondria	Hsp60	Ø2	Ø2	Ø2	Ø2
	Hsp10	↓1,4,6	↑1,4	↓1,4,6	Ø1,4
Mito/ER	Hsp32	↓3,4	Ø4	↓3,4	Ø4

*Numbers designate significant main effects or interactions where 1= sex, 2 = strain, 3 = injury, 4 = sex by strain, 5 = sex by injury, 6 = strain by injury, 7 = sex by strain by injury.

4.1.2 Hippocampus at 24 h post-mTBI

Results for the following section are summarized in table 4.2, and graphs are available in Appendix B.

4.1.2.1 Hsp90aa1

A three-way ANOVA revealed a trend towards main effect of sex, F (1, 28) = 5.02, p = .06, indicating that males had higher expression levels of *Hsp*90aa1 than females. Results

also demonstrated a sex by injury interaction, F (1, 28) = 7.03, $p < .05$ where mTBI had opposite effects on males versus females. In males, mTBI lowered *Hsp*90aa1 expression levels, but caused an increase in females. Lastly, main effects of strain and injury were not statistically significant, nor were the other interactions, p's > .05.

4.1.2.2 Hsp90b1

*Hsp*90b1 expression was significantly increased in Kir6.2-KO mice relative to C57BL/6J controls, and was reflected as a main effect of strain, F (1, 27) = 10.80, $p < .01$. Main effects of sex and injury were not statistically significant, nor were any interactions, p's > .05.

4.1.2.3 Hsp70

Analysis revealed a main effect of strain, F (1, 28) = 7.68, $p = .01$, and trend towards a main effect of injury, F (1, 28) = 3.57, $p = .06$. Kir6.2-KO mice had higher *Hsp*70 expression relative to C57BL/6J, and mTBI tended to decrease expression. However, a significant sex by injury interaction, F (1, 28) = 5.93, $p < .05$ demonstrated that the effect of injury was specific to females and did not result in significant changes for males. No main effect of sex was observed, nor were the other interactions significant, p's > .05.

4.1.2.4 Hsp60

*Hsp*60 expression was significantly higher in Kir6.2-KO mice compared to C57BL/6J controls, as demonstrated by a significant main effect of strain, F (1, 28) = 7.85, $p < .01$. However, a sex by strain interaction F (1, 28) = 7.99, $p < .01$, revealed that this effect was specific to females. A sex by injury interaction, F (1, 28) = 5.19, $p < .05$ showed that the effect

of mTBI on *Hsp*60 expression was opposite for males and females. In males, injury tended to increase expression, compared to females where this was decreased compared to shams. Lastly, an interaction between strain and injury, F (1, 28) = 5.36, $p < .05$ was also significant. Results demonstrated that the effect of mTBI increased *Hsp*60 expression in Kir6.2-KO mice, but decreased expression in C57BL/6J mice. No main effects of sex or injury were statistically significant, nor was the sex by strain by injury interaction, p's > .05.

4.1.2.5 Hsp40

A three-way ANOVA revealed a main effect of sex, F (1, 28) = 10.14, $p < .01$, and strain, F (1, 28) = 23.17, $p < .01$, indicating that males had increased expression relative to females and that Kir6.2-KO mice had higher expression compared to C57BL/6J controls. A strain by injury interaction, F (1, 28) = 10.14, $p < .05$ also demonstrated that mTBI had opposite effects in Kir6.2-KO mice versus C57BL/6J controls. In Kir6.2-KO mice, it increased *Hsp*40 expression, whereas in C57BL/6J controls mTBI decreased expression. A main effect of injury was not statistically significant, nor were the other interactions, p's > .05.

4.1.2.6 Hsp32

Statistical analysis revealed that Kir6.2-KO mice had significantly higher *Hsp*32 expression compared to C57BL/6J controls, as demonstrated by a main effect of strain, F (1, 28) = 25.73, $p < .01$. A significant sex by injury interaction was also observed, F (1, 28) = 8.49, $p < .01$, where mTBI had a greater effect on females compared to males, and resulted in decreased *Hsp*32 expression. In males, expression increased, between mTBI and sham control

groups though not to the same degree of change as observed in females. Analysis did not reveal main effects of sex or injury, nor were there other interactions, *p*'s > .05.

4.1.2.7 Hsp27

Following mTBI, males and females had opposite changes in *Hsp*27 expression. Injury increased expression in males, but reduced levels in females. Results demonstrated a significant sex by injury interaction, F (1, 27) = 6.38, $p < .05$ in a three-way ANOVA. However, analysis did not reveal main effects of sex, strain, or injury, nor did the other interactions reach statistical significance, *p*'s > .05.

4.1.2.8 Hsp10

Statistical analysis revealed a main effect of sex, F (1, 27) = 5.76, $p < .05$ where males had higher *Hsp*10 expression compared to males. Results also demonstrated a significant sex by strain interaction, F (1, 27) = 9.58, $p < .01$. The effect of strain on *Hsp*10 expression was opposite for males and females. In males, Kir6.2-KO mice had lower expression compared to C57BL/6J controls. This pattern was opposite for females; Kir6.2-KO mice had higher expression compared to C57BL/6J controls. A strain by injury interaction was also evident following statistical analysis, F (1, 27) =11.90, $p < .01$, which demonstrated that the effect of mTBI was specific to C57BL/6J mice and caused a decrease in *Hsp*10 expression. Main effects of strain and injury were not significant, nor were the other interactions, *p*'s > .05.

4.1.2.9 Hsp5

A three-way ANOVA revealed a main effect of strain, F (1, 27) =8.44, $p < .01$, where Kir6.2-KO mice had higher expression compared to C57BL/6J controls. A sex by injury interaction was also evident following analysis, F (1, 27) =7.12, $p = .01$, demonstrating that the effect of mTBI was specific to females, and let to a reduction in *Hsp*5 expression. Lastly, there was a significant sex by strain by injury interaction, F (1, 27) =4.11, $p = .05$. Following mTBI, male Kir6.2-KO mice had an increase in *Hsp*5 expression, whereas other groups had a reduction in expression following injury. Main effects of sex and injury were not statistically significant, nor were sex by strain or strain by injury interactions, p's > .05

Table 4.2 Hsp Expression in the Hippocampus at 24 h post-mTBI

		Male		Female	
Brain Region	**Gene**	**C57BL/6J**	**KATP-KO**	**C57BL/6J**	**KATP-KO**
Nucleus / Cytosol	Hsp90aa1	Ø1	↓1,5	Ø1	Ø1
	Hsp70	Ø2	Ø2	↓2,3,5	Ø2
	Hsp40	Ø1,2	↑1,2,6	↓1,2,6	Ø1,2
	Hsp27	Ø5	Ø5	Ø5	Ø5
ER	Hsp90b1	↓2	Ø2	Ø2	↓2
	Hsp5	↓2,5	↑2,5	↓2,5	↓2,5
Mitochondria	Hsp60	Ø2,4	↑2,4,5,6	↓2,4,5,6	Ø2,4
	Hsp10	↓1,4,6	↑1,4,6	↓1,4,6	↓1,4,6
Mito/ER	Hsp32	Ø2	Ø2	↓2,5	Ø2

*Numbers designate significant main effects or interactions where 1= sex, 2 = strain, 3 = injury, 4 = sex by strain, 5 = sex by injury, 6 = strain by injury, 7 = sex by strain by injury.

4.1.3 Prefrontal Cortex at 14 days post-mTBI

Results for the following section are summarized in table 4.3, and graphs are available in Appendix C.

4.1.3.1 Hsp90aa1

A three-way ANOVA revealed a main effect of sex, F (1, 18) =6.04, $p < .05$, and strain, F (1, 18) =6.04, $p < .01$. Males tended to have higher *Hsp*90aa1 expression compared to females, and Kir6.2-KO mice had higher expression compared to C57BL/6J controls. A main effect of injury was not revealed, nor were any interactions statistically significant, p's > .05.

4.1.3.2 Hsp90b1

*Hsp*90b1 expression was higher in males compared to females, and was reflected in the analysis as the three-way ANOVA demonstrated a main effect of sex, F (1, 18) =10.62, $p < .01$. Results also revealed a main effect of strain, F (1, 18) =10.52, $p < .01$, where Kir6.2-KO mice had higher expression compared to C57BL/6J controls. Though not significant, there was a trend towards a sex by strain interaction, F (1, 18) = 3.81, $p = .07$, where the effect strain was specific to females. Kir6.2-KO females tended to have higher *Hsp*90b1 expression compared to C57BL/6J mice. This difference in expression between strains was not evident in males. Lastly, a significant sex by strain by injury interaction was revealed F (1, 18) =8.42, $p = .01$. Following mTBI, male Kir6.2-KO mice had an increase in *Hsp*90b1 expression, whereas other groups had a reduction in expression following injury. A main effect of injury was not revealed, nor were the other interactions statistically significant, p's > .05.

4.1.3.3 Hsp70

The effect of strain on *Hsp*70 expression was sex dependent. Kir6.2-KO females tended to have higher *Hsp*70 expression compared to C57BL/6J controls. However, there was no difference evident in males. These results were demonstrated as a significant sex by strain interaction, F (1, 18) =7.02, $p < .05$. A trend towards a main effect of sex was also revealed though it did not reach statistical significance, F (1, 18) = 3.81, $p = .07$. Overall, males tended to have higher *Hsp*70 expression compared to females. Main effects of strain and injury were not revealed, nor were the other interactions statistically significant, p's > .05.

4.1.3.4 Hsp60

A main effect of strain, F (1, 18) = 6.95, $p < .05$ revealed that Kir6.2-KO mice had higher *Hsp*60 expression compared to C57BL/6J controls. However, this effect was driven by a significant sex by strain interaction, F (1, 18) = 8.86, $p < .01$ where Kir6.2-KO females tended to have higher *Hsp*60 expression compared to C57BL/6J controls. No differences evident in males. Main effects of sex and injury were not statistically significant, nor were the other interactions, p's > .05.

4.1.3.5 Hsp40

A three-way ANOVA revealed main effects of sex, F (1, 18) =9.63, $p < .01$, and strain, F (1, 18) = 6.80, $p < .05$. However, these main effects were driven by a sex by strain interaction, F (1, 18) = 7.77, $p = .01$ where the effect of strain on *Hsp*40 expression was specific to females. Kir6.2-KO females tended to have higher *Hsp*40 expression compared to C57BL/6J controls but in males, there were no significant differences in expression between strains. A significant strain

by injury interaction was also observed, F (1, 18) =5.09, $p < .05$ where the effect of mTBI was opposite in Kir6.2-KO mice compared to C57BL/6J controls. In Kir6.2-KO mice, mTBI increased *Hsp*40 expression relative to shams, but in C57BL/6J mTBI decreased expression. Lastly, a main effect of injury was not statistically significant, nor were the other interactions, p's > .05.

4.1.3.6 Hsp32

A three-way ANOVA revealed a main effect of strain, F (1, 18) = 9.98, $p < .01$ where Kir6.2-KO mice had higher *Hsp*32 expression compared to C57BL/6J controls. However, this strain effect was driven by a sex by strain, F (1, 18) = 7.44, $p < .05$, indicating that the effect of strain on *Hsp*32 expression was specific to females. Kir6.2-KO females tended to have higher *Hsp*32 expression compared to C57BL/6J controls but in males, there were no significant differences in expression between strains. Though not significant, a trend towards a sex by injury interaction was observed F (1, 18) = 3.87, $p = .06$. The effect of mTBI was greater in females and caused a decrease in *Hsp*32 relative to shams. In males, there was no difference between mTBI and sham groups. Lastly, no significant main effects of sex or injury were observed, nor were any additional interactions, p's > .05.

4.1.3.7 Hsp27

Statistical analysis did not reveal main effects of sex, strain, or injury, nor did it indicate any significant interactions, p's > .05.

4.1.3.8 Hsp10

Statistical analysis revealed a main effect of strain, F (1, 18) = 5.86, $p < .05$ where Kir6.2-KO mice had higher *Hsp*10 expression compared to C57BL/6J controls. However, this strain effect was driven by a sex by strain interaction, F (1, 18) = 9.36, $p < .01$, indicating that the effect of strain on *Hsp*10 expression was more prominent females. Kir6.2-KO females tended to have higher *Hsp*10 expression compared to C57BL/6J controls. In males, there were no significant differences in expression between strains. A significant sex by strain by injury interaction was also observed, F (1, 18) = 5.11, $p < .05$, where C57BL/6J females that received a mTBI had the lowest *Hsp*10 expression. Statistical analysis did not reveal main effects of sex, or injury, nor did it indicate significance in the other interactions, p's > .05.

4.1.3.9 Hsp5

*Hsp*5 expression was higher in males compared to females, and was reflected in the analysis as a main effect of sex, F (1, 18) = 23.07, $p < .01$. A sex by strain interaction was also evident, F (1, 18) = 43.64, $p < .01$, which indicated that the effect of strain was opposite for males versus females. Kir6.2-KO males tended to have lower expression than their C57BL/6J counterparts, whereas in females, Kir6.2-KO mice had higher *Hsp*5 expression than controls. Statistical analysis did not reveal main effects of strain or injury, nor did it indicate any other significant interactions, p's > .05.

Table 4.3. Hsp expression in the prefrontal cortex at 14 days post-mTBI

		Male		Female	
Brain Region	**Gene**	**C57BL/6J**	**KATP-KO**	**C57BL/6J**	**KATP-KO**
Nucleus / Cytosol	Hsp90aa1	Ø1,2	Ø1,2	Ø1,2	Ø1,2
	Hsp70	Ø4	Ø4	Ø4	Ø4
	Hsp40	Ø1,2,4	Ø1,2,4	↑1,2,4,6	↓1,2,4,6
	Hsp27	Ø	Ø	Ø	Ø
ER	Hsp90b1	↓1,2,4	↑1,2,4,7	Ø1,2,4	↓1,2,4
	Hsp5	↓1,4	Ø1,4	↓1,4	Ø1,4
Mitochondria	Hsp60	Ø2,4	Ø2,4	↑2,4	Ø2,4
	Hsp10	↑2,4,7	Ø2,4	↓2,4,7	Ø2,4
Mito/ER	Hsp32	Ø2,4	Ø2,4	↓2,4,5	Ø2,4

*Numbers designate significant main effects or interactions where 1= sex, 2 = strain, 3 = injury, 4 = sex by strain, 5 = sex by injury, 6 = strain by injury, 7 = sex by strain by injury.

4.1.4 Hippocampus at 14 days post-mTBI

Results for the following section are summarized in table 4.4, and graphs are available in Appendix D.

4.1.4.1 Hsp90aa1

Following mTBI, *Hsp*90aa1 expression was reduced relative to sham controls, and was reflected in the statistical analysis as a main effect of injury, F (1, 18) = 6.99, $p < .05$. A significant sex by strain interaction was also revealed, F (1, 18) = 19.74, $p < .01$. The effect of strain on *Hsp*90aa1 expression was opposite for males and females. In males, Kir6.2-KO mice had lower expression compared to C57BL/6J controls. This pattern was opposite for females; Kir6.2-KO mice had higher expression compared to C57BL/6J controls. Though not significant, a trend towards a sex by strain by injury interaction was also observed, F (1, 18) = 3.85, $p = .06$ where the effect of mTBI was greatest in male C57BL/6J mice. Statistical analysis did not reveal main effects of sex or strain, nor did it indicate any other significant interactions, p's > .05.

4.1.4.2 Hsp90b1

A three-way ANOVA revealed a significant sex by strain by injury interaction, F (1, 18) = 4.94, $p < .05$, where the effect of mTBI was greatest in female C57BL/6J mice, causing a reduction in *Hsp*90b1 expression. A trend towards a sex by strain interaction, F (1, 18) = 4.12, $p = .06$ indicated that the effect of strain was opposite for males and females. In males, Kir6.2-KO mice tended to have lower expression compared to C57BL/6J controls, whereas in females, expression was higher in Kir6.2-KO mice. A trend towards a strain by injury interaction was also observed, F (1, 18) = 3.84, $p = .06$. The effect of mTBI was opposite for Kir6.2-KO versus C57BL/6J mice. mTBI increased *Hsp*90b1 expression in Kir6.2-KO mice, but caused a decrease in C57BL/6J controls. Statistical analysis did not reveal main effects of sex, strain, or injury, nor did it reveal a significant sex by injury interaction, p's > .05.

4.1.4.3 Hsp70

Statistical analysis revealed main effects of sex, F (1, 18) = 13.28, $p < .01$, and strain, F (1, 18) = .45, $p < .01$. Males had higher *Hsp*70 expression than females, and Kir6.2-KO mice had lower expression than C57BL/6J controls. A significant sex by strain interaction was also observed, F (1, 18) = 13.47, $p < .01$ where the effect of strain was more prominent in males than females, and led to lower *Hsp*70 expression in male Kir6.2-KO mice compared to C57BL/6J controls. Lastly, the main effect of injury was not statistically significant, nor were the other interactions, p's > .05.

4.1.4.4 Hsp60

A three-way ANOVA revealed a sex by strain interaction, F (1, 18) = 5.19, $p < .05$, indicating that differences in *Hsp*60 expression between Kir6.2-KO mice and C57BL/6J controls was sex dependent. In females, Kir6.2-KO mice had higher expression than C57BL/6J controls, whereas in males no significant difference was observed. Statistical analysis did not reveal a main effect of sex, strain, or injury, nor did it reveal any other interactions, p's > .05.

4.1.4.5 Hsp40

*Hsp*40 expression tended to be higher in males compared to females, and was reflected in the analysis as a main effect of sex, F (1, 18) = 7.99, $p = .05$. A sex by strain interaction was also evident, F (1, 18) = 11.16, $p < .01$, which indicated that the effect of strain was opposite for males versus females. Kir6.2-KO males had lower expression than their C57BL/6J counterparts, whereas in females, Kir6.2-KO mice had higher *Hsp*40 expression than controls. Statistical analysis did not reveal a main effect of strain or injury, nor did it indicate any other significant interactions, p's > .05.

4.1.4.6 Hsp32

Following mTBI, *Hsp*32 expression was reduced relative to sham controls, and was demonstrated by a main effect of injury, F (1, 18) = 14.43, $p < .01$. A significant sex by strain interaction was also revealed, F (1, 18) = 12.56, $p < .01$. The effect of strain on *Hsp*32 expression was opposite for males and females. In males, Kir6.2-KO mice had lower expression compared to C57BL/6J controls. In females, Kir6.2-KO mice had higher expression compared to

C57BL/6J controls. Statistical analysis did not reveal a main effect of sex or strain, nor did it indicate any other significant interactions, *p*'s > .05.

4.1.4.7 *Hsp27*

Statistical analysis revealed a main effect of sex, F (1, 18) = 4.33, p = .05, and trend towards a main effect of strain, F (1, 18) = 3.86, p = .06. Males had higher expression than females, and Kir6.2-KO mice tended to have lower expression compared to C57BL/6J controls.

4.1.4.8 *Hsp10*

Following mTBI, *Hsp*10 expression was reduced relative to sham controls, and was reflected in the statistical analysis as a main effect of injury, F (1, 18) = 18.01, p = .01. A significant sex by strain interaction was also revealed, F (1, 18) = 9.19, p < .01. The effect of strain on *Hsp*10 expression was opposite for males and females. In males, Kir6.2-KO mice had lower expression compared to C57BL/6J controls. In females, Kir6.2-KO mice had higher expression compared to C57BL/6J controls. A sex by strain by injury interaction was also observed, F (1, 18) =10.45, p < .01 where the greatest reduction in *Hsp*10 expression was found following mTBI in C57BL/6J males. Statistical analysis did not reveal a main effect of sex or strain, nor did it indicate any other significant interactions, *p*'s > .05.

4.1.4.9 *Hsp5*

*Hsp*5 expression was higher in males compared to females, and was reflected in the analysis as a main effect of sex, F (1, 18) =6.26, p < .05. There was also a sex by strain interaction, F (1, 18) =6.55, p < .05, where effect of strain on *Hsp*5 expression was opposite for

males and females. In males, Kir6.2-KO mice had lower expression compared to C57BL/6J controls. In females, Kir6.2-KO mice had higher expression compared to C57BL/6J controls. A strain by injury interaction was also observed, F (1, 18) =6.55, $p < .01$. The effect of mTBI was opposite for Kir6.2-KO versus C57BL/6J mice. mTBI increased *Hsp*5 expression in Kir6.2-KO mice, but caused a decrease in C57BL/6J controls. Statistical analysis did not reveal a main effect of strain or injury, nor did it indicate any other significant interactions, p's > .05.

Table 4.4. Hsp Expression in the Hippocampus at 14 days post-mTBI

		Male		Female	
Brain Region	**Gene**	**C57BL/6J**	**KATP-KO**	**C57BL/6J**	**KATP-KO**
Nucleus / Cytosol	Hsp90aa1	↓3,4,5	Ø4	Ø4	↓3,4,5
	Hsp70	↓1,2,4	Ø1,2,4	Ø1,2,4	Ø1,2,4
	Hsp40	Ø1,4	Ø1,4	↓1,4	Ø1,4
	Hsp27	↑1,2	Ø1,2	Ø1,2	Ø1,2
ER	Hsp90b1	Ø4	Ø4	↓3,6,7	↑3,6
	Hsp5	↓1,4,6	↑1,4,6	Ø1,4	↑1,4,6
Mitochondria	Hsp60	↓6	Ø	↓6	Ø
	Hsp10	↓3,4,7	Ø4	↓3,4	Ø4
Mito/ER	Hsp32	↓3,4	Ø4	Ø4	↓3,4

*Numbers designate significant main effects or interactions where 1= sex, 2 = strain, 3 = injury, 4 = sex by strain, 5 = sex by injury, 6 = strain by injury, 7 = sex by strain by injury.

4.2 Discussion

One of the most significant challenges in developing treatment plans for patients following TBI is being able to predict long-term outcomes. The past decade of brain injury research has been focused on the development of a biomarker that could (1) reliably diagnose TBI, (2) accurately predict patient outcome, (3) provide a target for potential treatment strategies and (4) serve as a way to measure therapeutic response (Dash, Zhao, Hergenroeder, & Moore, 2010). Unfortunately, there are currently no biomarkers available for human use, and over 200 potential targets have failed to make it through clinical trials (Zhang et al., 2010). Part of this failure may stem from our lack of a complete understanding about the pathophysiology of TBI, as well as how these processes may be impacted by susceptibility factors such as sex or genetics. TBI is also a very heterogeneous injury that involves many interconnecting cellular pathways, making it difficult to find a biomarker capable of accommodating for this diversity. However, further insights into the molecular underpinnings of TBI, including factors affecting inherent susceptibility/resistance could aid in the development of novel biomarkers to help predict injury outcome as well as guide research into new therapeutics.

The second component of this thesis was designed to assess the cellular stress response in the face of both an inherent genetic susceptibility and an induced mTBI. A family of proteins, known as heat shock proteins (Hsp), plays a significant role in the cellular stress response. While Hsps were originally discovered in *Drosophila busckii* following heat shock (Ritossa, 1962), it was later discovered that these proteins respond to a variety of stressors, including TBI (Tissieres et al., 1974). Therefore, the term “heat shock protein” has become somewhat of a misnomer, and thus they are more commonly reffered to as molecular chaperones.

Following stress, Hsps have a multitude of functions. They act as molecular chaperones to prevent protein aggregation, ensure proteins are located in the correct cellular compartment, refold proteins that have become misfolded, and target misfolded proteins for degredation. These processes are key to the cellular stress response, as improperly folded proteins in the cell can be toxic and lead to cell death. There are over 80 Hsps in the cellular stress response pathway, as well as a number of other proteins and transcription factors (Kampinga et al., 2009). However, due to time constraints, we decided to analyze only nine of these genes using qRT-PCR. Specific *Hsps* were chosen based on their subcellular location in order to span three major cellular compartments- the mitochondria, endoplasmic reticulum, and nucleus, all of which are critical for protein handling, and have been implicated in the pathophysiology of TBI and other neurodegenerative disorders (Imaizumi et al., 2001; Ross & Poirier, 2004; Truettner et al., 2007; Wang et al., 2013). We sought to determine whether *Hsp* expression was altered by mTBI at two time points, acutely (24 h) and more long term (14 days), and whether sex or genetic susceptibility have an effect on the response of these cellular stress mediators. We found that *Hsp* expression varied according to time, brain region, sex, genetic make-up, and whether or not the animals sustained a mTBI. Overall, our results indicated that the cellular stress response to mTBI is influenced by a multitude of factors, which may or may not interact with each other to determine outcome. The sheer complexity of this pathway provides a sound example of why, at least historically, it has been so difficult to find a unified single biomarker for TBI that is consistent despite individual heterogeneity, but that is also sensitive enough to predict injury outcome.

4.2.1 Regional Differences

PCS is often associated with a sequelae of symptoms that involve impairments of executive functioning as well as learning and memory (Ryan & Warden, 2003). In order to assess possible molecular correlates of these deficits, we chose to examine the cellular stress response in two brain regions- the prefrontal cortex (PFC) and the hippocampus. The PFC plays an important role in cognitive flexibility, decision-making, and attention, whereas the hippocampus is involved in memory formation, learning, and spatial navigation (Kolb & Whishaw, 2009; Ryan & Warden, 2003). In our behavioural test battery we observed deficits in many of these functions following mTBI. Therefore, we also wanted to determine if any molecular changes were associated with these areas post-injury.

When controlling for time-point, we found that brain injury affected three subcellular compartments- the mitochondria, endoplasmic reticulum (ER), and nucleus/cytoplasm, but the degree to which each compartment was affected depended on brain region. At 24h in the PFC, all three compartments showed *Hsp* changes following mTBI, whereas in the hippocampus, *Hsps* in the mitochondria and endoplasmic reticulum (ER) showed the majority of changes. This suggests that the subcellular response to injury is brain region-dependent. In line with our results, Truettner and colleagues (2007) also demonstrated that the effect of TBI on subcellular compartment, using *Hsp* changes, depended on the brain region analyzed. Furthermore, Rall and colleagues (2003) also showed that mRNA expression of many genes differed between the PFC and hippocampus, both basally and after TBI. Owing to the different functions these brain regions have, it is not surprising that gene expression would differ within subcellular compartments.

Regional differences in the effect of mTBI may also simply reflect differences in the way the actual physical injury affects these two areas. The model we used to induce mTBI caused injury through rapid acceleration/deceleration and rotational forces that were applied to the dorsal surface of the head. These forces are thought to affect the integrity of fiber tracts within the brain, and lead to the entity referred to as diffuse axonal injury (DAI). The PFC is composed of nuclei (grey matter) as well as many long association white matter tracts to other brain regions. In comparison, the hippocampus is rich with tightly packed nuclei and shorter connections; it is also located deep within the brain. Owing to differences in the connections, orientation of white matter tracts, and brain location (deep versus more superficial), a DAI would likely affect these two brain regions differently.

We also found that regional differences in *Hsp* expression post-mTBI depended on the animal's genetic background. While ubiquitous changes in *Hsp* expression were found in both regions for C57BL/6J mice, we found that post-injury, Kir6.2-KO mice had greater changes in *Hsp* expression in the hippocampus compared to PFC. This is interesting as it suggests that susceptibility/resiliency to poor outcomes may be region-dependent. Extrapolating these findings to clinical populations, regional differences in susceptibility could account for the heterogeneity observed in symptom presentation. Differences in regional susceptibility/resiliency could lead to different symptom presentation.

Lastly, differences in regional *Hsp* expression may also exemplify why the research community has been unable to find reliable molecular biomarkers or single-molecule therapeutics for mTBI. Biomarkers for TBI are usually measured from the blood or cerebrospinal fluid (Zetterberg, Smith, & Blennow, 2013), with the rationale that measurements from these fluids would accurately reflect levels in the brain. However, our results, in combination with

other studies, highlight a crucial problem with this idea; how can a single biomarker be reflective of overall brain function when biomarker levels between brain regions differ?

4.2.2 Time Differences

We also assessed *Hsp* expression at two different time points in an effort to examine the temporal differences in the acute (24 h) and longer-term (14 days) post-injury periods. Due to the accelerated brain development in mice, a two-week period following mTBI at P45 correlates with human development occurring between adolescence and young adulthood (P59) (Semple et al., 2013). Analysis at 14 days would therefore allow us to assess *Hsp* expression during a time window equivalent to when a diagnosis of PCS could be made clinically.

Indeed, we found that the *Hsp* expression profile changed over time. At 24 h following mTBI, changes in *Hsp* expression were detected in the three subcellular compartments. However, by 14 days the effect of injury persisted mainly in the mitochondria and ER, but dissipated in the nucleus/cytosol. This is particularly interesting, as the mitochondria and ER have key roles in the pathophysiology of TBI, and it suggests that they may be affected past the acute injury.

The ER is responsible for the processing and folding of approximately one-third of cellular proteins (Ghaemmaghami et al., 2003). It contains numerous enzymes and chaperones that are constitutively active as well as induced, such as *Hsp90b1* and *Hsp5 (Grp78).* They ensure that proteins are processed correctly, and remain in the correct conformation. However, despite the presence of these chaperones, the ER is still at high risk for the accumulation of aggregated proteins, due to its inherently high concentration of proteins. Studies have shown that following mTBI, the number of aggregated proteins within the ER increases over time (Uryu et

al., 2007). The persistent changes in ER-resident *Hsps* that we observed at 14 days post-mTBI may therefore be indicative of prolonged ER stress. If the ER is incapable of processing these proteins, it may have important consequences in the long-term. For example, protein aggregation is a hallmark trait of neurodegenerative diseases such as Alzheimer's (AD) or Parkinson's Disease (PD) (Hyman et al., 2012). In fact, studies have shown that the protein aggregation observed in AD and PD may be attributed to a dysfunctional heat shock protein response in the ER (Hoozemans et al., 2007; Imaizumi et al., 2001). Recent literature has also suggested that a history of TBI could increase the risk of developing such age-related brain disorders, even if the injury is mild (Guo et al., 2000; Johnson, Stewart, & Smith, 2010; Lee et al., 2013).

We also found that mitochondrial *Hsps* were altered out to 14 days following mTBI. Persistent changes in the cellular stress response of this organelle suggest that it continues to be affected by the injury longer than 24 h. This is interesting, as mitochondrial dysfunction is considered to be one of the hallmark pathophysiological processes of TBI (Barkhoudarian et al., 2011). Our results may therefore suggest that changes in *Hsp* expression following mTBI could be indicative of mitochondrial stress. Because the mitochondria is considered the "powerhouse" of the cell, disruptions in its functioning may lead to impairments in the production of ATP, the main energy currency of the cell. This is particularly problematic following brain injury, as the cell requires increased energy in order to re-establish ionic homeostasis. Ultimately, disruption of the mitochondria could lead to an energy deficit and activation of apoptotic pathways (Lewen et al., 2001; Robertson, 2004).

Together, our results suggest that the effect of mTBI on *Hsp* expression changes over time. At the outset, the three major subcellular compartments are affected by injury. However, as time progresses, it appears that changes to the cellular stress response persist in certain

organelles, such as the mitochondria and ER. These compartments are also the same ones that have been implicated in the pathophysiology of TBI (Guo et al., 2000, Barkhoudarian et al., 2011).

4.2.3 Genetic Susceptibility

Interestingly, we also found that Kir6.2-KO mice showed either opposite or no changes in mitochondrial and ER *Hsp* expression when compared to C57BL/6J mice following mTBI. The few changes that were evident in *Hsps* at 24 h usually dissipated by 14 days post-injury. This suggests that not only does genetic make-up affect the expression of *Hsps*, but it also affects the time course of these changes. Assuming that results in C57BL/6J mice reflect the normal cellular stress response, this would indicate that the *Hsp* component of the ER and mitochondrial response might be perturbed, at least at the gene expression level, in Kir6.2-KO mice. These mice could either be particularly susceptible or resilient, depending on the functional consequences of these differences, to ER and mitochondrial dysfunction following mTBI. However, the behavioural outcomes we observed in the previous component of this study suggest that Kir6.2-KO mice have worse outcomes following mTBI than C57BL/6J controls. Therefore, differences observed in the cellular stress response more likely indicate susceptibility as oppose to resilience. However, we can only suggest an association between the behavioural outcomes and *Hsp* response; our results do not imply causality.

Overall, differences in *Hsp* expression between C57BL/6J and Kir6.2-KO mice following mTBI indicate that genetic background can influence the way cells respond to stress. The idea that differences in genetic background leads to altered responses following TBI may help explain why outcomes in the clinical population are so heterogeneous. Future studies using Kir6.2-KO

mice may wish to explore whether differential *Hsp* expression persists for longer than 14 days, and whether these perturbations correlate with a consistent behavioural phenotype, an increase or decrease in protein aggregation, or an association with biomarkers for AD and PD, such as amyloid-β plaques or tau.

4.2.4 Sex differences

While baseline differences in *Hsp* expression between C57BL/6J and Kir6.2-KO mice were found in both sexes, the specific response was opposite between males and females. For the majority of *Hsps*, female Kir6.2-KO mice had higher expression than C57BL/6J mice, whereas in the males, Kir6.2-KO mice tended to have lower expression than C57BL/6J controls. Sex differences may be related to differences in levels of sex hormones, such as estrogen, between males and females. Studies have shown that K_{ATP} channels play a key role in the regulation of the female reproductive hormones (Huang, Acosta-Martinez, & Levine, 2008; Zhang, Bosch, Levine, Ronnekleiv, & Kelly, 2007). Huang and colleagues (2007), demonstrated that in hypothalamic neurons, K_{ATP} channels were found to regulate the release of gonadotropin releasing hormone (GnRH), which is important for downstream signaling of estrogen production. Cells that expressed K_{ATP} channels were also responsive to the ovarian hormones 17β-estradiol (E_2) and progesterone, which provided a negative feedback loop from the ovaries to the hypothalamus. When K_{ATP} channels were pharmacologically blocked, it led to increased GnRH pulsatile release, and deregulation of the hypothalamic-pituitary-gonadal axis. Therefore, researchers concluded that K_{ATP} channels were key mediators of this neuroendocrine feedback loop. Knocking out these channels, such as in our study, could disrupt the production of ovarian hormones such as E_2 and alter their levels in the brain. In turn, altered levels of E_2 could have

specific effects on the cellular stress response, as previous studies have also shown that E_2 modulates Hsp levels (Krebs, Jarvis, & Pfaff, 1999; Olazabal, Pfaff, & Mobbs, 1992; Voss et al., 2003). In summary, higher levels of *Hsps* that were observed in female Kir6.2-KO mice may be due to a compromised feedback loop of E_2 production that is caused by the absence of K_{ATP} channels. Future studies may wish to look at E_2 levels in Kir6.2-KO mice compared to C57BL/6J mice to validate whether this could be the cause of altered *Hsp* expression in females.

Sex differences were also observed in the *Hsp* response, as well as behavioural response, to mTBI in both C57BL/6J and Kir6.2-KO mice. As previously stated, these sex differences may, in part, be due to sex differences in levels of E_2 within the brain. Many studies have implicated estrogen as a neuroprotective molecule within the brain following injury (Bramlett & Dietrich, 2001; Roof & Hall, 2000). This could partially account for the sex differences we observed in behavioural outcomes as well as in the *Hsp* response. Additionally, some have shown also that part of its neuroprotective functions could be mediated through an up-regulation of the Hsps following injury (Kiang, Gist, & Tsokos, 1997; Lu et al., 2002; Y. Zhang et al., 2004).

However, the combined effects of sex and mTBI were most prominent, and in some cases sexually dimorphic, in the Kir6.2-KO mice. For example, *Hsp*70 expression in the PFC at 24h post-mTBI was higher in Kir6.2-KO males following mTBI, but significantly lower in Kir6.2-KO females. Again, this is similar to behavioural outcomes, where we observed that sex differences post-TBI were often specific to Kir6.2-KO mice. It is interesting to consider that this pattern in behaviour and *Hsp* expression may reflect an association between E_2 and the K_{ATP} channel as Zhang and colleagues (2012) demonstrated that the protective effects of E_2 administration during stroke was diminished if applied when K_{ATP} channels were

pharmacologically blocked. These results suggest that the presence of K_{ATP} channels may be important for estrogen-mediated neuroprotection, and studies conducted in the heart have also produced similar results (Gao et al., 2014). Together, our results suggest that there is an interaction between sex and genetic background following mTBI that alters the cellular stress response.

Lastly, the marked sex differences that we observed in *Hsp* expression following mTBI present another potential reason why scientific community has been unsuccessful in therapeutic development for TBI. Research from numerous other fields consistently indicates that there are fundamental sex differences in many diseases (Khosla, Melton, & Riggs, 1999; Mahmoodzadeh, Fliegner, & Dworatzek, 2012; R. Schmidt et al., 2008; Zandman-Goddard, Peeva, & Shoenfeld, 2007). Yet, the majority research aimed at discovering novel biomarkers and therapeutics for TBI is conducted in males only (Xiong et al., 2013). How can we expect to design successful treatment options when these sex differences are rarely taken into consideration?

4.3 Conclusions

Overall, this study has demonstrated that mTBI is an incredibly complex and heterogeneous injury. While a reductionist approach to mTBI research is necessary to understand the basic role that certain molecule may play in the brain, the pathophysiology of TBI is ultimately the result of many factors that interact with each other. Brain injury results not only in perturbations of single molecules, but also changes to entire networks within the brain. This study has clearly demonstrated that genetic make-up, sex, and injury all influence the brain at a molecular level, and more specifically, they affect the cellular stress response. We have shown that the degree of influence these factors have on *Hsp* expression is dependent on both region

and subcellular compartment, which could have particular effects on symptomology and long-term outcomes. Lastly, we were able to show that *Hsp* expression may change over time, suggesting that gene expression following mTBI is dynamic. Together, these factors all interact to influence molecular outcomes following mTBI, which could have particular implications for the development of therapeutics in the future. It suggests that research geared towards drug development may benefit from incorporating additional variables beyond the injury itself in order to tests how therapeutic targets may influence outcome.

4.4 Implications of Study

Results from both the behavioural and molecular components of this study have demonstrated an important trend following mTBI; that is, outcomes following mTBI are influenced by a multitude of factors such as sex, as well as genetic background. However, the ways these factors interact and determine outcome following injury depend on the specific behavior measured, and from a molecular standpoint, the specific gene analyzed. Furthermore, the relative influence of these factors may change throughout the recovery process. These findings therefore have important implications for future drug and biomarker discovery as they suggest that finding one "magic bullet" for therapeutics, or a single-molecule biomarker may not be the best approach. If responses of single molecules vary on such a variety of levels (ie. sex, genetic background, brain region, and time), such as this study has shown, finding molecules that are consistent yet sensitive enough across these factors will be difficult. Furthermore, our study has also helped to explain why individuals vary so greatly in terms of symptom presentation. Many factors influence the degree to which certain behavioral abnormalities may present following injury, and these differences may also reflect differences at the molecular level as shown by the *Hsp* analysis component of this study. Perhaps in the future, research should be

directed towards finding ways to alter brain function on a network or systems level, as oppose to targeting a single molecule. It will be difficult to identify a single "magic bullet" for therapeutics, if the responses of single molecules as well as behaviors differ on such a variety of levels, as our study has shown.

4.5 Limitations

4.5.1 Use of a Global Knockout Mouse

Although there are many benefits to using a global knockout mouse such as the Kir6.2-KO, there are some important limitations that should be addressed. First, using global knockout bioengineering results in the gene of interest being absent from the time of conception. This presents a complicated problem in terms of the impact that losing a naturally occurring gene may have on normal events in neurodevelopment. That is, if the gene is knocked-out does the organism compensate by altering the expression of other genes and, is the organism "normal"? In terms of this model, the K_{ATP} channel has important roles in glucose metabolism, heart function, and regulation of excitation in the brain; all of which are important processes for normal development. If this channel is not present during development, the mice could exhibit compromised functioning within these processes or alternatively, they may have developed compensatory mechanisms to overcome the absence of the K_{ATP} channel. The presence of compensatory mechanisms or compromised systems would have substantial impact on the outcomes measured and the conclusions that could be drawn from the findings.

Second, using Kir6.2-KO mice as a model of inherent susceptibility also assumes that the only abnormalities these mice possess are directly related to those structures affected by

mTBI. However, as previously stated, the K_{ATP} channel has many physiological roles within the body, and a global knockout may affect many other systemic functions that could also have an indirect impact on the brain. Thus, it is possible that the results obtained from this study do not necessarily only reflect the role of K_{ATP} in the brain but may also be confounded by other functions of this channel elsewhere in the body.

Lastly, this study makes the assumption that prior to mTBI K_{ATP} –KO animals function normally on behavioural tests, as well as in terms of their cellular stress response. In essence, one of the assumptions with the K_{ATP} –KO mice, as well as all other KO strains, is that the animals are otherwise normal and that their vulnerability is related to the outcome studies. The hypothesis thus assumes that the susceptibility will only be unmasked following mTBI. However, one study has characterized the behaviours of Kir6.2-KO mice and has shown that their baseline behavioural phenotype is actually quite differently than C57BL/6J controls on measures of activity, motor coordination, and emotional reactivity (Deacon et al., 2006). Furthermore, multiple observations during handling suggested that these mice were abnormal at baseline. First, we discovered that the way Kir6.2-KO mice were housed pre-weaning was differently than the way C57BL/6J mice were handled. In control mice, before dams gave birth, the male partner was removed. Therefore, the biological mother as well as another dam in the cage provided the majority of offspring care. In contrast, Kir6.2-KO males were left in the cage with two dams after weaning. While we are unsure of whether the males provided paternal care, it is interesting that they did not negatively impact the offspring. Second, K_{ATP} –KO mice have small litter sizes, usually between 1-4 mice, compared to C57BL/6J mice which have litters between 6-10 mice. This made it difficult to conduct behavioural tests, as only small cohorts could be tested at a given time. Furthermore, we found that singly housed K_{ATP} –KO mice were

hyper-anxious, and were antisocial during tests of sociability. When an experimenter tried to handle a singly housed K_{ATP} –KO mouse, they became quite verbal and tried to bolt when being caught. In contrast, group-housed K_{ATP} –KO mice were much easier to handle, even more than C57BL/6J controls. They did not bite, and would not run when experimenters pulled them from the cage. These abnormal behaviours, in addition to the statistical differences we observed between K_{ATP} –KO and C57BL/6J shams, make it difficult to interpret the results in response to a mild TBI.

4.5.2 Behavioral Test Order

The behavioural tests in this study were performed in a specific order that was held constant for each cohort of animals. The order of the behavioural test battery was chosen to ensure that most stressful tests were conducted last in effort to limiting the effects of stress on performance in the other measures. However, it is well documented that experience in previous testing environments can affect he results on future behavioural measures (McIwain et al., 2001; Blokland et al., 2012). The effects of mTBI on test performance were expected to change over time as animals recover from the injury. Furthermore, the nature of the behavioural tests could be considered cognitive or physical exercise, and a wealth of literature has indicated that exercise may be beneficial to recovery (Grealy, Johnson, & Rushton, 1999; Griesbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004). It is therefore possible that tests conducted earlier may have revealed more profound deficits than those conducted later on, due to natural recovery and the effect of behavioural testing on this recovery. Ideally, multiple test orders would be run to

evaluate these effects but this would significantly increase the number of animals required. Therefore, it was decided to run one test battery order and use caution when interpreting results.

4.5.3 Controlling for Estrous Cycles

One of the underlying themes throughout this thesis is the substantial effect that sex differences have on outcomes following mTBI. We postulated, based on an abundance of previous literature, that sex differences could at least partially stem from differences in sex hormones, such as estrogen, found between males and females, as they can be neuroprotective following brain injury (Bramlett & Dietrich, 2001; Roof & Hall, 2000). While mice do not go through menstrual cycles, they do have an estrous cycle of approximately 3-4 days where levels of estrogen and progesterone oscillate (Caligioni, 2009). It has been documented that estrous cycles in group-housed female mice can become synchronized and decrease in frequency, and is known as the "Lee Boot Effect" (Van Der Lee & Boot, 1956). We therefore felt that within each cage, estrous cycles were indirectly controlled for. However, other studies have shown that the strain as well as number of females per cage can influence the frequency of these cycles (Champlin, 1971). It is therefore possible that estrous cycles in K_{ATP} –KO would have been different compared to C57BL/6J mice, due to differences in litter size. Furthermore, there may have been differences in cycling between cages of the same strain. Perhaps it would have been beneficial to control for estrogen levels in the female mice, as this may have confounded our results, or at the very least, contributed to variance both within and between groups.

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APPENDIX A: HSP EXPRESSION IN THE PREFRONTAL CORTEX AT 24 H POST-MTBI

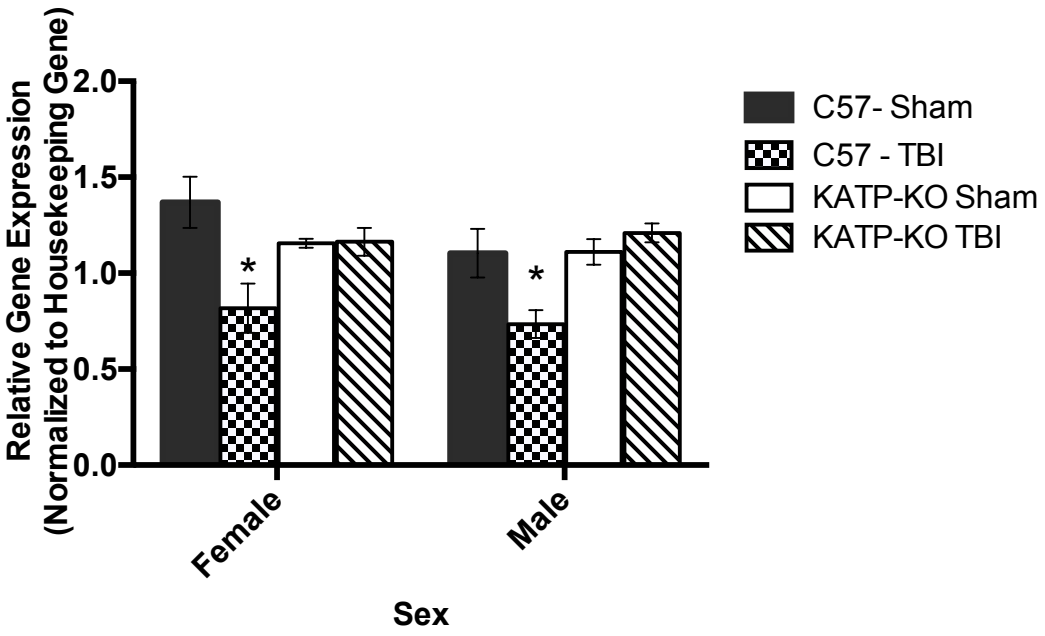


Figure A.1. Hsp90aa1 Expression.

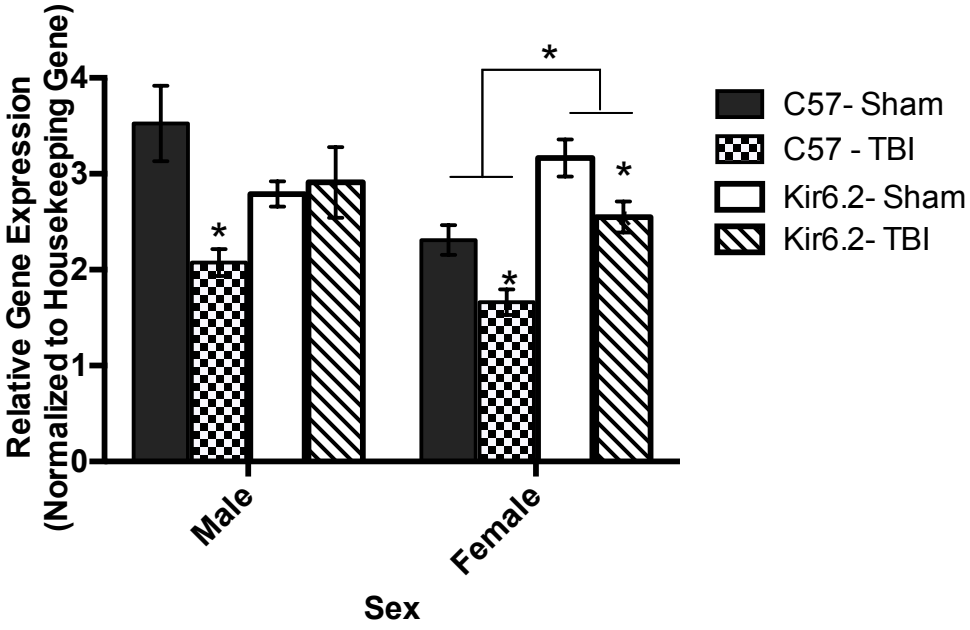


Figure A.2. Hsp90b1 Expression.

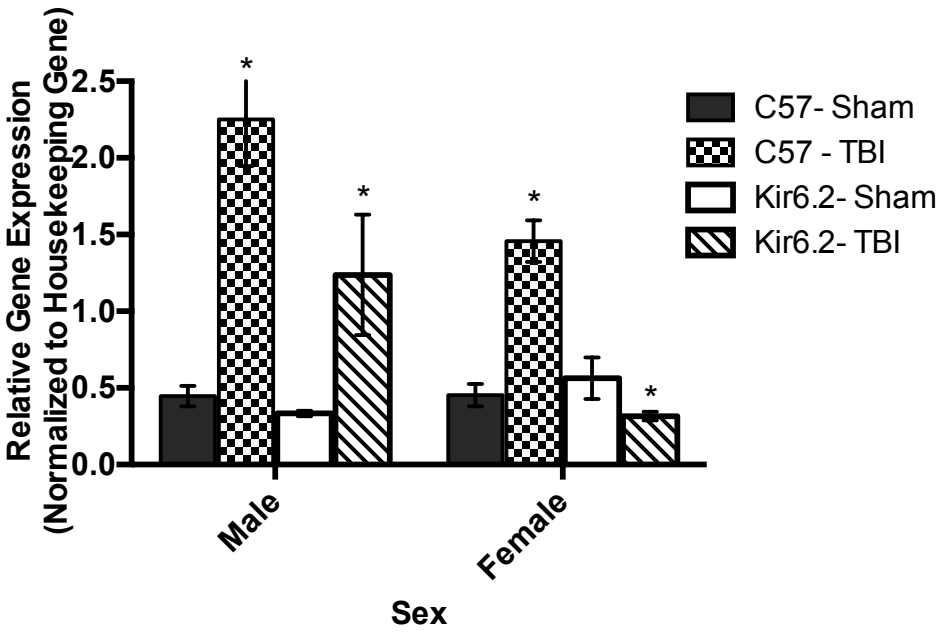


Figure A.3. Hsp70 Expression.

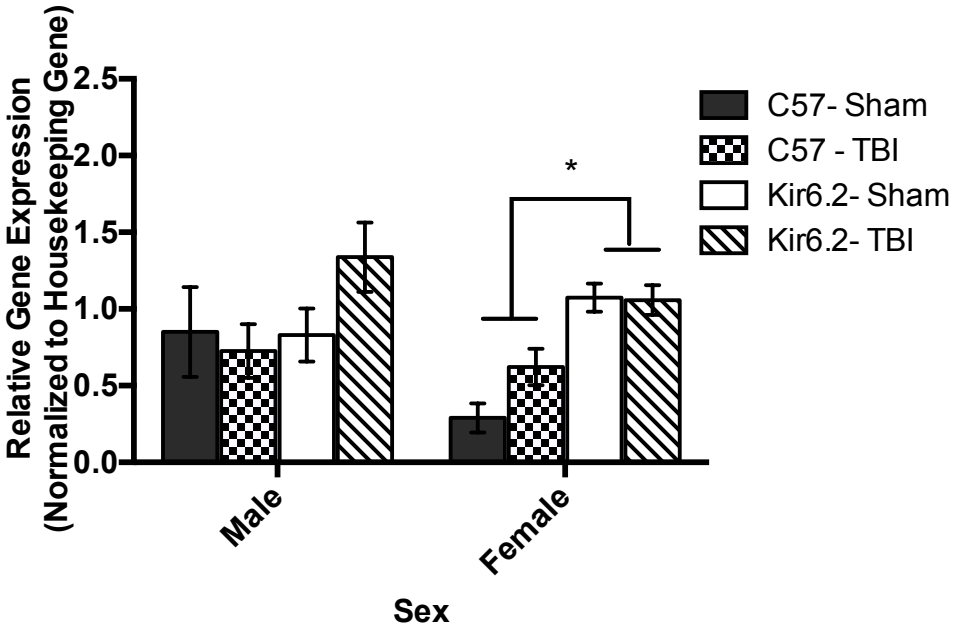


Figure A.4. Hsp60 Expression.

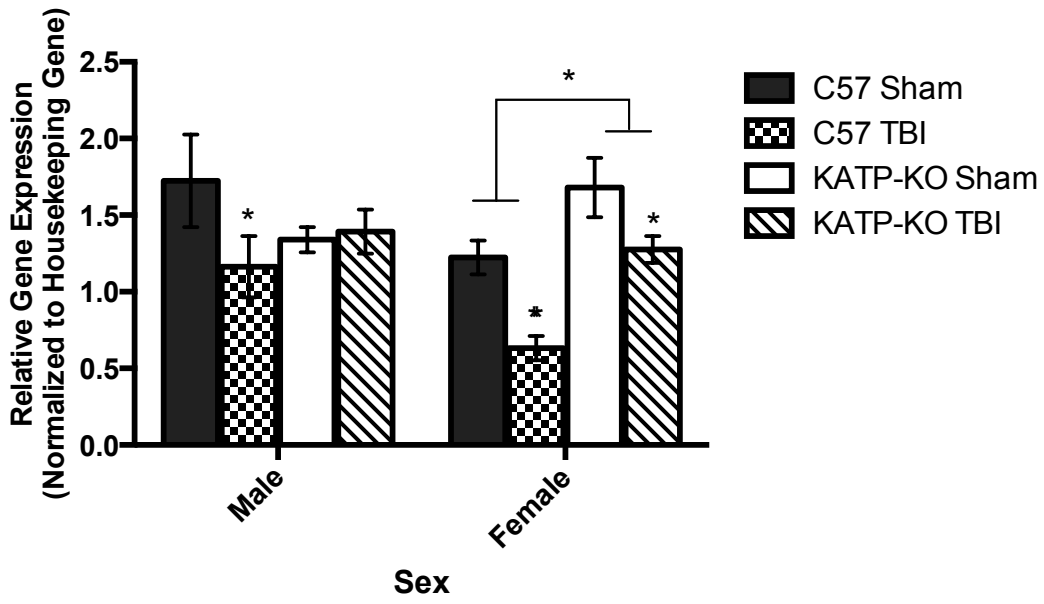


Figure A.5. Hsp40 Expression.

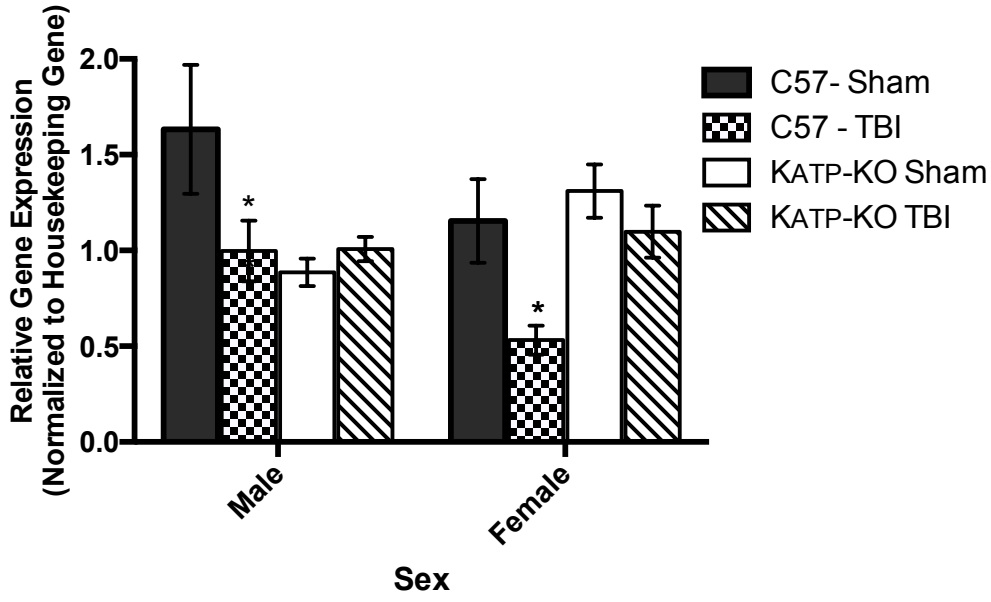


Figure A.6. Hsp32 Expression.

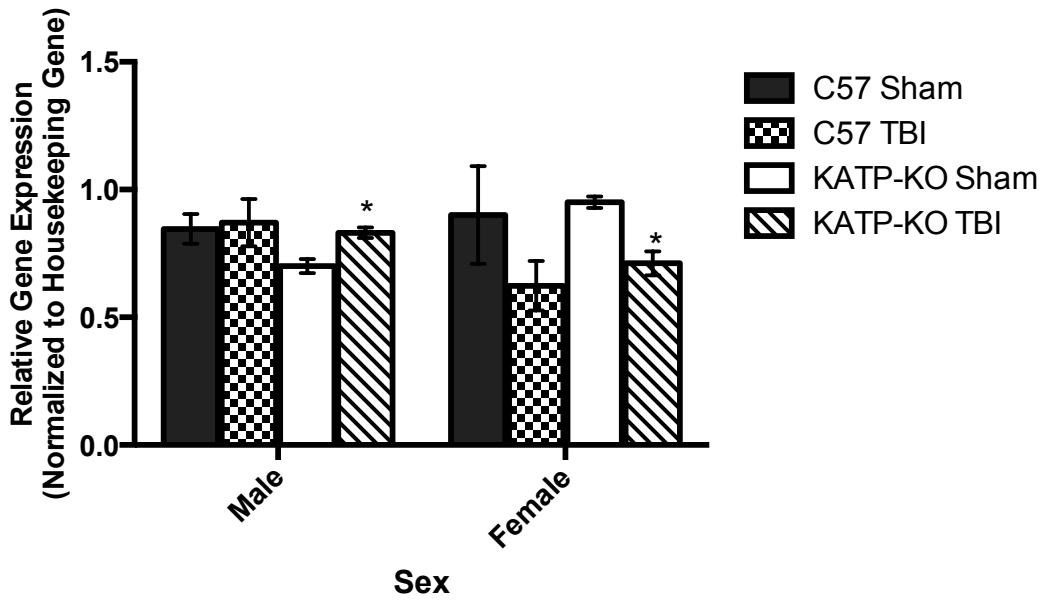


Figure A.7. Hsp27 Expression.

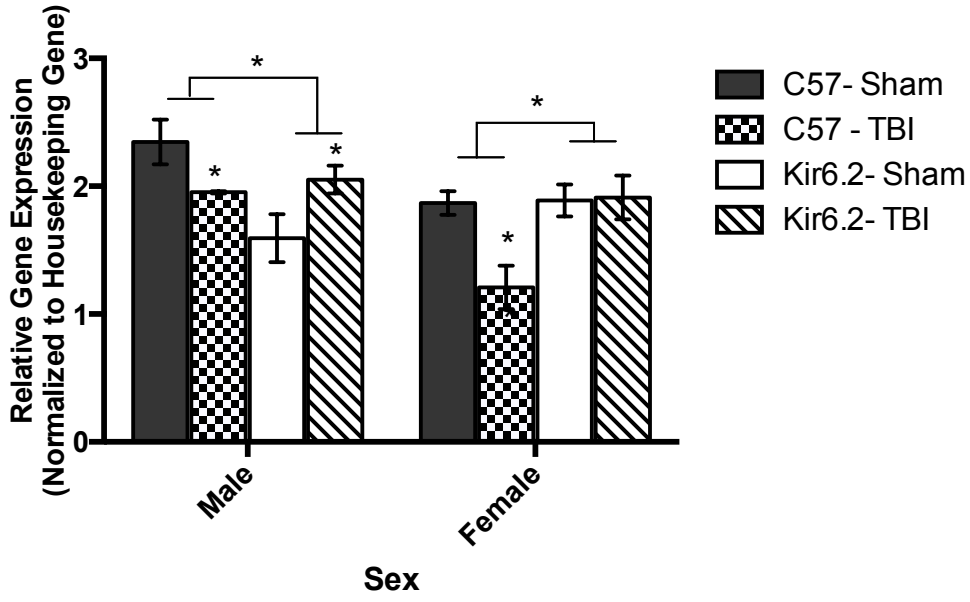


Figure A.8. Hsp10 Expression.

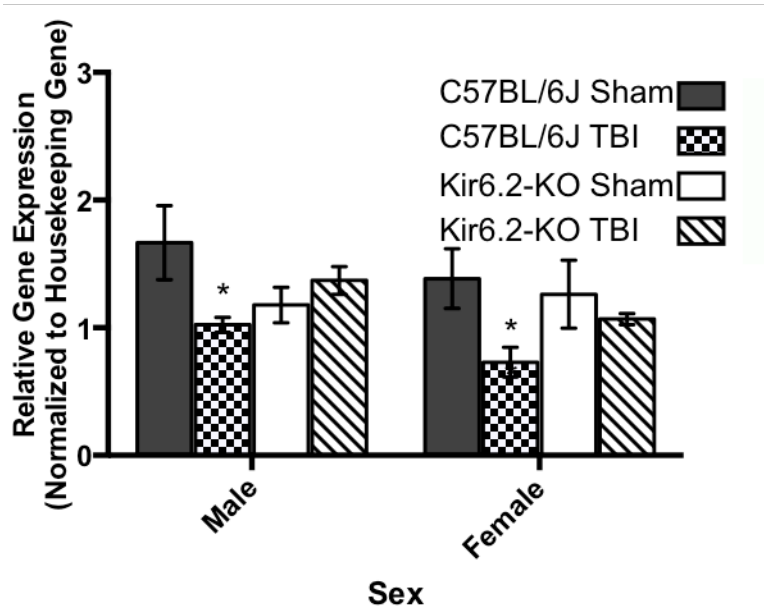


Figure A.9. Hsp5 (Grp78) Expression.

APPENDIX B: HSP EXPRESSION IN THE HIPPOCAMPUS AT 24 H POST-MTBI

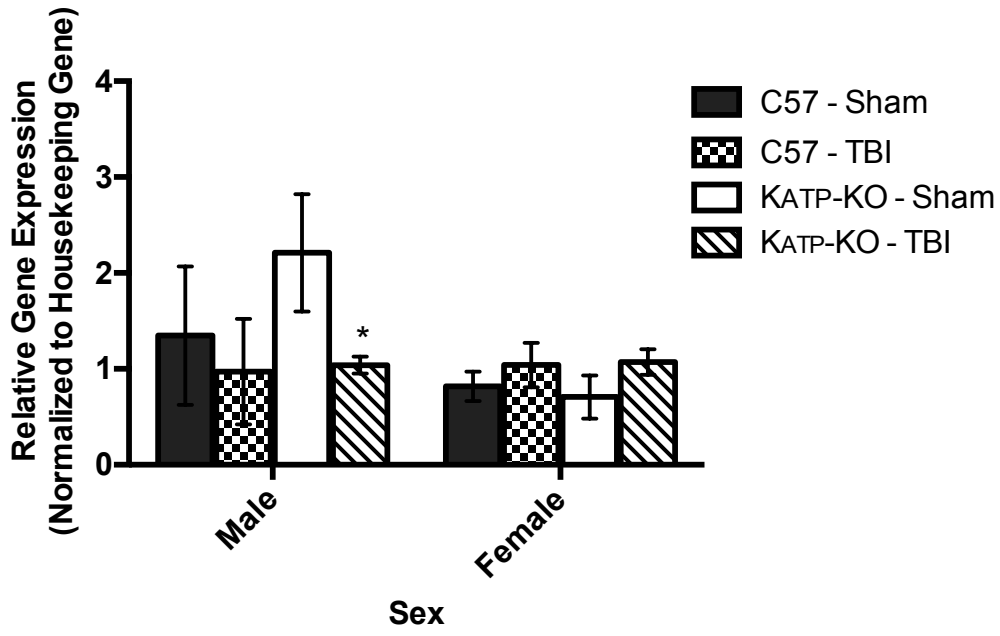


Figure B.1. Hsp90aa1 Expression.

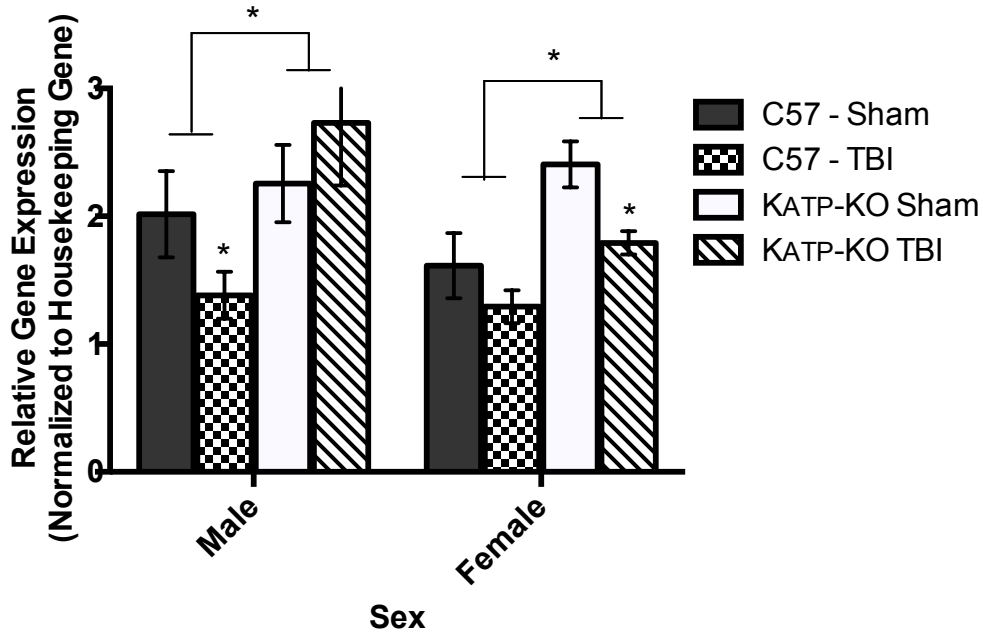


Figure B.2. Hsp90b1 Expression.

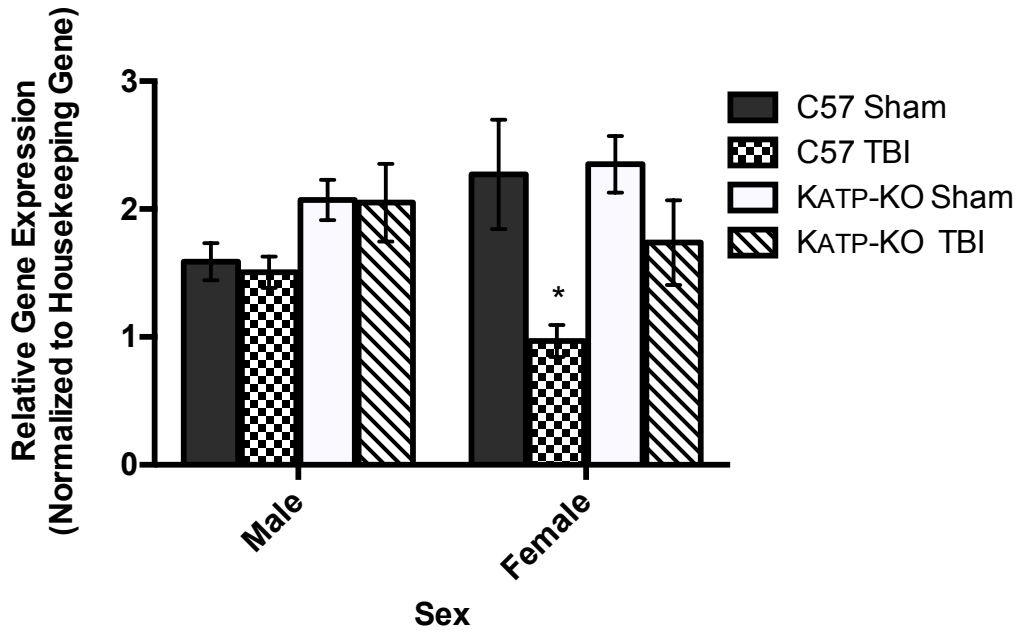


Figure B.3. Hsp70 Expression.

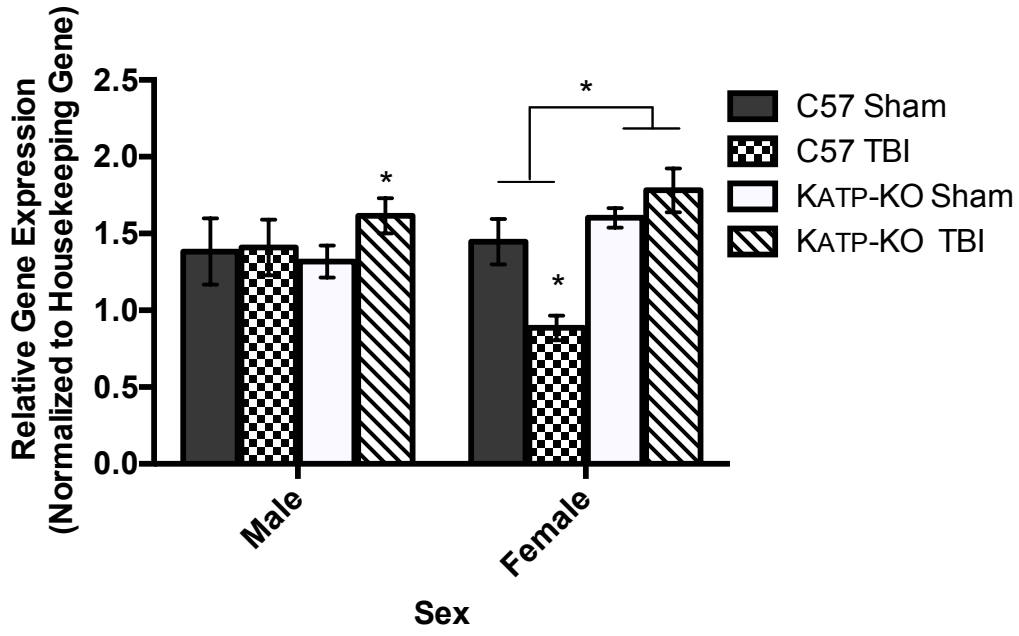


Figure B.4. Hsp60 Expression.

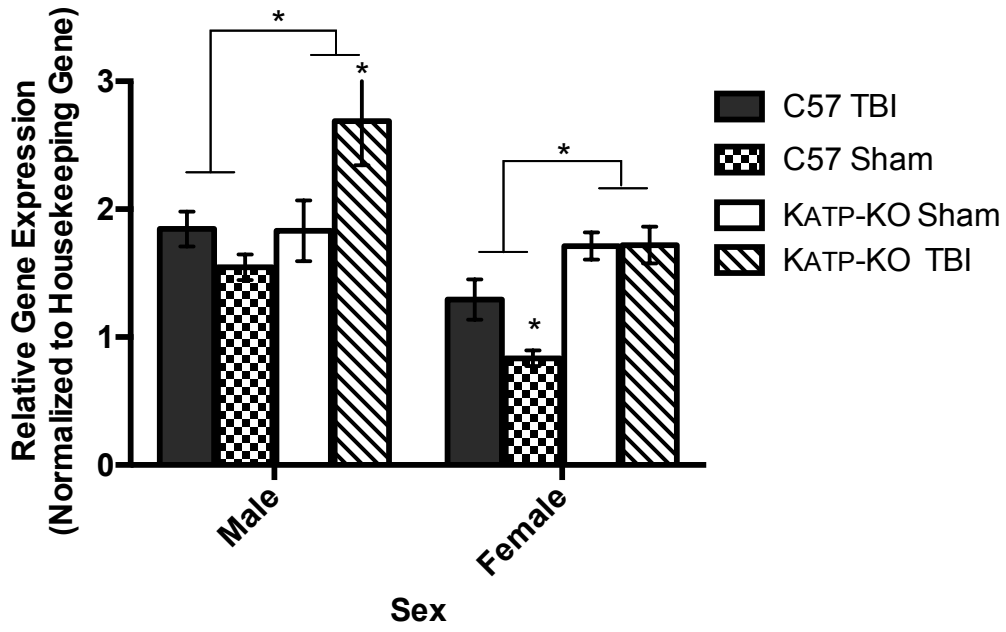


Figure B.5. Hsp40 Expression.

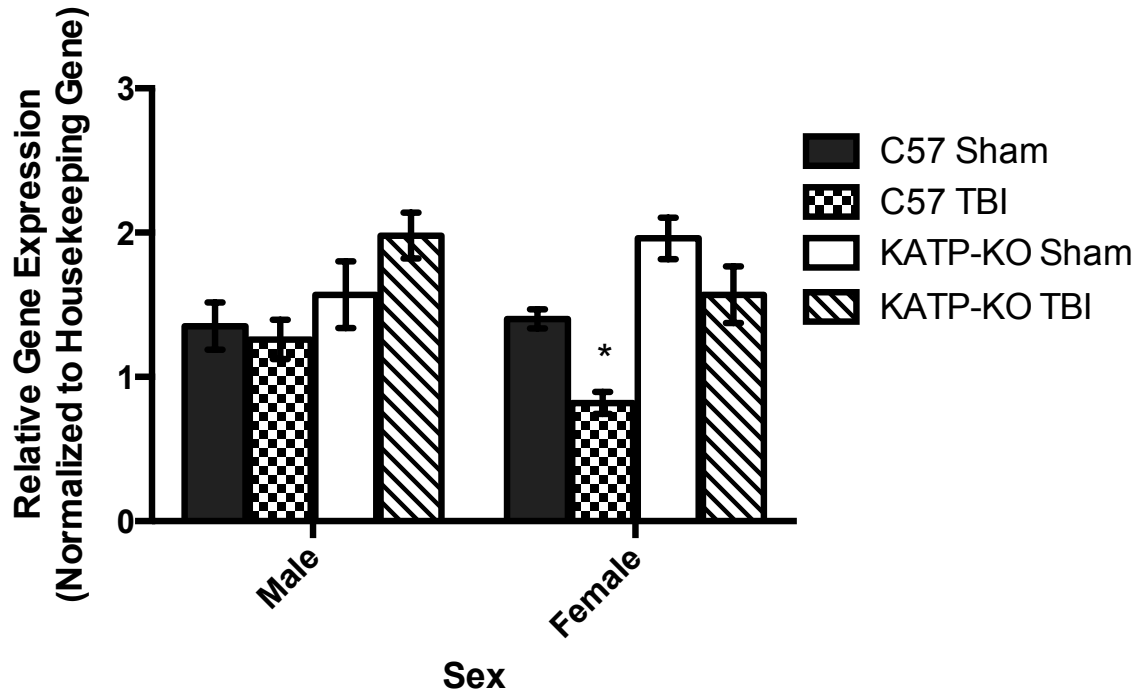
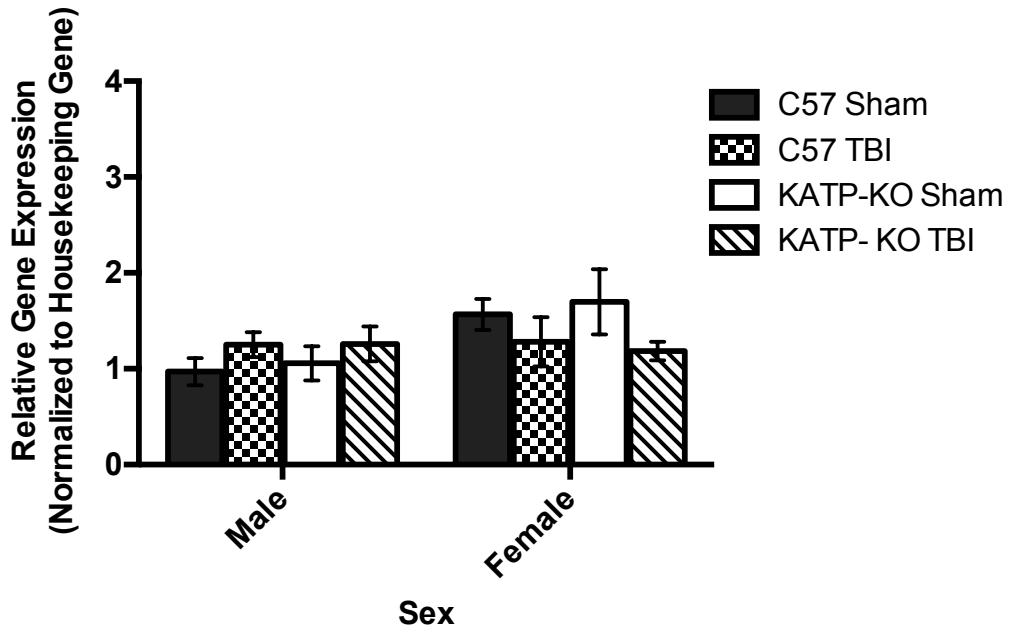


Figure B.6. Hsp32 Expression.



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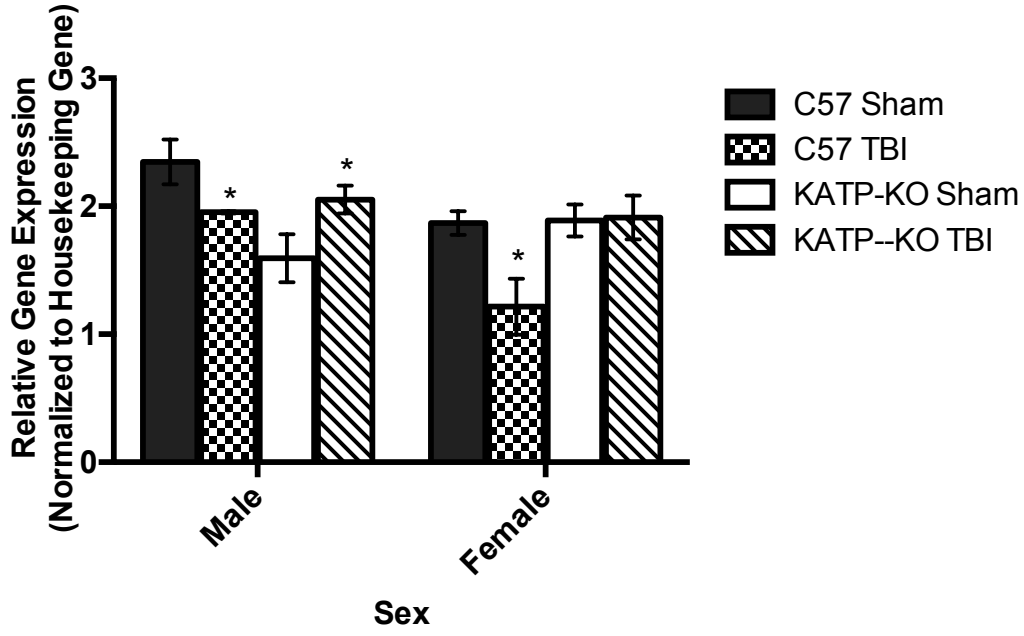

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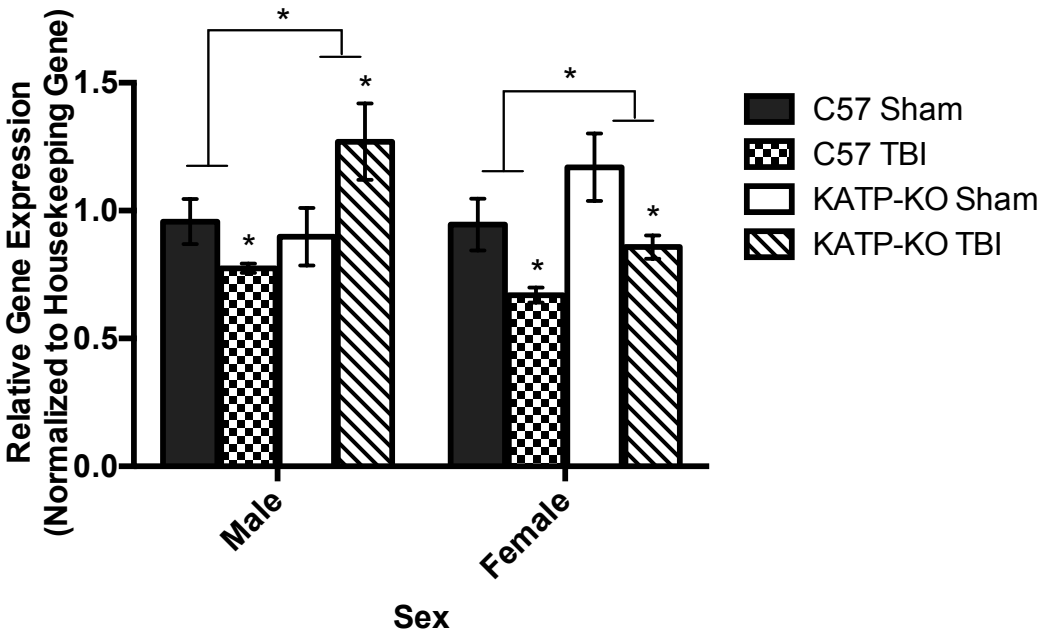

Figure B.9. Hsp5 Expression.

APPENDIX C: HSP EXPRESSION IN THE PREFRONTAL CORTEX AT 14 DAYS POST-MTBI

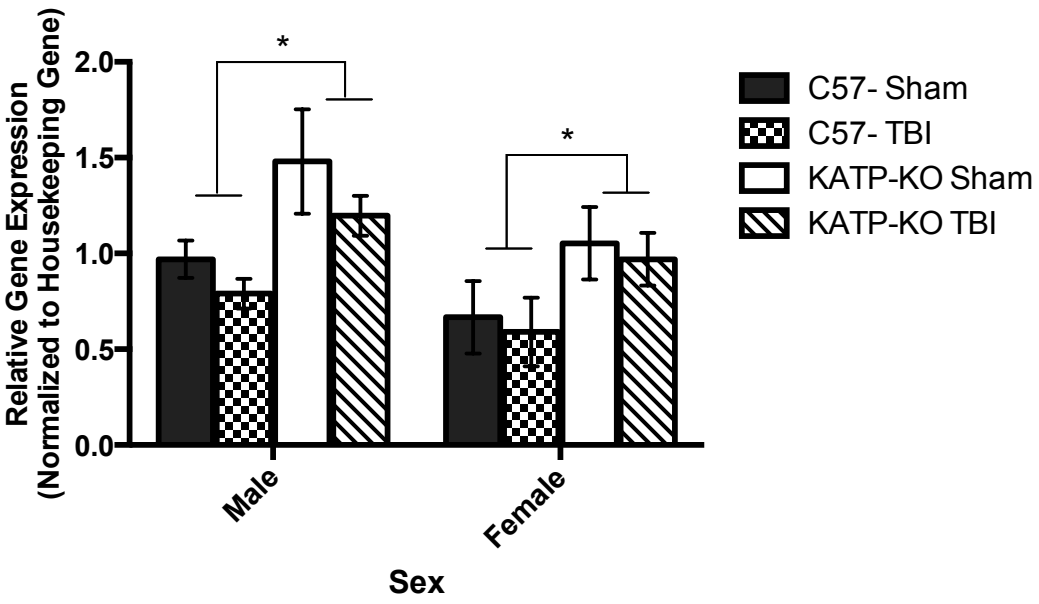

Figure C.1. Hsp90aa1 Expression.

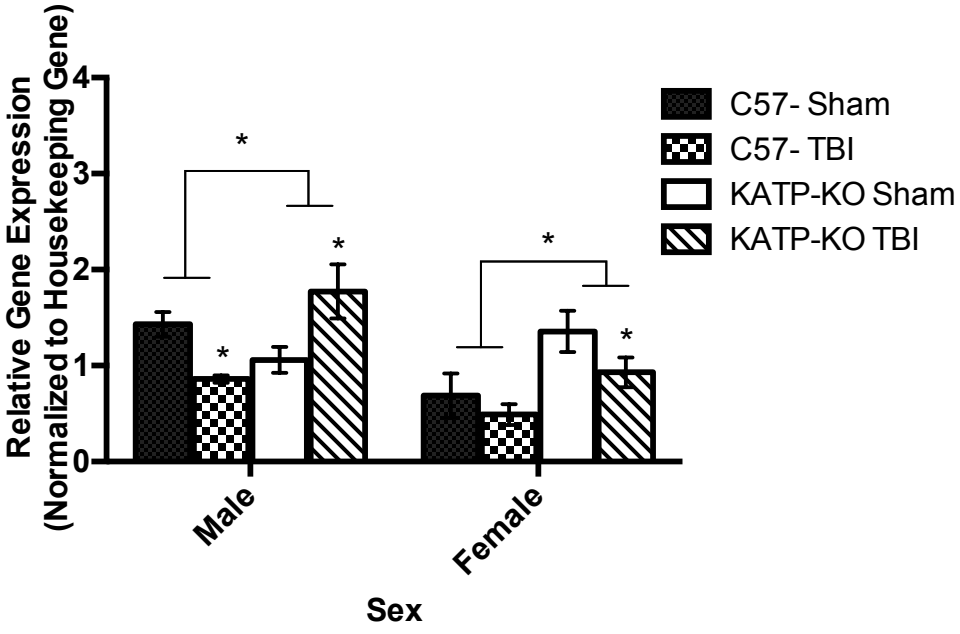

Figure C.2. Hsp90b1 Expression.

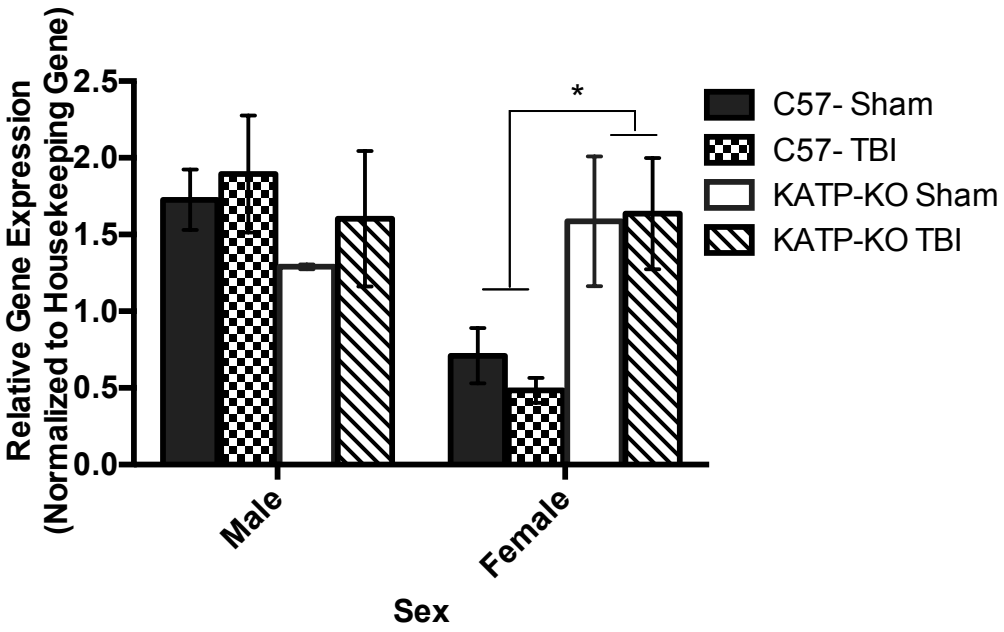

Figure C.3. Hsp70 Expression.

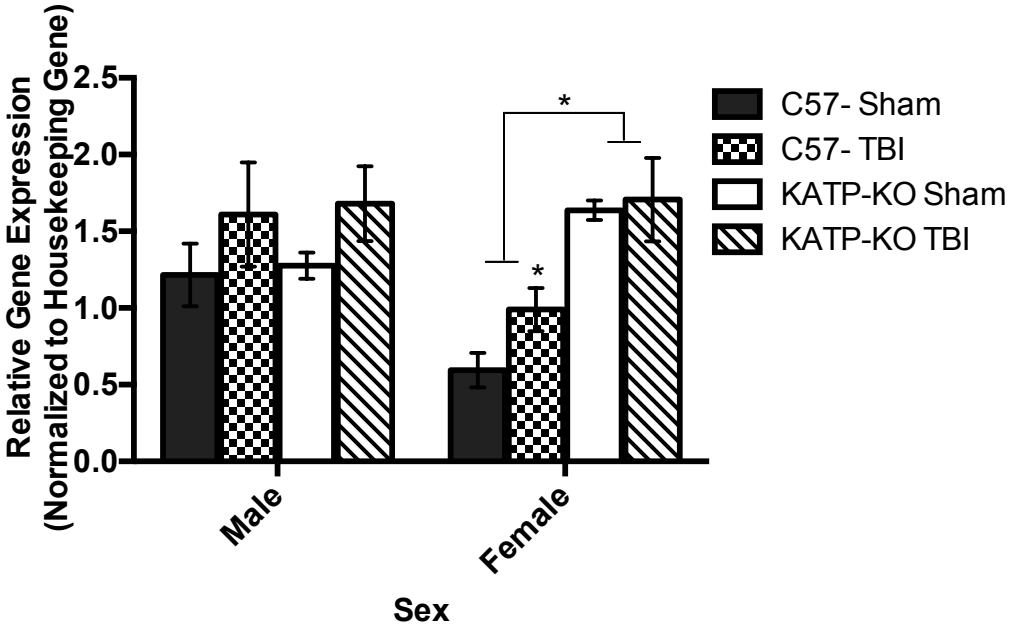

Figure C.4. Hsp60 Expression.

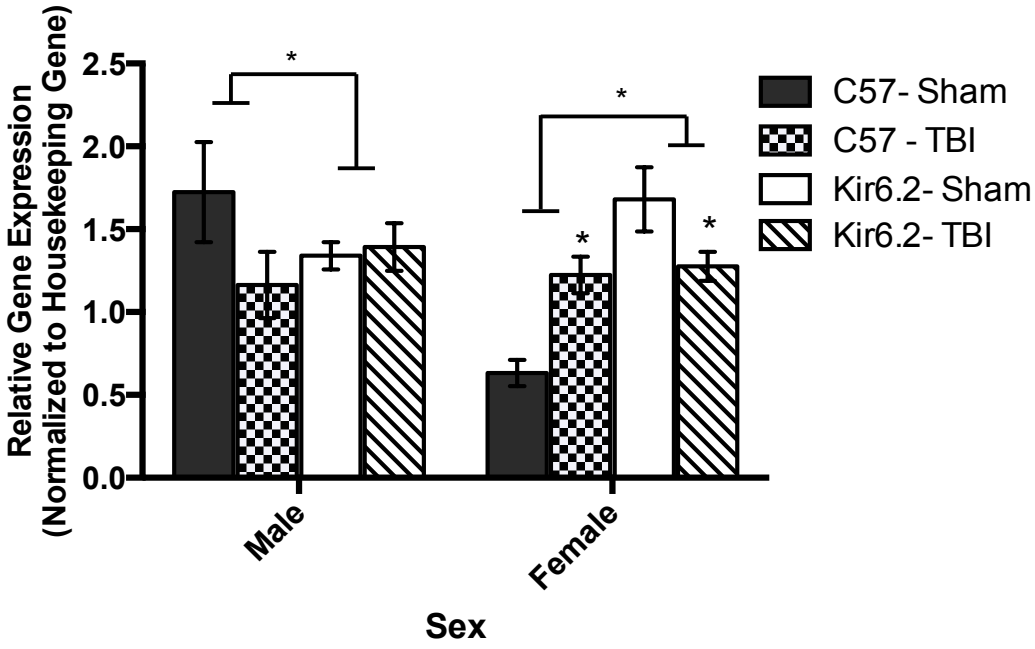

Figure C.5. Hsp40 Expression.

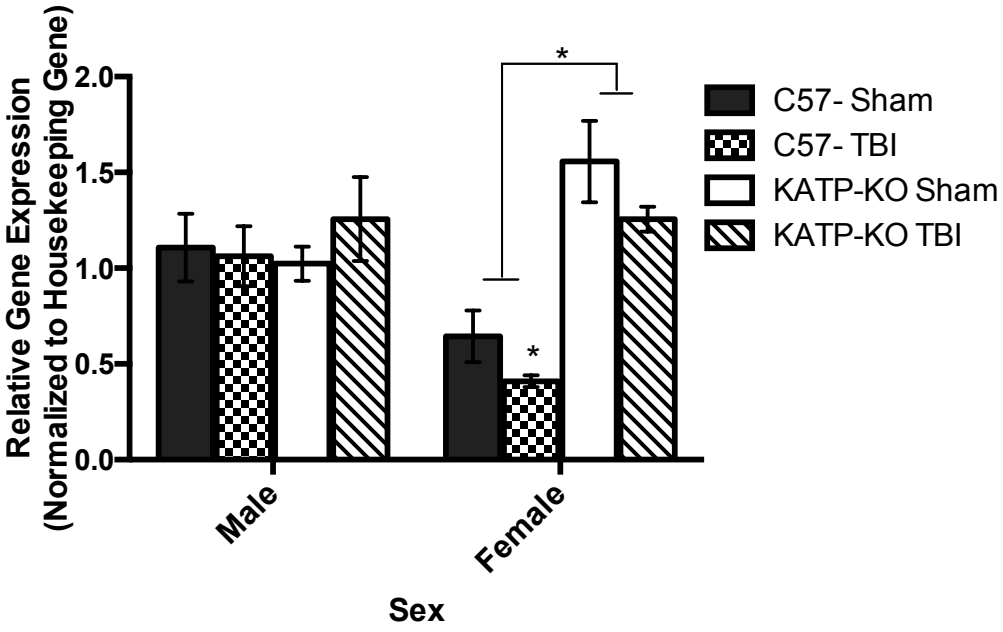

Figure C.6. Hsp32 Expression.

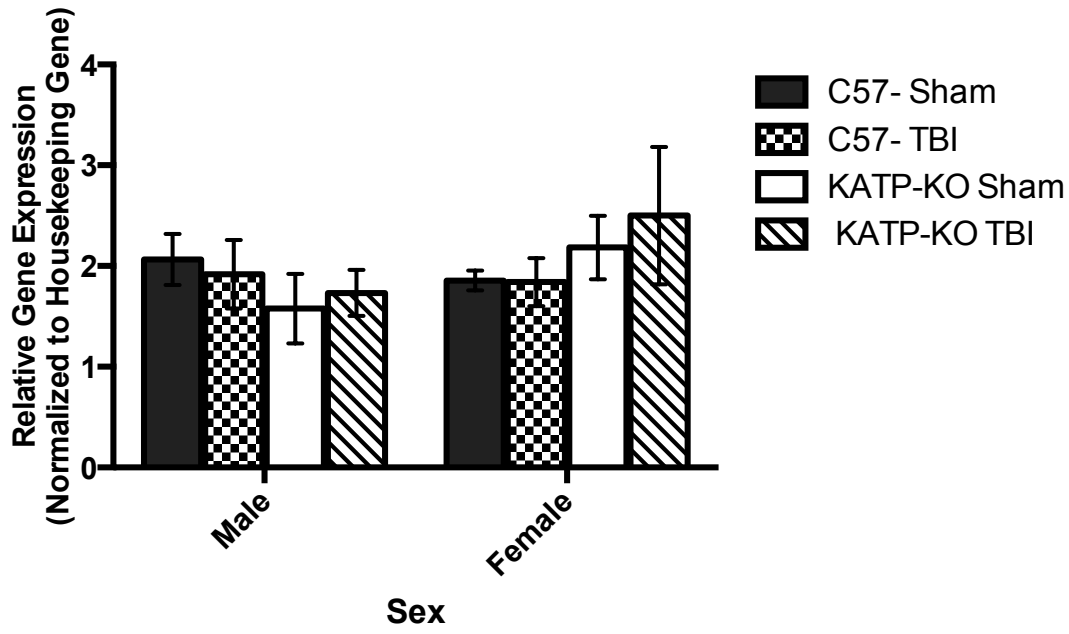

Figure C.7. Hsp27 Expression.

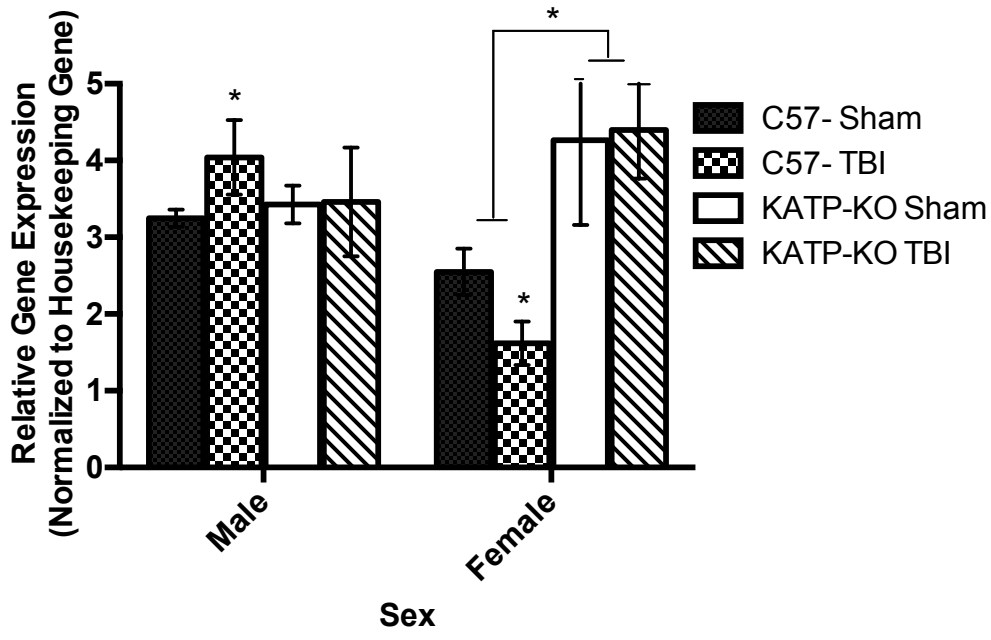

Figure C.7. Hsp10 Expression.

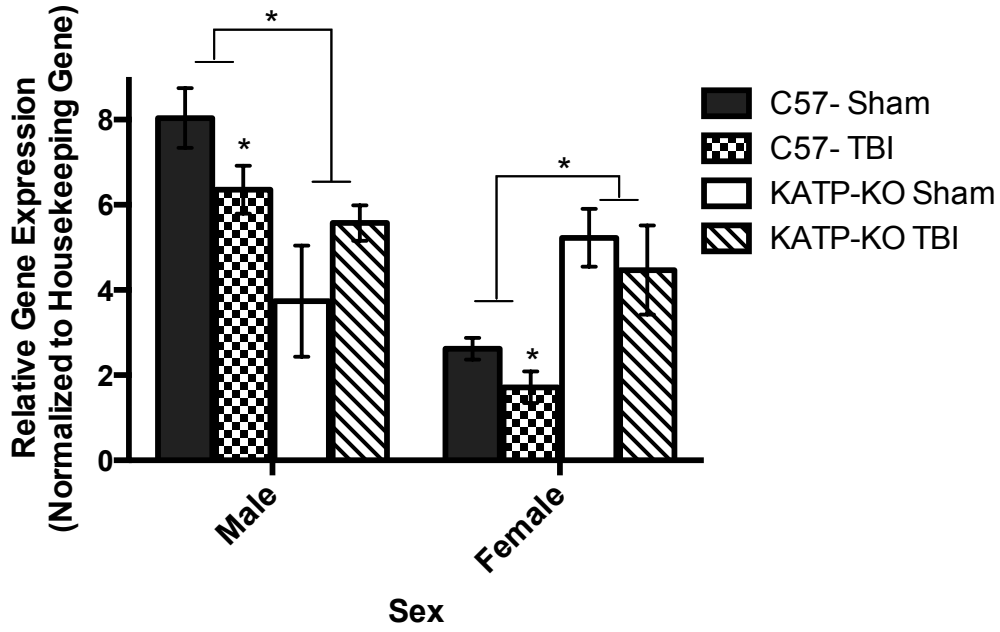

Figure C.8. Hsp5 Expression.

APPENDIX D: HSP EXPRESSION IN THE HIPPOCAMPUS AT 14 DAYS POST-MTBI

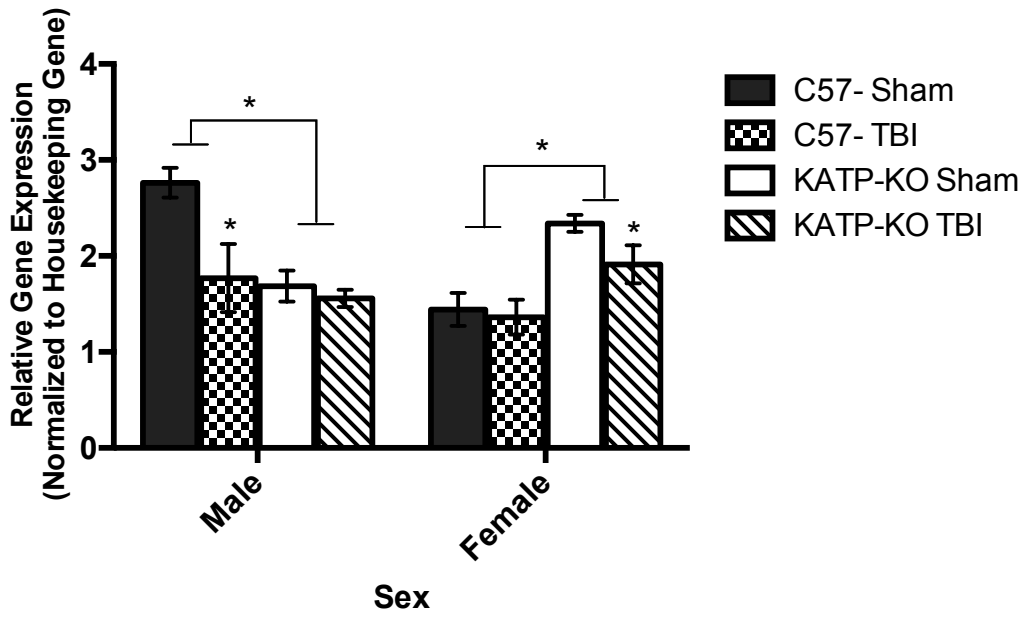

Figure D.1. Hsp90aa1 Expression.

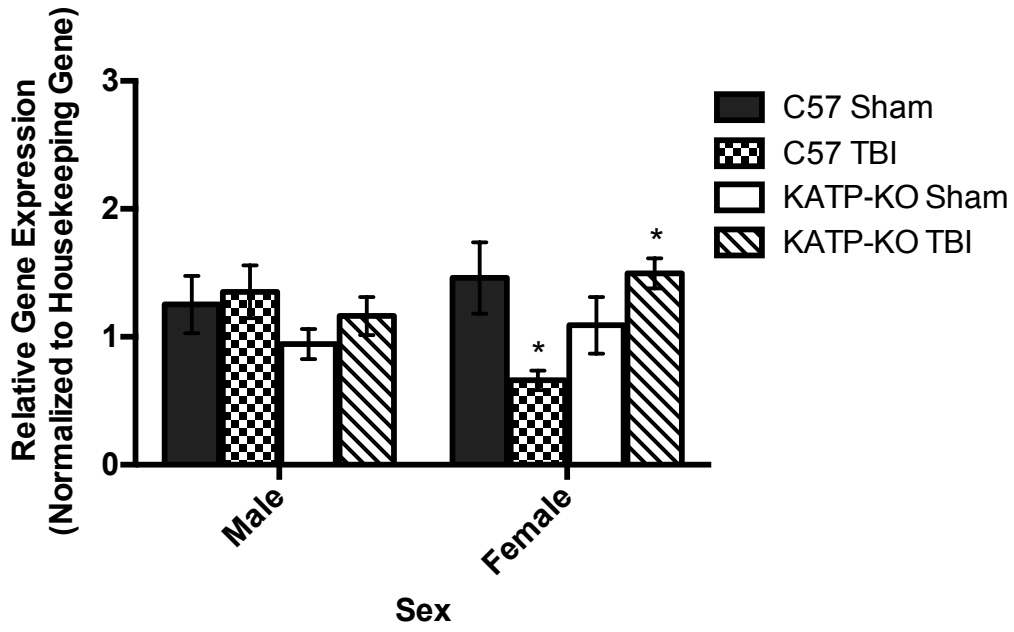

Figure D.2. Hsp90b1 Expression.

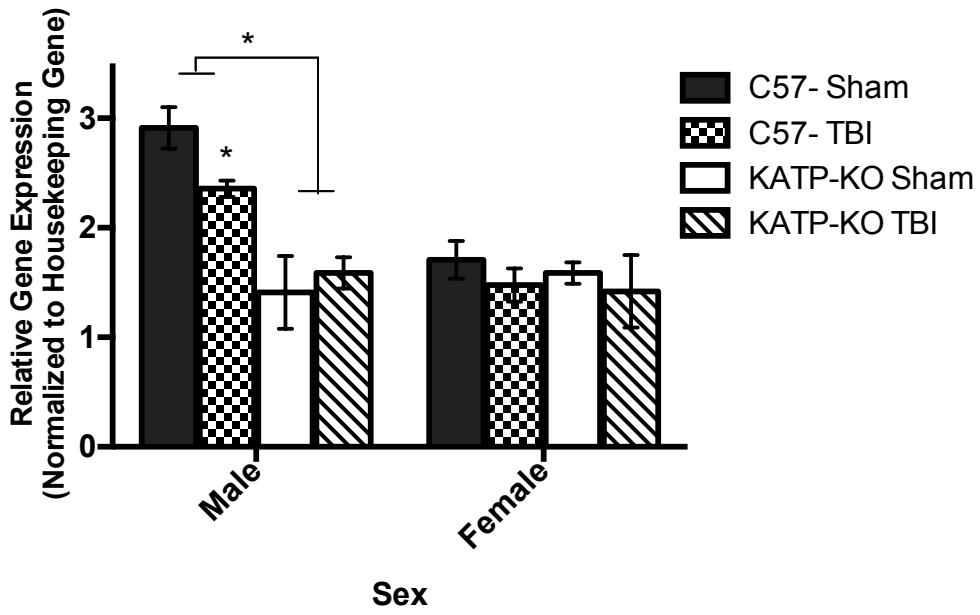

Figure D.3. Hsp70 Expression.

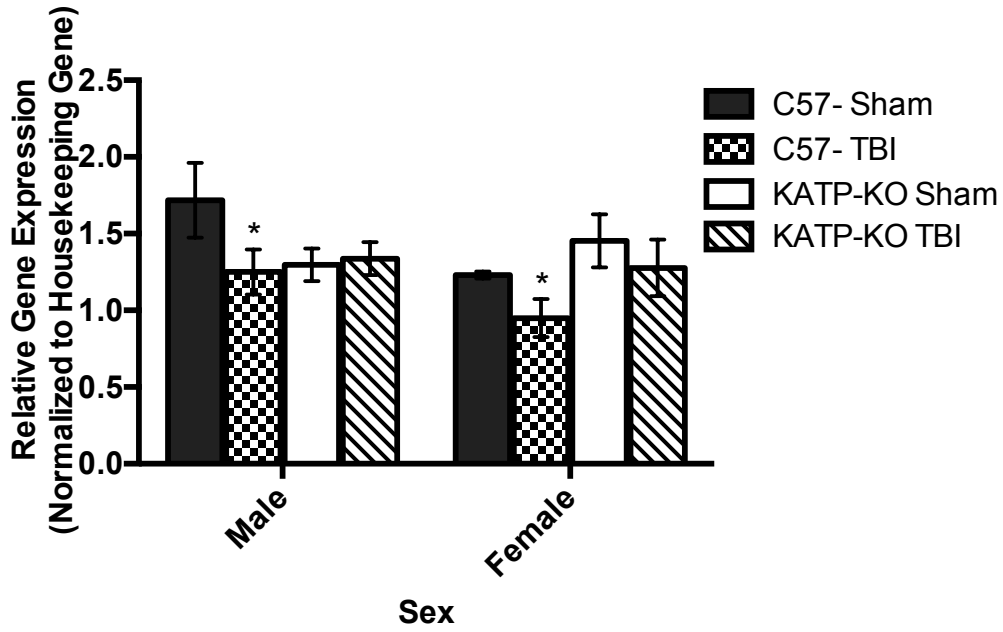

Figure D.4. Hsp60 Expression.

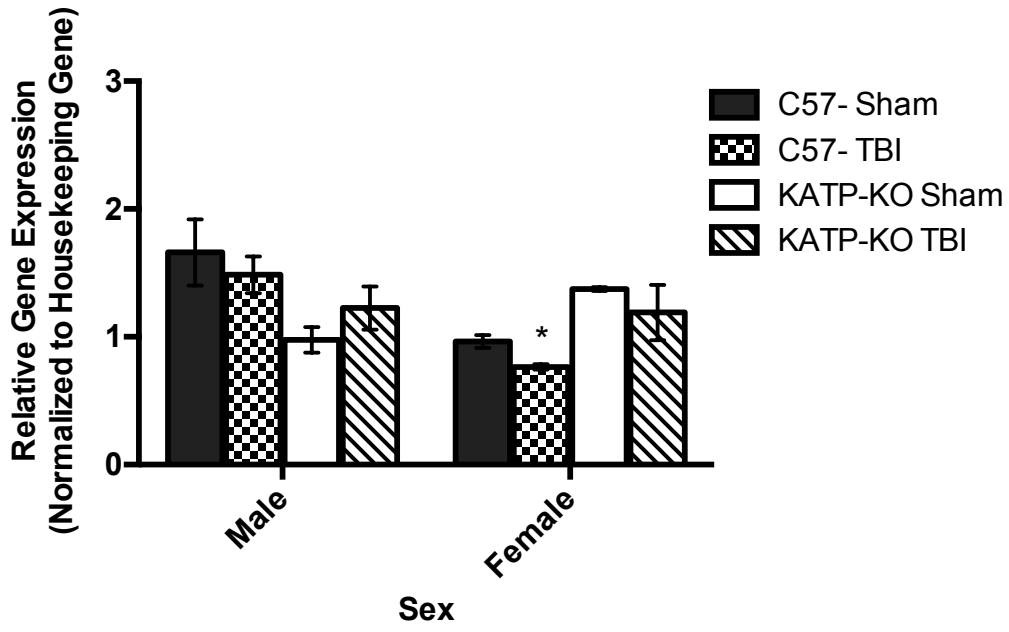

Figure D.5. Hsp40 Expression.

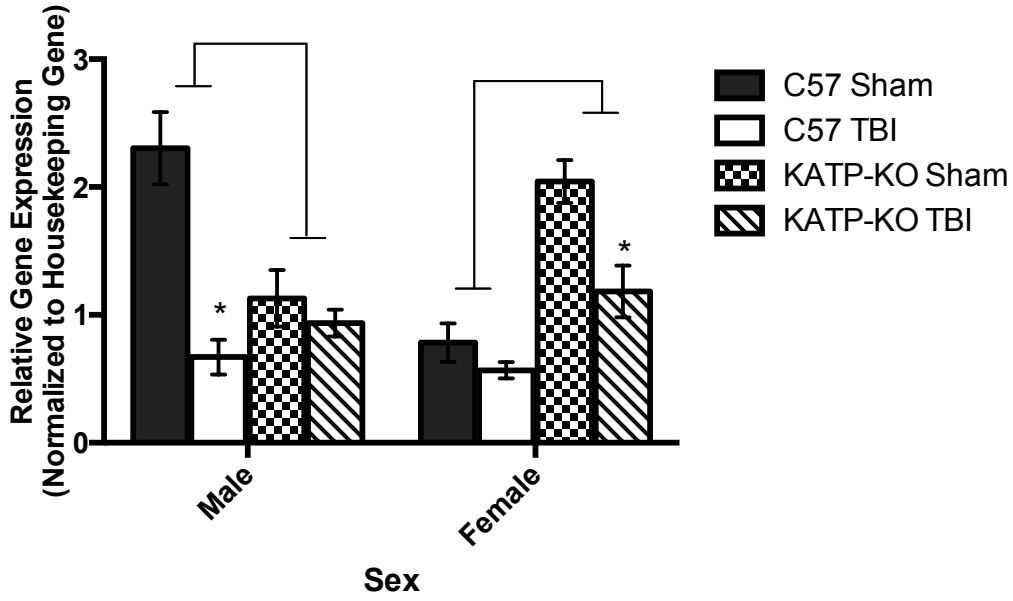

Figure D.6. Hsp32 Expression.

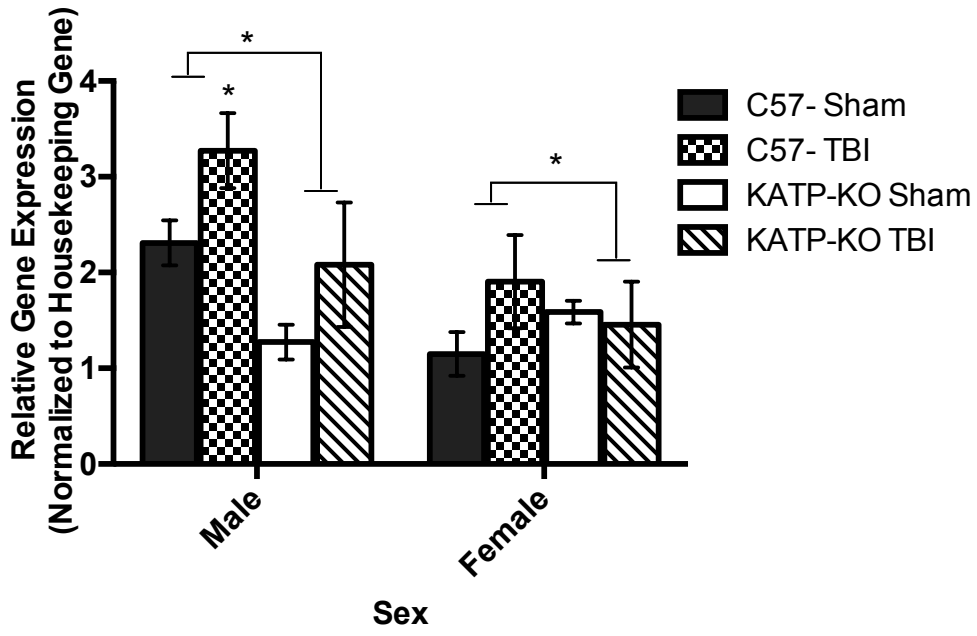

Figure D.7. Hsp27 Expression.

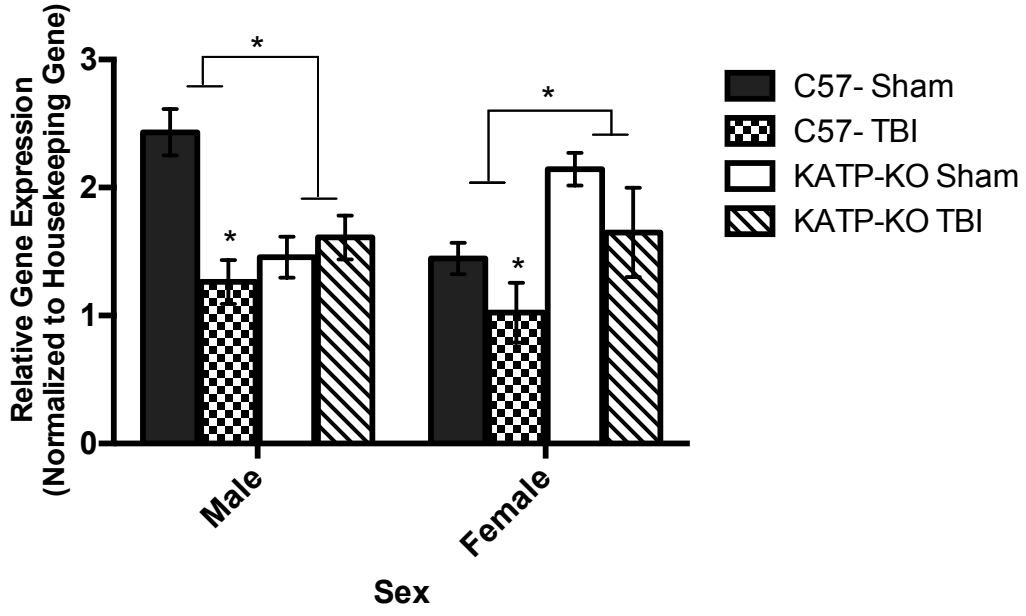

Figure D.8. Hsp10 Expression

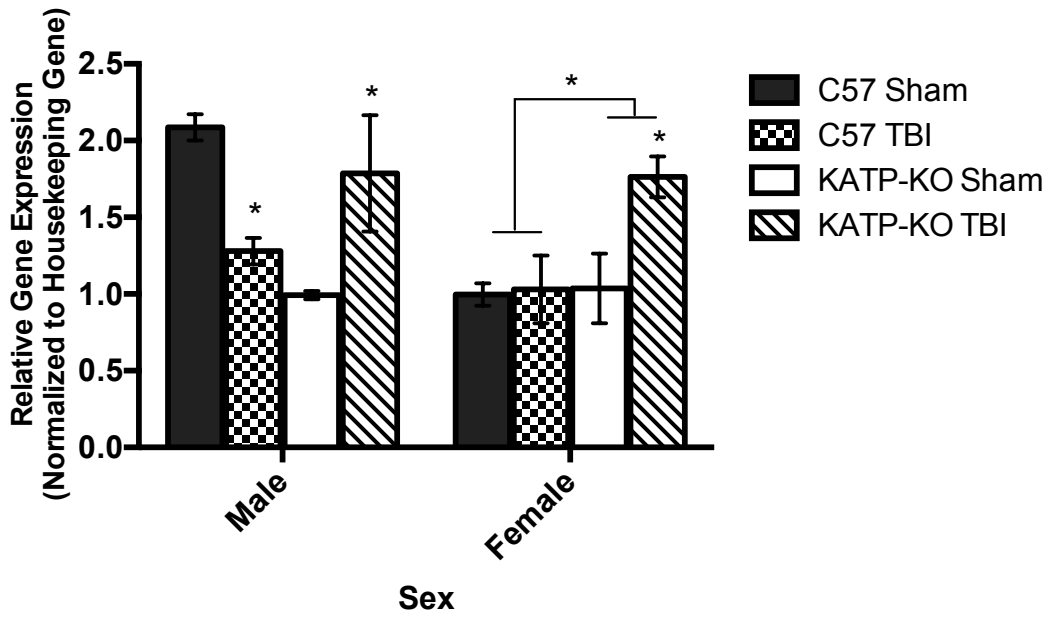

Figure D.9. Hsp5 Expression.