Transient Receptor Potential Melastatin 2 (TRPM2) Channel Plays a Role in Neonatal Hypoxic-Ischemic Brain Injury

by

Sammen Huang

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology
University of Toronto

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Abstract

Recent advances in stroke research have identified non-glutamate mechanisms associated with ischemic neuronal death in adults. Among the molecules involved in non-glutamate mechanisms, transient receptor potential melastatin 2 (TRPM2), a calcium-permeable non-selective cation channel, is reported to mediate brain damage following ischemic insults in adult mice. However, the role of TRPM2 channels in neonatal hypoxic-ischemic brain injury remains unknown. Here, we propose that TRPM2-null mice (TRPM2\(^{+/−}\) and TRPM2\(^{−/−}\)) reduces neonatal hypoxic-ischemic brain injury in postnatal 7-day-old mice. We report that the infarct volumes are significantly smaller and behavioral outcomes are improved in neonatal TRPM2\(^{+/−}\) and TRPM2\(^{−/−}\) mice compared to wild type mice. Next, we found that TRPM2-null mice down-regulated GSK-3β activity following HI. We subsequently tested the role of GSK-3β in neonatal HI brain injury using the pharmacological blocker TDZD-8. We showed that GSK-3β activity is involved in apoptotic neuronal cell death that could link the Akt/GSK-3β/caspase-3 pathway.
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# Table of Contents

Abstract................................................................................................................................. ii

Acknowledgements.............................................................................................................. iii

Table of Contents.................................................................................................................... iv

List of Tables.......................................................................................................................... vii

List of Figures........................................................................................................................ viii

List of Abbreviations............................................................................................................ x

1 Introduction......................................................................................................................... 1

1.1 Neonatal hypoxic-ischemic brain injury................................................................. 1

1.1.1 Current therapies............................................................................................... 2

1.2 Animal models of neonatal hypoxic-ischemic brain injury.................................. 2

1.2.1 Difference in vulnerability to ischemic injury between adult and neonatal brain ........................................................................................................... 3

1.3 Ischemic cascade........................................................................................................... 4

1.4 Failure in targeting NMDA channels with pharmacological antagonists........... 6

1.5 Targeting non-glutamate channels............................................................................ 6

1.5.1 Acid sensing ion channels................................................................................ 8

1.5.2 Volume regulated anion channels..................................................................... 8

1.5.3 Hemichannel....................................................................................................... 9

1.5.4 ATP-sensitive potassium channel....................................................................... 9

1.5.5 Transient receptor potential melastatin channels........................................... 10

1.6 TRP Channels and classifications.............................................................................. 10

1.7 TRPM2......................................................................................................................... 13

1.7.1 Gene.................................................................................................................... 13

1.7.2 Protein structure and distribution...................................................................... 13

1.7.3 Biophysical properties and gating mechanisms............................................. 16

1.7.4 Pharmacological interactions............................................................................ 19
1.7.5 Physiological and pathophysiological role .................................................19
1.7.6 Interaction with GSK-3β ......................................................................19
1.8 GSK-3 .........................................................................................................20
1.8.1 Protein structure and distribution ..........................................................20
1.8.2 Physiological and pathophysiological role .............................................20
1.9 Rationale and Hypothesis .........................................................................21

2 Materials and Methods ..................................................................................23
2.1 Development of TRPM2-null mouse ..........................................................23
2.2 Animals and genotyping .............................................................................23
2.3 Hypoxic-ischemic brain injury model ..........................................................24
2.4 Drug administration .....................................................................................25
2.5 Behavioral assessments ..............................................................................26
2.6 Morphological and histological assessments ..............................................26
2.7 Western blot ...............................................................................................27
2.8 Immunohistochemistry and confocal imaging .............................................27
2.9 In situ hybridization and 3D imaging ..........................................................28
2.10 Data analysis .............................................................................................28

3 Results ............................................................................................................29
3.1 The role of TRPM2 in neonatal HI brain injury .........................................30
  3.1.1 Knock-out of TRPM2 reduces brain infarct volume 24 hours following
       hypoxic-ischemic brain injury .................................................................30
  3.1.2 Brain damage is reduced in TRPM2+/-(HET) and TRPM2-/- (KO) mice 7
       days following hypoxic-ischemic brain injury .......................................32
  3.1.3 Sensorimotor function is improved in TRPM2+/-(HET) and TRPM2-/- (KO)
       mice 7 days after hypoxic-ischemic brain injury ....................................34
  3.1.4 p-Akt/t-Akt and p-GSK3β/t-GSK3β ratio is elevated in TRPM2+/-(HET)
       and TRPM2-/- (KO) mice following hypoxic-ischemic brain injury but not
       in sham treatment ..................................................................................36
3.2 The role of GSK-3β in neonatal HI brain injury .........................................38
  3.2.1 Developmental expression of GSK-3β and Akt ....................................38
  3.2.2 TDZD-9 as a pharmacological inhibitor of GSK-3β ..................................41
3.2.3 TDZD-8 pre-treatment attenuates infarct volume following hypoxic-ischemic brain injury

3.2.4 TDZD-8 improves neurobehavioral outcome following hypoxic-ischemic brain injury

3.2.5 TDZD-8 pre-treatment increases Serine-9 phosphorylated GSK-3β (p-GSK-3β) and phosphorylated Akt (p-Akt) levels after hypoxic-ischemic brain injury

3.2.6 TDZD-8 pre-treatment suppresses apoptotic signaling after hypoxic-ischemic brain injury

3.2.7 TDZD-8 reduces GFAP-positive cells and maintains NeuN-positive cells following hypoxic-ischemic brain injury

4 Discussion

4.1 Knock-out of TRPM2 reduces neonatal HI brain injury

4.1.1 TRPM2 HET/KO mice up-regulates Akt activity and down-regulates GSK3β activity following HI

4.2 Pharmacologically inhibiting GSK-3β activity is neuroprotective against HI injury

4.2.1 Changes in Akt/Akt and GSK3β activity after HI is developmentally regulated

4.2.2 GSK-3β activity following HI is involved in apoptotic signaling

4.2.3 The involvement of astrocytes following HI

4.3 Proposed model

4.4 Future Directions

References
List of Tables

Table 1: Primers for PCR detection of TRPM2.............................................................24
Table 2: Drug..................................................................................................................25
List of Figures

Figure 1. Representative pathway of the apoptosis-related ischemic cascade ......................5
Figure 2. Glutamate and non-glutamate channels involved in hypoxic-ischemic injury ..........7
Figure 3. TRP Superfamily classification...........................................................................12
Figure 4. TRPM2 protein structure and variants..............................................................15
Figure 5. Metabolic pathway of ADPr production..............................................................17
Figure 6. Biophysical properties of TRPM2 channel and activators/inhibitors.....................18
Figure 7. Timeline of neonatal hypoxic-ischemic injury and experimental procedures. ..........29
Figure 8. TRPM2+/−(HET) and TRPM2−/−(KO) mice had reduced brain infarct volume following
HI injury.........................................................................................................................31
Figure 9. TRPM2+/−(HET) and TRPM2−/−(KO) mice had reduced brain damage and maintained
brain weights 7 days following HI injury.......................................................................33
Figure 10. Sensorimotor function was improved in TRPM2+/−(HET) and TRPM2−/−(KO) mice
assessed 7 days after HI injury.....................................................................................35
Figure 11. Expression ratios of p-Akt/t-Akt and p-GSK3β/t-GSK3β were increased in HI
TRPM2+/−(HET) and TRPM2−/−(KO) mice but not in sham groups...............................37
Figure 12. Developmental gene changes in GSK-3β levels...............................................39
Figure 13. Developmental protein changes in GSK-3β and Akt levels.................................40
Figure 14. Chemical structure of TDZD-8...........................................................................41
Figure 15. TDZD-8 pre-treatment reduced infarct volume 24 hours following neonatal hypoxic-
ischemic brain injury.................................................................................................43
Figure 16. TDZD-8 pre-treatment reduced infarct volume 7 days following neonatal hypoxic-
ischemic brain injury.................................................................................................44
Figure 17. TDZD-8 pre-treatment improved neurobehavioral outcome assessed at 7 days following neonatal hypoxic-ischemic brain injury.................................................................46
Figure 18. Western blot showing the effects of TDZD-8 on the expression levels of cell survival and apoptotic markers following HI.................................................................48
Figure 19. Immunohistochemistry staining showing the effects of TDZD-8 on cleaved caspase-3 staining following hypoxic-ischemic brain injury.........................................................50
Figure 20. Immunohistochemistry staining showing the effects of TDZD-8 on NeuN-positive cells and GFAP-positive cells.................................................................52
Figure 21. Proposed mechanism for TRPM2 and TDZD-8 regulation of GSK-3β after hypoxic-ischemic brain injury.................................................................58
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ADPr</td>
<td>Adenosine diphosphate ribose</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>ASICs</td>
<td>Acid-sensing ion channel</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCAO</td>
<td>Bilateral cerebral artery occlusion</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cADPR</td>
<td>Cyclic adenosine diphosphate ribose</td>
</tr>
<tr>
<td>CCR</td>
<td>Coiled coil region</td>
</tr>
<tr>
<td>C38</td>
<td>Cluster of differentiation 38</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E17</td>
<td>Embryonic day 17</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphatae dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>GSK-3α</td>
<td>Glycogen synthase kinase 3 alpha</td>
</tr>
</tbody>
</table>
GSK-3β  Glycogen synthase kinase 3 beta
HET  Heterozygous
HI  Hypoxic-Ischemic
IHC  Immunohistochemistry
i.p.  Intraperitoneal
i.v.  Intravenous
IV  Current-voltage
K$_{\text{ATP}}$  ATP-sensitive potassium channel
K$^+$  Potassium ion
KO  Knockout
MAPK  Mitogen-activated protein kinases
Na$^+$  Sodium ion
NAD  Nicotinamide adenine dinucleotide
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA  N-methyl-D-aspartate receptor
NUDT9-H  Nudix (Nucleoside Diphosphate Linked Moiety X)-Type Motif 9
PARG  Poly (ADP-ribose) glycohydrolase
PARP  Poly (ADP-ribose) polymerase
PCR  Polymerase chain reaction
PI3K  Phosphoinositide 3-kinase
PIP2  Phosphatidylinositol 4,5-bisphosphate
P7/P14  Postnatal day 7/14
TDZD-8  4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione
TRPA  Transient receptor potential ankyrin
TRPC  Transient receptor potential canonical
TRPM  Transient receptor potential melastatin
TRPV  Transient receptor potential vanilloid
TRPP  Transient receptor potential polycystin
TRPML  Transient receptor potential mucolipin
TRPM2  Transient receptor potential melastatin 2
TTC  Tetrazolium chloride
VRACs  Volume-regulated anion channel
WT  Wildtype
Chapter 1

1 Introduction

Neonatal hypoxic-ischemic (HI) brain injury, also termed neonatal stroke, is a major cause of neurological impairment in infants. Ionic imbalance following injury has been strongly implicated as an important component of the pathophysiology in hypoxic-ischemic brain damage. One of these ion channels, the transient receptor potential melastatin 2 (TRPM2) channel, is a calcium-permeable non-selective cation channel. This channel is of particular interest because it has been shown that there is an accumulation of hydrogen peroxide in the neonatal brain following hypoxic-ischemic brain injury in both the cortex and hippocampus. Since, hydrogen peroxide is a direct and indirect activator of TRPM2, we expect TRPM2 to play an important role in neuronal injury following HI.

Glycogen synthase kinase-3beta (GSK-3β) has been implicated as a downstream target of TRPM2. Dysregulation of GSK-3β is known to regulate apoptotic cell death, a hallmark of neonatal HI brain injury. Here, I investigated the role of TRPM2 in neonatal hypoxic-ischemic brain injury in vivo. I hypothesized that TRPM2 is involved in neuronal death following neonatal hypoxic-ischemic brain injury and deletion of the channel elicits neuroprotection through GSK-3β mediated downstream signaling. To test this hypothesis, I proposed two objectives: 1. investigate the effect of TRPM2 on brain damage and sensorimotor function following neonatal HI injury and 2. identify GSK-3β-regulated potential signaling pathways that are involved with TRPM2-mediated neuronal death following HI.

1.1 Neonatal hypoxic-ischemic brain injury

Neonatal hypoxic-ischemic (HI) brain injury, also known as neonatal stroke, and its related disease hypoxic-ischemic encephalopathy (HIE) have become one of the leading causes of neurological impairment in children with a reported incidence of 2-4 cases per 1000 live births. This condition can develop during the pregnancy, labor or following birth and the early risk period has been identified as early as 20 weeks of gestation. There is an estimated 15% to 20%
mortality rate for children affected by neonatal HI injury\(^\text{10}\). Of the remaining survivors, approximately 25% suffer from lifelong neurological deficits including increased muscle tone, hemiparesis and seizures\(^\text{10}\).

### 1.1.2 Current Therapies

Currently, mild to moderate hypothermia is the widely used resuscitation treatment for affected patients which has shown success in a clinical setting\(^\text{11,12}\). However, approximately 40% of hypothermia-treated patients still suffer from death or severe disabilities\(^\text{12}\). Other neuroprotective strategies that has shown promise in clinical trials include prophylactic barbiturates\(^\text{13}\), erythropoietin\(^\text{14}\) and allopurinol\(^\text{15}\). None of these agents can directly target TRPM2 or one of its downstream targets involved in stroke, GSK-3\(\beta\), so we are aiming to identify new therapeutic targets that underlie neonatal HI brain injury and its related neurological disorders.

### 1.2 Animal models of neonatal hypoxic-ischemic brain injury

Traditionally, research has focused on studying adult ischemic stroke. In 1981, Rice and Vannucci was the first to develop a model modified from the Levine procedure that could be used to study neonatal hypoxic-ischemic brain injury\(^\text{10}\). Specifically, it involved the unilateral ligation of the common carotid artery followed by a period of systemic hypoxia. Unilateral ligation of the common carotid artery stops the main blood flow to the ipsilateral side of the brain hemisphere and results in focal ischemia. However, unilateral ligation of the common carotid artery alone is not sufficient to cause histological damage likely because there is collateral circulation of blood flow from the contralateral hemisphere through the Circle of Willis. Therefore, systemic hypoxia is used in conjunction with ischemia to decrease the blood pressure and further reduce cerebral blood flow by an addition 40-60% which is enough to induce an infarct core\(^\text{16}\). Subsequent ischemic cascade reactions cause the spread of the infarct core to the vulnerable brain tissue around the damage called the “penumbra.” Rescuing the penumbra is the primary goal of neuroprotection and is the focus of my thesis.
Other models of neonatal stroke include systemic hypoxia alone and bilateral occlusion of the carotid arteries (BCAO), where BCAO has been reported to be useful for studying white matter injury in neonatal rats\textsuperscript{17,18}. Here, I used the Rice and Vannucci model to study the neuroprotective effect caused by deletion of the TRPM2 channel following hypoxic-ischemic brain injury in neonatal mice. I chose this procedure over the other models because the Rice and Vannucci method provides reproducible results that affect only one hemisphere of the brain and have reduced mortality rate. This allows for the contralateral hemisphere to act as a control for hemispheric swelling. Bilateral hemispheric damage through ligation of both common carotid arteries is more likely to increase the mortality rate of the animals due to the severe nature of the damage.

1.2.1 Differences in vulnerability to ischemic injury between adult and neonatal brain

Based on the differences between adult and neonatal development, most of our understanding of stroke in adults is not transferrable to neonates. In fact, the vulnerability to ischemic damage in neonates is not compatible to adults and it is affected by their sensitivity to energy failure, glutamate excitotoxicity and oxidative stress which are hallmarks of hypoxic-ischemic injury. Neonatal rodents are well-adapted to tolerate hypoxic-ischemic-induced anaerobic conditions because of their ability to utilize ketone bodies as an alternative cerebral energy source\textsuperscript{19}. In comparison to adults, this offers an alternate metabolic pathway to glycolysis in anaerobic conditions that could counteract lactate accumulation and depletion of energy\textsuperscript{20,21}. In addition, neonatal pups have a reduced metabolic rate compared to adults conferring resistance to energy failure. Contrarily, neonates can have periods of vulnerability to hypoxic-ischemic damage because they express high levels of the NMDA receptor subtype N-methyl D-aspartate 2B (NR2B) during development\textsuperscript{22}. NR2B subunit expression can increase the duration of opening of NMDA channels and this can contribute to a heightened calcium influx in response to hypoxic-ischemic conditions\textsuperscript{23}. Furthermore, the accumulation of hydrogen peroxide coupled with low antioxidant activity in neonates make them sensitive to oxidative stress induced by hypoxic-ischemic brain injury\textsuperscript{5}. 
1.3 Ischemic Cascade

The ischemic cascade is a pathway of biochemical reactions that occurs following a lack of blood flow to the brain which ultimately leads to cell death. This cascade is composed of three stages that starts immediately after ischemia and persists long after the reperfusion of blood (see Yenari and Han 2012 for review\(^8\)). The first stage of the ischemia is the “primary energy failure stage” where a low supply of oxygen to brain hinders the production of ATP. Next, ATP-dependent ion transporters such as the \(\text{Na}^+/\text{K}^+\) pump fail to function and initiate a depolarization wave across neurons causing the release of glutamate into the synaptic cleft. Excess release of glutamate into the synaptic cleft activates AMPA and NMDA channels and causes an overload of calcium influx into the cell; this is also known as excitotoxicity. The excess amount of calcium inside the cell activates several pathways that lead to the generation of reactive oxygen species as well as activation of the apoptosis pathway (Figure 1). Recent advances in the field have implicated several non-glutamate dependent ion channels that are also implicated in HI injury. These are further discussed later on. Following primary energy failure, neonates can temporarily restore cellular metabolism to a certain degree by suppression of neuronal activity, initiation of anaerobic respiration and reducing energy consumption. This marks the second stage of the ischemic cascade also known as the “latent” phase\(^8\). Depending on the severity of the ischemic insult, this latent stage can result either in recovery or continued apoptosis, accumulation of excitotoxins, initiation of inflammation and cerebral edema which results in delayed neuronal death that persists days after the initial insult \(^8\). Delayed neuronal death following a secondary energy failure is final stage of the ischemia cascade.
Figure 1. Representative pathway of the apoptosis-related ischemic cascade

Following ischemia, apoptosis can be activated through metabolic acidosis, mitochondrial dysfunction, caspase activation and reactive oxygen species production which are mediated mainly by primary energy failure in conjunction to reperfusion injury.
1.4 Failure in targeting NMDA channels with pharmacological antagonists

Pharmacologically targeting NMDA channels has been a strategy used to treat acute ischemic stroke because it was previously found that there was an excessive rise in the neurotransmitter, glutamate, following injury. Glutamate is an activator of NMDA channels which in high concentrations can cause excessive NMDA stimulation resulting in toxic calcium influx that leads to neuronal injury. Specifically, neuronal toxic calcium influx affects a myriad of proteins including phospholipase, calcium binding proteins, proteases and endonucleases that contribute to neurotoxicity. Activation of phospholipases is involved in dysregulation of biomembranes through enzyme catalyzed peroxidation reactions. Calcium can also bind to calcium binding proteins such as calmodulin which can in turn increase the production of nitric oxide and free radical production. Proteases and endonucleases can be involved through degradation of cytoskeletal structures and DNA. Taken together, blocking NMDA-mediated calcium influx was thought to be an effective strategy in reducing neuronal damage against stroke injury. Indeed, early animal studies using NMDA antagonists including MK 801, selfotel (CGS 19755), cerestat and dextrometorpahan as a treatment for ischemic stroke was promising. However, further testing of these compounds was found to be unsuccessful in clinical studies.

1.5 Targeting non-glutamate channels

Previous attempts to pharmacologically block glutamate-mediated excitotoxicity failed to achieve success in clinical trials and this could have been attributed to multiple reasons. One major reason is that NMDA receptors play an essential role in normal neurophysiological processes and it’s been reported that its role following hypoxic-ischemic injury is not only a deleterious one, but also beneficial. Blocking NMDA receptors also caused side effects seen in patients including drowsiness, hallucinations and comas which caused much controversy. Since then, research has directed its focus to ameliorate toxic calcium influx through non-glutamate targets of hypoxic-ischemic cell death (Figure 2).
Glutamate receptors include: N-methyl-D-aspartate receptor (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA). Non-glutamate receptors include: Acid sensing ion channels (ASICs), Volume regulated anion channels (VRACs) ATP-sensitive potassium channels (K$_{ATP}$), Transient receptor potential melastatin channels (TRPM) and hemicannels.
1.5.1 Acid sensing ion channels

Acid sensing ion channels (ASICs) are glutamate independent calcium permeable channels that have been implicated in ischemic stroke. ASICs are subdivided into six isoforms including ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 where ASIC1a, 2a and 3b are found in the brain. Specifically, it has been shown that ASIC1a is a mediator of ischemic stroke and neuroprotection was achieved when this channel was blocked with psalmotoxin 1 in adult mice. ASIC1a is activated by acidosis and the half-maximal pH activation is 6.2. Following ischemia, there is a depletion of oxygen to the brain which causes neurons to undergo anaerobic respiration. As a result, there is an accumulation of lactate and lactic acid from the conversion of pyruvate, the end product of glycolysis. This releases an excess of hydrogen protons inside neurons causing lactic acidosis which subsequently can activate ASIC1a leading to toxic calcium influx. Recently, it has been shown that blocking ASIC1a in neonatal hypoxic-ischemic brain injury using psalmotoxin 1 has also elicited neuroprotection in newborn piglets.

During development, the brain has the ability to use ketone bodies as a source of energy and it is expected that this reduces the severity of acidosis following HI. Expectedly, it has been reported that following HI, the cerebral pH of newborn piglets dropped as low as 6.7; in adults, it can fall as low as 6.0 during severe ischemia. Nonetheless, the relatively weaker state of acidosis still had a significant effect on ASIC1a channel activation in neonatal HI implicating an important role of this channel in this condition.

1.5.2 Volume regulated anion channels

Brain edema and cell swelling are hallmarks of stroke that has been implicated as one of the major causes of brain injury. Volume regulated anion channels (VRACs) are volume sensors that regulate increased cell volume through efflux of ions and other solutes, driving water out of the cell. Following ischemia, depolarizing waves can lead to persistent cell swelling. Specifically, cell swelling of astrocytes have been shown to release excess excitatory amino acids (EAA) through VRACs which has been shown to be involved in neuronal injury. Two major EAs include glutamate and aspartate that can cause increased calcium influx through activation of EAA receptors consequently leading to excitotoxicity. It was recently reported that inhibition of
VRACs using DCPIB has been shown to be neuroprotective against adult and neonatal rodents\textsuperscript{38,39}. This suggests that VRACs contribute a significant amount of extracellular EAA and reduction of the swelling-induced activation of VRACs can be a treatment against neonatal hypoxic-ischemic brain injury.

1.5.3 Hemichannel

Hemichannels are transmembrane proteins that form the functional subunits of a gap junction. Specifically, each hemichannel is composed of six connexin units and two hemichannels comprise of a gap junction. Following ischemia, ionic imbalance has been implicated as one of the main component of neuronal death\textsuperscript{40}. In particular, gap junctions have been shown to contribute to a large current amplitude following ischemia\textsuperscript{40}. Furthermore, it was reported that opening of hemichannels may result in efflux of glutamate, glucose and ATP which further worsens energy failure following ischemia and limiting chances of recovery\textsuperscript{40}. Lastly, hemichannels have been proposed to contribute to anoxic depolarization which is a recognized component of ischemic cell death in the penumbra\textsuperscript{41}. The neuroprotective effect of blocking or deleting expression of hemichannels has been seen in oxygen glucose deprivation, fetal ischemia, and adult ischemia models implicating this channel as an important therapeutic target for treatment\textsuperscript{42-44}.

1.5.4 ATP-sensitive potassium channel

ATP-sensitive potassium channels (K\textsubscript{ATP}) are weak inward rectifying potassium permeable channels that are regulated by intracellular nucleotides, namely ATP and ADP. K\textsubscript{ATP} is a heterooctamer composed of four Kir6.x subunits that form the channel pore and this is surrounded by four sulfonylurea receptor (SUR) subunits that modulate channel activity; the specific isoform Kir6.2/SUR1 is found in the brain\textsuperscript{45}. These channels are activated by a high ADP and inactivated with high ATP implicating K\textsubscript{ATP} as a metabolic sensor involved in insulin secretion\textsuperscript{46}. Following ischemia, there is a marked drop in the ADP/ATP ratio which activates K\textsubscript{ATP} channels resulting in hyperpolarization and suppression of neuronal excitability. This was previously proposed as a cellular mechanism that underlies ischemic tolerance and neuroprotection follow hypoxic-ischemic brain injury\textsuperscript{47}. K\textsubscript{ATP} has also been shown to be
involved in neuroprotection mediated by hypoxic preconditioning in adult and neonatal mice\textsuperscript{47,48}. The involvement of $K_{\text{ATP}}$ in insulin secretion and hypoxic-ischemic injury suggests a role of the channel in prospects of linking diabetes and stroke.

1.5.5 Transient receptor potential melastatin channels

Transient receptor potential melastatin channels are calcium permeable ion channels that have been implicated in calcium dysregulation following ischemic injury in rodents. In particular, protein expression of TRPM7 were found to be up-regulated following ischemia reperfusion injury\textsuperscript{49}. Currently, the calcium conducting activity of TRPM channels have been attributed as one of the main mechanisms that mediate neuronal toxicity following stroke. However, a recent study has reported that TRPM2 expression modulates NMDA subunit expression activity suggesting that TRPM2 act through multiple pathways in ischemic conditions\textsuperscript{50}. To our knowledge, there are currently no studies that report the involvement of TRPM channels in neonatal hypoxic-ischemic brain injury.

1.6 TRP Channels and Classifications

TRP channels were a recently discovered class of channels found in \textit{Drosophila melanogaster}\textsuperscript{51}. They were classified into six subfamilies based on amino acid sequence homology including: TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPC (canonical), TRPM (melastatin), and TRPML (mucolipin). To date, there have been 28 identified mammalian TRP channels (Figure 3). Mammalian TRP channels are formed from 4 subunits each containing six transmembrane domains. Although these channels belong to the same family, they share a huge range of functional properties. In general, they are non-selective cation ion channels expressed in excitable and non-excitable cells.

TRP channel activation has been broadly divided into three categories namely: receptor activation, ligand activation, and direct activation (see Ramsey et al., 2006 for review\textsuperscript{52}). Receptor activation of TRP channels are largely mediated by G protein-coupled receptors (GPCRs) that can regulate TRP channel activity through activation of phospholipase C that hydrolyzes phosphatidylinositol 4, 5 bisphosphate (PIP2) and release diacyl glycerol (DAG)\textsuperscript{53}. Ligand activation of TRP channels are mediated by a plethora of molecules that can bind to TRP channels that modulate the activity of the
channel. These ligands can be natural or synthetic products originated from an exogenous or endogenous source and have organic or inorganic properties. The most well-known activators of TRP channels include: PIP2, DAG, adenosine diphosphate ribose (ADPr) and nicotinamide adenine dinucleotide (NAD). Lastly, certain sub-types of TRP channels namely TRPV1-V3 and TRPM8 can be affected by direct activation that is mostly attributed to changes in temperature and mechanical stress\textsuperscript{54}. 
Figure 3. TRP Superfamily classification

The TRP superfamily is subdivided into 6 subfamilies. There are a total of 28 TRP channels. The phylogenetic tree of the TRPM subfamily is shown here. This image is modified from Nilius and Voets 2005\textsuperscript{1}.
1.7 TRPM2

1.7.1 Gene

The transient receptor potential melastatin 2 (TRPM2) channel is a calcium-permeable non-selective cation channel and the gene in humans is located on the 21q22.3 chromosome (see Faouzi and Penner 2014 for review\textsuperscript{2}). The human \textit{TRPM2} gene has two forms: the first is the full length long form (TRPM2-L), and the second known as the striatum short form TRPM2 (TRPM2-SSF). The TPRM2-L form codes for 1503 amino acids and TRPM2-SSF has 1289 amino acids. The difference between the two forms is the lack of the N-terminal side containing 214 amino acids in the TRPM2-SSF form. In contrast with the human gene, the mouse \textit{TRPM2} gene consists of 34 exons that span approximately 61kb. It is important to note that the mouse TRPM2 form still mediates calcium influx induced by hydrogen peroxide. At the transcription level, four post-transcriptional splice variants of the full length TRPM2 have been identified and they include: TRPM2-ΔN, TRPM2-ΔC, TPRM2ΔNΔC and TRPM2-S\textsuperscript{2}(Figure 4).

1.7.2 Protein Structure and Distribution

The TRPM2 channel is a tetramer containing 24 transmembrane segments. At the TRPM2 N terminus, there are four homologous domains and a calmodulin binding IQ-like motif that acts as a calcium sensor modulating TRPM2 activation\textsuperscript{55}. It is notable that this calmodulin motif is located from the 406-416 amino acid sequence which is present in all known TRPM2 variants. Following the homology region, there are six transmembrane segments and each subunit has a pore-forming region between segment 5 and 6. In the TRPM2-S splice variant, the protein is ended at transmembrane segment 2 implicating it as a negative regulator of TRPM2 activity. After the transmembrane segments, there is a coiled-coil region (CCR) that spans approximately 100 amino acids implicated to play a role in channel assembly and gating. The CCR region is connected to the C-terminus region that is uniquely characterized by a NUDT9-H domain in TRP family\textsuperscript{2,56}. The NUDT9-H region is subdivided into two domains: the CORE domain and an N-terminal CAP domain. The NUDT9-H domain is known for its role in TRPM2 channel assembly and trafficking to the membrane surface. In addition, it is linked to TRPM2 gating via binding of
ADPr (TRPM2 activator). The CORE domain contains the pyrophosphatase activity and the N-terminal CAP domain plays a role in increasing the binding affinity of the CORE domain to ADPr\textsuperscript{57}. It was previously reported that the **TRPM2-ΔC splice variant contains missing regions in the NUDT9-H region** and expectedly, it is not responsive to ADPr activation\textsuperscript{58}. By extension, **TRPM2ΔNΔC also contains the same missing regions in the NUDT9-H region** so it is expected that this channel is also not responsive to gating by ADPr.

TRPM2 is widely expressed throughout the central nervous system, liver, pancreas, lungs and heart\textsuperscript{59}. At a cellular level, TRPM2 is found in neurons, microglia, neutrophils, macrophages, **pancreatic β-cell and endothelial cells**. TRPM2 channels were first discovered as a plasma membrane channel, but recent studies report that TRPM2 is also located in lysosomes. Here, they act as a calcium release channel found in pancreatic β cells that is activated by intracellular ADPr that contributes to calcium induced apoptotic signaling\textsuperscript{60}. Currently, the factors that determine the subcellular localization of TRPM2 remain unknown but this phenomenon was also seen in TRPM8 channels; TRPM8 channels are found in subcellular ER membranes and are implicated as a calcium release channel\textsuperscript{61}. While the roles of these channels in subcellular locations remain unclear, it is suggested that they are modulators of intracellular calcium levels.
Figure 4. TRPM2 protein structure and variants

A. Representative structure of the TRPM2 channel. The N-terminus contains a TRPM homology domain with a calmodulin binding IQ-like motif. This is followed by 6 transmembrane segments with a pore-forming loop found between S5-S6. The coiled-coil region is adjacent to the transmembrane segments followed by a NUTD9-H at the C-terminus. B. Forms of TRPM2. These include: full-length long form TRPM2 (TRPM2-L), TRPM2 cleavage from N terminus K538-Q557 (TRPM2-ΔN), TRPM2 cleavage from C terminus T-1292-L1325 (TRPM2-ΔC), TRPM2 cleaved from both C and N terminus (TRPM2-ΔN ΔC), TRPM2 short striatum variant that has 214 residues missing from C terminus (TRPM2-SSF) and TRPM2 short variant formed from a premature stop codon at the first two transmembrane segments (TRPM2-S). This image was adapted from Faouzi and Penner 2014\(^2\).
1.7.3 Biophysical properties and gating mechanisms

TRP channel activation has been broadly divided into three categories namely: receptor activation, ligand activation, and direct activation. TRPM2 has a linear IV curve suggesting that the channel activity it is not voltage-gated. Instead, TRPM2 gating by large falls into the ligand activation category. Specifically, it has been reported that TRPM2 displays a concentration dependent activity based on ADPr, hydrogen peroxide, NAD$^+$ and cADPR concentrations where ADPr is the most potent activator of TRPM2$^{62}$. Hydrogen peroxide, NAD$^+$ and cADPR are linked together during TRPM2 activation since they share the same metabolic pathway that produces ADPr (Figure 5). Specifically, NAD$^+$ and cADPR can be hydrolyzed via coenzyme C38’s NADase and ADPr hydrolase activity to produce ADPr, respectively. In addition, NAD$^+$ can be converted to cADPr through CD38’s ADP-ribosyl cyclase activity. Furthermore, NAD$^+$ can be converted to ADPr polymers via poly ADPr polymerase (PARP) activity. Subsequent cleaving of the polymer with poly ADPr glycohydrolase (PARG) can generate free ADPr. While hydrogen peroxide is able to directly activate TRPM2 independent of ADPr, it can also work synergistically with ADPr to regulate channel activity$^{63}$. Specifically, hydrogen peroxide can mediate the conversion of NAD$^+$ to ADPr$^{64}$.

ADPr activation of TRPM2 is gated by the NUDT9-H domain. The NUDT9-H region has multiple active sites that bind to ADPr and these acts as a reserve pool for TRPM2 during channel activation$^{65}$. The NUDT9-H pyrophosphatase activity acts to convert ADPr to ribose 5-phosphatae and adenosine monophosphate (AMP), a negative regulator of TRPM2. Interestingly, it has been reported that the enzymatic function of the domain is not important for channel gating suggesting the binding of intracellular ADPr alone regulates TRPM2 activity$^{56,57}$. Another molecule involved in TRPM2 gating is intracellular calcium. Previously, it was found that there are four sites near the intracellular pore of the TRPM2 channel that specifically bind calcium and are not sensitive to other divalent ions$^{66}$. In addition to these intracellular sites, calcium binding to the CaM-binding motif in the N-terminus of TRPM2 is another mechanism of gating that is independent of ADPr$^{67}$.

Negative regulators of TRPM2 that act to inhibit channel activity include acidification/high concentrations of protons, low levels of intracellular calcium and extracellular divalent copper
ions. Pathological conditions that disrupt the regulation of TRPM2 gating can potentially lead to cell injury (Figure 6).

Figure 5. Metabolic pathway of ADPr production

NAD$^+$ and cADPR can be hydrolyzed via coenzyme C38’s NADase and ADPr hydrolase activity to produce ADPr, respectively. NAD$^+$ can be converted to cADPr through CD38’s ADP-ribosyl cyclase activity. NAD$^+$ can be converted to ADPr polymers via poly ADPr polymerase (PARP) activity. Cleaving of the poly-ADPr with poly ADPr glycohydrolase (PARG) can generate free ADPr. ADPr is subsequently able to bind to TRPM2 NUDT9-H domain and be degraded to ribose 5-phosphate and AMP. Image is adapted from Faouzi and Penner 2014$^2$. 
Figure 6. Biophysical properties of TRPM2 channel and activators/inhibitors

A. Representative IV curve of TRPM2 channel. TRPM2 channel activity has a linear IV curve and is not voltage gated. B. List of activators and inhibitors of TRPM2 channel activity.

<table>
<thead>
<tr>
<th>Activators</th>
<th>Inhibitors</th>
</tr>
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<tbody>
<tr>
<td>Adenosine diphosphate ribose (ADPr)</td>
<td>Adenosine monophosphate (AMP)</td>
</tr>
<tr>
<td>Cyclic adenosine diphosphate ribose (cADPr)</td>
<td>Low pH or high protons (H+)</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide (NAD⁺)</td>
<td>Divalent heavy metal cations(Cu²⁺, Hg²⁺, Pb²⁺, Fe²⁺, Se²⁺ and Zn²⁺)</td>
</tr>
<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td></td>
</tr>
<tr>
<td>Intracellular calcium (Ca²⁺)</td>
<td></td>
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</table>
1.7.4 Pharmacological interactions

At present, there has not been a report of a characterized pharmacological inhibitor specific for the TRPM2 channel. Pharmacological attempts of inhibiting TRPM2 have resorted to targeting the related pathways of the channel. Namely, there are PARP inhibitors (SB750139-B, DPQ)\(^6\), Janus kinase 2 inhibitor (AG490)\(^6\), and phospholipase A\(_2\) inhibitor (N-(p-amylcinnamoyl) anthranilic acid)\(^7\). Other reported inhibitors include antifungal agents (clofimazole, econazole)\(^1\), 2-aminoethoxydiphenyl borate\(^2\) and flufenamic acid\(^3\). A reported activator of TRPM2 is alloxan. Alloxan is used for induction of diabetes and it causes calcium influx through hydrogen peroxide-induced TRPM2 activation\(^4\). Taken together, there are an abundance of pharmacological agents that can modulate TRPM2 activity albeit in a non-specific manner. Hence, transgenic knock-out of TRPM2 in animal models provides a useful tool to study the physiological and pathological role of the channel.

1.7.5 Physiological and pathophysiological role

In normal conditions, the calcium-conducting activity of TRPM2 has been shown to be involved in several physiological processes including inflammation through cytokine release, synaptic transmission in the hippocampus, microglial activation and insulin secretion\(^2\). In pathological conditions, TRPM2 has been reported to be involved in triggering neuronal cell death leading to central nervous system diseases including neuropathic pain, bipolar disorder, amyotrophic lateral sclerosis, Alzheimer’s disease and stroke\(^2\). It was previously demonstrated that there are many mechanisms of TRPM2 action in pathological conditions and it involved oxidative stress, tumor necrosis factor signaling and amyloid beta mediated pathological activation of TRPM2 channels\(^2\).

1.7.6 Interaction with GSK-3β

One possible downstream signaling pathway of TRPM2 activity could be mediated through GSK-3β signaling. TRPM2 has been identified as a susceptibility gene for bipolar disorder\(^7\). Specifically, TRPM2-defecient mice were shown to exhibit increased anxiety and poor social behavior and this was coupled with significantly increased Serine 9 phosphorylation of GSK-3β.
in adult mice\textsuperscript{75}. Serine 9 phosphorylation of GSK-3β is the inactive form of GSK-3β and this was thought to contribute to the pathophysiology of bipolar disorder and impairment of NMDA-dependant long term depression\textsuperscript{6}. The regulation of GSK-3β in neonatal mice by TRPM2 has yet to be reported.

1.8 GSK-3

1.8.1 Protein structure and distribution

GSK-3 is composed of two isoforms, GSK-3α and GSK-3β, and was first described as a regulatory protein for glycogen metabolism. GSK-3 is involved in numerous cellular processes in the brain including cytoskeletal reorganization\textsuperscript{76}, synaptic plasticity\textsuperscript{77}, neuronal survival and apoptosis\textsuperscript{78} mainly through the mitogen-activated protein kinase (MAPK)\textsuperscript{79}, Wnt-1/β-catenin\textsuperscript{80}, and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathways\textsuperscript{7}. GSK-3α and GSK-3β are both constitutively active kinases that share a high structural homology with different regulatory sites. The most well-known negative regulatory residues include the serine 21 (Ser21) site for GSK-3α and serine 9 (Ser9) site for GSK-3β. Phosphorylation of GSK-3α (p-GSK-3α) at the Ser21 site and GSK-3β (p-GSK-3β) at the Ser9 site inhibits kinase activity\textsuperscript{81}. While GSK-3α and GSK-3β are both found broadly in many tissues, GSK-3β is highly expressed in brain regions including the cerebral cortex, hippocampus and cerebellum\textsuperscript{82-84}. GSK-3β is also involved in neuronal development processes, including neuronal polarization\textsuperscript{85,86}, neurogenesis\textsuperscript{87,88} and axonal growth\textsuperscript{89,90}. These processes play an important role in neuronal recovery following ischemic insults. GSK-3β is composed of two splice variants, GSK-3β1 and GSK-3β2, where GSK-3β2 has a 13 residue insert in the C-terminus region. While GSK-3β1 is found in all tissue, GSK-3β2 is expressed only in neurons\textsuperscript{91}

1.8.2 Physiological and pathophysiological role

Glycogen synthase kinase 3 (GSK-3) is a constitutively active serine/threonine kinase that is expressed in all tissues. Under physiological conditions, it plays a critical role in neurodevelopment through the modulation of several transcription factors including cAMP response element binding protein, nuclear factor of activated T-cells c, c-Jun, and β-catenin\textsuperscript{92}. It
is also involved in numerous cellular processes in the brain including cytoskeletal reorganization\textsuperscript{76}, synaptic plasticity\textsuperscript{77}, neuronal survival and apoptosis\textsuperscript{78} mainly through the mitogen-activated protein kinase (MAPK)\textsuperscript{79}, Wnt-1/β-catenin\textsuperscript{80}, and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathways\textsuperscript{7}. Under pathological conditions, over-activation of GSK-3 is also involved in pro-apoptosis of neurons\textsuperscript{7} and dysregulation of the transcription factors important for neurodevelopment\textsuperscript{93}.

GSK-3β is an important kinase that is regulated in stroke pathology. In transient middle cerebral artery occlusion models, GSK-3β expression levels increased following 90 minutes of ischemia and 4 hours of reperfusion\textsuperscript{94}. Selective inhibition of the GSK-3β isoform using pharmacological tools (i.e. TDZD-8, Chir025) led to an increase in p-GSK-3β(Ser9) following cerebral ischemia-reperfusion injury and resulted in reduction of cerebral infarction, oxidative stress and apoptosis, thus suggesting the GSK-3β isoform as a drug target for neuroprotection\textsuperscript{95,96}. Inhibition of GSK-3β is thought to mediate neuroprotection against stroke through several mechanisms where the PI3K/Akt/GSK-3β, MAPK and nuclear factor-kappa B signaling pathways has been most studied\textsuperscript{95-99}. Other conditions that involve targeting GSK-3β as a therapeutic strategy include diabetes, Alzheimer’s disease, Parkinson’s disease, mood disorders, schizophrenia and cancer.

1.9 Rationale and Hypothesis

Based on the current literature, it is known that TRPM2 channels play an important role in several neurodegenerative diseases. Specifically, TRPM2 has been shown to be involved in adult ischemic injury through modulation of NMDA receptor subunit expression. However its effect in neonatal mice remains unclear. It was previously reported that following hypoxic-ischemic injury, there is accumulation of hydrogen peroxide in the neonatal brain at postnatal day 7 that is not present in the postnatal day 42 mouse brain\textsuperscript{5}. Since hydrogen peroxide is a potent activator of TRPM2, it is expected that TRPM2 may play an even greater role in neonatal mice compared to adult mice. In particular, it has been shown in an in vitro model that TRPM2 plays a critical role in neuronal death following activation by hydrogen peroxide\textsuperscript{100}. One downstream target of TRPM2 is the GSK-3β signaling pathway. Deletion of TRPM2 was shown to inhibit GSK-3β activity in adult mice\textsuperscript{75}, but its regulation during development has not been reported. Since
inhibition of GSK-3β activity is crucial during periods of neurodevelopment and neuroprotection following hypoxic-ischemic brain injury, it is important to understand whether TRPM2 activity is involved in regulating this process.

Here, I investigated the role of TRPM2 in neonatal hypoxic-ischemic brain injury in vivo. I hypothesized that inhibition of TRPM2 via TRPM2 knockout will provide neuroprotection against brain damage and improve behavioral outcome in neonatal mice following HI injury. To test this hypothesis, I proposed two objectives: 1. investigate the effect of TRPM2 on brain damage and sensorimotor function following neonatal HI injury and 2. study GSK-3β as a potential signaling pathway involved with TRPM2-mediated neuronal death.
Chapter 2

2 Materials and Methods

In my thesis, I performed experiments using hypoxic-ischemic brain injury on two sets of neonatal mice. The first set of experiments (Chapter 3.1) involved using genetically modified TRPM2 mouse to investigate the effect of TRPM2 on brain damage and sensorimotor function following neonatal HI injury. The second set of experiments (Chapter 3.2) used wild type CD1 mice administered with vehicle or TDZD-8, to investigate GSK-3β as a possible downstream signaling pathway of TRPM2 following HI.

2.1 Development of TRPM2-null mouse

Development of the TRPM2-null mouse was described in Yamamoto et al., 2008\textsuperscript{101}. Briefly, a targeting vector of approximately 200bp containing the neomycin coding region replaced the exon encoding the pore forming subunits of the TRPM2 channel. The resultant mice had a nonfunctional TRPM2 channel with disruption in mRNA expression. It has been reported that these mice do not show any differences in physical appearance, body weight and locomotion from the WT mice.

2.2 Animals and genotyping

All protocols were performed in accordance to the Canadian Council on Animal Care (CCAC guidelines) and all animal treatments were approved by the local Animal Care and Use Program Committee (Office of Research Ethics at the University of Toronto). Postnatal day 7(P7) C57B6J pups [wild type (WT), \textit{TRPM2}^+/-(HET) and \textit{TRPM2}^-/(KO)] were used for TRPM2 experiments. Timed-pregnant CD1 mice were purchased from Charles River Laboratories (Sherbrooke, Quebec, Canada) and were used for TDZD-8 experiments. All animals were housed at an ambient temperature of 23 ± 1°C and a 12-hour light/dark cycle with food and water fed ad libitum. Pups were timed as postnatal day 0, P0, on their day of birth and P7 pups were used for experiments. The genotype of the TRPM2 mice were determined using polymerase chain reaction (PCR) modified from protocol developed by Yamamoto et al., 2008\textsuperscript{101}. Briefly, ear
clippings were taken from the mice and placed in a Proteinase K and Tris-EDTA ear buffer (50mM Tris-HCl/10mM EDTA). Next, ear samples were mixed with phenol: chloroform and TE buffer and DNA was cleaned using 100% ethanol. Following DNA extraction, PCR primers designed by Mori, was used in conjunction with the KAPA HOT star kit for PCR. The final PCR products were ran on a 1% agarose gel mixed with ethidium bromide for 60 minutes and DNA was visualized with ultraviolet light. The PCR primers are shown in Table 1. The band size of the WT, TRPM2 KO is 514bp and 740bp respectively. Pups that exhibited symptoms of illness or disease were excluded from the experiment. Also, body weight was used as a general indicator of health and any pups below 4 grams at P7 were not used.

<table>
<thead>
<tr>
<th>PCR Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTRPM2-13F</td>
<td>CTTGGGTTGCAGTCATATGCAGGC</td>
</tr>
<tr>
<td>PTRPM2-10R</td>
<td>GCCCTCACCATCCGCTTCACGATG</td>
</tr>
<tr>
<td>Pneo-5’a</td>
<td>GCCACACGCACACCTAATATGCG</td>
</tr>
</tbody>
</table>

2.3 Hypoxic-ischemic brain injury model

Hypoxic-ischemic brain injury was induced in (WT, HET and KO) C57B6J pups and WT CD1 at age P7 using a previously described protocol with modifications. Briefly, anesthesia was induced and maintained in P7 pups with 3% and 1.5% inhaled isoflurane in oxygen, respectively. This method of anesthesia is preferred because we can control the amount of isoflurane that is inhaled. The right common carotid artery (CCA) was exposed, separated from surrounding tissue and nerves, and ligated using a bipolar electrocoagulation device (Vetroson V-10 Bi-polar...
electrosurgical unit, Summit Hill Laboratories, Tinton Falls, NJ, USA). Body temperature was maintained during the surgery with a heat blanket. Following the surgery, pups were placed in a recovery cage under a heat lamp for 5 minutes until they regained consciousness and was returned to their home cage for 90 minutes. After the recovery period, they were placed in an airtight hypoxic chamber (A-Chamber A-15274 with ProOx 110 Oxygen Controller/E720 Sensor, Biospherix, NY, USA) fed with humidified 7.5% oxygen in mixture with 92.5% nitrogen gas for 60 minutes. The chamber temperature was maintained at 37°C using a homoeothermic blanket control unit (K-017484 Harvard Apparatus, Massachusetts, USA). After the hypoxia, the pups were recovered under a heat lamp for 5 minutes and were returned to their home cage. Sham animals were anesthetized and their CCA was exposed and separated but no ligation or hypoxia took place. C57B6J pups that underwent this procedure were genotyped after.

2.4 Drug administration

CD1 pups were randomly selected to receive TDZD-8 or vehicle treatment. TDZD-8 (5mg/kg) or vehicle control containing 5% DMSO and 5% Tween-80 (P-8074) in 0.9% saline\(^{102,103}\) was administered to the pups 20 minutes prior to ischemia induction (Table 2). These compounds were administered intraperitoneally (i.p.) with a volume to body weight injection ratio of 20µL/g.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Function</th>
<th>Dose/Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDZD-8 with 5% DMSO and Tween-80 in 0.9% saline</td>
<td>Specific GSK-3â inhibitor</td>
<td>5mg/kg 0.25mg/mL</td>
<td>Gifted from Consejo Superior de Investigaciones Científicas – CSIC</td>
</tr>
</tbody>
</table>
2.5 Behavioral assessments

Behavioral outcomes were assessed 7 days following hypoxic-ischemic brain injury in the TRPM2 mice and drug administered mice and the whole brains were retrieved and assessed for damage. These reflex tests were chosen because they are strong indicators of sensorimotor function following brain damage. Moreover, these are reflexes that are present during early stages of development that are not strain or sex specific. All behavioral assessments were performed in a blinded fashion with the experimenter unaware of the genotypes of the animals.

Geotaxis reflex: This sensorimotor task assessed the animal’s vestibular and proprioceptive function. The mice were placed on a 45° inclined wooden board such that the animal’s head was pointed downward, and the time it took for them to rotate 90° either side was measured. The maximum time scored for each trial assessment was 20 seconds.

Cliff avoidance reflex: This test was used to assess the animal’s proprioception and maladaptive impulse response. Mouse pups were placed on the wooden board such that their front paws hanged over the cliff of the board. The amount of time taken for the mouse to avoid the cliff by turning the body 90° to either side was measured. Similar to the geotaxis reflex, 20 seconds was the maximum time scored for any trial.

2.6 Morphological and histological assessments

TTC staining/Infarct Volume Measurement\textsuperscript{104-106}: Twenty-four hours after HI injury, whole brains were removed from the pups and sectioned coronally into ~1mm slices used for TTC staining. Slices were stained with 1% TTC and placed in a dark incubator maintained at 37°C for 20 minutes.

Whole Brain Imaging/Nissl Staining: Seven days after HI injury, whole brains were removed, fixed, imaged, and sectioned coronally into ~100 µm slices used for Nissl staining (1% cresyl violet).

The corrected infarct volumes for TTC and whole brain imaging were calculated as follows: Corrected infarct volume (%) = (contralateral hemisphere volume – ipsilateral hemisphere +
The hemispheric and infarct volumes for the whole brains were traced and then quantified using the ImageJ software (National institute of Health, Bethesda, MD, USA). For TTC quantification, the hemispheric/infarct areas of each brain slice was traced and quantified using the same software. Assuming a thickness ~1mm for each slice, the areas were then summed up to find the respective volumes.

### 2.7 Western blot

Western blot procedures were performed as previously described\textsuperscript{104-106}. Twenty-four hours after HI, the ipsilateral hemispheres of the mouse brain was removed and frozen in dry ice. To study the developmental expression of GSK-3β, protein was extracted from cortex. The brain samples were homogenized in RIPA buffer with a cocktail of proteinase and phosphatase inhibitors, then incubated at 4°C for 1h and centrifuged for 15 min at 13000rpm. The protein concentrations were measured using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Samples of the mouse brain (30 μg) were separated on a 10% SDS-PAGE gel that was transferred to a nitrocellulose membrane (350 mA, 90 minutes). Blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS), and incubated with primary and secondary antibodies at 4°C overnight and room temperature, respectively [anti-cleaved caspase-3 (#9664S, 1:1000); anti-phospho-Akt (#9271S, Ser473, 1:1000); anti-Akt (#9272S, 1:1000); anti-phospho-GSK-3β (#9323S, Ser9, 1:1000); anti-GSK-3β (#9832S, 1:1000); anti-GAPDH (#2118S, 1:10,000)]. Protein signals of interest were visualized using enhanced chemiluminescent reagents (PerkinElmer, Mass, USA) and analyzed by exposure to film (HyBlot CL, NJ, USA).

### 2.8 Immunohistochemistry and confocal imaging

Brain samples were collected 7 days after HI at P14 and fixed in 4% paraformaldehyde/30% sucrose solution at 4°C overnight. Brains were coronally sectioned into ~50μm slices using a vibratome (Tissue Sectioning System Microtome Vibratome, HuiYou, China) and immunohistochemically stained as previously described\textsuperscript{106}. In brief, samples were probed with mouse anti-NeuN antibody(MAB377, 1:500, Chemicon, CA, USA), anti-GFAP
(ab7260, 1:1000, Abcam, USA), anti-caspase-3 (#9661, 1:200, Cell Signaling Technology, Mass, USA) antibodies overnight, and labeled with DAPI (1 µg/mL, Cell Signaling Technology, Mass, USA). Next, the sections were incubated with secondary antibodies Alexa 488 and 568 (#835724, #632115, 1:200, Cell Signaling Technology, Mass, USA) for 1h at room temperature and mounted on glass coverslips. Confocal laser scanning microscope (LSM700 Zeiss, Germany) was used to image the immunolabeled brain slices.

2.9 In situ hybridization and 3D imaging

In situ hybridization (ISH) data was collected from Allen Brain Atlas-Developing Mouse Brain website (http://developingmouse.brain-map.org/). Data was taken from C57BL/6J mice at developmental time points: E18.5, P4, P14 and P28. 3D imaging was performed using the Allen Developing Mouse Brain Atlas- Brain Explorer 2 software. Gene expression data measured as intensity level was plotted as a series of grid points for each brain slices super imposed on each other.

2.10 Data analysis

All statistical analysis was performed using the statistical software SigmaPlot (Systat Software, San Jose, CA). Data are presented as mean ± SEM. Student’s t-test was used to determine the statistical difference between two groups. Multiple group statistical analysis was performed using one-way ANOVA test followed by Fisher LSD Method for multiple pair wise comparisons. A p-value of less than 0.05 (p < 0.05) was considered statistically significant. All experiments were conducted in a blinded manner; experimenters did not know the treatment conditions. All experiments were repeated at least three times.
Chapter 3

3 Results

Neonatal hypoxic-ischemic injury model was performed on seven day old CD1 mice. The maturity of the mouse brain at this age resembles that of a human fetus at a gestation period of around 32-34 weeks, an early risk period for neonatal stroke.\textsuperscript{108,109} Subsequent experimental procedures and timeline of experiments are shown in Figure 7A.

![Figure 7A](image)

Figure 7A. Timeline of neonatal hypoxic-ischemic injury and experimental procedures. A. Seven day old pups (P7) were randomly selected to be injected with either TDZD-8 or vehicle solution 20 minutes prior to ischemia induction. This is followed by 90 minutes of recovery and 60 minutes of hypoxia with 7.5% O\textsubscript{2} and 92.5% N\textsubscript{2}. TTC staining was performed 24 hours after HI (P8). Neurobehavioral assessment, whole brain imaging, Nissl staining and immunohistochemistry was performed 7 days after the HI (P14). Sham animals did not receive injections and did not undergo HI.
3.1 The role of TRPM2 in neonatal HI brain injury

3.1.1 Knock-out of TRPM2 reduces brain infarct volume 24 hours following hypoxic-ischemic brain injury

We first investigated the brain infarct volumes in the WT, $TPRM2^{+/-}$ (HET) and $TPRM2^{-/-}$ (KO) groups 24 hours following HI injury. Experimenters did not know the genotype of the mice during the time of the surgery. Brain samples were coronally sectioned into 4 slices with ~1mm thickness and stained with TTC, an indicator of metabolic activity. The white area is the infarct area and it represents the damaged tissue which is not metabolically active (Figure 8A.). We found that the $TPRM2^{+/-}$ (HET) (36.92 ± 4.06%, $n=14$, *p < 0.05) and $TPRM2^{-/-}$ (KO) (26.67 ± 4.53%, $n=9$, *p < 0.05) mice had significantly reduced infarct volumes compared to the WT (53.60 ± 9.02%, $n=9$, *p < 0.05) mice (Figure 8B). There were no significant differences found between the $TPRM2^{+/-}$ (HET) and $TPRM2^{-/-}$ (KO) mice. Thus, this suggests that the absence of TRPM2 may have a neuroprotective effect against HI injury.
Figure 8. *TRPM2*<sup>+/−</sup>(HET) and *TRPM2*<sup>−/−</sup>(KO) mice had reduced brain infarct volume following HI injury.

Brains were collected 24 hours after HI and coronally sliced into 1mm sections for TTC staining. A. Representative TTC images for WT, *TRPM2*<sup>+/−</sup>(HET) and *TRPM2*<sup>−/−</sup>(KO) groups. White area represented the brain infarct area. B. Measurement of corrected infarct volume derived from the four infarct areas calculated in each sample. The WT group had significantly higher brain infarct volume compared to the *TRPM2*<sup>+/−</sup>(HET) and *TRPM2*<sup>−/−</sup>(KO) group (*p < 0.01 versus *TRPM2*<sup>+/−</sup>(HET) group; *p < 0.05 versus *TRPM2*<sup>−/−</sup>(KO) group, One-way ANOVA followed by Fisher LSD Method); the number of animals tested are shown on the bar graph.
3.1.2 Brain damage is reduced in $TRPM2^{+/\text{-}}$ (HET) and $TRPM2^{-/-}$ (KO) mice 7 days following hypoxic-ischemic brain injury

Next, we explored whether this neuroprotective effect lasted 7 days after HI injury. Whole brains were fixed 7 days after HI injury and imaged for morphological assessment. Samples from the sham group were undamaged and were used as a comparison for the WT, $TRPM2^{+/\text{-}}$ (HET) and $TRPM2^{-/-}$ (KO) brains. At 7 days after HI, the brain infarct has already undergone liquefactive necrosis resulting in the loss of brain weight due to liquidated brain mass (Figure 9A). Brain weight was used as indicator of the liquefactive infarct volume and was measured in all groups. We found that WT brains subjected to HI had a significantly lower brain weight compared to the $TRPM2^{+/\text{-}}$ (HET) and $TRPM2^{-/-}$ (KO) brains subjected to HI; sham brains had the highest brain weight of all groups (Figure 9B). This suggests that $TRPM2^{+/\text{-}}$ (HET) and $TRPM2^{-/-}$ (KO) reduced the liquefactive necrosis due to HI injury and maintained brain weight. Consistent with this data, morphological analysis of the brain infarcts in the whole brain images show that the WT mice have a significantly higher brain infarct volume compared to the $TRPM2^{+/\text{-}}$ (HET) and $TRPM2^{-/-}$ (KO) mice (Figure 9C). Whole brains were coronally sliced into ~100μm sections for Nissl (cresyl violet) staining and we found that $TRPM2^{+/\text{-}}$ (HET) and $TRPM2^{-/-}$ (KO) mice sustained less damage compared with the WT mice (Figure 9A).
Figure 9. TRPM2^{+/-}(HET) and TRPM2^{-/-}(KO) mice had reduced brain damage and maintained brain weights 7 days following HI injury

Brains samples were fixed, imaged and coronally sliced into 100μm sections for Nissl staining A. Representative whole brain images and Nissl staining for Sham, WT, TRPM2^{+/-}(HET) and TRPM2^{-/-}(KO) groups. B. Measurement of brain weight 7 days after HI. The WT group had significantly lower brain weight compared to the sham, TRPM2^{+/-}(HET) and TRPM2^{-/-}(KO) group C. Analysis of brain damage for whole brain images. TRPM2^{+/-}(HET) and TRPM2^{-/-}(KO) group had significantly lower long-term brain damage compared to WT group. (*p < 0.01 versus sham group; *p < 0.01 versus TRPM2^{+/-}(HET) group; *p < 0.01 versus TRPM2^{-/-}(KO) group , One-way ANOVA followed by Fisher LSD Method)
3.1.3 Sensorimotor function is improved in $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice 7 days after hypoxic-ischemic brain injury

To determine the functional significance of the neuroprotective effects from $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice, we used the cliff avoidance test and geotaxis reflex test to assess neurobehavioral outcome. These tests are suitable for our study because they are reflexes that are present during early stages of development and are a strong indicator of sensorimotor function. The mice were tested 7 days after HI injury and the sham group was used as a comparison for the WT, $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice. In the cliff avoidance test, the $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice had a significantly lower latency to complete the task compared to the WT mice suggesting an improved reflex to impulse behavior (Figure 10A). Consistent with the results of the cliff avoidance test, the geotaxis reflex assay also revealed that the $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice had improved reflex scores compared to the WT mice (Figure 10B). These results suggest that $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice have improved vestibular and proprioceptive function.
Figure 10. Sensorimotor function was improved in $TRPM2^{+/−}$ (HET) and $TRPM2^{-/-}$ (KO) mice assessed 7 days after HI injury.

The cliff avoidance and geotaxis reflex was used to measure vestibular and proprioceptive ability of the mice. A. Latency for the mice to complete the cliff avoidance test. B. Latency for the mice to complete the geotaxis reflex test. In both behavioral assessments, wild type took significantly longer to complete the sensorimotor task compared to the sham and $TRPM2^{+/−}$ (HET) group. (*p < 0.05 versus sham; *p < 0.05 versus $TRPM2^{+/−}$ (HET) group, One-way ANOVA followed by Fisher LSD Method)
3.1.4 p-Akt/t-Akt and p-GSK3β/t- GSK3β ratio is elevated in TRPM2+/−(HET) and TRPM2−/−(KO) mice following hypoxic-ischemic brain injury but not in sham treatment

To study the downstream signaling pathways of TRPM2 in normal and pathophysiological conditions, we measured the protein expression levels of p-Akt/t-Akt and p-GSK3β/t- GSK3β in sham and HI groups. Phosphorylated Akt is the active form of the kinase and it is known to be involved in pro-survival signaling pathways. In the sham group, there is no change in p-Akt/t-Akt levels between the WT, TRPM2+/−(HET) and TRPM2−/−(KO) mice; however, after HI, there is a significant increase of p-Akt/t-Akt levels in the TRPM2+/−(HET) and TRPM2−/−(KO) mice compared to the WT mice (Figure 11A, C). This suggests that TRPM2 may not regulate Akt in normal conditions but will suppress cell survival pathways after HI. GSK3β is an important regulator of neuronal development and it is known to be involved in neuronal cell death following HI. Here we report that TRPM2+/−(HET) and TRPM2−/−(KO) rescued the reduction of p-GSK3β/t- GSK3β levels shown in the WT group following HI suggesting that it could elicit neuroprotective effects through the Akt/ GSK3β signaling pathway (Figure 11A, B). There were no differences in p-GSK3β/t- GSK3β levels between the WT, TRPM2+/− (HET) and TRPM2−/− (KO) groups in sham conditions. This finding is seemingly inconsistent from another study that showed TRPM2 is a regulator of GSK3β in adult mice. However, it is notable that protein expression of GSK3β is elevated in neonatal mice which could mask any regulatory effects by TRPM2.
Figure 11. Expression ratios of p-Akt/t-Akt and p-GSK3β/t-GSK3β were increased in HI TRPM2+/- (HET) and TRPM2-/- (KO) mice but not in sham groups. A. Representative western blot for WT, TRPM2+/- (HET) and TRPM2-/- (KO) groups following sham and HI conditions. B. p-Akt/t-Akt ratio levels following sham or HI treatment in WT, TRPM2+/- (HET) and TRPM2-/- (KO) groups. C. p-GSK3β/t-GSK3β ratio levels following sham or HI treatment in WT, TRPM2+/- (HET) and TRPM2-/- (KO) groups. (*p < 0.05 versus TRPM2+/- (HET) group; *p < 0.05 versus TRPM2-/- (KO) group, One-way ANOVA followed by Fisher LSD Method). The numbers of animals tested are shown on the bar graph.
3.2 The role of GSK-3β in neonatal HI brain injury

3.2.1 Developmental expression of GSK-3β and Akt

To understand the role of GSK-3β in neuronal death following hypoxic-ischemic injury in neonatal mice, we studied the physiological gene expression of the kinase in development. In situ hybridization data obtained from Allen Brain Atlas: Developing Mouse Brain107 revealed that GSK-3β gene expression is localized in the mouse cortex and hippocampus during early and late stages of brain development measured at E18.5, P4, P14 and P28 (Figure 12A). Expectedly, the cortex and hippocampus are brain areas most vulnerable to hypoxic-ischemic injury in rodents110. This indicates that GSK-3β plays an important role in cell death following stroke in the cortex and hippocampus for adult and neonatal mice. While gene expression of GSK-3β was similar in E18.5, P14 and P28 times points, highest intensity levels were seen at P4 (Figure 12A). This supports the notion that GSK-3β plays an important role in neurodevelopment following birth which gradually declines during old age.

Next, we studied the protein expression levels of total GSK-3β at E17, P7 and P14 time points (Figure 13B-D). The level of phosphorylated GSK-3β (p-GSK-3β), the inactive form of the protein, was labeled with an antibody binding to the Serine 9. As shown in (Figure 13B), the p-GSK-3β level was significantly higher at E17 (1.41 ± 0.10) and P7 (1.65 ± 0.40) than at P14 (0.64 ± 0.23). We found that total GSK-3β protein levels was highest at P7 (1.36 ± 0.14) compared to E17 (1.07 ± 0.02) and P14 (1.02 ± 0.03) time points (Figure 13C); there was no significant difference between E17 and P14. The p-GSK-3β to t-GSK-3β ratio was compatible between E17 (1.32 ± 0.07) and P7 (1.16 ± 0.20) animals, but decreased significantly in P14 (0.61 ± 0.20) (Figure 13D), indicating that GSK-3β activity was low from prenatal to postnatal day 7 but increased on the 14 days following birth. We also measured the changes in Akt expression levels at these time points (Figure 13E-G). The p-Akt to t-Akt ratio was significantly higher at age P7 (0.94 ± 0.07) compared to E17 (0.61 ± 0.09) and P14 (0.51 ± 0.13) (p < 0.05; n = 5). The p-Akt to t-Akt ratios between E17 and P14 were compatible (Figure 13G).
**Figure 12. Developmental gene changes in GSK-3β levels**

A. 3D gene expression data of GSK-3β in coronal and sagittal slice in C57BL mice was determined at E18.5, P4, P14 and P28, using in situ hybridization. Images were obtained from Website: © 2015 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org.
Figure 13. Developmental protein changes in GSK-3β and Akt levels

A. Western blots showing protein levels of GSK-3β and Akt at E17, P7 and P14. Analysis of band intensity showing the ratio of B. p-GSK3β to GAPDH, C. t-GSK-3β to GAPDH, D. p-GSK3β to t-GSK3β, E. p-Akt to GAPDH, F. tAkt to GAPDH and G. p-Akt to t-Akt. (*p<0.05 versus sham group or TDZD-8 treatment group; One-way ANOVA followed by Fisher LSD Method). The numbers of samples used are shown on the bar graph.
3.2.2 TDZD-8 as a pharmacological inhibitor of GSK-3β

TDZD-8 is the first non-ATP competitive inhibitor developed that is specific for GSK-3β\textsuperscript{111}. Although the effect of TDZD-8 on the splice variants of GSK-3β have not been fully investigated, TDZD-8 has been proposed to bind residues, Lys205, Tyr216 and Arg96, (IC\textsubscript{50} of 2μM) that are required for stabilizing the active conformation of GSK-3β\textsuperscript{111}. These residues are present in both variants, GSK-3β\textsubscript{1} and GSK-3β\textsubscript{2}, thus TDZD-8 may inhibit both forms of GSK-3β in the brain. TDZD-8 has been previously reported to possess neuroprotective, anti-inflammatory and antioxidative effects\textsuperscript{112-114}. While it has been show that TDZD-8 reduced brain injury following cerebral ischemic injury in adult mice\textsuperscript{96}, its effect in neonatal mice remains unclear. As part of our second aim, we pre-treated postnatal day 7 CD1 mice with TDZD-8 and tested in our neonatal hypoxic-ischemic brain injury model. The chemical structure of the compound is shown in Figure 14.

![Chemical structure of TDZD-8.](image)

Figure 14. Chemical structure of TDZD-8.
Non-ATP competitive inhibitor specific for GSK-3β
3.2.3 TDZD-8 pre-treatment attenuates infarct volume following hypoxic-ischemic brain injury

We investigated if TDZD-8 can reduce brain damage using a hypoxic-ischemic brain injury model in neonatal mice. All experiments were conducted in a blinded manner. All pups used for HI were subjected to the same hypoxic conditions (7.5% O2 for 60 minutes). Twenty-four hours after HI, brains from the pups were coronally sectioned and stained with TTC; the brain damage is shown in white (Figure 15A). The infarct volume for the vehicle-treated HI group was 44.76 ± 3.11% (n=9); this was significantly reduced in the TDZD-8-treated HI group (33.79 ± 2.90%, n=10, *p < 0.05) (Figure 15B). These results indicate that TDZD-8 pre-treatment attenuates brain damage induced by HI in neonatal mice.

Whole brains were imaged, and then sectioned and stained with cresyl violet 7 days after HI to reveal morphological changes. While the sham group had fully intact brains, the vehicle-treated HI brains had a considerable portion of the brain missing indicating severe brain injury from HI. Brains from the TDZD-8-treated group were less morphological damaged compared to that of the vehicle-treated brains (Figure 16A). Subsequent staining with cresyl violet showed that the TDZD-8-treated group sustained less brain damage compared to the vehicle-treated HI group (Figure 16A). Analysis of the liquefaction corrected infarction volumes in the whole brains revealed that hypoxic-ischemic injury induced long-term brain damage in the vehicle-treated groups (53.25 ± 5.10%, n = 18) that was significantly attenuated in the TDZD-8-treated group (36.13 ± 3.84%, n = 20; *p < 0.01) (Figure 16B). These results demonstrated that TDZD-8 pre-treatment reduces long-term brain damage induced by HI brain injury in neonatal mice.
Figure 15. TDZD-8 pre-treatment reduced infarct volume 24 hours following neonatal hypoxic-ischemic brain injury

A. Effect of TDZD-8 pre-treatment on infarct volume. Representative TTC images of coronal brain slices in 24 hours after HI injury show brain damage areas in white color. B. Summary of brain infarction volume of TTC images. Pre-treatment with TDZD-8 significantly reduced the infarct volume compared to the vehicle-treated group (*p < 0.05, Student t test); the number of animals tested are shown on the bar graph.
Figure 16. TDZD-8 pre-treatment reduced infarct volume 7 days following neonatal hypoxic-ischemic brain injury

A. Long-term effect of TDZD-8 on brain damage. Representative whole brain images and Nissl staining taken 7 days after HI injury are shown. B. Analysis of brain damage for whole brain images. Hypoxic-ischemic injury induced significant long-term brain damage in the vehicle-treated group compared to the sham group. Pre-treatment with TDZD-8 significantly reduced the infarct volume and improved whole brain morphology compared to the vehicle-treated group (*p < 0.01 versus sham group or vehicle group; One-way ANOVA followed by Fisher LSD Method); the number of animals tested are shown on the bar graph.
3.2.4 TDZD-8 improves neurobehavioral outcome following hypoxic-ischemic brain injury

We next asked whether the reduction in brain damage observed in the TDZD-8-treated HI group can lead to improved long-term sensorimotor function in these mice. Seven days after HI, we assessed the neurobehavioral function of these mice using the cliff avoidance test and geotaxis reflex.

Cliff avoidance test: The vehicle control group (7 days: 6.14s ± 1.63 s) displayed a significantly longer latency to complete the test in comparison to the sham group (7 days: 2.03s ± 0.15 s). TDZD-8 pre-treatment (7 days: 3.02s ± 0.45 s) significantly reduced the latency to complete the test in comparison to the vehicle group (Figure 17A, *p < 0.05). These results indicate that TDZD-8 improves the long-term locomotion and reduces maladaptive behavior response of the mice following HI.

Geotaxis reflex test: Mice in the vehicle-treated HI group (7 days: 5.80s ± 1.96s) took significantly longer to complete the geotaxis test in comparison to the sham group (7 days: 2.26s ± 0.26s). Mice that were pre-treated with TDZD-8 (7 days: 2.02 ± 0.14s) had significantly improved geotaxis test scores compared to the vehicle-treated group (Figure 17B, *p < 0.05). This shows that TDZD-8 improves long-term balance and proprioception in these mice following HI.
Figure 17. TDZD-8 pre-treatment improved neurobehavioral outcome assessed at 7 days following neonatal hypoxic-ischemic brain injury

A. Cliff avoidance test. B. Geotaxis Reflex test. TDZD-8 pre-treatment significantly improved cliff avoidance and geotaxis test scores in comparison to the vehicle control group but had no difference compared to the sham group (*p < 0.05 versus sham group or vehicle group; One-way ANOVA followed by Fisher LSD Method); the number of animals tested are shown on the bar graph.
3.2.5 TDZD-8 pre-treatment increases Serine-9 phosphorylated GSK-3β (p-GSK-3β) and phosphorylated Akt (p-Akt) levels after hypoxic-ischemic brain injury

We next asked whether TDZD-8 treatment would affect the level of p-GSK-3β after the HI injury. Phosphorylated GSK-3β at the Ser9 site is the inactivated form of GSK-3β which is up-regulated by p-Akt. We found that the ratio of p-GSK-3β/GSK-3β in our Western blot was significantly increased in the TDZD-8-treated HI group (0.69 ± 0.02) compared to the vehicle-treated HI group (0.36 ± 0.13, p<0.05, Figure 18A, B). This finding is consistent with another study that used TDZD-8 in an adult model of cerebral ischemia96, and supports the idea that TDZD-8 mediates GSK-3β inactivation via Ser9 phosphorylation in addition to its direct binding to the enzyme. To investigate the potential signaling pathways of TDZD-8 mediated neuroprotection, we studied the involvement of p-Akt in the p-Akt/GSK-3β pathway following HI injury. It is well-established that p-Akt is an important marker for cell survival that is transiently suppressed following HI115. Our Western blot results show that p-Akt/t-Akt expression ratio was significantly increased when pre-treated with TDZD-8 (0.85 ± 0.11) compared to the vehicle-treated group (0.47 ± 0.12, p<0.05, Figure 18C). This finding supports the idea that TDZD-8 promotes pro-survival signaling by either directly or indirectly activating p-Akt following the HI injury.
Figure 18. Western blot showing the effects of TDZD-8 on the expression levels of cell survival and apoptotic markers following HI

Samples were collected 24 hours after HI from the ipsilateral brain hemisphere. A. Representative Western blot gel showing protein expression levels from the sham, vehicle-treated HI and TDZD-8-treated HI groups. Analysis of band intensity showing the ratio of B. p-GSK-3β to t-GSK-3β, C. p-Akt to t-Akt or D. cleaved caspase-3 to GAPDH. (*p<0.05 versus sham group or TDZD-8 treatment group; One-way ANOVA followed by Fisher LSD Method). The number of samples tested are shown on the bar graphs.
3.2.6 TDZD-8 pre-treatment suppresses apoptotic signaling after hypoxic-ischemic brain injury

We have previously shown that mitochondrial-mediated apoptosis is one of the main mechanisms for cell death following neonatal hypoxic-ischemic brain injury\textsuperscript{104-106}. Here we asked if TDZD-8 could rescue neuronal cells from apoptosis by first measuring the expression levels of cleaved caspase-3. Cleaved caspase-3 is a recognized marker for apoptosis that is known to be up-regulated by upstream activation of GSK-3β. Our results show that the expression levels of cleaved caspase-3 levels were significantly reduced in the TDZD-8 treatment group (0.01 ± .01) compared to the vehicle-treated group (0.68 ± 0.34, p*<0.05, Figure 18D). Consistent with our Western blot data, immunohistochemistry (IHC) results show that there was a significant decrease in DAPI-positive cells containing cleaved caspase-3 marker in the ipsilateral hemisphere of the TDZD-8 treatment group (39.43 ± 3.93) compared to the ipsilateral hemisphere of the vehicle-treated group(68.00 ± 7.13); the sham group had the lowest levels of capase-3 (Figure 19A, B).
Figure 19. Immunohistochemistry staining showing the effects of TDZD-8 on cleaved caspase-3 staining following hypoxic-ischemic brain injury

Brain samples were collected 7 days after HI and sliced coronally with a thickness of ~50µm. A. Representative images for cleaved caspase-3 and DAPI staining in sham, vehicle and TDZD-8 groups following HI. B. Analysis of cleaved caspase-3 count in DAPI positive cells. (p<0.05 for all comparisons; One-way ANOVA followed by Fisher LSD Method). The numbers of samples used are shown on the bar graph.
3.2.7 TDZD-8 reduces GFAP-positive cells and maintains NeuN-positive cells following hypoxic-ischemic brain injury

To investigate the non-neuronal cell involvement in hypoxia-ischemia, we stained for GFAP-positive cells in brains 7 days following HI. GFAP is a marker for astrogliosis (reactive astrocyte), which is an indicator of glial scar formation and astrocyte hypertrophy following HI\textsuperscript{116-120}. Our IHC results show that there was a significant reduction in GFAP-positive cells in the ipsilateral hemisphere of the TDZD-8 treatment group (15.80 ± 3.34%) in comparison to the ipsilateral hemisphere of the vehicle-treated group (39.80 ± 4.00%); the contralateral hemisphere and sham brains had the lowest number of GFAP-positive cells (Figure 20A, C). In conjunction with a lower number of GFAP-positive cells, there were significantly more NeuN-positive cells in the TDZD-8 treatment group (58.63 ± 6.12%) compared to the vehicle-treated group (31.30 ± 4.25%, Figure 20A, B). This indicates that fewer neurons were lost to injury in the TDZD-8 treated group as compared to that of the vehicle-treated group. These findings support the notion that TDZD-8 enhanced a number of surviving neurons following HI injury which may correlate with improved functional outcomes.
Figure 20. Immunohistochemistry staining showing the effects of TDZD-8 on NeuN-positive cells and GFAP-positive cells

Brain samples were collected 7 days after HI and sliced into coronal sections with a thickness of ~50µm. A. Representative images for NeuN and GFAP staining. B. Analysis of NeuN-positive cells as a percentage of DAPI-positive cells in sham, vehicle and treatment groups. C. Analysis of GFAP-positive cells as a percentage of DAPI-positive cells. (p<0.05 for all comparisons; One-way ANOVA followed by Fisher LSD Method). The numbers of samples used are shown on the bar graph.
4 Discussion

4.1 Knock-out of TRPM2 reduces neonatal HI brain injury

In Chapter 3.1, we used a genetically modified mouse to investigate the role of TRPM2 in neonatal hypoxic-ischemic brain injury. Here we report that, knock-out of TRPM2 is neuroprotective against hypoxic-ischemic injury in neonates based on the findings that TRPM2 HET and KO mice 1) had reduced cerebral infarct volumes at 24 hours and 7 days compared to the WT mice following HI injury; 2) showed improved neurobehavioral outcome assessed 7 days after injury in vivo; and 3) increased p-GSK-3β/t- GSK-3β and p-Akt/t-Akt ratio after HI but not in the sham group. These findings suggest that deletion of TRPM2 proteins provides neuroprotection against neonatal hypoxic-ischemic brain injury through modulation of GSK-3β and Akt activity ultimately promoting pro-survival signaling and suppressing pro-death signaling.

4.1.1 TRPM2 HET/KO mice up-regulates Akt/Akt and down-regulates GSK3β activity following HI

Glycogen synthase kinase 3β (GSK-3β) is a serine threonine kinase involved neurodevelopment and apoptotic signaling. It was previously reported that TRPM2 activation regulates GSK-3β activity. Specifically, application of a TRPM2 activator, H$_2$O$_2$, significantly decreased the levels of phosphorylated (Ser9) GSK-3β, inactive form of GSK-3β, while co-application of H$_2$O$_2$ with a TRPM2 antagonist, econazol or clotrimazol, reversed these effects on GSK-3β. In our results, we found there were high p-GSK-3β levels in the TRPM2$^{+/−}$ and TRPM2$^{-/-}$ (KO) mice following HI, but no observable changes were seen in TRPM2 groups following sham. The differences between the modulatory effects of GSK-3β activity by TRPM2 could be addressed by the age of the mice used for experiments. In Jang et al., 2015, they used 8-12 week old mice whereas in our study we used 7 day old pups. Based only on our results in the sham group, we
cannot rule out the possibility that that TRPM2 is not a regulator of GSK-3β in neonates during development. It is well-known that GSK-3β is highly expressed in the neonatal stage and gradually declines during development\textsuperscript{92}. So, it is possible that the level of GSK-3β is high enough in our neonatal mice that it masks any effect that TRPM2 has on the protein. On the other hand, p-GSK-3β levels showed changes in the TRPM2-deficient mice after HI. One possible explanation of this is the fact that p-GSK-3β is very high during the early stages and since HI causes a drop in GSK-3β expression levels, the regulatory effects of TRPM2 could be seen more evidently. The other explanation of this effect is that hypoxic-ischemic brain injury activates another molecule that facilitates the interaction between TRPM2 and GSK-3β. One such molecule could be calcineurin\textsuperscript{75}. Future experiments should be performed to understand the interaction between TRPM2, calcineurin and GSK-3β following HI.

4.2 Pharmacologically inhibiting GSK-3β activity is neuroprotective against HI injury

In chapter 3.2, we used a specific GSK-3β inhibitor, TDZD-8, to test the role of GSK-3β in neonatal hypoxic-ischemic brain injury. We showed that the levels of GSK-3β mRNA and total protein were significantly higher in P7 pups than at E17 and 14 days; the ratio of p-GSK-3β/t-GSK-3β reduced significantly at P14 compared to E17 and P7. We also demonstrated that treatment with TDZD-8 1) reduced cerebral infarct volume following the HI injury; 2) improved long-term neurobehavioral recovery outcomes after the HI injury \textit{in vivo}; and increased the p-GSK-3β; 3) reduced caspase-3 proteins and increased pro-survival molecule p-Akt; and 4) reduced HI-induced up-regulation of GFAP-positive cells and improved neuronal survival. These findings suggest that TDZD-8 elicits neuroprotection and neurobehavioral recovery \textit{in vivo} by restoring p-GSK-3β and p-Akt expression levels, thus suppressing apoptotic signaling through the Akt/GSK-3β/caspase-3 pathway after HI injury. In addition, our results indicate that TDZD-8 may reduce HI-activated astrocytes, a marker for neurological injury\textsuperscript{119}. 
4.2.1 Changes in Akt activity and GSK3β activity after HI is developmentally regulated

During development, the p-Akt/t-Akt and p-GSK-3β/t-GSK-3β levels are high during age P7, which corresponds to a high Akt and low GSK-3β activity (Figure 13A-G). Following HI, the p-Akt/t-Akt and p-GSK-3β/t-GSK-3β levels decreased significantly in our control group, which indicates that these molecules play an important role in the pathology of HI. The reduction of p-Akt and p-GSK-3β levels was found to cause an increase in cleaved caspase-3 levels indicating the involvement of apoptotic signaling. Taken together, we propose that pre-treatment with TDZD-8 elicited neuroprotection through restoring p-Akt/t-Akt and p-GSK-3β/t-GSK-3β levels that leads to suppression of cleaved caspase-3 mediated apoptotic signaling. Akt signaling is an upstream modulator of GSK-3β. We found that pro-survival Akt signaling was increased by TDZD-8, suggesting that Akt levels might be regulated via a feedback system mediated by GSK-3β signaling.

4.2.2 GSK-3β activity following HI is involved in apoptotic signaling

Neuronal death following HI injury is closely tied to the apoptosis pathway. In particular, caspase-3 has been a prominent marker of apoptosis that has been up-regulated following neonatal HI where inhibition of caspase-3 resulted in neuroprotection against hypoxic ischemia in neonatal mice. Previous studies have shown that phosphorylation of Akt (p-Akt) inhibited GSK-3β leading to the suppression of caspase-3 mediated apoptotic signaling; this suggests a possible link between GSK-3β regulation and cell death in our model. Specifically, upstream activation of GSK-3β after hypoxic-ischemic injury leads to decreased Bcl-2 expression, an anti-apoptotic protein, which in turn activates caspase-3. Caspase-3 is a member of the cysteine aspartyl-specific protease family involved in the programmed cell death that plays a key role in neuronal apoptosis and delayed neuronal death following traumatic brain injury in stroke for adult and neonatal mice. In this study, we found that cleaved caspase-3 expression levels were very low in the treatment group compared with the cleaved caspase-3
containing cells in our immunohistochemistry data (19A, B). The differences seen in cleaved caspase-3 levels could be attributed to the differences in the age of the brain samples, timing of caspase-3 expression and the pharmacological half-life of TDZD-8. Specifically, the brain samples used for Western blot analysis were post 1 day following HI whereas the brain samples used for immunohistochemistry were post 7 days following HI. While caspase-3 levels are present during both the early and late phases of ischemia, caspase-3 levels are predominately expressed from 72 hours to 7 days after ischemia injury\textsuperscript{126}. Moreover, TDZD-8 has a half-life of 120 minutes\textsuperscript{127}, thus it is likely that TDZD-8 exerted a greater effect during early onset of injury (24 hours) compared to a later stage (7days). This supports the idea that during early stages of ischemia, TDZD-8-mediated phosphorylation of GSK-3β leads to decrease of caspase-3 expression seen in our Western blot. However, during later stages of ischemia, it is possible that p-GSK-3β is dephosphorylated as the effect of TDZD-8 becomes diminished resulting in a relative increase in caspase-3 levels seen in the immunohistochemistry data.

4.2.3 The involvement of astrocytes following HI

Hypoxic-ischemic injury has also been known to affect astrocytes. Under physiological conditions, astrocytes are traditionally known for providing a host of supportive features for neurons including structural integrity and metabolic support as well as central nervous system repair following injury. However, HI injury can compromise the normal function of astrocytes leading to detrimental secondary effects on neuronal cells. Specially, pathologically activated astrocytes (reactive astrocytes) have been shown to mediate the inflammatory response\textsuperscript{128-130} and express caspase-3 apoptotic markers\textsuperscript{131} leading to secondary cerebral damage. In this study, we used GFAP, an indicator for reactive astrogliosis, as a marker for neurological damage following neonatal HI injury. Since GFAP is not a sensitive marker for healthy astrocytes and is required for the progression into reactive astrocytes, it is a reliable method to detect reactive astrocytes\textsuperscript{132,133}. Reactive astrogliosis can be categorized into three groups according to the severity of brain damage including: mild to moderate, severe diffuse and severe reactive astrogliosis with compact glial scar formation\textsuperscript{132}. In Figure 20A, it can be seen that the vehicle group had severe reactive astrogliosis with compact glial scar formation surrounding overlapping and hypertrophy astrocytes. In the TDZD-8-treated group, reactive astrocytes were less
proliferated and shared less overlapping regions. Further studies needs to address the link between GSK-3β and reactive astrogliosis following HI injury.

4.3 Proposed model

The Akt/GSK-3β/Caspase-3 pathway is a well-established pathway involved in apoptosis\textsuperscript{123,134}. Phosphorylated Akt levels also followed the same trend as GSK-3β suggesting that TRPM2 could also directly or indirectly regulate pro-survival signaling. In our study we found that, TRPM2-deficient mice up-regulated pro-survival signaling through Akt activation and GSK-3β inhibition. GSK-3β has been strongly implicated in apoptotic signaling, and it was required for Bax translocation which subsequently causes release of cytochrome c that activates caspase 3 ultimately resulting in cell death\textsuperscript{121}. Therefore, TRPM2-deficient mice can cause suppression of apoptotic signaling involving regulation of the Akt/GSK-3β/caspase-3 pathway. A representative pathway of the proposed model is shown in Figure 21.
Figure 21. Proposed mechanism for TRPM2 and TDZD-8 regulation of GSK-3β after hypoxic-ischemic brain injury

Hypoxic-ischemic injury inhibits p-Akt and up-regulates GSK-3β leading to activation of the mitochondrial apoptotic pathway mediated by caspase-3. This leads to cellular apoptosis following HI injury. TDZD-8 is non-ATP competitive inhibitor specific for GSK-3β that up-regulates p-Akt. This leads to the down-regulation of caspase-3. Dotted line indicates the neuroprotective pathway of TDZD-8
4.4 Future Directions

In my thesis I reported that knockout of TRPM2 is neuroprotective against brain damage and improve behavioral outcome in neonatal mice following HI injury; this effect is mediated at least in part by GSK-3β signaling. I performed two separate studies that individually support these conclusions; however some questions remain to be answered. Here I propose two directions to extend my thesis work.

First, it is important to understand the interaction between TRPM2 and GSK-3β. It was previously reported that calcineurin, a calmodulin-dependent serine threonine phosphatase, is a possible downstream target of TRPM2 and activation of calcineurin dephosphorylates GSK-3β. Specifically, pharmacologically blocking calcineurin prevented the dephosphorylation of GSK-3β following TRPM2 activation. In our model, I propose to inhibit calcineurin to see if it is a direct target of TRPM2 following HI. Furthermore, if inhibition of calcineurin can prevent the dephosphorylation of GSK-3β following HI, we want to test if it can elicit any neuroprotective effects.

Second, our evidence using genetic knockout of TRPM2 is largely inherently phenomenological. To extend the application side of this research, I propose to identify and use specific TRPM2 blockers to test the effect of pharmacologically inhibiting TRPM2 before and after HI. In addition, in vitro assays with a specific TRPM2 blocker such as oxygen glucose deprivation and H₂O₂-induced cell death should be performed.
References


