IN VIVO STUDY OF
THE MITOCHONDRIAL DYSFUNCTION
DURING ISCHEMIA
AND
THE EFFECT OF OXIDATIVE STRESS ON
CELL PROLIFERATION

by

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ABSTRACT

Ion influx and water imbalance are major causes of injury during ischemia. Knowledge of the instantaneous subcellular structural and functional changes occurring in vivo, both during ischemia and immediately after the onset of reperfusion, have not been well characterized, mainly due to the extremely rapid progression of these events.

To better understand the mechanisms underlying injury during ischemia in vivo, here, we examine mitochondrial function using the bilateral common carotid artery occlusion model of stroke. Mitochondrial membrane potential ($\varphi_m$) was examined using two-photon fluorescence imaging of the dye Rhodamine123, as an indicator of mitochondrial function. We demonstrate that mitochondrial permeability transition pore-induced $\varphi_m$ collapse occurs during ischemia concurrently with plasma membrane potential depolarization, and repolarized rapidly during reperfusion. Furthermore, we show that inhibition of $\varphi_m$ collapse with cyclosporine A does not result in any detectable attenuation of dendritic structural damage–either during the stroke event or 2 hours afterwards. Thus, these data suggest that mitochondrial dysfunction is an early event during stroke and could contribute to delayed injury at later time points.

Oxidative stress is another proposed mechanism of ischemic injury, given that anti-oxidant proteins play a vital role in brain cell survival. To study the chronic effect of elevated oxidative stress in vivo, we examined cell proliferation within neurogenic regions of the brain of adult mice with compromised anti-oxidant defences (Sut mice). Sut mice possess a natural truncation mutation in the gene Slc7a11 resulting in a malfunctional cystine/glutamate exchanger (xCT). Under normal conditions, xCT supply
intracellular cyst(e)ine for the production of glutathione, a major cellular anti-oxidant. Using bromodeoxyuridine labelling as an indicator of newborn cells, we found that the rate of subventricular-zone (SVZ) cell proliferation when normalized to tissue area was comparable between Sut and control mice. However, the cell proliferation rate within the dentate gyrus (DG) was elevated in Sut mice. These results demonstrate that xCT expression plays a role in regulating cellular proliferation in the DG, but not the SVZ of adult mice. Furthermore, our in vivo observations clearly indicate that in the absence of xCT ongoing cellular proliferation can still persist.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii

TABLE OF CONTENTS ............................................................................................... iv

LIST OF ABBREVIATIONS ......................................................................................... xi

ACKNOWLEDGEMENTS .............................................................................................. xii

CO-AUTHORSHIP STATEMENT ................................................................................. xiii

CHAPTER 1 ................................................................................................................... 1

INTRODUCTION .......................................................................................................... 1

1.1.1 What is stroke? .................................................................................................. 1

1.1.2 Stroke prevalence .............................................................................................. 2

1.1.3 Ischemic stroke .................................................................................................. 2

1.1.4 “Focal vs. global” ischemia .............................................................................. 2

1.1.5 Ischemic stroke prevention and treatment ....................................................... 4

STUDYING STROKE ................................................................................................... 5

1.2.1 Studying stroke: whole animal versus reductionist modeling ....................... 5

1.2.2 Studying stroke: in vivo modeling of transient global ischemia ................. 6

1.2.3 Studying stroke: common carotid artery occlusion model ......................... 7

1.2.4 Studying stroke: pathological changes after transient global ischemia ....... 9

1.2.5 Studying stroke: cell death ............................................................................... 9

1.2.6 Studying stroke: problems studying stoke ...................................................... 10

1.2.7 Studying stroke: *in vivo* two-photon time-lapse imaging ......................... 11
1.2.8 Studying stroke: early events during transient global ischemia ......................... 12

ISCHEMIA MECHANISMS ........................................................................................................ 13

1.3.1 Ischemia mechanisms: energy failure, ion gradient loss, and glutamate release. ................................................................................................................................. 13

1.3.2 Ischemia mechanisms: excitotoxicity hypothesis ....................................................... 14

1.3.3 Ischemia mechanisms: ion imbalance ......................................................................... 15

1.3.4 Ischemia mechanisms: role of Ca\textsuperscript{2+} toxicity in cell death .......................... 15

1.3.5 Ischemia mechanisms: mitochondrial dysfunction ..................................................... 16

1.3.6 Ischemia mechanisms: oxidative stress hypothesis .................................................... 17

1.3.7 Ischemia mechanisms: reduced GSH attenuates cell proliferation \textit{in vitro} .... 18

OVERVIEW .................................................................................................................................. 19

PROJECT 1: .................................................................................................................................. 20

Objective ...................................................................................................................................... 20

Hypothesis .................................................................................................................................. 20

PROJECT 2: .................................................................................................................................. 22

Objective: ..................................................................................................................................... 22

Hypothesis .................................................................................................................................. 22

REFERENCES .................................................................................................................................. 23

CHAPTER 2 .................................................................................................................................. 30

Project 1: Reversible cyclosporine A sensitive mitochondrial depolarization occurs within minutes of stroke onset in mouse somatosensory cortex \textit{in vivo}, a two-photon imaging study.
INTRODUCTION ......................................................................................................................... 31
MATERIALS AND METHODS................................................................................................. 32
RESULTS ................................................................................................................................. 34
Mitochondrial depolarization during global ischemia ......................................................... 34
Role of mPTP in mitochondrial depolarization during global ischemia ......................... 35
The role of mitochondrial depolarization in dendritic blebbing during transient global ischemia ..................................................................................................................................... 36
DISCUSSION ......................................................................................................................... 37
mPTP opening is an early event associated with ischemia and is a mediator of Ψm collapse ............................................................................................................................................. 37
Mechanisms of mPTP opening ........................................................................................... 38
mPTP activation is a reversible event associated with ischemia in vivo ......................... 39
mPTP activation and dendritic structural damage are mechanistically separable .... 40
CONCLUSION ....................................................................................................................... 41
FIGURES ................................................................................................................................. 42
Figure 2.1 ............................................................................................................................ 42
Figure 2.2 ............................................................................................................................ 44
Figure 2.3 ............................................................................................................................ 46
REFERENCES ....................................................................................................................... 47

CHAPTER 3 .......................................................................................................................... 50
Project 2: Differential regulation of cell proliferation in neurogenic zones in mice.
CHAPTER 4 ........................................................................................................................................... 67

DISCUSSION ........................................................................................................................................... 67

Discussion overview ................................................................................................................................. 67

PROJECTION 1 DISCUSSION ...................................................................................................................... 68

4.1.1 Advantages of experimental approach .......................................................................................... 68
4.1.2 Possible limitations ....................................................................................................................... 68
4.1.3 Timing $\psi_m$ depolarization ....................................................................................................... 69
4.1.4 $Ca^{2+}$ involvement in $\psi_m$ depolarization .................................................................................. 71
4.1.5 Resolving inconsistent findings in brain mPTP studies ................................................................. 71
4.1.6 Mitochondrial depolarization $in vivo$ is mono-phasic ................................................................. 73
4.1.7 Therapeutic mechanism of mPTP inhibition ................................................................................. 74
4.1.8 Significance of mitochondrial findings .......................................................................................... 75
4.1.9 Significance of findings with respect to ischemia research ............................................................ 75
4.1.10 Implications of finding: $\psi_p$ depolarization .............................................................................. 76
4.1.11 Implications of findings: oxidative stress during ischemia ......................................................... 77
4.1.12 Implications of findings: dendritic blebbing ............................................................................... 78
4.1.13 Implications of findings: $Ca^{2+}$, apoptosis and delayed cell death ............................................. 80
4.1.14 Reperfusion is associated with the recovery of mitochondrial function ..................................... 81
4.1.15 Proposed $in vivo$ mechanism ..................................................................................................... 82
4.1.16 Future experiments and directions: examining mitochondria morphology during ischemia $in vivo$ ........................................................................................................................................... 82
4.1.17 Future experiments and directions: mitochondria and free radicals ........................................ 84
4.1.18 Broader applications of techniques utilized .......................................................... 85
4.1.19 Biological relevance ................................................................................................ 85

PROJECT 2 DISCUSSION ................................................................................................... 86

4.2.1 Advantages of experimental approach ....................................................................... 86
4.2.2 Possible limitations ................................................................................................... 86
4.2.3 Anti-oxidant production in Sut mice in vivo ............................................................... 87
4.2.4 The role of cell death in xCT deficient mice .............................................................. 88
4.2.5 Significance of findings ............................................................................................ 89
4.2.6 Implications of findings: oxidative stress during ischemia ........................................ 89
4.2.7 Implications of findings: the role of xCT in excitotoxicity .......................................... 90
4.2.8 Future experiment 1: examining long-term survival rates and cell fates of newly generated cells in Sut mice ................................................................................. 90
4.2.9 Future experiment 2: examining the role of xCT in neuronal injury during transient global ischemia in vivo ....................................................................................... 91
4.2.10 Broader implications of findings ............................................................................. 92

REFERENCES ....................................................................................................................... 93

APPENDIX 1 .......................................................................................................................... 98

Improvements to the common carotid artery occlusion model for in vivo imaging

A1.1 Technical improvements ............................................................................................. 98
A1.2 Surgery improvements ............................................................................................... 98
A1.3 Selective breeding ..................................................................................................... 99
A1.4 Temperature regulation

REFERENCES

APPENDIX 2

Project 1 supplementary material

SUPPLEMENTARY METHODS

A2.1 Animals
A2.2 Surgery and imaging procedures
A2.3 Common carotid artery occlusion and stroke induction
A2.4 Electroencephalogram recording
A2.5 Mitochondria membrane potential imaging
A2.6 Assessing dendritic changes
A2.7 Pharmacology
A2.8 Intrinsic optical signal imaging

SUPPLEMENTARY FIGURES

Figure A2.1
Figure A2.2

REFERENCES

APPENDIX 3

UBC Research Ethics Board’s Certificates of Approval
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
</tr>
<tr>
<td>CCAO</td>
<td>Common Carotid Artery Occlusion</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>DCFDA</td>
<td>Dichlorofluorescein Diacetate</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>GCL</td>
<td>Granule Cell Layer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial Permeability Transition Pore</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Rh123</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>Sut</td>
<td>Subtle Gray Pigmentation Mutant Phenotype</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Florescent Protein</td>
</tr>
<tr>
<td>xCT</td>
<td>Cystine/Glutamate Exchanger</td>
</tr>
<tr>
<td>( \Psi_m )</td>
<td>Mitochondrial Membrane Potential</td>
</tr>
<tr>
<td>( \Psi_p )</td>
<td>Plasma Membrane Potential</td>
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</table>
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**CO-AUTHORSHIP STATEMENT**

**Chapter 2 Contributions**

Identification and design of research program

Ran Liu, Timothy Murphy

Performing the research

Ran Liu

Data analyses

Ran Liu

Manuscript preparation

Ran Liu, Timothy Murphy

**Chapter 3 Contributions**

Identification and design of research program

Ran Liu, Craig Brown, Timothy Murphy

Performing the research

Ran Liu

Data analyses

Ran Liu

Manuscript preparation

Ran Liu, Craig Brown, Timothy Murphy
CHAPTER 1

INTRODUCTION

1.1.1 What is stroke?

Stroke is a neurological disorder characterized by the abrupt loss of brain function. Stroke can be caused by a blockage of blood flow to the brain in the case of ischemic stroke or a blood vessel rupture in the case of hemorrhagic stroke. In both cases, cells within the affected area undergo a complex series of events often leading to cell injury or death, resulting in a loss of function. Consequently, the effects and severity of stroke is specific to the brain region injured and extent of damage. Effects may vary vastly, affecting different functions such as motor skills, language, or memory. Severe cases may lead to death (Rijntjes and Weiller, 2002; Lapchak and Araujo, 2007; Myers et al., 2008).

Numerous diagnostic tools are used to diagnose stroke. These include a blood test (Lynch et al., 2004), electroencephalogram (Jordan, 2004), neurological exam (Gocan and Fisher, 2008) and magnetic resonance imaging (Hacke and Warach, 2000). These techniques detect strokes after they have occurred. Current treatments often have restricted therapeutic time windows (Fisher and Bastan, 2008); therefore it is important to recognize the warning signs of stroke before full onset to permit timely treatment. These signs are weakness, difficulty speaking, vision impairment, headache and dizziness. Immediate treatment can significantly improve the chance of survival and recovery (Ellis and Egede, 2009).
1.1.2 Stroke prevalence

Stroke is a leading cause of death around the world (Donnan et al., 2008). In Canada, stroke accounts for 7% of all deaths. Most strokes occur in the elderly. Over 4 percent of Canadians 65 years of age or older are living with the effects of stroke (Wilson et al., 2001). In addition, stroke victims have an increased likelihood of having another stroke, further increasing stroke prevalence in the elderly (Sylaja and Hill, 2007).

1.1.3 Ischemic stroke

87% of all strokes are ischemic strokes (Rosamond et al., 2007). During an ischemic stroke, blood flow is reduced, resulting in a decrease in supply of oxygen and glucose to brain tissue. The energy impairment caused by an ischemic stroke triggers a series of complex and interrelated events leading to cell dysfunction and ultimately cell death in the brain region affected. Our current understanding of the complex interaction between ischemia-induced events and stroke injury is limited. Thus, it is important to enhance our understanding of the mechanism underlying ischemic stroke damage in order to improve methods of stroke prevention and stroke treatment (Hossmann, 2006).

1.1.4 “Focal vs. global” ischemia

Ischemic strokes are generally classified into two types. Focal ischemic strokes are the result of a reduction of blood flow to a local region of the brain due to a thrombosis or embolism. The tissue at the centre of the stroke is called the “ischemic core” and undergoes functional and metabolic failure as well as cell death. The tissue surrounding
the core called the “penumbra” contains live tissue with partial blood flow and oxygenation with passive reperfusion. The penumbra shows acute and chronic plasticity and is a target for therapeutic reversal (Carmichael, 2003; Hossmann, 2006; Li and Murphy, 2008). In global ischemia, therapeutic interest lies within transient global ischemia, since permanent global ischemia results in death. Transient global ischemia occurs when total blood supply to the brain is reduced such as in the case of reduction in cardiac output, for example during cardiac arrest (Traystman, 2003). The entire brain undergoes morphological and functional alterations during the ischemic episode. Reperfusion is generally active as the entire brain regains blood supply and some morphological and functional damage are often reversible upon reperfusion (Gao et al., 1998; Murphy et al., 2008). However, after ischemia seemingly recovered tissue often shows delayed injury possibly due to signaling pathways triggered during ischemia (Gao et al., 1998).

Some similarities exist between tissue in global ischemia and in the penumbra of focal ischemia. 1) Both tissues show some degree of reperfusion (Hossmann, 2006). 2) Acutely, both regions show reversible structural and functional plasticity (Li and Murphy, 2008; Murphy et al., 2008). 3) Over a period of days to months both tissues show delayed plasticity likely due to events induced during the initial period of ischemia (Carmichael, 2003; Traystman, 2003). Consequently, studies in transient global ischemia can also shed light on therapeutic strategies for both types of ischemia.
1.1.5 Ischemic stroke prevention and treatment

There are controllable risk factors that can be avoided to reduce the chance of stroke. These include high blood pressure, obesity, and physical inactivity. In addition, other environmental factors include stress, alcohol consumption and smoking (Leys et al., 2002). Moreover, surgery can be done to remove plaques (Alhaddad, 2004) or bypass narrowing blood vessels (Serruys et al., 2009).

In Canada, stroke costs 2.7 billion dollars annually in physician services (Wilson et al., 2001). Treatment is focused on neuroprotection which is the protection of structural and functional integrity of the brain from stroke. Once an ischemic stroke has occurred the two main approaches to neuroprotection are 1) improving blood supply restoration and 2) stopping ischemia induced molecular cascades leading to cell death (Hossmann, 2006).

The severity of tissue damage depends on the duration and severity of ischemia such that improvement in blood flow alone can result in neuroprotection. Most of the currently available treatments are focused on breaking the blood clot and recovering blood supply. Thrombolytic drugs such as tissue plasminogen activator can be given to break up blood clots. Surgically, a thrombectomy can mechanically remove the clot. These treatments have a limited therapeutic time window and are only effective immediate to when stroke occurs (Hossmann, 2006; Suwanwela and Koroshetz, 2007; Fisher and Bastan, 2008).

The current abilities to block ischemia induced molecular cascades are very limited.
Therapeutic hypothermia has generated clinical success; however, its mechanism of action is yet to be understood and its clinical application is far from optimized (Hoesch and Geocadin, 2007). Novel pharmacological therapies such as anti-oxidant treatments and glutamate blockers are some alternative strategies under investigation with limited clinical success. Physiotherapy such as constraint-induced movement therapy is also under investigation but is not widely used (Rijntjes and Weiller, 2002; O'Collins et al., 2006; Ginsberg, 2008). The ischemia induced molecular mechanism which leads to the adverse effects of stroke is extremely complicated and our understanding of it is limited. Consequently, our current ability to inhibit or reverse the molecular cascade is very limited and further research is needed to expand our understanding of ischemia to allow for more effective ischemic stroke treatments.

STUDYING STROKE

1.2.1 Studying stroke: whole animal versus reductionist modeling

To understand the mechanisms underlying injury during ischemia, experimental models have been developed in vitro (cultured neurons), ex vivo (brain slices) and in vivo (whole animal preparations). Much of the mechanisms proposed for ischemic injury have been generated from studies in vitro and ex vitro. In these settings, oxygen glucose deprivation is employed to simulate ischemia.

Although it is easier to control and manipulate variables as well as visualize changes in vitro and ex vitro, there are many differences between conditions during oxygen glucose
deprivation versus ischemia *in vivo* (Iijima, 2006; Greenwood et al., 2007; Murphy et al., 2008). Stroke in the whole animal occurs in the context of dynamic physiological changes such as pressure and temperature changes as well as reperfusion of blood flow. Other environmental changes such as pH (Combs et al., 1990; Sheldon and Church, 2002), reactive oxygen species (ROS) (Abramov et al., 2007) and ion dynamics (Murphy et al., 2008) are also very intricate. Furthermore, cell-to-cell interaction in the brain, brain vasculature and brain regional selective vulnerability can also play a role in ischemia. These complex interactions are very difficult to mimic in reductionist models. Thus it is unclear how these proposed *in vitro* mechanisms can be extrapolated to stroke *in vivo*. Consequently, ischemia studies *in vivo* are necessary to examine the dynamic interactions within the intact ischemic brain and to validate proposed mechanisms from reductionist modeling.

1.2.2 *Studying stroke: in vivo modeling of transient global ischemia*

Various models of global ischemia have been developed *in vivo*. To induce cerebral forebrain ischemia, the most frequently used animal models employ mechanical occlusion of cortical blood supply (Ginsberg and Busto, 1989; Block, 1999). Cerebral blood supply is provided by the common carotid arteries (CCAs) and the basilar trunk which joins with the Circle of Willis at the base of the brain. The Circle of Willis then branches out into the anterior, middle and posterior cerebral arteries which subsequently supply blood to the surface of the cortex (Hossmann, 2006). Due to redundancy in blood supply provided by the Circle of Willis, typically both the CCAs and the basilar trunk
would have to be occluded to generate ischemia. Alternatively, clamping the CCAs combined with hypotension can also achieve ischemia. In animals with an incomplete Circle of Willis, occluding of the CCAs alone is sufficient (Ginsberg and Busto, 1989; Block, 1999).

Depending on the vasculature of the species, animal models utilize either four vessel occlusion (Pulsinelli et al., 1982), two vessel occlusion plus systemic hypotension (Smith et al., 1984) or two vessel occlusion alone (Kirino, 1982; Yang et al., 1997). Less utilized global ischemia models include cardiac vessel compression (Kawai et al., 1992), neck tourniquet (Siemkowicz and Hansen, 1978), and intracranial pressure elevation (Duhaime and Ross, 1990). The global ischemia models that permit active reperfusion can also provide insight into the dynamics of the penumbra in focal ischemia (similarities discussed above).

1.2.3 Studying stroke: common carotid artery occlusion model

In the studies described here, we employ the common carotid artery occlusion (CCAO) model, where two-vessel occlusion alone simulates transient global ischemia. First proposed in 1972 (Eklof and Siesjo, 1972), bilateral CCAO was commonly used in gerbils because these organisms have an incomplete Circle of Willis (Kirino, 1982). It was subsequently shown that some strains of mice also have this property. Here, we utilized C57Bl6 mice which have weak posterior communicating arteries resulting in an incomplete Circle of Willis (Yang et al., 1997). Subsequently, bilateral occlusions of the
CCAs in these mice are sufficient to generate ischemia to the anterior and middle cerebral arteries, while the posterior cerebral arteries are unaffected as they obtain blood supply from the basilar trunk. As a result, the CCAO model when utilized in C57Bl6 mice can generate sufficient forebrain ischemia specifically to the areas supplied by anterior and middle cerebral arteries. However, because of the overlap in cortical areas covered by the posterior cerebral arteries, in our CCAO model, the anterior lateral areas generally are more ischemic (Yang et al., 1997; Murphy et al., 2008).

An advantage of this model is the rapid induction of forebrain ischemia, defined periods of occlusion, and precisely controlled reperfusion (Yang et al., 1997; Murphy et al., 2008). The use of sutures to constrict the CCAs confers a high degree of control over ischemia and reperfusion, and additionally, this can be performed while anaesthetised animals are mounted onto a microscope stage. In addition, a high grade of forebrain ischemia can be induced without the requirement for induced hypotension (Murphy et al., 2008). Furthermore, this model is a one stage surgical preparation with lower experimental failure rates compared with the four-vessel occlusion model. Lastly, this model is also suitable for chronic studies (Ginsberg and Busto, 1989).

A potential confounding factor of the CCAO model preparation is that anaesthesia is required, which may interfere with particular aspects of experimental observations (Ginsberg and Busto, 1989). Moreover, the success of this model depends on the loss of redundant blood supply in the Circle of Willis; however, a sub-population of the mice
does have partially functional posterior communicating arteries. Consequently, internal inconsistency with respect to cerebral blood flow decrease and pathological outcome is large (Ginsberg and Busto, 1989). Blood flow monitoring must be done to confirm and standardize the degree of ischemia (Murphy et al., 2008).

1.2.4 Studying stroke: pathological changes after transient global ischemia
Previous research examining transient global ischemia in vivo has reported a wide range of pathological outcomes. Elevated oxidative stress has been observed hours to days after transient global ischemia (Friberg et al., 2002). Inflammation also persists for days after the ischemic episode (Orzylowska et al., 1999; Langdon et al., 2008). Various delayed cellular changes have also been shown to occur in response to ischemia induced insults. Within days, glial activation occurs throughout the brain (Pforte et al., 2005; Salazar-Colocho et al., 2008). Concurrently, selected neurons show changes in dendritic aborization (Ruan et al., 2006) and spine density fluctuation (Ruan et al., 2009). Gliogenesis (Pforte et al., 2005) and neurogenesis (Kokaia and Lindvall, 2003) are also elevated days following transient global ischemia, possibly in response to cell injury or cell death (Salazar-Colocho et al., 2008).

1.2.5 Studying stroke: cell death
Cell death plays a critical role in the pathological outcome of transient global ischemia. Previous research has shown that within days following transient global ischemia, various cell death pathways are activated (Domanska-Janik et al., 2004; Zhao et al., 2005).
Indeed, delayed cell death after transient global ischemia has been observed in various brain regions such as the hippocampal CA1 region (Yonekura et al., 2004) and the cortex (Olsson et al., 2003). In global ischemia, synaptic activity is transiently suppressed; however, after ischemia neurons that have seemingly recovered synaptic properties go on to die days later (Gao et al., 1998). These results suggest that while some immediate events during ischemia may be reversible, during this time other pathways are activated that are responsible for triggering pathological outcomes, ultimately leading to cell death. Thus it is important to study the initial events during ischemia and shortly after reperfusion to understand the mechanisms underlying ischemia induced cell death.

1.2.6 Studying stroke: problems studying stoke

Transient global ischemia is an abrupt and transient occurrence, with a complex series of events occurring within the initial minutes of stroke (Gao et al., 1998; Murphy et al., 2008; Risher et al., 2009). Most of the previous in vivo studies of transient global ischemia have examined only a single time point after stroke (Friberg et al., 2002; Olsson et al., 2003; Domanska-Janik et al., 2004; Yonekura et al., 2004; Zhao et al., 2005). The recent development of in vivo imaging has advanced our ability to examine the sequence of immediate events during transient global ischemia (Svoboda and Yasuda, 2006). Further in vivo time-lapse studies are needed to confirm results from in vitro studies, in order to better understand the early events of global ischemia.
1.2.7 Studying stroke: in vivo two-photon time-lapse imaging

Two-photon microscopy is a fluorescent imaging technique first developed by Denk, Webb and others (Denk et al., 1990), which has many advantages in imaging living tissues. In the present experiments, we have combined two-photon microscopy with the CCAO model to generate a setup that allows in vivo time-lapse imaging of transient global ischemia (Murphy et al., 2008). By imaging through a cranial window, this combinatorial technique allows us to visualize cellular and sub-cellular events over the time scale of seconds.

There are three main advantages of two-photon microscopy over conventional microscopy techniques. In two-photon microscopy, two low energy photons excite a fluorophore resulting in the emission of a fluorescence photon at a higher energy than either of the two individual excitatory photons. Photons with low energy have longer wavelengths, allowing for better penetration deep into the brain tissue. Consequently, the use of long wavelength photons make two-photon microscopy ideal for in vivo imaging. Secondly, two-photon microscopy only excites the specimen within a precise and small volume. Therefore, very little out-of-focus excitation occurs, greatly improving the signal-to-noise ratio of acquired images. Lastly, since at any instant in time only one plane of focus is excited, two-photon microscopy also produces less photo-toxicity and fluorophore bleaching when compared to confocal microscopy (Svoboda and Yasuda, 2006). As a result, this method is well suited for studying the early events of stroke because it allows for prolonged monitoring of events deep in the brain with high spatial
and temporal resolution immediately after the onset of stroke and reperfusion.

1.2.8 Studying stroke: early events during transient global ischemia

Previous studies from our laboratory, using two-photon microscopy to image early events in transient global ischemia, have reported intracellular Ca$^{2+}$ elevation and dendritic structural damage concurrent with the onset of plasma membrane potential ($\Psi_p$) depolarization. These events were found to be reversible upon reperfusion. Following the onset of reperfusion, $\Psi_p$ depolarization and intracellular Ca$^{2+}$ elevation reverses rapidly, while dendritic structural recovery occurs over a slower time scale (Murphy et al., 2008). Similarly, astrocyte swelling is also observed in global ischemia induced by a short period of cardiac arrest (Risher et al., 2009). On the functional level, slow wave cortical activity, evoked hemodynamic response as measured by intrinsic optical imaging, and evoked action potentials as measured by voltage sensitive dye, are all suppressed during ischemia and reversible over time after reperfusion onset (Murphy et al., 2008).

New imaging techniques reveal that many of these early events are reversible upon reperfusion (Li and Murphy, 2008; Murphy et al., 2008). However, the mechanisms underlying these events and how these early events trigger delayed injury, detected at later times (Olsson et al., 2003; Yonekura et al., 2004), is far from clear.
ISCHEMIA MECHANISMS

1.3.1 Ischemia mechanisms: energy failure, ion gradient loss, and glutamate release

The principle consequence of ischemia is a failure of energy production. Exhaustion in oxygen and glucose causes an inhibition of energy production resulting in ATP depletion. Consequently, membrane ATPases cease to function resulting in the loss of ionic gradients across the plasma membrane (Hansen et al., 1982; Jiang et al., 1992; Saeed et al., 2007). This causes $\Psi_p$ depolarization which triggers the opening of voltage gated ion channels (Hansen et al., 1982; Fujiwara et al., 1987; Silver and Erecinska, 1990).

The loss of ionic gradient across the plasma membrane and the opening of voltage gated ion channels also trigger extracellular glutamate elevation, causing harm to cells. The early phase of glutamate release is Ca$^{2+}$ dependent, as intracellular Ca$^{2+}$ elevation triggers exocytosis of glutamate vesicles (Katayama et al., 1991; Pocock and Nicholls, 1998). Ca$^{2+}$ independent, non-exocytotic glutamate release mechanisms take part with prolonged energy depletion (Pocock and Nicholls, 1998). It is suggested that these are due to activation of volume-sensitive channels (Phillis et al., 2000) as well as intracellular Na+ elevation which reverses Na+/ glutamate transporters (Szatkowski et al., 1990; Taylor et al., 1995; Phillis et al., 2000). Glutamate activation of kainate receptors is also observed to cause presynaptic glutamate release forming a positive feed-forward cycle (Kimura et al., 1998).
1.3.2 Ischemia mechanisms: excitotoxicity hypothesis

Glutamate is the primary excitatory neurotransmitter within the central nervous system, acting through ionotropic and metabotropic glutamate receptors. Excitotoxicity is the concept that excessive extracellular glutamate accumulation causes injury, primarily to neurons (Olney, 1994). This hypothesis is one proposed mechanism of neuronal injury during ischemia (Hossmann, 2006). It has been demonstrated that the mechanism of cell death depends the concentration of glutamate; high concentrations cause necrosis while low concentrations cause apoptosis (Choi, 1996).

Experimental (Benveniste et al., 1984) and clinical (Bullock et al., 1995) studies have shown that extracellular concentration of glutamate during ischemia elevates to toxic levels. In addition, neurons in energy deficient environments are more vulnerable to excitotoxicity (Kimura et al., 1999), being susceptible to lower levels of glutamate. In laboratory studies, glutamate receptor antagonists have been shown to reduce ischemic damage (Ginsberg, 1996; Grotta, 1996). Unfortunately, however, little such success has been observed in clinical studies (De Keyser et al., 1999; O'Collins et al., 2006).

After the ischemic episode, extracellular glutamate is rapidly reduced (Benveniste et al., 1984). However, in vivo (Andine et al., 1991) and in vitro (Kimura et al., 1999) studies have shown that glutamate antagonist administered after ischemic insult can reduce cell death. These findings show that delayed glutamate release may occur after reperfusion onset and may lead to secondary excitotoxicity, ion gradient impairment, and Ψ̄p
depolarization.

### 1.3.3 Ischemia mechanisms: ion imbalance

Ischemia-induced energy failure leads to an initial ion imbalance. In addition, ionotropic glutamate receptor activation associated with excitotoxicity triggers an abrupt and massive ion flux across the plasma membrane. At this time, a substantial Na\(^+\) and Ca\(^{2+}\) influx occurs due to their electrochemical gradients, resulting in rapid \(\Psi_p\) depolarization (Nishizawa, 2001; Camacho and Massieu, 2006). This massive ion influx drives a series of complex and interrelated pathological events, including intracellular signaling, Ca\(^{2+}\) toxicity (Sattler and Tymianski, 2001), water influx and dendritic structural damage (Greenwood et al., 2007). Concurrently, mitochondrial and endoplasmic reticulum (Paschen, 1996) dysfunction occurs, as well as ATP depletion (Henrich and Buckler, 2008), and protein synthesis inhibition (Paschen, 2003). Additionally, ROS production (Frantseva et al., 2001) and alterations in pH (Sheldon and Church, 2002) also occur. Although numerous events have been identified and characterized, how these events interact collaboratively to induce damage in stroke is still not clear.

### 1.3.4 Ischemia mechanisms: role of Ca\(^{2+}\) toxicity in cell death

High Ca\(^{2+}\) levels are cytotoxic. Ca\(^{2+}\) toxicity is a chief mechanism for linking excitotoxicity with cell death. Under physiological conditions, cytosolic free Ca\(^{2+}\) is typically maintained at extremely low levels by calcium transport systems. During ischemia, energy failure and ionotropic glutamate receptor activation results in a massive
influx of Ca\textsuperscript{2+} into the cell. The rise in cytoplasmic Ca\textsuperscript{2+} results in activation of Ca\textsuperscript{2+} dependent catabolic enzymes (Won et al., 2002) as well as mitochondrial (Starkov et al., 2004) and endoplasmic reticulum dysfunction (Paschen, 1996). Strong evidence suggests a role of Ca\textsuperscript{2+} toxicity in ischemic injury. However, the mechanisms leading to cell death are complex and a more complete understanding of these events is necessary for the development of therapeutic interventions.

Delayed calcium de-regulation is a process where Ca\textsuperscript{2+} homeostasis is lost at some point after the initial insult. Delayed calcium de-regulation has been observed in glutamate-induced excitotoxicity. This phenomenon appears to be closely linked with cell death, and may be a chief mechanism in delayed cell death after transient ischemia (Randall and Thayer, 1992; Alano et al., 2002; Abramov and Duchen, 2008).

Interestingly, excitotoxic injury show selective regional vulnerability, likely due to differences in intracellular Ca\textsuperscript{2+} elevation within cells of a particular brain region. This may likely be due to difference in glutamate receptor expression or cell specific Ca\textsuperscript{2+} regulation which results in differences in intracellular Ca\textsuperscript{2+} concentration during ischemia (Mitani et al., 1992).

\subsection{1.3.5 Ischemia mechanisms: mitochondrial dysfunction}

Mitochondrial dysfunction is a hallmark of neuro-toxicity and is a possible link between Ca\textsuperscript{2+} toxicity and cell death in ischemia. It is characterized by mitochondrial membrane
potential (∆ψm) depolarization and mitochondrial membrane morphology collapse (Starkov et al., 2004; Halestrap, 2006).

In vitro studies suggest that during ischemia, elevated cytoplasmic Ca\(^{2+}\) is sequestered within mitochondria due to their high ∆ψm (Alano et al., 2002; Abramov and Duchen, 2008). Mitochondrial Ca\(^{2+}\) overload, in combination with other ischemia-induced factors, results in increased permeability of the mitochondrial membrane, a process called mitochondrial membrane transition. This occurs via Ca\(^{2+}\)-activation of the mitochondrial permeability transition pore, a protein pore in the membrane of the mitochondria allowing molecules less than 1500 daltons to flow freely between the cytoplasm and the mitochondrial matrix. As a consequence of mitochondrial membrane transition, the loss of ∆ψm halts mitochondrial respiration and ATP production. Furthermore, the loss of mitochondrial membrane integrity results in the release and activation of various apoptotic factors. Therefore, mitochondrial dysfunction is suggested to play a key role in linking Ca\(^{2+}\) toxicity with delayed cell death (Starkov et al., 2004; Halestrap, 2006).

1.3.6 Ischemia mechanisms: oxidative stress hypothesis

Oxidative stress is another major class of events contributing to injury in ischemia. Previous evidence suggests that ROS can be generated during ischemia and reperfusion (Frantseva et al., 2001; Friberg et al., 2002; Abramov et al., 2007). ROS can cause plasma membrane damage (Chan, 1996), mitochondrial (Siesjo et al., 1999) and ER disturbances (Paschen, 2003), as well as DNA fragmentation (Lipton and Nicotera,
Consequently, ROS likely play a role in ischemic cell death. However, in ischemia the exact mechanism of ROS generation and how ROS cause ischemic injury is unclear.

The brain has an intricate system of defences against oxidative stress during pathological conditions. Glutathione (GSH), a low molecular weight thiol is a major cellular antioxidant molecule in this system (Slemmer et al., 2008). A decrease is GSH increases the brain’s susceptibility to oxidative stress. Indeed, GSH deficiencies have been associated with neuronal injury in ischemia (Mizui et al., 1992; Bobyn et al., 2002).

1.3.7 Ischemia mechanisms: reduced GSH attenuates cell proliferation in vitro

Na+ independent cystine–glutamate exchange antiporter (xCT) supplies intracellular cyst(e)ine for GSH production (Miura et al., 1992). xCT is expressed in astrocytes and developing neurons, as well as within the meninges and ependymal cells (Sato et al., 2002; Shih et al., 2006). Indeed, mutant mice with malfunction of xCT have reduced cellular GSH levels (Sato et al., 2005; Shih et al., 2006). xCT expression modulates cell proliferation and neuroprotection against oxidative stress. An important observation is that xCT malfunction largely attenuates cell proliferation in cell cultures (Shih et al., 2006). Interestingly, in the brain, xCT expression and GSH concentration are elevated in neurogenic zones (subventricular zone and denate gyrus) (Shih et al., 2006). These lines of evidence suggests the xCT expression and production of intracellular GSH is important for protecting proliferating cells in the brain from oxidative stress.
OVERVIEW

This thesis work encompasses two separate projects. The first project examines an *in vivo* mechanism of mitochondrial dysfunction during transient global ischemia. The second project examines the role of anti-oxidant production on cell proliferation in the brain.
PROJECT 1:

Mitochondrial dysfunction occurring early in transient global ischemia may be the cause of delayed cell death of seemingly recovered neurons (Gao et al., 1998; Olsson et al., 2003; Yonekura et al., 2004; Murphy et al., 2008). The process of mitochondrial dysfunction and mitochondrial membrane transition is extremely complex and is affected by a wide range of factors that are dynamic during transient ischemia (Halestrap, 2006). Therefore it is difficult to extrapolate early mitochondrial events in transient ischemia from in vitro studies or histological/biochemical in vivo studies. To date, no in vivo time-lapse studies have examined the timing and mechanism of mitochondrial dysfunction during ischemia and immediately after reperfusion in transient ischemia. An in-depth understanding of the early events in mitochondrial function during ischemia will be important in defining the link between Ca\(^{2+}\) toxicity and delayed cell death in ischemic injury.

Objective:

We used two-photon time-lapse imaging to examine \( \mathcal{V}_m \) as an indicator of mitochondrial function during transient global ischemia induced by CCAO. Pharmacological manipulations were used to explore the mechanisms of ischemia induced \( \mathcal{V}_m \) depolarization.

Hypothesis:

\( \mathcal{V}_m \) depolarization occurs during ischemia concurrently with \( \mathcal{V}_p \) depolarization.
Furthermore, \( \psi \) depolarization is driven by mPTP (mitochondrial permeability transition pore) activation. Lastly, inhibition of \( \psi \) depolarization will reduce dendritic structural damage.
PROJECT 2:

xCT is vital for cell proliferation \textit{in vitro} (Shih et al., 2006). However, the effect of xCT malfunction and GSH reduction on cell proliferation \textit{in vivo} is unclear. Examining cell proliferation in a GSH deficient system is important because oxidative insults on cell proliferation maybe a critical component of ischemia-induced injury. Therefore, understanding the interactions between oxidative stress, GSH intracellular levels and cell proliferation \textit{in vivo} may provide further insights to the mechanism of ischemic injury.

\textit{Objective:}

Subtle gray pigmentation mutant phenotype (Sut) mice have a truncation mutation in \textit{slc7a11} gene resulting in a malfunction of xCT (Chintala et al., 2005), and reduced intracellular GSH levels (Shih et al., 2006). We use BrdU (bromodeoxyuridine) to study the effect of reduced GSH production on \textit{in vivo} cell proliferation in the neurogenic zones of adult Sut mice.

\textit{Hypothesis:}

xCT malfunction will result in significant reduction in cell proliferation in neurogenic zones \textit{in vivo}. 
REFERENCES


CHAPTER 2

Project 1: Reversible cyclosporine A sensitive mitochondrial depolarization occurs within minutes of stroke onset in mouse somatosensory cortex in vivo, a two-photon imaging study. ¹

¹ A version of this chapter has been submitted for publication. Liu, R. and Murphy, T. Reversible cyclosporine A sensitive mitochondrial depolarization occurs within minutes of stroke onset in mouse somatosensory cortex in vivo, a two-photon imaging study.
INTRODUCTION

Ion and water imbalance during stroke are widely accepted as chief contributors to acute ischemic injury, but how these events are linked to more slowly activated cell death pathways is unclear (Hossmann, 2006). Neurons within the cortex and the hippocampus can recover both structure and function within hours after brief ischemia of less than 10 min duration (Gao et al., 1998; Murphy et al., 2008), but are still subject to later cell death with apoptotic features days later (Yonekura et al., 2004). One mechanism described *in vitro* that may link intracellular Ca\(^{2+}\) elevation to cell death pathways is the activation of mitochondrial permeability transition pore (mPTP) in response to mitochondrial Ca\(^{2+}\) overload (White and Reynolds, 1996; Khaspekov et al., 1999; Schinzel et al., 2005; Abramov and Duchen, 2008). MPTP activation can lead to mitochondrial membrane potential (\(\Psi_m\)) collapse and excessive reactive oxygen species (ROS) generation (White and Reynolds, 1996; Frantseva et al., 2001; Abramov and Duchen, 2008). Mitochondrial dysfunction and specifically mPTP opening has been suggested as a hallmark of ischemic injury (Starkov et al., 2004; Halestrap, 2006), although its exact role and timing are unclear. *In vitro* studies in neurons suggest that mitochondrial dysfunction occurs during ischemia and excitotoxic ion overload (White and Reynolds, 1996; Alano et al., 2002; Rintoul et al., 2003; Abramov et al., 2007; Greenwood et al., 2007; Abramov and Duchen, 2008), however, other evidence suggests that mitochondrial dysfunction may be delayed and occur during reperfusion, or after a period of ionic stress (Matsumoto et al., 1999; Frantseva et al., 2001; Domanska-Janik et al., 2004). To complicate matters, there are differences between conditions during *in vitro*
ischemia-like events and stroke in vivo (Greenwood et al., 2007; Murphy et al., 2008). Previously, the role of mitochondrial dysfunction has been addressed in stroke in vivo using endpoint measures of histological and biochemical markers (Matsumoto et al., 1999; Schinzel et al., 2005), or by using potentially less-direct methods such as time-lapse imaging of NADH fluorescence (Mayevsky and Chance, 2007). To date, no real-time monitoring of mitochondrial function in vivo during stroke and reperfusion has been performed. Consequently, when and where mitochondrial dysfunction occurs in vivo during stroke is unclear.

Here bilateral occlusion of the common carotid arteries (CCAs) was used to produce rapid and reversible forebrain ischemia (Murphy et al., 2008). When combined with two-photon imaging of rhodamine 123 (Rh123) fluorescence this model allowed us to monitor $\Psi_m$ with high temporal and spatial resolution during ischemia and reperfusion. We show that mPTP activation is an early event during stroke that occurs within about 2 min of occlusion onset in parallel with plasma membrane potential ($\Psi_p$) depolarization.

MATERIALS AND METHODS

For details see appendix 2 (supplementary methods), brief methods appear here. Experimental protocols were approved by the University of British Columbia Animal Care Committee. Urethane-anesthetized, 2-5 month old green fluorescent protein (GFP-M) and yellow fluorescent protein (YFP-H) transgenic mice (Feng et al., 2000) and C57BL/6 wild-type mice were used. A cranial window was created over the forelimb-
hindlimb somatosensory cortex for two-photon imaging and the dura was removed to facilitate penetration of Rh123 and CsA. γPm was examined by monitoring Rh123 fluorescence within layer-I of the cortex, typically within 50μm of the pial surface. Rh123 (20μM) was applied directly to the cortex and washed before imaging. CsA (20μM) or FK-506 (50μM) was pre-incubated directly to the cortex for 1 h and mixed into the agarose and applied for sustained application (CsA at 10μM and FK-506 at 50μM); note concentrations within the brain are expected to be lower. The core temperature was maintained at 37±0.5°C using a heat-pad and a heat exchanger warmed the craniotomy to ~37 °C. To induce stroke sutures were looped around each CCA and tightened as previously described (Murphy et al., 2008). Suppression of slow wave cortical activity was used to define the onset of occlusion (Supplementary Fig. A2.1C top). Abrupt changes in the DC EEG potential were indicative of γP depolarization (Supplementary Fig. A2.1C bottom). All dendritic structural analysis of dendrites were done within 50μm of the pial surface. Intrinsic optical imaging was performed prior to stroke to locate the forelimb-hindlimb somatosensory cortex.

Image analysis was done with ImageJ software. Image filtering and dendritic bleb quantification was done as previously described (Murphy et al., 2008). For Rh123 fluorescent intensity analysis, the percent change from a pre-ischemia control stack taken before stroke was calculated by a frame-by-frame ratio of region of interest values. Statistical analysis comparing two groups was made by t-tests. One-way ANOVA was used when comparing repeated measures within the same group (time course data).
followed by a post hoc Bonferroni’s multiple comparison test. When comparing repeated measures between two groups, a two-way ANOVA was used.

RESULTS

Mitochondrial depolarization during global ischemia.

To address the role of \( \mathcal{V}_m \) depolarization during stroke we performed in vivo imaging within the mouse forelimb somatosensory cortex using a model that permits rapid stroke induction, defined periods of ischemia, and controlled reperfusion. In this model the CCAs were occluded bilaterally for about 6.5 min resulting in a reduction in blood flow to the anterior and middle cerebral arteries (Murphy et al., 2008). Within 2 min of stroke induction a massive \( \mathcal{V}_p \) depolarization occurred (within 91±12s, \( n=15 \) ischemic trials), that was previously shown to be closely associated with intracellular Ca\(^{2+} \) elevation and dendritic structural damage (Murphy et al., 2008). Here we examine mitochondrial function during these early stroke-dependent events. Rh123 was applied to the cortex to monitor \( \mathcal{V}_m \) as an indicator of mitochondrial function (Benel et al., 1989). During stroke, synchronized \( \mathcal{V}_m \) collapse occurred within minutes after occlusion (average of 86±11 s, \( n=14 \) ischemic trials) as indicated by a rapid elevation of Rh123 fluorescence (135±20\% above baseline, \( n=14 \), Fig. 2.1A and B, 2.2B). The \( \mathcal{V}_m \) collapse coincided closely with \( \mathcal{V}_p \) depolarization (Fig. 2.1 C) and occurred 6±4 s before the time-point when the \( \mathcal{V}_p \) reached the maximal rate of depolarization (\( n=14 \) ischemic trials; Fig. 2.1 A, C). Contrary to the hypothesis of \( \mathcal{V}_m \) depolarizing at reperfusion and contributing to reperfusion injury (Matsumoto et al., 1999; Frantseva et al., 2001; Domanska-Janik et al., 2004;
Halestrap, 2006), we observed no additional $\Psi m$ depolarization during reperfusion. Quite the opposite, $\Psi m$ repolarization occurred rapidly after the start of reperfusion (fluorescence reduced by 63% from the maximum increase 145±53s after the onset of reperfusion, n=11 ischemic trials) as indicated by a sharp drop in Rh123 fluorescence that remained at baseline for the entire duration of monitoring usually up to 2 h after stroke. The kinetics of $\Psi m$ repolarization were similar to the recovery of intracellular Ca$^{2+}$ levels and preceded the recovery of dendritic blebbing which occurred over 13-41 min (Murphy et al., 2008). After reperfusion, $\Psi m$ depolarization can be re-triggered by a second stroke (~120 min later) resulting in a similar fluorescent elevation (Fig. 2.1 D). This result indicates that the reduction in fluorescence during reperfusion is reflective of $\Psi m$ repolarization and not dye loss or bleaching and therefore possible delayed $\Psi m$ depolarization would have been detectable.

**Role of mPTP in mitochondrial depolarization during global ischemia.**

*In vitro* studies suggest that the mPTP contributes to the collapse of $\Psi m$ (White and Reynolds, 1996; Alano et al., 2002; Halestrap, 2006; Abramov and Duchen, 2008). Here, bath application of CsA (a mPTP inhibitor) locally within the craniotomy largely abolished $\Psi m$ collapse as evident by the absence of Rh123 fluorescence elevation (control n=11 versus CsA n=5, P<0.0001 two-way ANOVA; control n=14, CsA n=5 P=0.0024 by t-test; Fig. 2.2A, B). In figure 2.2A, animals with prolonged survival after ischemia were plotted and compared in the ANOVA. It is conceivable that the effects of CsA on the inhibition of $\Psi m$ collapse were due to inhibition of calcineurin. Therefore,
we also applied FK-506 a calcineurin inhibitor that does not affect the mPTP to control for non-specific effects of CsA. FK-506 treated animals showed no reduction of Rh123 fluorescence elevation during stroke when compared with the controls (FK-506 n=5, P=0.74 Fig. 2.2B), and exhibited a significant Rh123 fluorescence increase when compared with the CsA treated animals (P=0.028 Fig. 2.2B). We found that the CsA inhibition of $\Psi_m$ collapse was selective as ischemia induced $\Psi_p$ depolarization still occurred at similar latency from the start of occlusion (control n=15, CsA n=11, p=0.19; Fig. 2.2C, D) and was also not affected by the FK-506 treatment (FK-506 n=11 P=0.83 Fig. 2.2D). However, inhibition of mPTP caused a small but significant reduction in the total amplitude of the $\Psi_p$ depolarization (control n=11, CsA n=9, 18% reduction compared to control, P=0.027; Fig. 2.2 E). This effect was not seen in FK-506 treated animals (FK-506 n=11, P=0.20 versus untreated controls) suggesting that mPTP inhibition was responsible for this reduction. These experiments suggest that in vivo the dominant mechanism of $\Psi_m$ depolarization is mediated by CsA-sensitive mPTP opening.

The role of mitochondrial depolarization in dendritic blebbing during transient global ischemia.

We assessed the effect of CsA-induced mPTP blockade on dendritic structural damage by examining the dendritic tufts of layer 5 neurons using in vivo imaging. Within minutes of ischemic induction apical dendrites were found to rapidly swell and “bleb” leading to a beads on a string appearance (Zhang et al., 2005). Dendritic blebbing was quantified before and during ischemia as well as during reperfusion. As previously established...
(Murphy et al., 2008), dendrites bleb during occlusion at the time of \( \Psi_p \) depolarization and recover during reperfusion (control \( n=4 \) animals, \( P=0.006 \) one-way ANOVA; Fig. 2.3 A, B). Animals treated with locally applied CsA (\( n=4 \), \( P=0.092 \) two-way ANOVA) showed no reduction in dendritic blebbing when compared to control animals (\( n=4 \)) during occlusion or within 2 h of reperfusion (Fig. 2.3 A, B). FK-506 a related molecule that also inhibits calcineurin, but does not affect mPTP was also without effect on ischemic changes to dendritic morphology (\( n=4 \), \( P=0.462 \) two-way ANOVA).

**DISCUSSION**

*mPTP opening is an early event associated with ischemia and is a mediator of \( \Psi_m \) collapse.*

mPTP opening has been suggested to play a significant role in excitotoxic ion overload (White and Reynolds, 1996; Alano et al., 2002; Abramov and Duchen, 2008), and ischemia/reperfusion damage (Khaspekov et al., 1999; Matsumoto et al., 1999; Frantseva et al., 2001; Domanska-Janik et al., 2004; Schinzel et al., 2005). We used Rh123 to monitor \( \Psi_m \) in both neurons and glia that undergo rapid swelling during ischemia in vivo (Risher et al., 2009). \( \Psi_m \) collapse in vivo occurred with a similar rapid time course as cytoplasmic \( \mathrm{Ca}^{2+} \) elevation and recovery during ischemia and reperfusion (Murphy et al., 2008). This supports previous in vitro findings (White and Reynolds, 1996; Alano et al., 2002; Greenwood et al., 2007; Abramov and Duchen, 2008) suggesting that intracellular \( \mathrm{Ca}^{2+} \) elevation and \( \Psi_m \) depolarization are closely related. Studies from non-neuronal cell types suggest that mitochondria depolarize, swell, and release cytochrome-C through
CsA-sensitive Ca\(^{2+}\) induced mPTP activation (Halestrap, 2006), with brain mitochondria sharing similar mechanisms (White and Reynolds, 1996; Alano et al., 2002; Domanska-Janik et al., 2004; Abramov and Duchen, 2008). However, other investigations of brain mitochondria have generated mixed results in regards to these mechanisms with some reports suggesting that the brain mPTP is relatively CsA-insensitive (Andreyev and Fiskum, 1999; Kobayashi et al., 2003). In other cases where brain and liver mitochondria were directly compared both were found to undergo Ca\(^{2+}\)-trigged depolarization that was delayed by CsA pretreatment (Vergun and Reynolds, 2005). These discrepancies may result from variation in experimental conditions for mitochondria in isolation, different conditions for \textit{in vivo} versus \textit{in vitro} experiments, as well as regional or subcellular heterogeneity of mitochondria within brain. Here we demonstrate \textit{in vivo} that rapid \(\psi_m\) collapse during ischemia is mediated by activation of a CsA-sensitive mPTP in intact brain mitochondria within minutes after the onset of ischemia (see Supplementary Fig. A2.2 for schematic).

\textit{Mechanisms of mPTP opening.}

\textit{In vitro} and brain slice studies suggest that there are two main mechanistic components behind \(\psi_m\) depolarization during excitotoxicity. The initial component of \(\psi_m\) collapse is believed to involve mitochondrial Zn\(^{2+}\) uptake (Medvedeva et al., 2009), mitochondrial Ca\(^{2+}\) overload and depletion of mitochondrial respiratory substrates which are reversible (Abramov and Duchen, 2008), while the later component of \(\psi_m\) collapse is thought to be associated with the activation of mPTP and was potentially irreversible (Abramov and
Evidence suggests that intracellular \( \text{Ca}^{2+} \) elevation triggers mPTP opening via binding to cyclophilin-D that subsequently binds in a CsA-sensitive manner to adenine nucleotide translocase and results in mPTP opening (Basso et al., 2005; Schinzel et al., 2005). However, alternative CsA-insensitive mechanisms could also promote mPTP opening during ischemia, such as ROS release, \( \Psi_m \) depolarization, adenine nucleotide depletion, or pH changes (Halestrap et al., 1997; Sheldon and Church, 2002; Basso et al., 2005) making it difficult for a single model to mimic \textit{in vivo} stroke parameters.

CsA can potentially have non-specific effects aside from mPTP inhibition (Halestrap, 2006). Importantly, we show that CsA does not affect \( \Psi_p \) depolarization, but does block the ischemia-induced Rh123 fluorescence elevation. There are concerns that the elevation of Rh123 fluorescence during occlusion could in part reflect \( \Psi_p \) depolarization (Nicholls and Ward, 2000). However, since CsA inhibited the Rh123 fluorescence elevation, but not ischemic \( \Psi_p \) depolarization, it is unlikely that the change in Rh123 fluorescence reflects activity in cytoplasmic compartments. This selective effect of CsA also suggests that mPTP activation and \( \Psi_m \) depolarization are not the triggers of \( \Psi_p \) depolarization.

\textbf{mPTP activation is a reversible event associated with ischemia} \textit{in vivo}.

\textit{In vitro} studies suggest that the activation of mPTP is associated with the triggering of irreversible processes leading to delayed \( \text{Ca}^{2+} \) deregulation and cell death (Khaspekov et al., 1999; Alano et al., 2002; Precht et al., 2005; Abramov and Duchen, 2008). Here, we
show that in vivo mPTP activation is reversible. Furthermore, contrary of the prediction that mPTP would open during reperfusion (due to elevation of ROS or Ca\textsuperscript{2+}) (Matsumoto et al., 1999; Frantseva et al., 2001; Domanska-Janik et al., 2004; Halestrap, 2006), our experiments demonstrate that mPTP opens during occlusion and closes during reperfusion. In our previous in vivo findings (Murphy et al., 2008), there was no secondary elevation of Ca\textsuperscript{2+} within 2 h of reperfusion consistent with the recovery of \(\Psi m\) we observe. Perhaps, only brief mPTP opening is enough to release factors from mitochondria (or allow factors to enter mitochondria) that may trigger later cell death.

\textit{mPTP activation and dendritic structural damage are mechanistically separable.}

Previous evidence has demonstrated that CsA is neuroprotective in ischemia models (Frantseva et al., 2001; Domanska-Janik et al., 2004). However, due to non-specific effects of CsA such as calcineurin inhibition (Halestrap, 2006), it is not clear if the neuroprotective effects are specifically due to the inhibition of mPTP. Our findings indicate that CsA inhibition of mPTP does not result in a measurable reduction in structural damage to dendrites within 2 h after stroke. Indeed most research on the neuroprotective effects of CsA examined animals at time-points days after stroke that may reflect slower apoptotic processes (Matsumoto et al., 1999; Kobayashi et al., 2003; Domanska-Janik et al., 2004). Our current findings combined with previous literature (Friberg et al., 1998; Abramov and Duchen, 2008) suggest that inhibition of mPTP activation during stroke does not reduce structural damage during occlusion or shortly after reperfusion. However, mPTP opening during ischemia may trigger delayed
mechanisms for cell injury or death. Indeed, evidence of mPTP opening leading to activation of apoptotic pathways supports this hypothesis (Domanska-Janik et al., 2004; Precht et al., 2005; Cheung et al., 2006) (see Supplementary Fig. A2.2 for schematic).

Recent in vivo evidence using FK-506 has suggested that calcineurin-induced actin depolymerization is the chief mechanism of dendritic blebbing observed after kainate-induced seizures (Zeng et al., 2007). However, here, using two separate compounds we demonstrate that calcineurin inhibition did not result in a significant reduction in dendritic structural damage. This suggests that calcineurin-induced actin depolymerization likely plays a minor role in dendritic blebbing in transient global ischemia, but may selectively affect other aspects of seizure-induced damage.

CONCLUSION

Reversible changes in mitochondrial function as well as synaptic structure and function occur in vivo during the first minutes of transient ischemia (Murphy et al., 2008). With prompt reperfusion dendritic structural abnormalities can exhibit significant recovery (Murphy et al., 2008; Li and Murphy, 2008). However, it is conceivable that mitochondrially-mediated signals that lead to later cell death are already initiated making it important for future neuroprotective treatments to be geared towards both management of acute ion and water imbalance that lead to deranged structure (Andrew et al., 2007; Greenwood et al., 2007; Li and Murphy, 2008; Risher et al., 2009) as well as blocking apoptotic events (Cheung et al., 2006; Iyirhiaro et al., 2008).
Synchronous $\Psi_m$ collapse occurs during ischemia and coincides with the $\Psi_p$ depolarization. A, B and D are recorded from the same animal. A) Top, change in Rh123 fluorescence within layer I (<50 μm from the pial surface). Rh123 fluorescence changes were calculated frame by frame relative to a control stack taken before ischemia. Cortical
DC EEG recording is shown on the same time scale. B) Two-photon images (2μm sections) of Rh123 fluorescence at the indicated times before (pre), during and after ischemia. In this animal reperfusion occurred at t=371s. C) Linear correlation plot of the latency from the time of ischemic onset to the onset of Rh123 fluorescence elevation versus the time point when \( \mathcal{V} \) depolarization reached maximal rate of depolarization \((n=14\) ischemic trials in 8 animals, \( R=0.96 \)). D) Graph of two strokes triggered in the same animal 2 h apart leading to a similar Rh123 fluorescence elevation.
Figure 2.2.

CsA inhibition of mPTP opening selectively blocks $\Psi_m$ depolarization during transient global ischemia. A) Rh123 fluorescence in the presence and absence of CsA. Fluorescence percentage change was calculated from frame by frame ratio relative to a control stack taken before ischemia (control n=11 stroke trials, CsA n=5; P < 0.0001 two-way ANOVA). B) Group data comparing the maximal change in Rh123 fluorescence during of ischemia (Control n=14, CsA n=5 **P = 0.0024 by two-way t-test). FK-506 treatment had no effect compared to control (FK-506 n=5 P=0.74). CsA treatment was significantly different compared to FK-506 treatment (*P=0.028). C) Example DC filtered EEG recording (bottom; 10 Hz lowpass) of $\Psi_p$ depolarization during ischemia in
an animal treated with CsA. D) Group data comparing the latency measured from the start of ischemia to the when $\Psi_p$ depolarization reached the maximal rate of depolarization. Application of CsA or FK-506 did not significantly change the latency of $\Psi_p$ depolarization (control n=13, CsA n=11 p = 0.19, FK-506 n=11 P=0.83). CsA treatment also showed no significant difference compared to FK-506 treatment (P=0.27).

E) Group data comparing the total amplitude of $\Psi_p$ depolarization. CsA application reduced the amplitude of the $\Psi_p$ depolarization (control n=11, CsA n=9 *P= 0.027, two sample t-test) when compared to controls while FK-506 treatment did not (FK-506 n=11 P=0.20).
No effect of mPTP inhibition by CsA on dendritic blebbing. A) Maximal intensity projection images (4μm) from a mouse with GFP labeled dendrites and treated with CsA. Dendrites blebbed during ischemia and recovered over time during reperfusion. B) Group data comparing dendritic blebbing in CsA treated, FK-506, treated and control mice. Dendritic blebs are quantified before occlusion (pre), immediately before the onset of reperfusion (occ), and at time points after reperfusion onset. By assessing non-drug treated controls, changes in blebed dendrite percentages during/after stroke were significantly different from before stroke (control n=4, one-way ANOVA, *p<0.05). No significant difference in dendritic blebbing in CsA (n=4 P=0.092 two-way ANOVA) or FK-506 (n=4 P=0.462 two-way ANOVA) treated animals compared to controls.
REFERENCES


CHAPTER 3

Project 2: Differential regulation of cell proliferation in neurogenic zones in mice lacking cystine transport by xCT. ²

² A version of this chapter has been published. Liu, R., Brown, C. and Murphy, T. (2007) Differential regulation of cell proliferation in neurogenic zones in mice lacking cystine transport by xCT, Biochem Biophys Res Commun. 364:528-33
INTRODUCTION

In mammals, neurogenesis persists throughout adulthood in two regions: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation (Kempermann et al., 1997) and the subventricular zone (SVZ) of the lateral ventricles (Luo et al., 2006). In both neurogenic regions, these cells arise from stem cell precursors, migrate towards their respective targets, differentiate and become integrated into the existing circuitry (Winner et al., 2002; Kempermann et al., 2003). Although it is not entirely certain what functional role these new neurons play, growing evidence suggest that these cells participate in normal processes such as spatial learning, memory storage, and olfactory discrimination (Gould et al., 1999; Tashiro et al., 2006; Shapiro et al., 2007). In addition, alterations in the rate of cellular proliferation have also been implicated in the etiology of psychiatric and neuropathological conditions such as depression (Sahay and Hen, 2007) and epilepsy (Parent et al., 1997). Considering the potential importance of neurogenesis in multiple aspects of normal and abnormal brain functioning, it is essential that we understand what molecular mechanisms regulate the ongoing proliferation of these cells.

Anti-oxidant proteins play an essential role in the survival of brain cells exposed to various metabolic and oxidative challenges (Orrenius et al., 2007), therefore it would seem likely that they could also influence the production of new cells (Sleeper et al., 2002). Indeed, studies have shown that certain conditions known to promote oxidative stress such as traumatic brain injury (Dash et al., 2001) and ischemia (Liu et al., 1998), greatly impact rates of adult neurogenesis. Glutathione (GSH) is a major cellular
antioxidant that plays a pivotal role in a cell’s defence against oxidative stress (Martensson et al., 1991). A decrease in cellular GSH levels increases the brain’s susceptibility to oxidative injuries (Mizui et al., 1992; Bains and Shaw, 1997). The Na\(^+\) independent cystine-glutamate exchange antiporter (xCT) uptakes cystine which subsequently participates in the maintenance of intracellular cyst(e)ine which is essential for GSH production (Miura et al., 1992; Sato et al., 2005; Shih et al., 2006). xCT can be detected in astrocytes and developing neuronal preparations, although its levels are a factor of 10 higher in the meninges and the ependymal cells of periventricular regions (Sato et al., 2002; Shih et al., 2006). Consistently, in vivo imaging of fluorescent indicators for GSH show that it is highly enriched in the ependymal cells of the lateral ventricle, and the subgranular cell layer of the hippocampus (Sun et al., 2006). Therefore, the abundance of GSH in the developing brain and its enrichment in neurogenic regions of the adult suggest that factors involved in the regulation of GSH, such as xCT, may be important for cell proliferation (Shih et al., 2006).

The subtle gray pigmentation mutant phenotype (Sut) is a natural truncation mutation in the gene Slc7a11 (gene coding for the light chain of xCT transporter), leading to non-functional xCT protein (Chintala et al., 2005). In vitro work suggests that xCT is critical for cell proliferation given that melanocytes (Chintala et al., 2005), fibroblasts (Sato et al., 2005), astrocytes and meningeal (Shih et al., 2006) cells do not proliferate without the addition of the antioxidant β-mercaptoethanol (β-ME) which reduces extracellular cystine to cysteine allowing it to bypass xCT transport. Here, we utilized Sut mice to determine
what role, if any the xCT exchanger protein plays in cellular proliferation in neurogenic regions of the adult brain *in vivo*.

**MATERIALS AND METHODS**

*Animals.*

All experiments were approved by the University of British Columbia Animal Care Committee and were conducted in strict accordance with guidelines set by the Canadian Council on Animal Care. Sut mice breeding pairs were obtained from Dr. R. Swank (Roswell Park Cancer Institute, Buffalo, NY) (Chintala et al., 2005). The C3H/HeSnJ control background strain was obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the University of British Columbia Animal Care Facility in a 12 h light/dark cycle with food and water ad libitum.

*Injections and tissue processing.*

Labelling of new cells in the adult brain was accomplished by administering two intraperitoneal injections (2 hours apart, see Fig. 3.1A) of the thymidine analog bromodeoxyuridine (BrdU, 100 mg/kg, Sigma-aldrich). BrdU was dissolved at a concentration of 10mg/ml in 0.9% NaCl (w/v).

To examine rates of cellular proliferation, all mice were sacrificed 24 h after the first BrdU injection. Mice were deeply anaesthetized using pentobarbital (100mg/kg) and perfused intracardially through the left ventricle with 10 ml of phosphate buffered saline
(PBS, 0.9% NaCl in 0.1 M phosphate buffer, pH 7.4) followed by 10 ml of phosphate buffered 4% paraformaldehyde (PFA). Brains were removed and post-fixed for 2 days in PFA at 4°C, then immersed in 30% sucrose solution for another 2 days. Brains were cut frozen, at 40 μm in the coronal plane on a sliding microtome and collected into a series of 6 wells containing PBS with 0.02% sodium azide.

BrdU immunohistochemistry.

Free-floating sections were denatured by incubation in a solution containing 50% deionized formamide (Sigma-Aldrich) and 50% 2X saline citrate buffer (SSC, 0.9% NaCl in 0.03 M saline citrate buffer, pH 7.6) at 65°C for 2 h. After a wash in SSC buffer, sections were immersed in 2N HCl at 37°C for 30 min. To eliminate endogenous peroxidase activity, sections were treated with 0.3% H$_2$O$_2$ in dH$_2$O for 10 min. For BrdU immunolabeling, sections were incubated in primary antibodies raised against BrdU (mouse anti-BrdU, 1:1000 dilution, Sigma-Aldrich) with 2% normal horse serum (Vectastain) in PBS containing 0.3% Triton X-100 (PBS+), overnight at room temperature. Sections were then immersed in secondary antisera (biotinylated horse-anti-mouse IgG, 1:1000 dilution, Vectastain ABC kit) in PBS+ at room temperature, followed by 1 hour incubation in avidin-horseradish peroxidase complex (1:1000 dilution, Vectastain ABC kit) at room temperature. BrdU labelled nuclei were visualized by incubating sections in chromagen solution containing 0.02% diaminobenzadine, 0.08% nickel chloride and 0.009% H$_2$O$_2$ in 0.1M Tris buffered saline for approximately 1-2 minutes at room temperature. Sections were then washed 3 times in PBS at room
temperature, mounted, dehydrated and coverslipped using Permount (Fisher Scientific).

Data analysis.

To assess the role of xCT in cellular proliferation, an observer blind to experimental condition would count BrdU positive nuclei in the SVZ and DG using a Zeiss Axiophot microscope in bright field mode using a 40X objective (NA = 0.75). Cells in the uppermost focal plane were excluded to reduce double counting of split nuclei. For the SVZ, labelled nuclei were counted along the lateral walls of the lateral ventricles for a total of 5 sections per mouse, beginning at 1.18mm anterior of bregma (every 6th section was counted). For the DG, all BrdU labelled cells within 2 cell diameters from the inner edge of the granule cell layer (GCL) of the DG were included in the analysis. Due to systematic differences in brain size between genotypes, quantification of cell proliferation rates were expressed as the total number of BrdU positive nuclei per unit area. Measurements of brain area and morphology were performed using NIH Image J software (v1.35) from images taken using 4X objective lens on a Zeiss Axiphot microscope equipped with a 12-bit digital camera (Retiga EXi, Q imaging).

Results are presented as the mean ± SEM. Statistical analysis of raw data was performed with Microsoft Excel. Comparisons between groups were done using two-tailed Students t-tests. Probability values less than 0.05 were deemed statistically significant.
RESULTS

Subventricular zone and dentate gyrus structure.

The Sut mice have previously been reported to have alterations in brain morphology (Sun et al., 2006). Therefore morphological measurements of the SVZ and DG GCL in 3 and 11 month old mice were first performed. There were no differences between mice in SVZ thickness, however, SVZ length and area were reduced in Sut mice relative to the wild type C3H control (Table 3.1, 3 month n = 8, 11 month n = 5). In the DG, Sut mice showed a reduction in GCL length, an increase in thickness, but were comparable in area when relative to C3H mice (Table 3.2, 3 month n = 8, 11 month C3H n = 5, Sut n = 4). We also measured cortical thickness, as well as hemispheric, hippocampal, and lateral ventricular area. Our results are in agreement with previous findings showing a reduction in the size of the Sut mouse brain compared to its background strain control C3H (Shih et al., 2006) (data not shown).

Cellular proliferation in the subventricular zone and dentate gyrus.

To examine the effect of xCT disruption on cellular proliferation, BrdU labelling was examined in the SVZ 24 h after two systemic injections of BrdU (Fig 3.1 A). BrdU is a thymidine analogue that becomes incorporated into the DNA of cells undergoing S phase of mitotic division (Gratzner, 1982). Consistent with previous reports (Luo et al., 2006), BrdU labelled nuclei were found throughout the lateral portions of the SVZ (Fig 3.1B-E). Quantitative analysis of BrdU labelling in the SVZ did not reveal a significant effect of genotype on cell proliferation in either 3 (t(14)=1.983, p=0.067, n = 8) or 11 (t(8)=1.336,
p=0.218, n = 5) month old mice (Fig. 3.1F,G). However, in both 3 and 11 month old Sut mice, there was a trend towards fewer BrdU labelled nuclei in the SVZ relative to wild-type C3H mice (12.1% and 9.4% reduction relative to controls in 3 and 11 month olds, respectively). The fact that proliferation rates did not differ significantly between genotypes (when normalized to area) suggests that xCT mediated cystine uptake is not an absolute requirement for cell proliferation in the SVZ in vivo.

Twenty-four hours after BrdU injection, immunopositive nuclei were also found along the subgranular zone of the dentate gyrus (Fig 3.2 A-D), a second major zone of neurogenesis in adult rodents. In sharp contrast to our results in the SVZ, labelling of proliferating cells in the SGZ was significantly increased in Sut mice at 3 months of age (Fig. 3.2E, 21.6% increase relative to controls, t(14)=2.978, p=0.01, n = 8). At 11 months, there was a trend towards more labelled nuclei in the SGZ of Sut mice, although this did not reach statistical significance (Fig. 3.2F, t(7)=2.232, p=0.061, C3H n = 5 Sut n = 4).

**DISCUSSION**

*Morphological and cell proliferation alterations.*

It is evident from *in vitro* studies that xCT plays a pivotal role in cellular proliferation (Chintala et al., 2005; Sato et al., 2005; Shih et al., 2006). The present study has examined the *in vivo* involvement of xCT in cell proliferation in neurogenic zones of the adult mouse brain. Despite an effect *in vitro*, we report relatively modest changes in the
rates of cell proliferation measured \textit{in vivo} using BrdU labelling after a 24 h period. A simple explanation for these findings is that compensatory mechanisms may support cell proliferation despite a lack of xCT mediated cystine transport.

Past research has shown that Sut mice display signs of atrophy such as enlarged ventricles and reduced overall brain size (Shih et al., 2006). We suspected that the morphology of neurogenic zones was also altered. The SVZ of Sut mice has the same thickness, but showed a reduction in length and consequently a reduction in overall area. In the DG, the GCL of Sut mice showed a reduction in length, an increase in thickness and a trend toward a reduction in overall area. Thus, these results parallel previous findings of brain atrophy observed in the Sut mice (Shih et al., 2006). In the SVZ, the absolute BrdU cell count is reduced in the Sut mice; however, changes in cell density are less prominent due to the reduced area of the Sut mice SVZ. On the contrary, in the DG Sut mice shown an increase in absolute BrdU cell count and a similar increase was seen in their overall GCL BrdU density.

\textit{Regional specific expression of xCT.}

xCT deficient Sut mice show a modest reduction in SVZ cell proliferation, but a significant increase in DG neurogenesis. These differential effects on proliferation in the two neurogenic regions studied may be accounted for by the specific distribution of xCT in the brain as well as the region specific differences in exposure levels to oxidative stress. Using in situ hybridization, RT-PCR and immunoblotting, xCT has been detected
in both ependymal cells of the lateral ventricles, as well as in the hippocampus (Sato et al., 2002; Burdo et al., 2006; Shih et al., 2006; La Bella et al., 2007). Interestingly, in regions bordering the CSF-brain barrier (ependymal cells), xCT expression levels were more than 10-folds higher than elsewhere in the brain possibly due to a requirement for local GSH production (Sato et al., 2002; Burdo et al., 2006; Shih et al., 2006; La Bella et al., 2007). Thus, xCT expression levels near the SVZ are much greater than that in the SGZ of the DG. Therefore, functional loss of xCT could have a larger impact on SVZ cell proliferation than SGZ cell proliferation as we observed in Sut mice.

Evidence supporting compensatory mechanism for oxidative stress.

The observation that cultured Sut mouse astrocytes, meninges (Shih et al., 2006) and fibroblasts (Sato et al., 2005) do not initially proliferate in vitro suggests that compensatory mechanisms are effective in vivo that allow xCT to be dispensable. Interestingly, with Sut astrocytes and meninges grown in vitro, compensatory mechanisms become effective after 1 week in culture, and the cells can survive independently of the reductant β-ME (Shih et al., 2006). Additionally, cultured xCT deficient mouse cells also showed a low velocity cystine transport via a Na+ dependent mechanism resistant to inhibition by glutamate an xCT blocker (Sato et al., 2005). Lastly, it is evident that xCT deficient mice have lower plasma (Sato et al., 2005) and cellular (Shih et al., 2006) GSH. This suggests that Sut mice are more likely to be in a state of oxidative stress that could induce antioxidant pathways such as Nrf2 activation. Nrf2 is a transcription factor, which upon activation will trigger anti-oxidant pathways (Shih et al.,
2003; Mann et al., 2007). Thus, the loss of xCT function could trigger a global compensatory mechanism, which would explain the elevation of cell proliferation in the DG SGZ. The elevation in oxidative stress in xCT deficient mice may also be associated with increased cell death. This may consequently trigger a regeneration mechanism as reflected by the elevation in cell proliferation in the DG of Sut mice. A similar phenomenon has been reported in ischemic stroke, where oxidative stress is elevated and DG neurogenesis is increased (Yagita et al., 2001; Zhang et al., 2001; Nakatomi et al., 2002).

**CONCLUSION**

In conclusion, we have shown that the natural loss of functional xCT transport activity in Sut mice was accompanied by relatively normal rates of cellular proliferation in the SVZ, and an enhancement of cell production in the DG. These results implicate xCT in the regulation of hippocampal neurogenesis and demonstrate that, unlike previous *in vitro* studies, the absence of cystine transport via xCT is not an essential component of ongoing cell production in the adult brain.
**FIGURES**

*Figure 3.1.*

BrdU labelling of proliferating cells in the SVZ 24 hours after injection. (A)

Experimental design for proliferation studies. All mice received two injections of BrdU (i.p. 100mg/kg) and were sacrificed 24 h from the time of the first injection. (B, C) Low-magnification brightfield images showing anterior-posterior profile of BrdU labelling in the SVZ of wild-type C3H and Sut mice (each section is approximately 480 μm apart). (D, E) Higher-magnification images (20X objective) of boxed regions in B and C. (F, G) Quantitative analysis of BrdU positive nuclei in the SVZ of 3 and 11 month old mice.
Figure 3.2.

Role of xCT transporter on cellular proliferation in the DG.

(A-D) Brightfield images showing BrdU immunopositive nuclei decorating the inner lining of the DG in both wild-type C3H and Sut mice. The granule cell layer is outlined for clarity (E, F). Histograms showing the significant increase in cell proliferation in the DG of Sut mice at 3 months, but not at 11 months of age. *p < 0.01.
**TABLES**

*Table 3.1.*

Subventricular zone area, length and thickness in 3 (n = 8) and 11 month (n = 5) old mice.

<table>
<thead>
<tr>
<th></th>
<th>SVZ area (mm$^2$)</th>
<th>SVZ length (mm)</th>
<th>SVZ thickness (mm)</th>
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</thead>
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<tr>
<td>3 month</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H</td>
<td>0.0838 ± 0.0016</td>
<td>1.85 ± 0.029</td>
<td>0.0453 ± 0.00086</td>
</tr>
<tr>
<td>SUT</td>
<td>0.0765 ± 0.0017</td>
<td>1.70 ± 0.050</td>
<td>0.0451 ± 0.00051</td>
</tr>
<tr>
<td>P value</td>
<td>0.0082</td>
<td>0.00088</td>
<td>0.86</td>
</tr>
<tr>
<td>11 month</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H</td>
<td>0.0785 ± 0.0024</td>
<td>1.85 ± 0.049</td>
<td>0.0425 ± 0.0013</td>
</tr>
<tr>
<td>SUT</td>
<td>0.0750 ± 0.0026</td>
<td>1.72 ± 0.026</td>
<td>0.0437 ± 0.0016</td>
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<tr>
<td>P value</td>
<td>0.343</td>
<td>0.106</td>
<td>0.562</td>
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Table 3.2.

Dentate gyrus granule cell layer area, length and thickness in 3 (n = 8) and 11 month old mice (C3H n = 5, Sut n = 4).

<table>
<thead>
<tr>
<th></th>
<th>SVZ area (mm²)</th>
<th>SVZ length (mm)</th>
<th>SVZ thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 month</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H</td>
<td>0.819 ± 0.015</td>
<td>4.16 ± 0.055</td>
<td>0.197 ± 0.0032</td>
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<tr>
<td>SUT</td>
<td>0.774 ± 0.016</td>
<td>3.74 ± 0.065</td>
<td>0.207 ± 0.0025</td>
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<tr>
<td>P value</td>
<td>0.0552</td>
<td>0.0002</td>
<td>0.0412</td>
</tr>
<tr>
<td>11 month</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H</td>
<td>0.778 ± 0.029</td>
<td>4.31 ± 0.105</td>
<td>0.180 ± 0.0035</td>
</tr>
<tr>
<td>SUT</td>
<td>0.738 ± 0.042</td>
<td>3.86 ± 0.075</td>
<td>0.191 ± 0.0079</td>
</tr>
<tr>
<td>P value</td>
<td>0.45</td>
<td>0.0137</td>
<td>0.20</td>
</tr>
</tbody>
</table>
REFERENCES


Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727-3738.
CHAPTER 4

DISCUSSION

Discussion overview

The general objective of project 1 was to determine the timing and mechanism of mitochondrial membrane potential (\( \psi_m \)) depolarization \textit{in vivo} during transient global ischemia. The general objective of project 2 was to determine the effect of reduced glutathione levels on cell proliferation within the neurogenic zones, using a xCT mutant mouse model. This study provides insights into cellular pathology induced by oxidative stress in animals with reduced oxidative defence, reflecting aspects of oxidative defence in stroke-related damage (Doyle et al., 2008). Therefore, while the two projects are independent, the general goals of the two projects intersect, as they both utilize a “systems” approach to better understand the pathological processes involved in ischemic injury.

The majority of the discussion focuses on project 1, as it constitutes the majority of the research contribution. The discussion for project 2 will therefore be brief.
PROJECT 1 DISCUSSION:

Reversible cyclosporine A sensitive mitochondrial depolarization occurs within minutes of stroke onset in mouse somatosensory cortex in vivo, a two-photon imaging study.

4.1.1 Advantages of experimental approach

A major advantage of our approach is that two-photon microscopy allows visualization of the sequence of cellular and subcellular events in vivo with high spatial and temporal resolution (Svoboda and Yasuda, 2006; Murphy et al., 2008). With this technique, we are able to map out the precise timing of $\Psi_m$ depolarization during transient global ischemia. Also, local drug bath applications directly over the cortex allowed us to examine the mechanism of $\Psi_m$ depolarization while minimizing interference from systemic side effects of drug application. The use of two-photon imaging to monitor blood flow allows accurate confirmation of ischemia and eliminates possible artefacts generated by inconsistency in ischemia severity (Zhang et al., 2005). Finally, our ability to examine multiple physiological parameters simultaneously allows an accurate assessment of the temporal relationship between interrelated events during stroke (Murphy et al., 2008).

4.1.2 Possible limitations

Rhodamine 123 (Rh123) is a lipophilic cationic probe that is non-selectively membrane permeating (Nicholls and Ward, 2000). Consequently, Rh123 labelling is not cell-type specific. Previous studies have shown heterogeneity in brain mitochondrial response when challenged with $\text{Ca}^{2+}$, possibly reflecting differences in mitochondrial responses in
differing cell types (Kristian et al., 2002). A limitation of our study is the inability to distinguish which cell types displayed $\Psi_m$ depolarization during ischemia.

Quantitative comparisons of the magnitude of Rh123 intensity change are difficult to make across animals. This is because various factors during stroke can change the relative signal, including changes in background fluorescence, differences in the alteration of vascular structure within the region of interest, and regional focus shifts. Consequently, our technique only reveals when $\Psi_m$ depolarization occurs. The magnitude of $\Psi_m$ loss cannot be determined.

Our EEG (electroencephalogram) recordings are not specific to the region of two-photon imaging, since the recording electrode is embedded in the agarose above the craniotomy. Since agarose has high conductance, the electrode detects signal from the entire cranial window, and likely from other brain regions. Therefore it is difficult to precisely determine when plasma membrane ($\Psi_p$) depolarization occurs within the two-photon imaging area using EEG data. It must therefore be noted that our EEG recordings are reflective of a large brain area, and that some events seen on the EEG may not necessarily reflect cortical activity of the area of cortex imaged by the two-photon microscopy.

4.1.3 Timing of $\Psi_m$ depolarization

The overall purpose of this experiment was to determine the timing and mechanism of
mitochondrial dysfunction during and immediately after transient global ischemia. $\Psi_m$ was used as an indicator of mitochondrial function (Benel et al., 1989). Our primary finding was that mPTP (mitochondrial permeability transition pore) drives mitochondrial depolarization during ischemia in vivo and recovers rapidly after reperfusion onset, these findings are supported by some previous in vitro findings (Frantseva et al., 2001; Alano et al., 2002; Abramov et al., 2007; Abramov and Duchen, 2008). However, a previous study demonstrated that mitochondria isolated from the brain after reperfusion onset generate reactive oxygen species (ROS) (Friberg et al., 2002). Moreover, pharmacological mPTP inhibition after reperfusion onset is neurprotective (Matsumoto et al., 1999) and reduces cytochrome C release (Domanska-Janik et al., 2004). These studies suggest that mPTP activation and mitochondrial dysfunction occurs during reperfusion. Indeed, activation of mPTP after ischemia is shown in cardiac myocytes (Hausenloy et al., 2003; Halestrap et al., 2004).

In our experiments, Rh123 is responsive during reperfusion as confirmed by its elevation during the second stroke. During this time, no $\Psi_m$ depolarization was detected. It is possible that there is a delayed $\Psi_m$ depolarization occurring at a time beyond our 2 h range of monitoring. Alternatively it is possible that in the study by Friberg and colleagues (Friberg et al., 2002), mitochondria once isolated from the brain behave differently than in vivo. Furthermore, in some studies (Matsumoto et al., 1999; Domanska-Janik et al., 2004) systemic administration of drugs to inhibit mPTP may have resulted in neuroprotective effects due to non-specific effects; subsequently, it is possible
that no mitochondrial dysfunction or mPTP activation occurred in the brain at any time after reperfusion onset in these studies.

4.1.4 $\text{Ca}^{2+}$ involvement in $\Psi_m$ depolarization

$\text{Ca}^{2+}$ influx during ischemia causing mitochondrial $\text{Ca}^{2+}$ overload has been proposed as a mechanism for $\Psi_m$ depolarization (Halestrap and Brenner, 2003; Starkov et al., 2004). In agreement with this, we observed that $\Psi_m$ depolarization occurred concurrently with $\Psi_P$ depolarization. Our observations that $\Psi_m$ depolarization had identical kinetics to intracellular $\text{Ca}^{2+}$ elevation (Murphy et al., 2008) also supports this proposed mechanism. In addition, previous research has showed that mPTP inhibition by cyclosporine (CsA) is $\text{Ca}^{2+}$ dependent (Halestrap, 2006). Therefore, inhibition of $\Psi_m$ depolarization by CsA further supports the role of $\text{Ca}^{2+}$ in $\Psi_m$ depolarization.

4.1.5 Resolving inconsistent findings in brain mPTP studies

Previous studies suggest that mPTP activation via $\text{Ca}^{2+}$ could be the predominant mechanism for $\Psi_m$ depolarization in vivo during transient global ischemia (Uchino et al., 1998; Baines et al., 2005; Schinzel et al., 2005). Moreover, CsA can inhibit this pathway. Much of this mechanism was initially discovered in non-neuronal cell types (Halestrap, 2006); nonetheless, there are previous in vitro studies in neurons that support this mechanism (Alano et al., 2002; Greenwood et al., 2007; Abramov and Duchen, 2008).

However, some in vitro studies provide evidence that do not support this mechanism;
these reports suggest that neuronal mitochondria and neuronal mPTP demonstrate unique pathologies. These studies show that isolated neuronal mitochondria, when challenged with Ca\(^{2+}\) insult, require far higher Ca\(^{2+}\) levels for mPTP activation compared to non-neuronal cell types. In addition, neuronal mitochondria appear to have a different depolarization profile and do not undergo swelling. Also, neuronal mitochondria seem to be less sensitive to CsA rescue. Lastly, CsA does not block neuronal mitochondria cytochrome C release, suggesting release is through an mPTP-independent mechanism (Andreyev and Fiskum, 1999; Kristian et al., 2002; Kobayashi et al., 2003; Vergun and Reynolds, 2005). These studies suggest the possibility that mitochondrial pathology in the brain deviates from the classic mechanism outlined in cardiac myocytes (Hausenloy et al., 2003; Halestrap et al., 2004). Furthermore, they raise the question whether mPTP activation, \(\Psi_m\) depolarization or mitochondrial swelling indeed occur in the brain during ischemia. Lastly, they question the role of mPTP activation in apoptotic pathways in the brain.

Activation of mPTP can be influenced by various physiological parameters including Ca\(^{2+}\) levels, ATP/ADP concentration, pH, \(\Psi_m\) and ROS. These parameters are dynamic and complex during ischemia (Halestrap et al., 1997; Sheldon and Church, 2002; Basso et al., 2005). Disparate results may be explained by the inability to produce experimental parameters that mimic the dynamic parameters generated during ischemia \textit{in vivo}. For example, differences in ATP/ADP concentration can drastically alter mPTP opening probabilities when challenged with Ca\(^{2+}\) (Halestrap et al., 1997). In addition, unique
mitochondria interaction with the host cell, interactions with surrounding cells, and brain regional specificity all can contribute to differences observed in studies *in vitro* versus *in vivo*. Thus, it is likely that parameters in ischemia *in vivo* are sufficient for mPTP activation and $\Psi_m$ depolarization. Furthermore, there is *in vivo* evidence suggesting that brain mitochondria mPTP activation indeed induces cytochrome C release (Domanska-Janik et al., 2004). Studies have also shown that mPTP activation also releases other apoptosis-inducing factors further supporting the role of mPTP activation in apoptosis (Precht et al., 2005).

**4.1.6 Mitochondrial depolarization *in vivo* is mono-phasic**

As briefly discussed in chapter 2, it is interesting that we only observed one phase of $\Psi_m$ depolarization during ischemia, since *in vitro* studies show that $\Psi_m$ depolarization during excitotoxic ion overload is biphasic (Alano et al., 2002; Abramov and Duchen, 2008). In our proposed mechanism, elevated intracellular cations are sequestered by the mitochondria, therefore this process will dissipate $\Psi_m$. Indeed, *in vitro* studies show that the initial $\Psi_m$ depolarization is gradual and is caused by $\Psi_m$ dissipation due to ion ($\text{Ca}^{2+}$ and $\text{Zn}^{2+}$) sequestering. The latter $\Psi_m$ collapse phase is abrupt and attributed to the activation of mPTP (Alano et al., 2002; Abramov and Duchen, 2008; Medvedeva et al., 2009).

In our experiments, complete inhibition of Rh123 elevation by CsA *in vivo* suggests that mPTP activation is the predominant contributor to $\Psi_m$ collapse, and the initial phase
observed in vitro does not appear to contribute to the Rh123 elevation we observed in vivo. However, in the presence of CsA, cations should still be sequestered by the mitochondria during ischemia (Alano et al., 2002; Abramov and Duchen, 2008); nonetheless, we do not observe a loss in $\Psi$m due to this cation sequestration. There are three possible explanations for this. 1) Lower levels of cations are sequestered by the mitochondria in vivo during ischemia than in vitro; this may be due to reduction in voltage potential difference between mitochondrial matrix and the cytosol, or differences in intracellular cation dynamics in vivo versus in vitro. 2) Mitochondria may also be better at maintaining $\Psi$m in vivo than in vitro upon sequestering of cations during ischemia. 3) Lastly, $\Psi$m change induced by cation sequestration in vivo may be too small, such that mitochondrial matrix is still relatively negative compared to the rest of the cell; consequently an insufficient amount of Rh123 is released and de-quenched for detection.

4.1.7 Therapeutic mechanism of mPTP inhibition

Previous research suggests that mPTP inhibition is neuroprotective during ischemia (Uchino et al., 1998; Khaspekov et al., 1999; Matsumoto et al., 1999; Frantseva et al., 2001; Schinzel et al., 2005). Our findings indicate that CsA inhibition of mPTP does not result in a measurable reduction in structural damage to dendrites within 2 h after ischemia. Indeed most research on the neuroprotective effects of CsA examined animals at time points in the order of days after stroke and may reflect slower apoptotic processes (Uchino et al., 1998; Khaspekov et al., 1999; Matsumoto et al., 1999; Frantseva et al., 2001; Schinzel et al., 2005). Interestingly, Friberg and colleagues (Friberg et al., 1998)
documented that when the mPTP was inhibited by CsA, animals killed immediately after insulin induced hypoglycemia showed mitochondria with healthy morphology but like the control group they had swollen dendrites. In this study, CsA treated animals examined 1 week after induced hypoglycaemia showed significant improvement in dendritic structure compared to controls. Our current findings combined with previous work (Friberg et al., 1998; Abramov and Duchen, 2008) suggest that inhibition of mPTP activation during stroke does not reduce structural damage during occlusion or shortly after reperfusion. Furthermore, our findings, combined with previous research (Domanska-Janik et al., 2004; Precht et al., 2005), suggest that mPTP activation plays a critical role in apoptosis which is detectable only at later time points.

4.1.8 Significance of mitochondrial findings

This study is significant in the field of mitochondria pathology because it is the first time-lapse study to directly assess the immediate responses of $\Psi_m$ in response to an abrupt insult, in \textit{in vivo} intact alive tissue. This study allows better understanding of when and how mitochondria dysfunction occurs in ischemia and brings previous \textit{in vitro} evidence into context of a whole animal setting. This work also helps us understand the early mitochondrial pathology that leads to the later endpoints observed with \textit{in vivo} histological and biochemical methods.

4.1.9 Significance of findings with respect to ischemia research

In the context of broader ischemia pathology, this research improves understanding of the
early events during stroke (discussed below). Stoke is a very abrupt occurrence and early events are complicated and interrelated. Our research helps elucidate how mitochondrial dysfunction fits in with mechanisms of other stroke events during this time. Moreover, it helps us understand how the mechanisms underlying these early events lead to delayed cell death in ischemia (Hossmann, 2006).

4.1.10 Implications of finding: $\Psi_p$ depolarization

A large $\Psi_p$ depolarization occurs as glutamate release activates ionotropic glutamate receptors resulting in a massive cation influx. Previous evidence suggests that massive $\text{Ca}^{2+}$ influx from this event causes mitochondrial $\text{Ca}^{2+}$ overload and dysfunction. However, since energy failure is the initial trigger for glutamate release and since $\Psi_m$ is required for energy production, an alternative proposal is that $\Psi_m$ depolarization is upstream of glutamate release (Di Filippo et al., 2008).

In our experiments, because the EEG electrode may receive signals from outside of the cranial window, we cannot distinguish which event ($\Psi_m$ or $\Psi_p$ depolarization) occurs first by comparing EEG data with Rh123 fluorescence. However, when $\Psi_m$ depolarization is inhibited by CsA, $\Psi_p$ still occurs and with unaltered latency from onset of ischemia. This suggests that $\Psi_m$ depolarization occurs downstream of glutamate release. This indicates that, in the presence of CsA, $\Psi_m$ was maintained but sufficient energy failure still occurred to cause glutamate release. This further suggests that early during ischemia, prior to $\Psi_m$ depolarization, significant energy failure has already started.
to disrupt $\mathcal{V}_p$. This also implies that during ischemia, sustained $\mathcal{V}_m$ prior to the $\mathcal{V}_m$ collapse is not an indicator of sustained energy production.

An alternative probability is that CsA may have inhibited or reduced energy failure and attenuated the initiation of $\mathcal{V}_p$ within the cranial window. However, since the CsA is applied locally to the cranial window, excitotoxic waves initiated from outside the cranial window can trigger ion influx and glutamate release (Nishizawa, 2001) in tissues inside the cranial window such that any effect of CsA is masked.

4.1.11 Implications of findings: oxidative stress during ischemia

When and how ROS are generated during ischemia is unclear. It is proposed that mitochondria play a role in ROS generation in ischemic injury (Starkov et al., 2004). In vitro time-lapse imaging in neurons demonstrates that there are three waves of ROS generation during anoxia and reoxygenation. $\mathcal{V}_m$ depolarization is suggested to be responsible for the first wave which occurs during anoxia (Abramov et al., 2007). A separate in vitro pharmacology study demonstrates that mPTP activation is responsible for mitochondrial ROS production in neurons (Frantseva et al., 2001). Our finding provides in vivo support for this hypothesis. Thus, in support of these in vitro studies, it is likely that mPTP induced $\mathcal{V}_m$ depolarization generates ROS in vivo during ischemia and this mechanism contributes to ischemic injury.

Other previous research show delayed generation of ROS by mitochondria at time points
of hours to days after transient ischemia (Friberg et al., 2002). It is possible that a delayed \( \Psi_m \) depolarization occurred at a time beyond our 2 h monitoring range. On the other hand, it is also possible that these observed delayed ROS generation does not require mPTP activation or significant loss of \( \Psi_m \).

4.1.12 Implications of findings: dendritic blebbing

The mechanism leading to dendritic damage in ischemia is clearly multi-factorial. In vitro, facing glutamate challenge, activation of any type of glutamate receptor can induce dendritic blebbing (Greenwood et al., 2007). This suggests that ion influx is likely the cause of dendritic blebbing. One in vitro study demonstrated that during glutamate challenge, dendritic blebbing can be caused by Na+ induced water influx resulting in microtubule damage (Greenwood et al., 2007). Alternatively, in a seizure study, Ca\(^{2+}\) activation of calcineurin inducing actin depolymerization was suggested to be the mechanism of dendritic blebbing (Zeng et al., 2007). The present experiments indirectly tested the calcineurin inducing actin depolymerization pathway for dendritic blebbing.

With the application of two calcineurin inhibitors (FK 506 and CsA), our results suggest that calcineurin-induced actin depolymerization likely does not play a major role in dendritic blebbing during transient global ischemia.

Previous studies suggest a relationship between mitochondrial function and dendritic blebbing (Friberg et al., 1998). In vitro, when challenged with glutamate, \( \Psi_m \) depolarization is temporally associated with dendritic blebbing. Moreover, this study also
demonstrated that maintaining $\psi_m$ improves the recovery of dendritic blebbing after glutamate insult (Greenwood et al., 2007). In our experiments, rescuing $\psi_m$ did not result in any structural protection during ischemia or improvement in dendritic structural recovery during reperfusion. Moreover, $\psi_m$ and dendritic blebbing occurs at a similar time but have different recovery kinetics. This suggests that there may be a common trigger mechanism for both events, but the two mechanisms are separable pathways.

Inhibition of glutamate receptors was insufficient to block dendritic blebbing in vivo. Previous attempts to inhibit NMDA or AMPA/kainate receptors have failed to inhibit blebbing during ischemia, in vivo (Murphy et al., 2008). A recent in vivo pilot study conducted in our laboratory, using a high concentration of MK801 and CNQX to simultaneously inhibit both NMDA and AMPA/kainate receptors still resulted in dendritic blebbing during ischemia (unpublished data). In these glutamate receptor antagonist studies, $\psi_p$ depolarization persisted during ischemia, suggesting that ion influx still occurred.

Previous failed attempts to inhibit dendritic blebbing in vivo during ischemia suggest that there are likely multiple mechanisms that induce ion influx and contribute to dendritic blebbing during ischemia. It is possible that ion influx must be inhibited in order to block dendritic blebbing. In all previous in vivo experiments mentioned above, $\psi_p$ depolarization still persisted. Other non-glutamate mediated pathways such as acid-sensing ion channels and volume-sensitive channels also may induce ion influx (Xiong et
al., 2004; Takano et al., 2005). Consequently, combinatorial pharmacology blocking multiple pathways simultaneously will likely be required to inhibit dendritic blebbing during ischemia.

4.1.13 Implications of findings: Ca^{2+}, apoptosis and delayed cell death

It is known that brief global ischemia can generate delayed cell death (Olsson et al., 2003; Yonekura et al., 2004). Recent research from our laboratory has shown that in transient global ischemia, various pathological parameters are reversible upon reperfusion (Murphy et al., 2008). However, seemingly recovered neurons with relatively normal synaptic properties still suffer delayed death via apoptosis (Gao et al., 1998). Recent experimental treatment with a cyclin-dependent kinase inhibitor (flavopiridol) combined with an anti-apoptotic/anti-inflammatory (minocycline) showed effective protection toward delayed cell death; however, synaptic impairment persists (Iyirhiaro et al., 2008). These findings suggest that although various ischemia induced parameters are reversible upon reperfusion, during brief periods of ischemia pathological mechanisms are activated that trigger delayed cell injury or death at a later time-points.

As previously discussed, mitochondrial dysfunction can lead to cell death. Various reports have shown that mPTP activation is associated with the release of cytochrome C and other apoptotic factors (Domanska-Janik et al., 2004; Precht et al., 2005). Our findings show that mPTP activation occurs during ischemia, and is reversible. Since mPTP activation occurs approximately 1-2 min after ischemia onset and we induced
ischemia of 6.5 min duration, it is conceivable that during a short period of 4-5 min, enough apoptotic factors are released through mPTP to cause delayed cell death.

This suggests two possibilities. 1) The amount of apoptotic factors released is directly proportional to the duration of mPTP opening. This would provide a direct link between stroke duration and the severity of delayed cell death. 2) Alternatively, apoptotic factors release could follow a complex threshold response of mPTP opening. In this case, our findings would suggest that 4-5 min of mPTP opening is adequate to reach the threshold to allow sufficient release of apoptotic factors to cause delayed cell death, similar to that observed in previous experiments of brief ischemia (Olsson et al., 2003; Yonekura et al., 2004).

4.1.14 Reperfusion is associated with the recovery of mitochondrial function

Reperfusion is proposed to be associated with additional injury after the initial ischemic episode (Sugawara et al., 2004). Immediate events during reperfusion have traditionally been difficult to study in vivo, due to the limitations of histological and biochemical techniques. Using novel two-photon in vivo time-lapse imaging techniques, previous findings in common carotid artery occlusion (CCAO) (Murphy et al., 2008) and middle cerebral artery occlusion (Li and Murphy, 2008) models in mice have shown that reperfusion is associated with recovery of various ischemia induced parameters such as Ca\textsuperscript{2+} elevation, dendritic structural damage, and cortical spontaneous activity. Our findings showing the rapid recovery of $\Psi$m and the closing of mPTP is consistent with
these previous observations. It is possible that other forms of injury, such as ROS generation, occurred during reperfusion but were not assessed. Indeed previous studies have attributed reperfusion with ROS production (Frantseva et al., 2001; Friberg et al., 2002). Alternatively, our relatively short time window of monitoring may have prevented observation of prolonged effects observed in other studies.

4.1.15 Proposed in vivo mechanism

We hypothesize that the transient $\Psi_m$ depolarization during ischemia could play a critical role in delayed cell death. During ischemia, energy impairment results in glutamate release leading to an aberrant ion influx, resulting in $\Psi_p$ depolarization (Nishizawa, 2001). Elevated intracellular $\text{Ca}^{2+}$ is sequestered by mitochondria, resulting in mitochondrial $\text{Ca}^{2+}$ overload and the activation of mPTP. Consequently, the activation of mPTP allows the exchange of molecules between mitochondrial matrix and cytoplasm resulting in the collapse of $\Psi_m$ (Halestrap, 2006). Previous studies suggest that ATP depletion results in failure of $\text{Ca}^{2+}$ ATPase which can further contribute to intracellular $\text{Ca}^{2+}$ elevation (Ohta et al., 1996). The transient period of mitochondrial dysfunction and mPTP opening results in the release of various pro-apoptotic factors, ultimately triggering delayed cell death (Halestrap, 2006).

4.1.16 Future experiments and directions: examining mitochondria morphology during ischemia in vivo

Previous in vitro evidence shows that neuronal mitochondria swell when challenged with
glutamate (Greenwood et al., 2007). Alternative studies show that when challenged with \( \text{Ca}^{2+} \) neuronal mitochondria uniquely do not swell (Andreyev and Fiskum, 1999; Kobayashi et al., 2003). Whether neuronal mitochondria undergo morphological change during transient global ischemia \textit{in vivo} is unclear.

Rh123 fluorescence cannot be used to examine mitochondria morphology. Rh123 is released from the mitochondria upon \( \psi_m \) depolarization leading to de-quenching (Benel et al., 1989). This property makes Rh123 unsuitable to examine mitochondria morphology during ischemia. An alternative fluorescent probe that may be used is mitotracker (M7514; Molecular Probes, Eugene, OR), a high affinity mitochondrial probe used to examine mitochondrial morphology \textit{in vitro} (Koopman et al., 2006). Our attempts to examine mitochondrial morphology \textit{in vivo} using the mitotracker probe were unsuccessful. The application of mitotracker resulted in no labeling beyond a depth of 1-2 \( \mu \text{m} \) of the cortex when bath applied directly to the cortex, and subsequent attempts with micro-injection resulted in insufficient signal intensity for two-photon imaging.

An alternative approach to examine mitochondrial morphology during ischemia \textit{in vivo} is through the utilization of recently developed transgenic mice (MitoMice) expressing CFP or YFP selectively in neuronal mitochondria. Transgene expression is found within neurons of the somatosensory cortex (Misgeld et al., 2007).

Therefore, using these transgenic mice a proposed series of experiments would be the
examination of changes in mitochondrial morphology during transient global ischemia using our in vivo imaging methodology. These mice may also aid in the study of mitochondrial mobility before, during and after transient ischemia.

4.1.17 Future experiments and directions: mitochondria and free radicals

Oxidative stress is suggested to contribute to ischemic injury, and mitochondrial dysfunction is linked to generation of free radicals (Frantseva et al., 2001; Friberg et al., 2002). The precise timing of free radical dynamics in vivo during ischemia and reperfusion is unknown. Furthermore, it is possible that the $\Delta m$ depolarization we observed contributes to the generation of ROS during ischemia.

Previous attempt to examine ROS using a free radical probe 2-Cl-dichlorofluorescein diacetate (DCFDA; C6827; Molecular Probes, Eugene, OR) have proven to be very difficult. DCFDA irreversibly becomes fluorescent when oxidized by ROS (Xie et al., 1999). Upon micro-injection into the brain, this probe was easily converted into the oxidized form and frequently became completely oxidized before ischemia was induced. Thus, for these experiments a more stable free radical indicator is required, which will remain responsive for a longer period of time.

The proposed experimental paradigm would involve micro-injection of an appropriate probe into the brain, and utilizing the CCAO model we can perform in vivo time-lapse imaging of ROS generation during ischemia and reperfusion using two-photon
Another proposed experiment would involve application of CsA to rescue $\Psi_m$ depolarization, allowing us to examine the effect on ROS generation during CCAO. This would allow us to examine the role of $\Psi_m$ in ROS generation during ischemia.

4.1.18 Broader applications of techniques utilized

The success of monitoring $\Psi_m$ in vivo during ischemia can be applied to examining mitochondrial function in other diseases. Mitochondrial dysfunction has been suggested to be involved in various other neurological disorders such as epilepsy (Kudin et al., 2009), Alzheimer’s disease (Gibson et al., 2008) and Parkinson’s disease (Gibson et al., 2008). Therefore, the techniques used here can easily be applied for time-lapse examination of mitochondrial behavior in various other animal models of neurological disorders in vivo.

4.1.19 Biological relevance

The physiological relevance of CCAO is similar to that of cardiac arrest and resuscitation. Our findings suggest that during a brief heart attack or medical procedures such as bypass surgery, short interruption of blood flow can lead to global mitochondrial dysfunction. Consequently, this suggests that even in these brief events, cell injury or death pathways are being activated. Indeed various neurological disorders are attributed to brief periods of cerebral ischemia (Roach et al., 1996; Newman et al., 2004).
PROJECT 2 DISCUSSION:

Differential regulation of cell proliferation in neurogenic zones in mice lacking cystine transport by xCT.

4.2.1 Advantages of experimental approach

The subtle gray pigmentation mutant phenotype (Sut) strain of mice used in these experiments have a natural truncation mutation resulting in the malfunction of the cystine-glutamate antiporter (xCT) (Chintala et al., 2005), allowing for a non-invasive, highly specific approach to inhibit a major pathway of glutathione (GSH) production. In addition, cystine/glutamate exchanger (xCT) is expressed in the neurogenic areas of the adult brain (Shih et al., 2006). Consequently, this transgenic mouse strain provides a unique model to examine of the effects of reduced plasma and cellular GSH on cell proliferation in adult neurogenic zones in vivo (Sato et al., 2005; Shih et al., 2006).

4.2.2 Possible limitations

Bromodeoxyuridine (BrdU) is an analogue of the nucleic acid thymidine, and is incorporated into growing DNA strands during DNA synthesis (Gratzner, 1982). Consequently, BrdU labels all proliferating cells regardless of cell type. Evidence suggests that different cell types have different degrees of vulnerability toward oxidative stress (Feeney et al., 2008). In our approach we cannot detect if any cell types are selectively affected in Sut mice.
We only examined cell proliferation within adult neurogenic zones 24 h after BrdU injection. Labelled cells therefore were less than 24 h in age. Any deleterious effects of reduced GSH during the cellular developmental phase after 24 h would have gone undetected in the present work.

4.2.3 Anti-oxidant production in Sut mice in vivo

Given that GSH is high in neurogenic areas, and that xCT is critical for cell proliferation in vitro (Shih et al., 2006), it was surprising that Sut mice showed no reduction in cell proliferation within neurogenic zones in vivo.

Induction of compensatory anti-oxidant pathways may contribute to rescuing of proliferating cells in vivo. Previous in vitro findings suggest that at one week after birth, cells with dysfunctional xCT can survive without exogenously applied anti-oxidants, suggesting that alternative anti-oxidant mechanisms can contribute to cell survival at early stages of cell development (Shih et al., 2006). Indeed, cultured xCT-deficient cells show a low velocity glutamate independent cystine transport mechanism (Sato et al., 2005).

Further, reduced plasma (Sato et al., 2005) and cellular (Shih et al., 2006) GSH results in elevated oxidative stress, likely inducing transcription factor Nrf2 which subsequently triggers various anti-oxidant pathways (Shih et al., 2003; Mann et al., 2007).

Consequently, this mechanism can cause xCT deficient proliferating cells to become more resistant to oxidative stress. This presents a possible explanation of our results (Chapter 3).
Another possible explanation of our results is that, in vivo, newly proliferating cells are surrounded by more mature cells which have functional alternative anti-oxidant mechanisms. Thus, it is likely that these alternative mechanisms are constantly generating anti-oxidants which stabilize extracellular redox buffering, ultimately contributing to the protection of newborn cells from oxidative stress.

4.2.4 The role of cell death in xCT deficient mice

Anti-oxidant proteins are important for the survival of brain cells exposed to various oxidative insults (Orrenius et al., 2007). Previous work has shown that various xCT deficient cell types demonstrate increased vulnerability to oxidative stress (Shih et al., 2006). Thus it is possible that in vivo, this deleterious effect becomes more prominent at later time-points in the cell’s survival. It is also possible that, although proliferation is not altered in the Sut mice, cell death is increased resulting in net loss of cells. Indeed, it has been shown that xCT deficiency is associated with the activation of the c-Jun N-terminal kinase pathway leading to apoptosis (Qiao et al., 2008). It is also possible that prolonged elevation of oxidative stress in Sut mice results in accumulation of DNA damage (or other accumulative injuries) leading to cell death (Mates et al., 2008). This could explain brain atrophy observed in Sut mice, as well as the atrophy of neurogenic zones (Shih et al., 2006).

The elevation in dentate gyrus (DG) neurogenesis in Sut mice may also be explained by
increased cell death, where increased cell death leads to reactive cell proliferation.

Indeed, it has been shown that after ischemia, cell proliferation increases only in areas of the brain where ischemia was severe enough to cause cell death (Salazar-Colocho et al., 2008). Thus this supports the possibility that elevated cell death may result in atrophy of the DG, and may also trigger an elevation in cell proliferation as a reactive mechanism to injury.

4.2.5 Significance of findings

This study contributes to the field of oxidative stress research since we demonstrate the considerable plasticity of anti-oxidant production pathways in response to oxidative stress within the living brain. In the context of broader ischemia pathology, this research sheds light on compensatory anti-oxidant defence which can play a neuroprotective role during ischemia.

4.2.6 Implications of findings: oxidative stress during ischemia

Oxidative stress is proposed to be a chief contributor to ischemic injury (Hossmann, 2006). During ischemia and reperfusion, GSH as well as other anti-oxidants levels are exhausted (Slemmer et al., 2008). Our research supports the hypothesis that during ischemia, oxidative stress and other ischemia induced events such as cell death likely triggers repair mechanisms such as alternative anti-oxidant production (Shih et al., 2003; Mann et al., 2007) and region-specific cell proliferation (Salazar-Colocho et al., 2008).
4.2.7 Implications of findings: the role of xCT in excitotoxicity

xCT may also play a critical role in excitotoxicity. Since xCT is a cystine-glutamate antiporter, its activity affects extracellular glutamate concentrations. It is possible that physiological or elevated xCT expression can contribute to excitotoxicity. During ischemia, depletion of GSH will cause xCT to increase cystine uptake in order to elevate GSH production; this will subsequently result in an increase in glutamate expulsion into the extracellular space (Warr et al., 1999).

Alternatively, it is possible that xCT deficiency can also enhance excitotoxicity. It has been previously shown in Drosophila that deficiency in xCT results in reduced extracellular glutamate levels, and leads to increase postsynaptic glutamate receptor expression (Augustin et al., 2007). Increases in postsynaptic glutamate receptor expression will likely result in an enhanced susceptibility to excitotoxicity.

4.2.8 Future experiment 1: examining long-term survival rates and cell fates of newly generated cells in Sut mice

Our experiments examined cell viability 24 h after birth. It is possible that elevated oxidative stress in Sut mice may result in altered survival rates at later time-points. Moreover, it is also possible that reduced oxidative defences may selectively induce proliferation of cell types that are more resistant to elevated oxidative stress, and reduce production of cell types that are less resistant.
To address these questions, future experiments which examine levels of newly generated cells weeks to months after initial BrdU labelling will shed light on the long-term survival of cells generated within neurogenic zones. The application of immunohistological double labelling with either neuronal or glial sub-type specific markers will reveal the cellular identity of labelled cells.

4.2.9 Future experiment 2: examining the role of xCT in neuronal injury during transient global ischemia in vivo

Anti-oxidant proteins are important in the survival of brain cells exposed to oxidative insults (Orrenius et al., 2007). Supporting this is the finding that xCT deficient cells are more sensitive to oxidative stress in vitro (Shih et al., 2006). An important question arising is whether xCT plays a neuroprotective role during ischemia in vivo.

An approach to directly address this question is to examine dendritic blebbing in vivo in response to transient global ischemia induced in xCT-deficient mice. To perform these experiments, however, Sut mice must be crosses with mice of C57Bl6 background expressing green florescent protein/yellow florescent protein (GFP/YFP) labeled dendrites. Using techniques discussed in Chapter 2, the role of xCT in dendritic damage during ischemia or recovery during reperfusion may be examined for the first time in vivo.
4.2.10 Broader implications of findings

Cyst(e)ine and intracellular GSH have been associated with the general rate of cell growth and proliferation (Noda et al., 2002). For instance, fast growing cancer cells, which typically demonstrate high levels of oxidative stress, show reduced proliferation rates upon inhibition of xCT (Chung et al., 2005). Consequently, our study sheds light on \textit{in vivo} compensatory anti-oxidant mechanisms that may play a role in general cell growth and proliferation throughout the body.
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APPENDIX 1

Improvements to the common carotid artery occlusion model for *in vivo* imaging

During the course of the work described here, I have made various improvements to improve the quality and the success rate of the common carotid occlusion experiments.

*A1.1 Technical improvements*

Previously, once mounted onto the microscope stage the mouse is oriented with its rostral end towards the microscope. Consequently, the experimenter cannot visualize the entire length of the constricting suture, making it difficult to estimate its tension. Too much tension applied leads to complete severing of the CCA, and conversely, when too little tension is applied the CCA is not properly occluded. For these experiments, I constructed an adapter plate that allows the mouse to be mounted such that it faces the experimenter. This adjustment allows the experimenter to directly view the suture, and greatly facilitate suture tension adjustments. This improvement has essentially eliminated irreversible damage to the CCAs and under-tensioning in my experiments - previously occurring in ~30% of experiments.

*A1.2 Surgery improvements*

Occasionally, mice die of heart failure during occlusion. Previously, during surgery
sutures were looped around the CCAs and then crossed over the trachea. This was performed to allow the CCA to be pressed against the trachea for optimal occlusion (Murphy et al., 2008). I found that animals with uncrossed sutures had reduced rates of heart failure during ischemia and comparable levels of ischemia. The reduced rate of heart failure is possibly due to reduced pressure on the carotid bodies at the CCA bifurcations (Torres, 2009). This improvement has significantly reduced the occurrence of heart failure from ~40% to ~20% of subjects.

A1.3 Selective breeding

I have observed that mice from parallel parental lines have similar vasculature. By examining the quality of blood-flow occlusion during two-photon imaging, I have subsequently bred mice with parents or littermates that showed substantial blockage. This has allowed us to select animals more likely to have an incomplete Circle of Willis. Therefore, selective breeding has led to a reduction in the number of experiments with incomplete occlusion of blood flow due to the existence functional posterior communicating arteries in a sub-population of C57Bl7 mice (Ginsberg and Busto, 1989).

A1.4 Temperature regulation

Differences in brain temperature have been previously shown to affect numerous parameters in stroke research (Hoesch and Geocadin, 2007). In my work, I have carefully documented the cortical temperature and further designed a thermo-regulation system to ensure consistent physiological cortical temperature. Brass tubing was constructed over
the head-plate to warm the head plate and the area around the craniotomy including the physiological solution surrounding the water immersion objective. Cortical surface temperature was maintained at 37°C by perfusing warm water through the brass tubing using a heating water pump. This system therefore minimizes potential non-specific effects caused by fluctuations in cortical temperature.
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SUPPLEMENTARY METHODS

A2.1 Animals

All mice were bred at the University of British Columbia animal facilities. All experimental anesthesia was achieved by intraperitoneal injections of urethane (0.12% w/w) as in (Zhang et al., 2005). A heat exchanger was used to warm the head plate and the area around the craniotomy including the physiological solution surrounding the water immersion objective. Cortical surface temperature was maintained at ~37 °C by pumping 38 °C water through the heat exchanger using a water pump (T/Pump; TP500; Gaymar, Orchard Park, NY). Urethane supplementation for maintenance of anesthesia as well as saline injections with glucose for hydration and nutrition were administered as previously described in (Murphy et al., 2008).

A2.2 Surgery and imaging procedures

Mice were attached to a custom made head-hold and a cranial window was made over the somatosensory cortex. Detailed surgical procedures for cranial window preparation for in vivo two-photon imaging are as previously described (Zhang et al., 2005). Due to the inability of rhodamine 123 (Rh123; R302; Invitrogen, Carlsbad, CA) to cross the dura (observed in our experiments) the dura was removed from all preparations to permit direct application of compounds to cortex. Instrumentation for two-photon laser scanning
and procedure for image acquisition was as previously described in (Murphy et al., 2008). All fluorophores were excited at 900nm. For all in vivo time-lapse imaging, multiple z-series of 20 images were taken during ischemia and reperfusion. Each image was the average of three frames taken over 5.5s.

A2.3 Common carotid artery occlusion and stroke induction

One suture (5–0, silk; Ethicon, Somerville, NJ) was surgically looped around each common carotid artery (CCA) as previously described in (Murphy et al., 2008). However, the sutures were not crossed over the trachea as previously performed (Murphy et al., 2008). We found that animals with uncrossed sutures had reduced rates of heart failure during stroke, possibly due to reduced pressure on the carotid bodies at the CCA bifurcation. Sutures were pulled by hand to apply pressure on the CCAs and secured with tape to induce occlusion and released for reperfusion. Over the duration of the stroke, two-photon imaging of texas-red dextran confirmed blood flow was blocked immediately upon occlusion of the CCAs and remained occluded for the duration of the stroke (Supplementary Fig. A2.1B). Our previous two-photon measurements indicated that the common carotid artery occlusion (CCAO) is sufficient to generate a >90% reduction in cortical blood flow within seconds of tensioning the sutures (Murphy et al., 2008). In some animals a second stroke was induced. For measurements of Rh123 fluorescence or EEG quantification both first and second stroke trials were pooled (Fig. 2.1,2.2). All statistics comparing data from first and second stroke trials showed no significant difference (P>0.05) supporting the pooling of two stroke trials within the same animal.
For dendritic structural analysis only the first stroke from each animal was used.

**A2.4 Electroencephalogram recording**

Electroencephalogram (EEG) recording was performed as previously described (Murphy et al., 2008). One electrode was inserted into the agarose within the craniotomy, one on the back between the scapula and one ground electrode on the foot. A surface DC potential EEG was recorded as previously described in (Murphy et al., 2008). After AC filtering (0.1-10Hz band pass) or collection of the EEG in an AC mode a large slow ripple oscillation on the EEG recording was temporally correlated with maximal rate of plasma membrane potential (\( \Psi_p \)) depolarization (Fig. A2.1C) (Murphy et al., 2008). In 4 stroke trials that lacked DC EEG recording (because of larger offsets) AC mode recording was used to estimate the time when \( \Psi_p \) depolarization reached maximal rate of depolarization. The Mouse-Ox pulse oximeter (Starr Life Sciences, Oakmont, PA) was used in some animals to monitor heart rate and oxygen saturation and was mounted on the tail (Murphy et al., 2008).

**A2.5 Mitochondria membrane potential imaging**

Rh123 fluorescence is used to determine mitochondrial membrane potential (\( \Psi_m \)) potential. Rh123 is concentrated by the mitochondria due to the negative \( \Psi_m \) and at high local concentration Rh123 fluorescence becomes auto-quenched. Upon \( \Psi_m \) collapse it is dispersed and becomes de-quenched leading to an increase in fluorescence. A stock solution of Rh123 20mg/ml (52.5 mM) was made in anhydrous DMSO and diluted in
HEPES-buffered artificial CSF to a working solution of 8.2 μg/ml (20μM). Rh123 is directly applied to the craniotomy for 1 h and washed before imaging. Imaging of Rh123 was done within the layer I of the cortex, typically within 50 μm of the pial surface. The maintained presence of Rh123 within mitochondria after stroke was confirmed by monitoring basal Rh123 fluorescence after reperfusion. We were also able to trigger a second Rh123 fluorescence elevation during a second stroke indicating the maintained presence of responsive dye.

A2.6 Assessing dendritic changes

The dendritic tufts of GFP and YFP labelled layer 5 neurons are examined for structural analysis. CCAO can induce shifts in the vertical position of the cortex making it difficult to examine only a single image plane. Therefore, to ensure that we examined the same dendrites throughout the experiment despite small vertical position shifts, stacks of 20 images with z-spacing of 2μm were taken.

A2.7 Pharmacology

Cyclosporine A (CsA; C 3662; Sigma, St. Louis, MO) was stored in solid form at 4°C. Immediately prior to an experiment, CsA was dissolved into anhydrous DMSO 25mg/ml and subsequently diluted in HEPES-buffered artificial CSF to a working concentration of 20μM. CsA at 20μM was bath applied directly to the craniotomy for 1 h and mixed into the agarose at 10μM for sustained application, given that we have only applied the drug to the surface we expect that the actual concentration within the tissue would be
significantly lower than 10 μM. An FK-506 (F4679; Sigma, St. Louis, MO) stock solution was prepared in anhydrous DMSO 20mg/ml and stored at 4°C. During the experiments, it was diluted to a working concentration of 50μM in HEPES-buffered artificial CSF. FK-506 was bath applied directly to the craniotomy for 1 h and mixed into the agarose at 50μM for sustained application.

A2.8 Intrinsic optical signal imaging

Intrinsic optical signal imaging (IOS) imaging was performed to map the somatosensory cortex of each animal so that all two-photon imaging could be done in the posterior region of the forelimb representation (Supplementary Fig. A2.1D). IOS recording procedures and instruments used were as previously described (Murphy et al., 2008). Contralateral forelimb and hindlimb functional maps were recorded at a focus depth of 200μm below the cortical surface.
Figure A2.1.

Experimental procedures and monitored parameters. A. Experimental schematics. Each incubation was 1 h and each period of ischemia was ~6.5 min with 2 h between strokes. B, Example of two-photon imaging of blood flow using texas-red dextran showing before (Pre) and immediately after the onset of occlusion (Occ). The movement of the red blood cells resulted in horizontal dark streaks when scanned by the laser (Pre). Upon occlusion, movement of the red blood cells significantly slowed down as indicated by the lack of dark streaks (Occ). C, Example AC filtered EEG recording (top; 0.1-10 Hz bandpass) showing the suppression of slow wave cortical activity at the immediate onset of ischemia. Example DC filtered EEG recording (bottom; 10 Hz lowpass) showing the $\Psi_p$ depolarization during ischemia and a period of repolarization after reperfusion onset. The appearance of the AC ripple (top) occurred at the time point when $\Psi_p$ depolarization (bottom) reached maximal rate of depolarization. D, Example of IOS image taken before
stroke, showing the sensory representation of forelimb (green) and hindlimb (red) over the somatosensory cortex. Two-photon imaging during CCAO was always performed in the bottom left corner of the forelimb representation as indicated by the black box.
Figure A2.2.

Proposed *in vivo* mechanism summary of mitochondrial depolarization in transient global ischemia. 1) Ischemia causes aberrant ion influx. Ion influx results in 2) $\Psi_p$ depolarization and 3) dendritic structural damage. 4) mitochondrial Ca\(^{2+}\) overload results in 5) activation of mPTP by CypD. 6) CsA can bind to CypD to inhibit the Ca\(^{2+}\) activation of mPTP. 7) activation of mPTP allows the exchange of molecules between mitochondrial matrix and cytoplasm resulting in the collapse of $\Psi_m$. 8) ATP depletion result in inactivation of Ca\(^{2+}\) ATPase. 9) Mitochondrial dysfunction could result in the activation of intracellular signals that trigger delayed cell death by apoptosis or necrosis.
REFERENCES


APPENDIX 3

UBC Research Ethics Board’s Certificates of Approval
ANIMAL CARE CERTIFICATE

Application Number: A05-0933

Investigator or Course Director: Timothy H. Murphy

Department:

Animals:

- Mice YFPH.2Jrs 150
- Rats Wistar 150
- Mice GFP-M 150
- Mice C57BL6, 50
- Mice Nrf/- 100

Start Date: July 1, 2005

Approval Date: September 16, 2008

Funding Sources:

Funding Agency: Canadian Stroke Network (CSN) - Networks of Centres of Excellence (NCE)
Funding Title: Neuroprotection: Preventing cell death and neuronal damage from stroke

Funding Agency: UBC Faculty of Medicine
Funding Title: Erika White Research Seed Funding: Laser based optical interrogation of brain circuits associated with recovery from stroke damage in live mice

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: In vivo 2-photon imaging of acute and chronic alterations in synapse structure associated with ischemia provides insight into mechanism of stroke damage and recovery

Funding Agency: Canadian Stroke Network (CSN) - Networks of Centres of Excellence (NCE)
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Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: In vivo 2-photon imaging of acute and chronic alterations in synapse structure associated with ischemia provides insight into mechanism of stroke damage and recovery

Funding Agency: Heart and Stroke Foundation of British Columbia and Yukon
Funding Title: Using in vivo imaging to resolve if, when, and where excitotoxicity and oxidative stress occur during stroke

Funding Agency: Canadian Institutes of Health Research (CIHR)
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<td><strong>Funding Title</strong></td>
<td>Structural plasticity and electrophysiology in cortical neurons imaged during recovery from stroke</td>
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<td><strong>Funding Agency</strong></td>
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<td><strong>Funding Title</strong></td>
<td>Determining the rules for high frequency synaptic signaling at CNS synapses</td>
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<td>Calcium dependent control of CNS synapse development and NMDA receptor function</td>
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The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

*A copy of this certificate must be displayed in your animal facility.*

Office of Research Services and Administration  
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3  
Phone: 604-827-5111 Fax: 604-822-5093
## ANIMAL CARE CERTIFICATE

**Application Number:** A05-0966  
**Investigator or Course Director:** Timothy H. Murphy  
**Department:** Psychiatry  
**Animals:**

<table>
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<td>Mice GFP-M 200</td>
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<td>Rats Wistar 500</td>
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**Start Date:** April 1, 2004  
**Approval Date:** March 5, 2009

**Funding Sources:**

**Funding Agency:** Canadian Institutes of Health Research (CIHR)  
**Funding Title:** Postsynaptic mechanisms that regulate synapse development  

**Funding Agency:** UBC Faculty of Medicine  
**Funding Title:** Erika White Research Seed Funding: Laser based optical interrogation of brain circuits associated with recovery from stroke damage in live mice

**Funding Agency:** Canadian Stroke Network (CSN) - Networks of Centres of Excellence (NCE)  
**Funding Title:** Neuroprotection: Preventing cell death and neuronal damage from stroke

**Funding Agency:** Canadian Stroke Network (CSN) - Networks of Centres of Excellence (NCE)  
**Funding Title:** Neuroprotection: Preventing cell death and neuronal damage from stroke

**Funding Agency:** Natural Sciences and Engineering Research Council of Canada (NSERC)  
**Funding Title:** LOI Insight into stroke damage and recovery from imaging the synapse

**Funding Agency:** Allon Therapeutics Inc.  
**Funding Title:** Evaluation of the Effect of AI-208 on Synaptogenesis and Dendritic Spine Foundation
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<td>Determining the rules for high frequency synaptic signaling at CNS synapses.</td>
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<td>CSN Project#9: Molecular mechanisms of neuronal injury: Development of a therapeutic strategy for stroke-induced damage</td>
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<td>Saving Neurons During Stroke by Activating a Multi-faceted Antioxidant Defense in the Brain</td>
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<td>In Vivo Imaging of Acute and Chronic Alterations in Synapse Structure Associated with Ischemia Provides Insight Into Mechanism of Stroke Damage and Recovery</td>
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**Unfunded title:** Neuroprotection: Preventing cell death and neuroal damage from stroke

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