MODERATE OR DEEP LOCAL HYPOTHERMIA DOES NOT PREVENT THE ONSET OF ISCHEMIA-INDUCED DENDRITIC DAMAGE

by

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Abstract

Apical dendrites of layer 5 pyramidal neurons can rapidly undergo a structural perturbation within minutes of ischemia onset. This "blebbing" is a morphological phenomenon in which dendrites display regions of spherical and/or ellipsoid swellings, resembling a "beads-on-a-string" appearance. We investigated the acute (up to 2 hours after reperfusion) effects of localised cortical hypothermia, a well-recognised neuroprotective strategy, on ischemia-induced dendritic structural damage. Using *in vivo* two-photon imaging combined with a global ischemia model of occluding the common carotid arteries in C57Bl/6 mice, we monitored in real time these dynamic structural alterations during ischemia and reperfusion. We show that moderate (31°C) and deep hypothermia (22°C) delays but does not block the onset of dendritic blebbing during global ischemia. Deep hypothermic treatment also tended to promote more consistent recovery of dendritic structure during reperfusion. These results suggest that those employing therapeutic hypothermia will need to consider that it does not spare neurons from structural changes that are the result of ischemia, but may interact with mechanisms that control the onset of damage and recovery during reperfusion.

Preface

Sections of this work has been submitted as a Brief Communication. I am the first author in this study having performed the majority of experiments, analysis, and writing up of the manuscript. Dr. Shangbin Chen, a visiting scientist in our lab and Richard Liu, a former graduate student are also co-authors in this work. Dr. Chen assisted with image analysis and Liu had initiated this research project three years ago. Dr. Albrecht Sigler had also assisted with making figures for the paper.

The original version of the image analysis algorithm used in this study as discussed in the Experimental Methods, Results, and Discussion has been published. I collected and performed manual blebbing analysis, contributed in algorithm optimisation, and writing up of the manuscript. The reference for this paper is:

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Experimental protocols were approved by the University of British Columbia animal care committee and are consistent with the Canadian Council on Animal Care and Use guidelines. Animal care training and certificates were obtained for Biology and Husbandry of the Laboratory Rodent (RBH-534-08), Anesthesia of the Laboratory Rodent (RA-275-08), and General Priniciples of Rodent Surgery (RSHX-209-08).

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List of Abbreviations

2P	Two-photon
2VO	Two-vessel occlusion
4VO	Four-vessel occlusion
AC	Alternating current
ACSF	Artificial cerebral spinal fluid
ATP	Adenosine triphosphate
CCA	Common carotid artery
CCAO	Common carotid artery occlusion
DEEP	Deep hypothermia
DC	Direct current
EEG	Electroencephalogram
FLIM	Fluorescence lifetime imaging microscopy
fMRI	Functional magnetic resonance imaging
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
HYPO	Moderate hypothermia
ICH	Intracerebral hemorrhagic stroke

IOS	Intrinsic optical signal
LED	Light-emitting diodes
MCAO	Middle cerebral artery occlusion
NORMO	Normothermia
PComA	Posterior communicating artery
PET	Positron emission tomography
RB	Rose Bengal
SD	Spreading depression
TR	Texas-Red
YFP	Yellow fluorescent protein

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1. INTRODUCTION

1.1 Stroke

Stroke is the leading cause of chronic adult disability and the third leading cause of death in North America. In Canada each year, 50 000 individuals suffer a stroke and over 300 000 Canadians are living with the effects of stroke (Heart & Stroke Foundation of Canada). A stroke results from a reduction in cerebral blood flow to the brain which can arise from thrombosis, embolic obstruction, or systemic hypoperfusion as in the case of ischemia (Doyle et al., 2008) or bleeding from a ruptured vessel in a hemorrhagic stroke (Traystman, 2003). In the case of ischemic strokes, disruption of blood flow can be focal, limited to a brain territory supplied by an artery resulting in an ischemic core surrounded by a hypoperfused penumbra (Hossmann, 2006). In global ischemia, disruption of blood flow occurs throughout most or all of the brain, as what occurs in cardiac arrest (Kawai et al., 1992; Siesjo et al., 1995; Traystman, 2003).

Brain tissue is highly dependent on oxygen and glucose. Weighing just 2% of total body weight, the brain accounts for nearly 20% of total oxygen consumption and utilises 25% of total glucose, requiring these metabolic substrates for energy production through oxidative phosphorylation, most of which is used to maintain neuronal transmembrane gradients by Na^+/K^+- ATPase (Edvinsson and Krause, 2002). Even a few minutes of ischemia is sufficient to deplete neuronal ATP (Folbergrova et al., 1997). Ischemia results in ATP depletion, depolarization of neuronal membrane potential, glutamate excitotoxicity, loss of ion homeostasis, and in particular, the rise in intracellular Ca^{2+} initiates of a series of complex cascades leading to apoptosis, necrosis and eventually functional impairment in affected brain regions (Dirnagl et al., 1999; Traystman, 2003; Hossmann, 2006; Zhao et al., 2007; Doyle et al.,

2008; Murphy and Corbett, 2009). Depending on the severity, duration, and location of ischemia, affected individuals can suffer mild to profound sensory, cognitive, and motor deficits.

1.2 Ischemia-induced dendritic structural plasticity

In addition to these detrimental events, structural changes to dendrites, the components of neural circuits, also result following stroke. A well studied form of ischemia-induced dendritic structural alteration is that of spine plasticity (Brown et al., 2007; Brown et al., 2008; Brown et al., 2009; Mostany et al., 2010). Dendritic spines, small protrusions along the length of dendrites, are the principle sites of excitatory synaptic input to neurons (Fiala et al., 2002). In the mature brain, spines are relatively stable, but show remarkable plasticity following sensory stimulation, environmental enrichment, and disease (Fiala et al., 2002; Alvarez and Sabatini, 2007). Especially in the diseased state, dendritic spines have been the focus of much work since they are believed to be substrates for recovery (Fiala et al., 2002; Alvarez and Sabatini, 2007). As such, studies have described changes in spine turnover and spine formation in the hours (Brown et al., 2007; Brown et al., 2008) and weeks (Brown et al., 2007; Brown et al., 2009; Mostany et al., 2010) after ischemia. Even the dendrite branch undergoes ischemia-induced structural plasticity (Brown et al., 2010). Brown and colleagues have shown that peri-infarct dendrites demonstrate a region-specific modelling such that the branch tips grow away from the site of infarction and those oriented towards the infarct retract in the weeks following stroke (Brown et al., 2010).

1.3 Ischemia-induced dendritic blebbing

Perhaps a more dramatic form of ischemia-induced structural plasticity is what occurs within the first minutes of global (Murphy et al., 2008) or focal (Zhang et al., 2005; Zhang and

Murphy, 2007; Li and Murphy, 2008) ischemia where apical dendrites of pyramidal neurons take on a "beads-on-a-string" appearance (Zhang et al., 2005; Murphy et al., 2008). This "blebbing" or "beading" of dendrites reflects a gross morphological change where the normal dendrite structure becomes "varicose" with localised regions of swelling. Dendritic spines appear to be absorbed into blebs in this process possibly because of ischemia-induced cytoskeletal breakdown (Zhang et al., 2005). A minimal level of circulation appears to be necessary in order to maintain structural integrity and prevent the onset of blebbing. Zhang and colleagues showed that dendrite structure remains unperturbed in the presence of endothelin-induced vasoconstriction which produces moderate reductions in blood flow (~50%) (Zhang et al., 2005). In fact, dendrite damage was not observed until severe reductions in blood flow (~90%) was induced using Rose-Bengal (RB) photothrombosis (Zhang et al., 2005). Additionally, the presence of a nearby flowing vessel (within 80 μm) is enough to keep dendrites intact (Zhang et al., 2007).

Reperfusion appears to aid recovery of dendrite structure following ischemia-induced damage (Li and Murphy, 2008; Murphy et al., 2008). In focal ischemia (MCAO or photothrombosis), blebbing is greatest in the ischemic core and this damage reduces with distance from the site of infarct (Enright et al., 2007; Li and Murphy, 2008). Within the penumbra, improvements in damaged structure can occur with reperfusion (Li and Murphy, 2008), but damage progresses in the core and can spread to the penumbra in the absence of reperfusion (Enright et al, 2007). Even in the case of global ischemia, partial recovery of structure is observed following reperfusion (Murphy et al., 2008).

Dendritic blebbing is not a newly observed phenomenon and was first described over a century ago by Ramon Y Cajal as "segmental swellings in [the] terminal field of hippocampal dendrites" (Cajal, 1959; Garcia-Lopez et al., 2007). When Cajal was initially confronted with

this, he was concerned the observed "segmental swellings" were an artefact due to his fixation method (Cajal, 1959). Recently, such "swellings" have also been visualised using modern imaging techniques combined with GFP-labeling of cells (Feng et al., 2000) and owing to the remarkable stability of GFP over a wide pH range (Cubitt et al., 1995; Ormo et al., 1996), blebs are unlikely to be an artefact of GFP clumping due to pathophysiological changes in pH. Our work and others show that blebbing is a real morphological change in response to neuronal stress and/or excitotoxicity (Park et al., 1996; Hasbani et al., 1998; Obeidat et al., 2000; Kirov et al., 2004; Zhang et al., 2005; Andrew et al., 2007; Zhang et al., 2007; Brown et al., 2008; Li and Murphy, 2008; Murphy et al., 2008; Risher et al., 2010). The observation of dendrite structural abnormality is substantiated through studies which describe blebbing with membrane-bound dyes (DiI) (Park et al., 1996; Hasbani et al., 1998) and histological stains (Golgi-Cox) (Brown et al., 2008). Dendritic blebbing has been observed in cultured neurons (Park et al., 1996; Hasbani et al., 1998) and brain slice where it occurs following hypoxia, oxygen-glucose deprivation, and glutamate excitotoxicity (Obeidat et al., 2000; Andrew et al., 2007). Other than cases of focal and global ischemia (Zhang et al., 2005; Zhang et al., 2007; Li and Murphy, 2008; Murphy et al., 2008), dendritic blebbing occurs between periods of spontaneous spreading depolarizations (Risher et al., 2010) and has also been observed in epilepsy (Isokawa et al., 1997).

Different mechanisms have been proposed to describe dendritic blebbing. The formation of blebs could reflect local changes in volume due to the excessive influx of water and ions following ischemia (Andrew et al., 2007). During glutamate excitotoxicity, the intracellular rise of Na^+ and Cl^- causes water to move intracellularly to maintain osmolarity (Andrew et al., 2007). Interestingly, as dendritic blebbing appears to be localised to apical dendrites in superficial cortical layers, it has been proposed that the surface area to volume ratio of apical dendritic tufts

predispose them to ion fluxes more than larger dendritic segments (Enright et al., 2007). Spatial selectivity in damage may also reflect a laminar distribution of blood vessels (Enright et al., 2007). It is also possible that such blebbing may be a cellular protective mechanism by localising excessive glutamate into dendritic compartments whereby protecting the remainder of the cell (Ikegaya et al., 2001; Greenwood and Connolly, 2007). Indeed, *in vitro* application of low Na^+ solution, ethacrynic acid (inhibitor of the HCO_3^-/Cl^- exchanger), or protease inhibitors which reduce bleb formation induced by glutamate excitotoxicity results in greater cell death (Ikegaya et al., 2001). A change in dendrite morphology likely involves a combination of these effects with an interaction with the neuronal cytoskeleton. In fact, energy deprivation alters the conformation and distribution of actin to focal dendritic swellings (Gisselsson et al., 2005) and loss of MAP-2 (a key component of the dendritic cytoskeleton) following ischemia coincides with the appearance of beaded dendrites (Matesic and Lin, 1994). Finally, from a bioenergetics standpoint, neurons must follow thermodynamic principles in order to maintain optimal functioning (Lyakhov et al., 2002). Thus, because of the high energetic costs associated with maintaining dendrites in their elongated shape (Wong-Riley, 1989), the adoption of spherical and ellipsoid blebs during ischemia may reflect the most stable and lowest energy configuration that a cell can adopt during a time of energy depletion (Dr. Mark Reimers, personal communication).

There are likely functional consequences from structural damage to dendrites. It has been proposed that blebbing acts as a marker for delayed neuronal death (Takeuchi et al., 2005) and may be a sign of involvement of cell death pathway activation (Enright et al., 2007). Indeed, apoptotic and necrotic markers are found to be localised to the same regions as blebbed dendrites (Enright et al., 2007). If there is a relationship between structural damage and apoptosis/necrosis of cells, then treatment with anti-apoptotic agents would be expected to at least rescue cells.

However, surviving cells which are blebbed would have compromised synaptic transmission due to altered morphology (Li and Murphy, 2008) since thin inter-bleb regions would disrupt dendritic cable properties for signal propagation (Segev and London, 2000). Also blocking apoptosis does not always restore synaptic transmission and behaviour after stroke (Iyirhiaro et al., 2008). Based on this evidence, interventions aimed either at preventing the onset of blebbing or recovering dendrite structure can be a viable target in ischemia.

1.4 Neuroprotection

Because of the diverse mechanisms involved in ischemic injury, as discussed herein, it is no wonder that approaches in treating or minimising the effects of ischemia are as diverse (O'Collins et al., 2006). Neuroprotection describes the approaches in protecting neuronal function and/or neurons from eventual apoptosis or necrosis by targeting biochemical and cellular processes both in the acute and chronic phases of ischemia (Hossmann, 2006; O'Collins et al., 2006). Unfortunately, many pharmacological approaches for ischemic neuroprotection have not been successful, especially in the clinical context (O'Collins et al., 2006), and the prevailing view attributes this to the targeting of a single molecule or molecular pathway when ischemia is a complex process involving the interplay of many biomolecules (Dirnagl et al., 1999; Hossmann, 2006; Zhao et al., 2007; Doyle et al., 2008).

1.5 Hypothermia

For over 50 years, hypothermia has been shown to have neuroprotective effects in experimental models of stroke and in the clinical setting (Mayer and Sessler, 2005). It has been considered the “gold-standard” for which other potential therapies and interventions are compared (Krieger and Yenari, 2004). An extensive literature describes a “pan-inhibiting” effect

(Zhao et al., 2007) of hypothermia. Contrary to pharmacological approaches, it is likely that the robust effects of hypothermia are conferred through the action on many ischemia-induced mechanisms (Krieger and Yenari, 2004). The broad-spectrum effects of hypothermia have been proposed to be achieved from the lowering of cerebral metabolic rate (a 1°C reduction in temperature reduces the rate of cellular respiration, oxygen demand and carbon dioxide production by approximately 10%) (Lo et al., 2003). Such metabolic suppression may be responsible for increasing the brain's tolerance to detrimental effects through the slowing or delaying of ischemic processes (Lo et al., 2003). Hypothermia inhibits or reduces detrimental immediate and chronic effects of stroke (Zhao et al., 2007). Hypothermia has been shown to delay ATP depletion and ischemic depolarization, reduce glutamate excitotoxicity, suppress oxidative stress, caspase activity and apoptotic pathways, and inflammation (Nakashima et al., 1995; Nakashima and Todd, 1996; Maier et al., 1998; Xu et al., 1998; Lo et al., 2003; Zhao et al., 2007). The attenuation of these molecular and cellular processes likely contributes to the observation of hypothermia in improving later functional and behavioural outcomes (Corbett et al., 1997; Colbourne et al., 2000; Corbett et al., 2000; Clark et al., 2008; Clark et al., 2009).

Results from studies describing hypothermic neuroprotection in ischemia has prompted work evaluating hypothermia for the treatment in other cases of neurologic injury (Mayer and Sessler, 2005). Hypothermia is effective in improving behavioural and histological outcome after intracerebral hemorrhagic stroke (IHS) (MacLellan et al., 2004). Hypothermia is used to treat fever following neurologic injury, traumatic brain injury, cardiac arrest, myocardial infarction, and to minimise neurologic injury during neurological and cardiac surgery (Mayer and Sessler, 2005). Hypothermic neuroprotection for spinal cord injury (Kwon et al., 2008; Dietrich et al., 2011) is also under investigation.

The extensive work describing the effectiveness of hypothermia has prompted research in optimising application and depth of hypothermia. Hypothermia can be induced either systemically (cooling the full body) or locally over affected brain regions. Systemic hypothermia can be induced chemically by inhalation of H_2S (Florian et al., 2008), by controlling room temperature (Yanamoto et al., 2001), or by spraying water and the use of fans (DeBow and Colbourne, 2003). Generally, whole-body cooling, even with mild hypothermia, is known to cause complications which range from mild (shivering) to more severe side effects on the immune and cardiovascular systems (Schubert, 1995). In contrast, localised hypothermia allows a reduction of brain temperature while maintaining the remainder of the body normothermic and avoids such complications. Because of this, localised hypothermia has been advocated for use clinically (Wagner and Zuccarello, 2005) and much research has been conducted in recent years to validate its safety and efficacy. Indeed, in the case of ischemia, it is brain temperature rather than body temperature that is most important for neuroprotection (Mayer and Sessler, 2005). Non-invasive and invasive methods exist for the induction of focal hypothermia and include the use of cooling blankets placed around the head (Nurse and Corbett, 1994), cooling helmet (Thoresen et al., 2001), and surgical implantation of cooling coils over the skull (Clark and Colbourne, 2007).

The brain is sensitive to small variations in temperature. Whereas reductions in brain temperature improve outcomes, increases (>37 °C) accentuate the effects of stroke (Busto et al., 1987; Dietrich et al., 1990; Ginsberg and Busto, 1998). Effective hypothermia is dependent upon the depth, duration, and time of intervention (Colbourne et al., 1997). The brain appears to tolerate a range in cooling and under controlled conditions, mild (33-36°C), moderate (28-33°C), and deep (~20°C) levels of hypothermia are safe and protective (Mayer and Sessler, 2005).

Acute (hours) and prolonged (days) cooling is effective, but the time of hypothermic intervention likely targets different mechanisms. Time windows for effective hypothermia can be induced before or during ischemia ("intraischemic") (Xue et al., 1992), intraischemic and delayed (Kawai et al., 2000; Hashimoto et al., 2008), and postischemic both early (minutes) (Yanamoto et al., 1996; Kawai et al., 2000; Hashimoto et al., 2008) or late (hours) (Colbourne et al., 2000) into reperfusion. However, intraischemic hypothermia appears to be more effective at neuroprotection than hypothermia induced at other timepoints (Dietrich et al., 1993) especially with respect to behavioural outcomes (Corbett et al., 1997).

1.6 Approaches for studying ischemia *in vivo*

Our lab uses a variety of imaging methods to study acute and chronic outcomes in stroke *in vivo.* The approaches utilized in this study are discussed below.

1.6.1 *In vivo* two-photon imaging

While a number of studies have described blebbing *in vitro* (Hori and Carpenter, 1994; Park et al., 1996; Hasbani et al., 1998; Obeidat et al., 2000; Andrew et al., 2007), we are interested in monitoring blebbing *in vivo*, specifically within apical dendrites of layer 5 pyramidal neurons. Imaging in living specimens allows the study of complex and dynamic biological processes within their native tissue environment (Pawley, 2006). Particularly within the nervous system, *in vivo* imaging is desirable to partial models such as slice preparations which while preserving local circuits, disrupt long range connections (Davies et al., 2007). To accomplish our goals, an imaging method should be as non-invasive as possible while still having good spatial and temporal resolution to monitor ischemia-induced dendritic blebbing and structural recovery. While various techniques exist for non-invasive *in vivo* imaging such as

functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) these methods are limited in their spatial resolution and are best suited for imaging whole brain regions and neuronal networks.

Fluorescence imaging techniques provide enhanced cellular and subcellular resolution required for the visualization and study of biological processes (Pawley, 2006). Optimising signal-to-noise in conventional confocal microscopy (one-photon excitation) by adjusting the detector pinhole and fluorescence excitation intensity permits high 3D spatial resolution, but imaging in deep tissue is limited due to strong scattering of light in the blue-green/UV range typically required for excitation of fluorophores (Helmchen and Denk, 2006; Pawley, 2006). Another important constraint for *in vivo* imaging is maintaining the viability of the tissue during imaging (Pawley, 2006). The use of visible-UV excitation light increases the probability of photodamage (Pawley, 2006). With confocal imaging, all tissue in the focused laser beam path above and below the focal plane becomes excited even if a single plane is being imaged (Denk and Svoboda, 1997; Helmchen and Denk, 2005; Pawley, 2006; Svoboda and Yasuda, 2006). As the photochemical properties of light can alter biological functions and compromise cell health, one-photon excitation which causes out-of-focus background fluorescence becomes a concern as the need to prevent and minimise phototoxicity and photobleaching is more critical *in vivo* (Pawley, 2006).

The development of two-photon (2P) imaging (Denk et al., 1990) offers distinct advantages over confocal microscopy. Some excellent reviews discuss in detail the components and features of 2P microscopes (Denk and Svoboda, 1997; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006). 2P imaging is based on the physical principle of simultaneous absorption of two long wavelength photons (usually infrared or near infrared) that combine their quantum

energies to produce molecular excitation that would normally be produced by a single photon of shorter wavelength (Pawley, 2006). Such 2P excitation is achieved through the use of high intensity, femtosecond pulsed-lasers which concentrate photons increasing the probability of a 2P event (Denk and Svoboda, 1997; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006). The benefits of 2P imaging arise from the nonlinear properties of 2P excitation, where photon absorbance varies with the square of the light intensity, so that outside of a strongly focused excitation beam, the excitation probability decreases quadratically with intensity (Pawley, 2006) and is of insufficient intensity to produce 2P excitation. This produces focal excitation within a small diffraction-limited focal volume, reduces background fluorescence, photodamage, and photobleaching outside the plane of excitation and gives 2P imaging an intrinsic optical sectioning capability without the need to adjust a pinhole or optics as in confocal microscopy (Denk and Svoboda, 1997; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006). Also, longer wavelengths penetrate deeper into tissue, allowing for greater imaging depth and have the added benefit of being less phototoxic than imaging with light in the visible-UV range (Denk and Svoboda, 1997; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006). Lastly, as 2P is usually coupled with a laser-scanning microscope, the speed of image acquisition is limited by the frame-scanning rate of galvanometer mirrors (~1-2 Hz) (Pawley, 2006). This temporal resolution is sufficient for visualising many cellular and biochemical processes (Pawley, 2006), and for our purposes is well within the required limits to accurately monitor dendritic blebbing.

Combined with the use of fluorescent probes or transgenic mice expressing fluorescent proteins in a subset of cells (Feng et al., 2000), 2P imaging has been the imaging method of choice in thick tissue and living animals. Applications of 2P imaging include monitoring blood flow dynamics (Kleinfeld et al., 1998), functional calcium imaging (Stosiek et al., 2003; Carter

and Sabatini, 2004), uncaging (Matsuzaki et al., 2001; Carter and Sabatini, 2004), fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) for the study of protein-protein interactions and enzyme activity (Yasuda, 2006). Advances in 2P imaging have included the miniaturization of 2P microscopes for head-mounting (Helmchen et al., 2001) and the combined use with a spherical treadmill (Dombeck et al., 2007) for imaging in awake and freely-moving animals (Helmchen et al., 2001; Dombeck et al., 2007).

1.6.2 Mouse models of stroke

Work *in vitro* in cultured neurons (Aarts et al., 2002; Liu et al., 2007; Taghibiglou et al., 2009), brain slice preparations (Aarts et al., 2002; Hori and Carpenter, 1994; Obeidat et al., 2000; Andrew et al., 2007) and *in vivo* (Corbett et al., 1997; Colbourne et al., 2000; Corbett et al., 2000; Zhang et al., 2005; Brown et al., 2007; Brown et al., 2008; Clark et al., 2008; Clark et al., 2009; Liu and Murphy, 2009; Sigler et al., 2009; Brown et al., 2010; Mostany et al., 2010; Risher et al., 2010; Mohajerani et al., 2011) has undeniably contributed to an improved understanding of the pathophysiological mechanisms of stroke. *In vitro* simulated models of ischemia such as oxygen-glucose deprivation, hypoxia, induced glutamate excitotoxicity and oxidative stress are well suited to address questions on specific mechanisms since such reductionist models replicate some aspects of ischemia *in vivo* and experimental manipulations are facilitated (Mattson et al., 2000). However, to understand the complex and dynamic processes which occur within a living animal, ischemia should ideally be studied *in vivo*. Several rodent models of ischemia have been developed (Ginsberg and Busto, 1989; Traystman, 2003) which reduce cerebral blood flow in a manner which replicates focal or global ischemia in a physiologically-controlled and reproducible manner *in vivo* (Ginsberg and Busto, 1989).

In focal or multi-focal models of stroke blood flow is interrupted using RB-photothrombosis, middle cerebral artery occlusion (MCAO), intraluminal suture advancement, microvascular clipping or cauterization, thrombin injection, and endothelin-induced vasoconstriction (Traystman, 2003; Murphy and Corbett, 2009). In global ischemia, widespread and global reductions in blood flow can be achieved by two-vessel occlusion (2 VO) of the common carotid arteries (CCAs) combined with systemic hypotension or four-vessel occlusion (4 VO) of carotid and vertebral arteries in the rat (Traystman, 2003). In mice, global ischemia can be induced using KCl-induced cardiac arrest (Traystman, 2003), but recent studies use common carotid artery occlusion (CCAO) (Murphy et al., 2008; Liu and Murphy, 2009).

It is important to note that not all mouse strains are equally susceptible to global ischemia by CCAO due to strain differences in cerebral vasculature (Yang et al., 1997; Kitagawa et al., 1998). Susceptibility to ischemia is determined by the patency of the posterior communicating arteries (PComA) which normally connect the carotid and vertebrobasilar arteries at the circle of Willis (Kitagawa et al., 1998). Anastomoses at the circle of Willis allow for collateral blood flow in the event of occlusion of one of the branching arteries (Liebeskind, 2003). C57Bl/6 mice have poorly developed PComAs and when subjected to CCAO, ischemia occurs in the territories supplied by the posterior (hippocampus and thalamus), middle, and anterior cerebral arteries (Yang et al., 1997; Kitagawa et al., 1998). Compared with other mouse strains subjected to the same period of CCAO, C57Bl/6 mice have greater reductions in cortical microperfusion and tissue ATP, larger ischemic lesions, more extensive neuronal death, and suffer larger neurological deficits (Yang et al., 1997; Kitagawa et al., 1998). Finally, unlike focal models of ischemia which have variable reperfusion, an advantage of using sutures for occlusion of the CCAs is the transient induction of ischemia with controlled reperfusion (Murphy et al., 2008).

1.7 Hypothesis and objective

While much research has investigated multiple sites of action for hypothermia, whether this treatment can affect ischemia-induced changes in neuronal structure is currently unknown. This study was undertaken to determine a role for hypothermia in protecting dendritic structure during ischemia. We hypothesize that hypothermia would act to improve the recovery of structure during reperfusion. To this end, we have used 2P imaging to compare the induction and recovery of ischemia-induced dendritic blebbing under normothermia (37°C), and moderate (31°C) and deep hypothermic (22°C) conditions.

2. EXPERIMENTAL METHODS

2.1 Transgenic mice

Adult male C57Bl/6 mice (2–5 months of age) expressing yellow fluorescent protein (YFP)-H and green fluorescent protein (GFP)-M (Feng et al., 2000) were used in this study. Transgenic mice were bred at the University of British Columbia animal facilities. Experimental protocols were approved by the University of British Columbia animal care committee and are consistent with the Canadian Council on Animal Care and Use guidelines.

2.2 Surgical procedures

For all surgical procedures, mice were anesthetized using an intraperitoneal injection of urethane (0.12% w/w) (Zhang et al., 2005). Body temperature was maintained at 37 ± 0.5°C using a heating pad and feedback regulation from a rectal temperature probe. A sufficient level of anesthesia was confirmed by the lack of a toe-pinch reflex, typically established within 90 minutes of the initial urethane injection and maintained with only minimal supplementation (<10% of the initial urethane dose) for up to 8 h during experiments. Hydration was maintained by intraperitoneal injection of saline (200–300 µl) with 20 mM glucose at 1–2 h intervals. To aid with ventilation during experimental procedures, a tracheotomy was performed in a subset of animals.

For surgical procedures placing sutures around the common carotid arteries, a local anesthetic (50 µl of lidocaine) was injected subcutaneously into the neck region before making an incision over the thyroid gland. While maintaining the surgical site moist with saline, the thyroid and associated musculature and connective tissue were dissected away from the trachea. The common carotid arteries were separated from the vagus nerves and muscles using blunt

forceps. Sutures (5–0, silk; Ethicon, Somerville, NJ) were looped around each CCA. The skin was closed using stitches, allowing enough space around the sutures for tensioning during occlusion.

Surgical procedures for preparing a cranial window for imaging have been described in detail previously (Zhang et al., 2005). Briefly, animals were fitted into a custom-made stainless steel head hold and 3x3 mm craniotomy over the right somatosensory cortex was performed as described by others in the past (Zhang et al., 2005). The cortex was covered with 1.3% low-melt agarose (at 37–38°C; Type 3-A; A9793; Sigma, St. Louis, MO) dissolved in HEPES-buffered artificial CSF (ACSF) and sealed with a glass coverslip (#1).

2.3 Cortical surface temperature regulation

In some animals, cortical surface temperature was measured using a thermocouple (IT-24P, Physitemp Instruments Inc, Clifton, NJ) placed over the cortical surface within agarose. A custom-made stainless steel headplate adhered to the skull was connected with tubing to a water pump (T/Pump TP500, Gaymar, Orchard Park, NY) which circulated water and regulated cortical surface temperature to 37°C (normothermia) or 31°C (moderate hypothermia). For deep hypothermia experiments, a separate cooling pump was used to maintain cortical surface temperature at 22°C. Cortical surface temperature regulation would begin following the completion of surgical procedures when the animal was transferred to the microscope rig for imaging and continue during the occlusion period and up to 2 hours during reperfusion.

2.4 *In vivo* 2-photon imaging

2P imaging of apical dendrites of layer 5 neurons and Texas-Red (TR) dextran (70kDa; Invitrogen, Eugene, OR)-labeled blood plasma was performed using a Coherent Mira 900 Ti-

sapphire laser pumped by a 5 W Verdi laser and tuned to 900 nm to excite YFP or GFP. Images were acquired by custom software (Igor) and by using an Olympus (Tokyo, Japan) IR-LUMPlanFl water-immersion objective [40x, 0.8 numerical aperture (NA)]. 3D image stacks (20 x 1 µm or 20 x 2 µm) was restricted to the first 70 µm of cortex where layer 5 neurons project laterally. Images were acquired before ischemia, repeatedly during the occlusion period, the first few minutes of reperfusion and less frequently thereafter to reduce photodamage.

2.5 Stroke model

CCAO, 4-7 minutes in duration, was used to induce global forebrain ischemia in mice whose cortical surface temperature was regulated at normothermia (NORMO), moderate hypothermia (HYPO), and deep hypothermia (DEEP). A shorter period of occlusion, 2-3 minutes in duration, was induced in a NORMO short group. In some cases the duration of ischemia was shortened if we observed a depression of the heart rate accounting for the range of ischemic durations. To induce stroke, tension was applied to both sutures and secured with tape, and removing tension restored reperfusion. Ischemia was confirmed by monitoring a slowing of surface blood flow through video microscopy, 2P imaging of blood flow, ischemic depolarization by DC EEG, and AC electroencephalogram depression.

2.6 Electroencephalogram recording

For EEG recording, a Teflon coated silver wire (0.125 mm; World Precision Instruments, Sarasota, FL) was placed on the surface of the cortex within agarose. A reference electrode was placed on the nasal bone under the skin and a second reference electrode placed in the animal's back allowed the measurement of heart rate electrophysiologically. The cortical signal was amplified and filtered (0.1–1000 Hz) using a differential alternating current (AC) amplifier

(Model 1700, A-M Systems) and digitized (1000 Hz) using a 1322A Digidata (Molecular Devices, Sunnyvale, CA). EEG was collected in Clampex 9.2 and analysed in Clampit 9.2 (Axon Instruments).

2.7 Intrinsic optical signal imaging

Intrinsic optical signal (IOS) imaging was performed to localise the forelimb and hindlimb representations within the somatosensory cortex enabling 2P imaging of dendrite structure within the forelimb region or along the forelimb/hindlimb border. Details of this procedure can be found in previous reports (Murphy et al., 2008). Briefly, the depth of focus within the right somatosensory cortex was set between 200-700 μm below the cortical surface. The cortical surface was illuminated by red light-emitting diodes (LEDs) mounted on a 2.5x Zeiss Neofluor 0.075 NA objective (Oberkochen, Germany) and driven by a regulated direct current (DC) power supply (Circuit Test, Burnaby, British Columbia, Canada). Piezoelectric devices driven by an isolated pulse stimulator (Model 2100; A-M Systems, Everett, WA) alternatively generated vibrotactile stimulation of the left forelimb and hindlimb. Images were acquired in XCAP-standard version 2.2 imaging software (EPIX, Buffalo Grove, IL). Image acquisition, limb stimulation, and LED illumination were synchronised using TTL signals. In cases where IOS was not performed, we determined the centre of forelimb and hindlimb using mouse atlas coordinates. The centre of forelimb was taken to be 2.5 mm lateral and 0.5 mm anterior of bregma and the centre of hindlimb to be 1.9 mm lateral and 0.6 mm posterior of bregma (Harrison et al., 2009).

2.8 Physiological measurements

In a subset of animals, physiological parameters such as oxygen saturation and heart rate were monitored throughout 2P imaging using a Starr Life Sciences (Oakmont, PA) Mouse-Ox pulse oximeter placed on the right hindlimb or the tail.

2.9 Image analysis

A modified version of a MATLAB (MATLAB R2008a, the MathWorks, Natick, MA)-based software program was used to perform automated image analysis (Chen et al., 2011). The original version of the algorithm has been shown to generate results (% of blebbed dendrites) with good correlation to blinded manual analysis (Chen et al., 2011). Because blebbing is preferentially localised to apical tufts near the cortical surface (Enright et al., 2007), image analysis was restricted to the top ~70 µm of cortex. Z-projections of small sections of cortex (5-7 µm) up to 20 µm within 3D image stacks (20 x1 µm or 20 x 2 µm) were aligned and comparisons between pre-ischemia (reference) images were made with images acquired during occlusion or reperfusion (test images). Blebs were detected based on identifying key morphological features such as eccentricity (ratio of minor to major axis of an ellipse or round object) and area compared to the parent dendrite. A blebbing percentage was derived for each section and the average of all sections was taken to gain the fraction of blebbed dendrites for a single image stack. For NORMO, HYPO, and DEEP groups, images acquired at 0-10, 10-40, 40-70, and 70-100 minutes of reperfusion were averaged together. For NORMO short, we only analysed a single image in the first 4 minutes of reperfusion in addition to later timepoints. For all groups, we used the image stack with the greatest % blebbing during occlusion, which was typically the last image stack acquired before reperfusion.

2.10 Statistical analysis

For the final analysis, only animals which had a confirmed reduction of blood flow during the occlusion period as seen on 2P images of TR dextran-labeled plasma were included. We also confirmed that all animals had recovery of blood flow in a timely manner with reperfusion. Statistical tests used a 1-way ANOVA to make comparisons across groups. A repeated measures 1-way ANOVA followed by Bonferroni's post-tests were used to compare differences in blebbed dendrites across time in each experimental group. F-tests were used to compare variance in the percentage of blebbed dendrites across experimental groups at different time points. Significance was set at $p<0.05$. Data are expressed as mean ± standard error of the mean (SEM).

3. RESULTS

3.1 Physiological parameters in experimental groups

In this study we investigated the effect of localised cortical hypothermia on ischemia-induced structural damage and structural recovery in reperfusion. Using a stainless-steel headplate to circulate heated or cooled water, we were able to induce stable cortical surface temperatures in mice (Figures 1A-C). Temperature ranges for therapeutic hypothermia can be categorised into mild (33-36°C), moderate (28-33°C), and deep (~20°C) (Mayer and Sessler, 2005). We verified in some animals that the average cortical surface temperature during experiments was consistent with normothermia (36.5 ± 0.2°C, n=6), moderate hypothermia (31.1 ± 0.2°C, n=4), and deep hypothermia (21.8 ± 0.8°C, n=5). Occlusion duration was measured as the time of onset where the filtered (0.1-10 Hz) AC EEG showed a ~90% suppression in power to the time of reperfusion as verified by 2P imaging as seen as the first image stack showing a return of blood flow. We found no significant differences in occlusion duration between NORMO (the mean duration of occlusion ± SEM was found to be 357.9 ± 20.7 s, n=5), HYPO (350.6 ± 40.6 s, n=5), and DEEP (433.8 ± 51.1 s, n=5). Animals in the NORMO short group received a significantly shorter period of occlusion (164.6 ± 18.2 s, n=4) compared to NORMO ($p<0.01$). No significant differences were found in the age or weight of mice across experimental groups (Table 1).

Figure 1 (next page)**. Experimental setup and timeline.** (A) Schematic of experimental setup for combined 2P imaging and temperature regulation. A pump circulates water through a stainless steel headplate to regulate surface cortical temperature under normothermia and moderate and deep hypothermia while body temperature is maintained at 37°C. A temperature probe provides a digital readout of surface temperature. (B) Cortical surface temperature measurements during experiments for NORMO+NORMO short, HYPO, and DEEP groups. (C) This method of temperature regulation produces a temperature gradient with cooler temperatures at the surface and temperatures approaching body temperature with increasing depth. (D) Experimental timeline. Regulation of cortical temperature to 37°C, 31°C, or 22°C and EEG recording begins following the completion of surgery. The DC and AC EEG traces shown are from an animal from the NORMO short group.

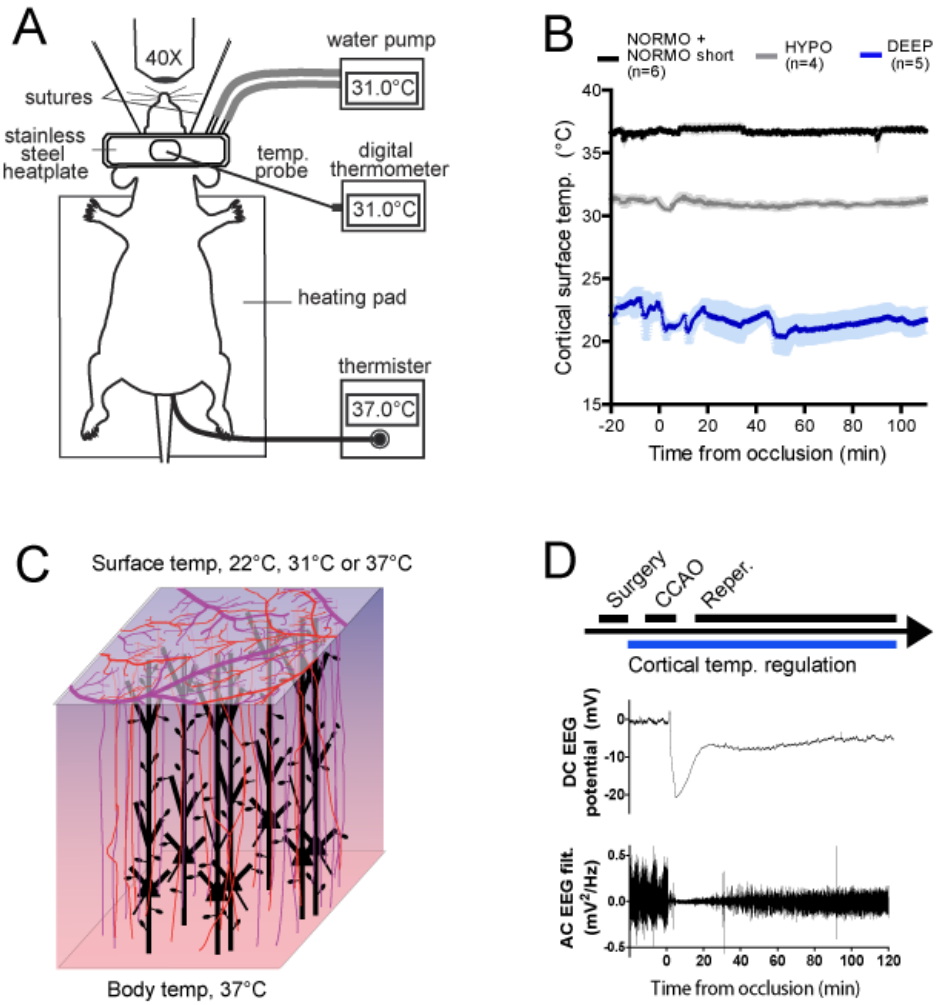
A
40X
sutures
stainless steel heatplate
water pump
31.0°C
temp. probe
digital thermometer
31.0°C
heating pad
thermister
37.0°C
B
NORMO + NORMO short (n=6)
HYPO (n=4)
DEEP (n=5)
Cortical surface temp. (°C)
Time from occlusion (min)
C
Surface temp, 22°C, 31°C or 37°C
Body temp, 37°C
D
Surgery
CCAO
Reper.
Cortical temp. regulation
DC EEG potential (mV)
AC EEG filt. (mV²/Hz)
Time from occlusion (min)

Figure 1

Experimental Group	Age (days)	Weight (g)	Occlusion duration (s)
NORMO	100.2 ± 8.0 (n=6)	29.8 ± 1.0 (n=5)	357.9 ± 20.7 (n=5)
NORMO short	91.5 ± 14.1 (n=4)	28.1 ± 1.3 (n=4)	164.6 ± 18.2 (n=4) *p <0.01*
HYPO	90.3 ± 8.5 (n=6)	25.6 ± 3.0 (n=3)	350.6 ± 40.6 (n=5)
DEEP	80.6 ± 10.31 (n=5)	26.9 ±0.6 (n=5)	433.8 ± 51.1 (n=5)

Table 1. Summary of age, weight, and occlusion duration for mice in experimental groups. No significant differences in occlusion duration were found between NORMO, HYPO, and DEEP. Animals in the NORMO short group had a significantly shorter period of occlusion ($p<0.01$) compared with NORMO. Analysis by 1-way ANOVA and Bonferonni's post-test with all groups compared to NORMO. Significance is set at $p<0.05$. Data are presented as mean ± SEM.

3.2 Onset of common carotid artery occlusion precedes ischemic depolarization

For all animals, successful CCAO preceded the onset of ischemic depolarization (Figure 2) and was typically associated with a ~90% or greater suppression of the spontaneous EEG power (Figure 1D). No differences were observed in amplitudes of ischemic depolarization across experimental groups (Figure 2A). The duration of depolarization increased with cortical surface temperature and was significantly longer ($p<0.01$) for DEEP as compared with NORMO (Figure 2B). Similarly, the rate of depolarization varied directly with surface cortical temperature (Figure 2C). Under NORMO and NORMO short, rates were 0.46 ± 0.09 mV/s and 0.52 ± 0.09 mV/s respectively ($p>0.05$). Compared to NORMO, the rate reduced to 0.26 ± 0.06 mV/s ($p>0.05$) under HYPO and was reduced further to 0.10 ± 0.03 mV/s ($p<0.01$) under DEEP. Consistent with previous reports (Nakashima et al., 1995; Nakashima and Todd, 1996), we found that cooler cortical temperatures delayed ischemic depolarization (Figure 2D). As measured from occlusion onset, the time to depolarise under NORMO and NORMO short occurred within tens of seconds. The times to depolarise were found to be 42.7 ± 8.5 s and 47.1 ± 10.5 s for NORMO and NORMO short respectively ($p>0.05$). Compared to normothermic conditions, the time to depolarise increased to 90.6 ± 14.4 s ($p>0.05$) under HYPO and to 213.1 ± 47.7 s ($p<0.001$) under DEEP.

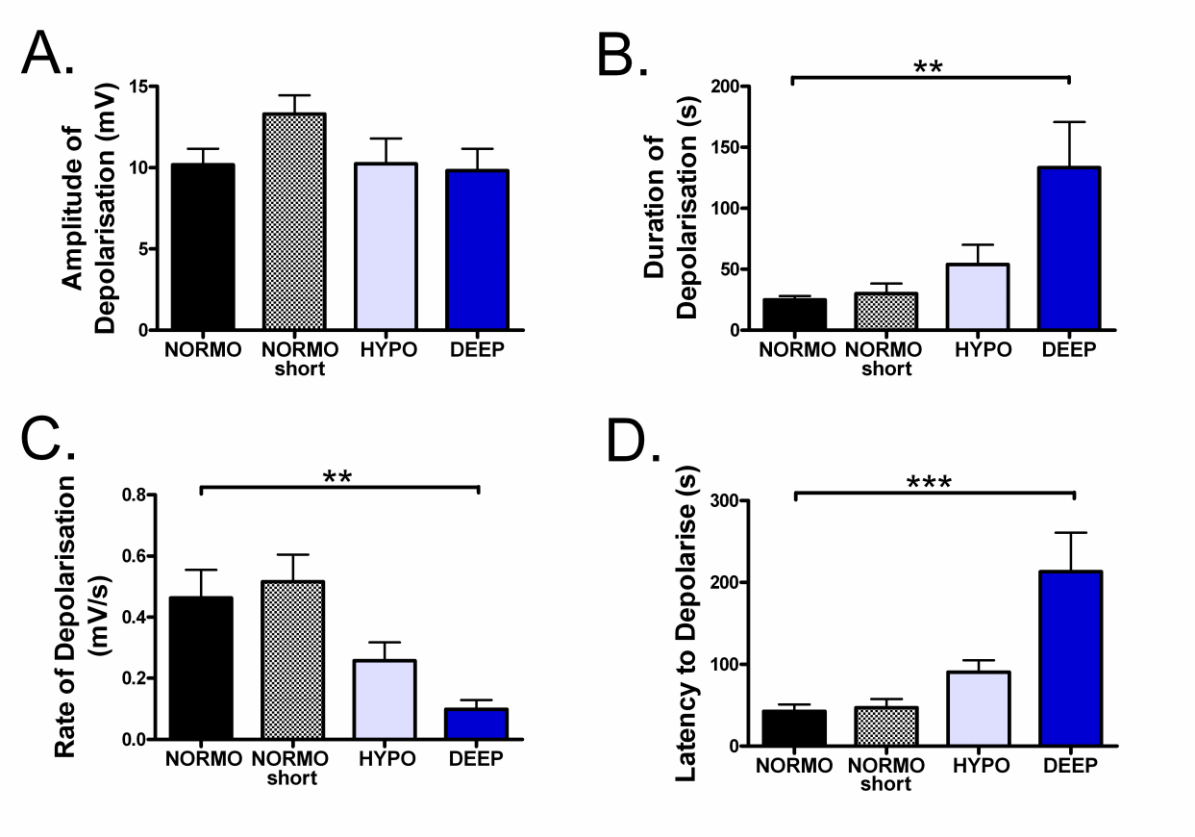

Figure 2. Summary of ischemic depolarization amplitudes, durations, rates, and latencies in experimental groups. Analysis by 1-way ANOVA and Bonferonni's post-test with all groups compared to NORMO. Significance is set at $p<0.05$. Data represent mean ± SEM.

3.3 Hypothermia does not prevent, but delays the onset of dendritic blebbing

In vivo 2P imaging of labeled GFP or YFP layer 5 apical dendrites was used to assess ischemia-induced dendritic structural damage and recovery. Successful occlusions had confirmed blood flow reductions as visualised by concurrent 2P imaging of TR-dextran labeled plasma (Figure 3). Here, we report that under all treatment conditions, dendrites blebbed with occlusion (Figures 4 and 5A). We also found that the latency to bleb was inversely related to surface cortical temperature (Figure 5B). The time to bleb (mean ± SEM) under NORMO and NORMO short was found to be 76.0 ± 10.0 s (n=8). This increased under HYPO to 140.5 ± 17.7 s, n=6 ($p<0.05$) and DEEP to 348.5 ± 26.9 s, n=5 ($p<0.001$). Once dendrites blebbed, there was no significant difference between the amount of blebbing across groups (Figure 5A).

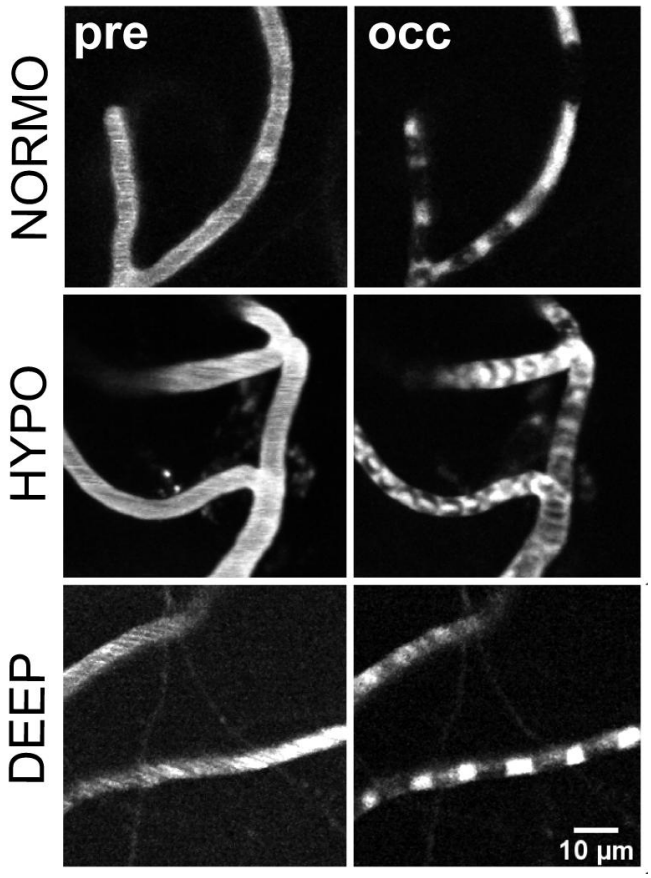

Figure 3. Representative two-photon images of Texas-Red Dextran labeled plasma for NORMO, HYPO, and DEEP before and during occlusion. Blood flow is visualised as streaking within vessels (high red blood cell velocity) and turbulence seen with occlusion indicates reduced velocity. Scale bar is 10 µm.

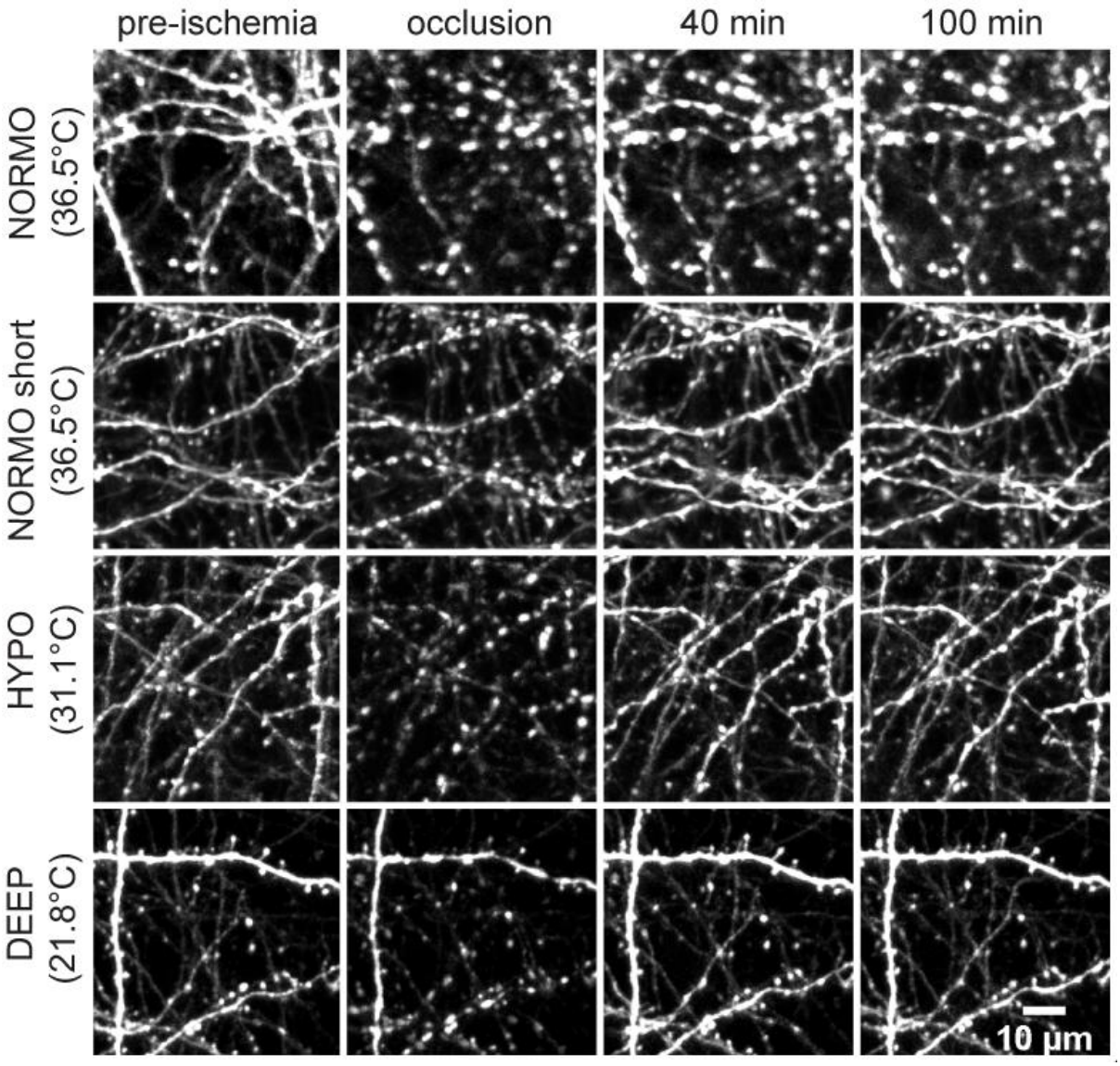

Figure 4. Representative two-photon images of GFP/YFP-labeled dendrites under NORMO, NORMO short, HYPO, and DEEP conditions. Shown are images acquired pre-ischemia, during occlusion, and at indicated times in reperfusion. Scale bar is 10 µm.

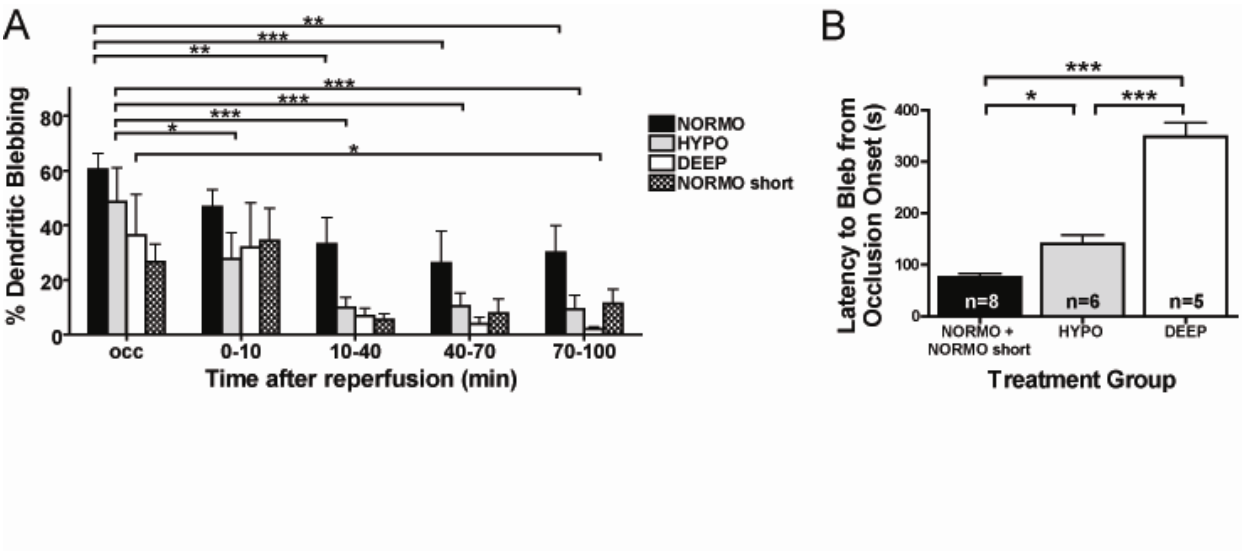

Figure 5. Quantification of dendritic blebbing and latency to bleb under experimental conditions. (A) Relative increases in dendritic blebbing as compared to pre-ischemia quantified at occlusion and indicated times of reperfusion (min). Recovery from blebbing is observed under NORMO, HYPO, and DEEP. Under NORMO, reductions in blebbed dendrites compared with occlusion were observed at 10-40 ($p<0.01$), 40-70 ($p<0.001$), and 70-100 min ($p<0.01$) following reperfusion. Under HYPO, reductions in blebbing were observed at 0-10 ($p<0.05$), 10-40 ($p<0.001$), 40-70 ($p<0.001$), 70-100 min ($p<0.001$) following reperfusion. Under DEEP, blebbing is reduced at 70-100 min ($p<0.05$) of reperfusion. Data represent mean ± SEM. Analysis by repeated measures 1-way ANOVA and Bonferonni's post-test. (B) Moderate and deep hypothermia delay the latency to bleb. An increase in the time to bleb is seen between NORMO + NORMO short and HYPO ($p<0.05$), NORMO + NORMO short and DEEP ($p<0.001$), and HYPO and DEEP ($p<0.001$). Data represent mean ± SEM. Analysis by 1-way ANOVA and Bonferonni's post-test. For all analysis, significance is set at $p<0.05$.

3.4 More consistent structural recovery in reperfusion is seen with deep hypothermia

To quantify the amount of dendritic blebbing at occlusion and during reperfusion, we used a MATLAB algorithm to automatically identify and quantify blebs in 5-7 µm z-sections. Using this approach we focused on dendritic blebbing near the cortical surface (<70 µm) when analysing a 3D image stack. As this approach determines relative changes in blebbing compared to pre-ischemia, we confirmed that reference (pre-ischemia) images contained little or no blebbing (Figure 4). Compared to occlusion, this analysis revealed that mice in NORMO, HYPO, and DEEP groups showed recovery of blebbed dendrites during reperfusion (Figures 4 and 5A). The percentage of blebbed dendrites under NORMO was reduced from 60.4 ± 5.8% at occlusion to 26.2 ± 11.6% ($p<0.001$) at 40-70 min of reperfusion and 30.0 ± 9.9% ($p<0.01$) by 70-100 min of reperfusion. No statistically significant reductions in blebbing were observed under NORMO short, however, this could be attributed to less extensive blebbing at occlusion and a relatively smaller number of animals lowering statistical power. Under HYPO, dendrites blebbed to 48.5 ± 12.6% at occlusion and in contrast to NORMO, recovery in blebbing was observed much more rapidly during reperfusion with significant reductions in blebbing apparent by just 0-10 min ($p<0.05$) of reperfusion. Under HYPO, by 40-70 min of reperfusion, blebbing was reduced to 10.5 ± 4.8% ($p<0.001$) by 40-70 min of reperfusion and to 9.4 ± 5.0% ($p<0.001$) by 70-100 min of reperfusion. Dendrites also blebbed under DEEP at occlusion to 36.3 ± 14.9% and this blebbing was reduced to 2.0 ± 0.8% ($p<0.05$) by 70-100 min of reperfusion. Statistical tests for variance indicated that the variability in blebbed dendrites during reperfusion was reduced for HYPO and DEEP as compared with NORMO, indicating that structural recovery is more uniform at lower cortical temperatures. For HYPO, an F-test for variance was significant

($p<0.05$) at 10-40 min of reperfusion and for DEEP, significance was observed at 10-40 ($p<0.05$), 40-70 ($p<0.05$) and 70-100 ($p<0.001$) min of reperfusion.

4. DISCUSSION

4.1 *In vivo* imaging reveals dynamic structural changes to dendrites

In this study, we investigated the role of hypothermia in ischemia-induced dendritic blebbing and structural recovery in reperfusion. A powerful advantage of our studies is the use of *in vivo* imaging for the visualization of dendritic structural alterations. "Static" imaging methods requiring animal sacrifice, tissue fixation, and histology (Park et al., 1996; Hasbani et al., 1998; Obeidat et al., 2000; Brown et al., 2008) to assess ischemic damage may miss important structural changes if specific timepoints are not sampled or, as in our case, if the onset and/or recovery of ischemic damage is rapid. In contrast to these techniques, we are able to simultaneously monitor in real time dendrite structure and its relationship with blood flow during ischemia and in the minutes to hours following reperfusion *in vivo*. This method allows us to chart the dynamic process of structural alterations in ischemia-induced damage. Our results show that hypothermia does not prevent the onset of dendritic blebbing (Figures 4 and 5A), but treatment with deep hypothermia does tend to favour more consistent recovery during reperfusion (Figure 5A).

Unlike the study of spine plasticity in ischemia which is extensive (Brown et al., 2007; Brown et al., 2008; Brown et al., 2009; Mostany et al., 2010), there are relatively few reports assessing dendritic blebbing, especially following ischemia (Zhang et al., 2005; Li and Murphy, 2008; Murphy et al., 2008). We find this to be unusual since it is widely believed that neuronal structure and function are intricately linked. However, the effect of mild or extreme hypothermia on dendritic structure has been investigated previously *in vitro* in the presence (Obeidat et al., 2000) and absence (Kirov et al., 2004) of anoxia. Here, we extend these results by investigating such effects *in vivo* with global ischemia. In studies utilising brain slices, exposure to ice-cold

ACSF (~6-15°C) caused blebbing and spine loss which was reversible with rewarming to 32°C (Kirov et al., 2004). While this finding indicates potentially detrimental effects of cooling on synaptic morphology, such cooling is reflective of extreme hypothermia which is typically not used clinically or in experimental models of stroke. Furthermore, under our experimental conditions, baseline dendritic structure was apparently not altered with HYPO or DEEP in that we did not observe hypothermia-induced structural alterations either in the form of beading or spine loss (Figure 4).

4.2 Comparisons to other methods of cooling

Our method of localised hypothermia allows the combination of cortical surface temperature regulation with head stabilisation for 2P imaging (Figure 1A). One question is whether surface cooling by our method is sufficient to induce cortical hypothermia. This method of cooling likely produces a temperature gradient with cooler temperatures at the cortical surface and warmer temperatures in deeper layers (Figure 1C) as systemic blood flow at body temperature would circulate throughout the brain. It has been shown that unilateral, hemispheric, focal cooling at the skull produces hypothermia which is greatest at the cortex and less so subcortically in the striatum (Clark and Colbourne, 2007). These results provide support that our approach is effective since the work by Clark and Colbourne (2007) was conducted in the rat which has a much thicker skull and larger cortical volume compared with the mouse. Nonetheless, all experiments assessing effects of hypothermia need to take into consideration measurement of brain temperature (DeBow and Colbourne, 2003). This is especially important in the context of localised hypothermia since rectal temperature does not necessarily reflect brain temperature (DeBow and Colbourne, 2003). In these experiments, we measured cortical temperature indirectly with a thermocouple placed within agarose over the brain and this non-

invasive method showed that cortical temperature remained stable during temperature regulation (Figure 1B). While obtaining direct temperature measurements in the region of cortex being cooled would be ideal, the large size of our temperature measurement device (tip diameter ~230 μm) relative to the size of the mouse brain makes this impractical and would cause brain damage (DeBow and Colbourne, 2003). In contrast, in rats and larger rodents, brain temperature can be measured experimentally by implanting a thermocouple or telemetry probe into brain structures of interest (DeBow and Colbourne, 2003; Clark and Colbourne, 2007).

4.3 Electrophysiological confirmation of cortical surface cooling

Ischemic depolarization is a critical event in the evolution of ischemia (Nedergaard and Hansen, 1993; Dirnagl et al., 1999; Doyle et al., 2008). In addition to 2P confirmation of reduced blood flow to verify CCAO, we also used the occurrence of ischemic depolarization as an additional marker of energy failure. For NORMO, NORMO short, HYPO, and DEEP groups, all animals experienced ischemic depolarization associated with CCAO (Figure 2). To give confidence in the validity of our cooling method, we also observed the reported changes in ischemic depolarization latency under hypothermia (Figure 2D) reported by others (Nakashima et al., 1995; Nakashima and Todd, 1996). Interestingly, the duration of ischemic depolarization for HYPO and DEEP groups was longer compared with NORMO and NORMO short (Figure 2B), but this did not result in more statistically significant blebbing at occlusion (Figures 4 and 5A). One would expect that a longer depolarization period would lead to more structural damage since it prolongs the time in which cells are exposed to glutamate excitotoxicity and ionic disequilibrium (Higuchi et al., 2002). Indeed, reports have described a correlation between total depolarization duration with histological assessment of neuronal damage (Higuchi et al., 2002).

In our case, it appears that the onset of structural damage to dendrites is independent of depolarization duration.

4.4 Implications for cardiac arrest

In our studies, hypothermia was initiated before ischemia onset and continued during the occlusion period and into reperfusion (Figure 1D). Our method of hypothermia administration combined with CCAO models prophylactic hypothermia used clinically to minimize brain injury during neurologic and cardiac surgeries and in predicted cases of ischemia (Mayer and Sessler, 2005). In fact, hypothermia has been used as an intervention during cardiac surgical procedures since the 1950s when it became apparent that mild to severe brain damage results from the lack of systemic circulation (Arrowsmith et al., 2000). While localised hypothermia has been the focus of this study and would be the preferred method of neuroprotection in ischemia, in the extreme case of cardiac arrest where blood flow is interrupted systemically, systemic hypothermia would be desirable (Bernard et al., 2002) over localised brain hypothermia since it allows the protection of other body organs. Our results suggest that more consistent dendritic structural recovery would be expected following ischemia induced by cardiac arrest upon recirculation when combined with deep hypothermia treatment. Future studies of systemic hypothermia combined with a model of cardiac arrest would be needed in order to validate this prediction.

4.5 Duration of occlusion

In this study, mice in the NORMO, HYPO, and DEEP groups underwent 4-7 minutes of occlusion and a shorter period of 2-3 minutes was induced for animals in the NORMO short group (Table 1). The 4-7 minute occlusion duration in this study is consistent with our previous

reports in which occlusion was maintained for up to ~8 min before a slowing of the heart rate was observed and reperfusion was induced to avoid compromising the health of animals (Murphy et al., 2008). CCAO produces a severe (>90%) reduction in cortical blood flow within seconds of tensioning sutures (Murphy et al., 2008). Compared to other reports using the same mouse strain as us, CCAO has been successfully maintained for much longer durations (15-30 minutes) (Yang et al., 1997; Terashima et al., 1998; Yoshioka et al., 2011b; Yoshioka et al., 2011a). In this work, we were unable to occlude mice for longer than ~7 minutes since this resulted in a high mortality rate. The difference here in occlusion duration within C57Bl/6 mice can be attributed to the manner in which CCAO is administered and the use of microaneurysm clips (Yang et al., 1997; Terashima et al., 1998; Yoshioka et al., 2011b; Yoshioka et al., 2011a) seems to allow for longer occlusion durations. As an improvement to the current method of generating global ischemia, our lab is currently experimenting with the use of vascular occluders (Access Technologies, Skokie, IL) surgically placed around each CCA to induce global ischemia. This method has been used successfully in the rat for unilateral occlusion of the CCA (Bluestone et al., 2004). Instead of applying tension to sutures, a water-filled syringe connected with tubing to each occluder would gradually inflate a cuff and constrict each artery until blood flow is blocked. Releasing water from the syringe would deflate the occluders and restore reperfusion. This method would allow for the same control in inducing ischemia and reperfusion during 2P imaging as with the current model using sutures.

4.6 Automated analysis of blebbed dendrites

In this study, an automated approach using a modified software program developed by us (Chen et al., 2011) was used for dendritic blebbing quantification. In the past, this analysis was done manually with the aid of a grid (20x20 μm) placed by ImageJ software

(http://rsb.info.nih.gov/ij/) over 2P acquired images to help identify and quantify the fraction of blebbed dendrites (Li and Murphy, 2008; Murphy et al., 2008; Liu and Murphy, 2009). In manual analysis, observers would identify dendritic blebbing (Zhang et al., 2005; Zhang and Murphy, 2007; Li and Murphy, 2008; Murphy et al., 2008; Liu and Murphy, 2009) by the presence of a "beads-on-string" appearance as described by us and other investigators as evidence of neuronal damage (Hori and Carpenter, 1994; Park et al., 1996; Obeidat et al., 2000; Zhang et al., 2005; Enright et al., 2007; Li and Murphy, 2008; Murphy et al., 2008; Andrew et al., 2007; Liu and Murphy, 2009). Manual analysis of beaded dendrites is time-consuming and can be biased by the observer and this prompted the development of a software program for image analysis.

We have reported good correlation in the results generated between manual and automated analysis (Chen et al., 2011). Compared with the original version of the program which relied on maximal intensity z-projections of entire image stacks (20 x 1 µm), z-projections of smaller sections of cortex (5-7 µm) up to 20 µm were analysed separately with our updated algorithm. We believe these changes improve the accuracy of bleb detection especially in mice with densely-labeled dendrites. While the program is accurate enough to identify increases and decreases in blebs with ischemia and reperfusion to chart the recovery of blebbing, there are limitations which contribute to the lack of perfect agreement with manual analysis. First, bleb detection is reliant on comparisons with a pre-ischemia reference image in order to determine changes in the geometry and size of objects from long and cylindrical (intact dendrite) to round or ellipsoid (bleb) (Chen et al., 2011). Because of this, we assume there is no damage pre-ischemia (i.e. dendrites are not blebbed and blebbing is set to 0%) (Chen et al., 2011). Thus only relative changes in blebbing during occlusion and reperfusion as compared with the reference

image are detected. Because of this caveat, we reviewed images acquired from experiments used in this study and can confirm that pre-ischemia images contained little or no blebbing (Figure 4). Secondly, bleb detection is reliant on good image alignment between reference and test images (i.e. blebs appearing in test images must fall along the axis of the parent dendrite in the reference image) in order to be counted (Chen et al., 2011) and the lack of proper alignment during and after stroke because of tissue warping (Brown et al., 2007) prevents identification. Because of these limitations, we typically found that the % blebbing obtained by the program is sometimes lower (especially for images acquired during occlusion) when compared with visual inspection. Improvements to the program would allow for blebs to be detected within a 3D image stack instead of detection from a series of 2D z-projections, making automated blebbing analysis independent of reference images, and improving bleb detection parameters so as to not incorrectly identify small, round structures such as the cross-sections of dendrites, spines, and axonal boutons to be blebs which sometimes occurs.

4.7 Hypothermia does not block mechanisms of blebbing onset

It was anticipated that hypothermia may prevent the onset of dendritic blebbing altogether or that moderate and deep levels of hypothermia would completely restore dendrite structure upon reperfusion. Even though hypothermia was administered before and during occlusion, our results show that moderate and deep hypothermia are only able to delay and not block the onset of ischemic depolarization (Figure 2) and dendritic blebbing (Figures 4 and 5), suggesting that hypothermia was only able to temporarily resist energy-dependent ischemic processes that would lead to swelling (Obeidat et al., 2000; Swann et al., 2000; Andrew et al., 2007). These immediate steps in the development of neuronal injury may be key in preventing blebbing altogether and if this is the case, an alternative approach would be to target these early

steps in the ischemic process. However, the solution appears more complicated since pharmacologic treatment with MK-801 or CNQX was unable to block ischemic depolarization and prevent blebbing *in vivo* (Murphy et al., 2008), confirming previous observations of the ineffectiveness of NMDA antagonists in preventing anoxia-induced swelling in brain slices (Obeidat et al., 2000).

While intraischemic hypothermia is studied experimentally, postischemic or delayed intraischemic hypothermia is of greater clinical relevance since treatment for stroke-affected individuals is typically delayed (Colbourne et al., 1997). Future studies comparing the effectiveness of induced hypothermia at different times from ischemic onset would resolve questions on optimal hypothermic administration for dendritic structural protection. Because improvements in structure were observed during reperfusion (Figures 4 and 5A), it is conceivable that facilitation of structural recovery requires metabolic substrates in order to restore normal dendrite structure. Indeed it has been shown that hypothermia effects the actin polymerisation-depolymerisation cycle important in maintaining dendrite structure (Gisselsson et al., 2005). While we did not address mechanisms of bleb formation and structural recovery, future experiments to visualise cytoskeletal proteins in the presence of ischemia would resolve this issue.

4.8 Acute assessment of hypothermia in dendritic blebbing

This study only examined the acute effects of hypothermia on ischemia-induced dendritic blebbing. Chronic *in vivo* imaging combined with hypothermia, which was not performed in this study, would reveal the fate of dendrites in the days and weeks following ischemia. It is unknown whether dendrites which showed only partial recovery by 2 hours of reperfusion, as in the NORMO group (Figures 4 and 5A), would continue to make further improvements in

structure, degenerate, or remain stable. If the brain continues to receive adequate blood flow in the hours and days beyond our 2 hour imaging window, we predict that it is unlikely that dendrite structure would continue to deteriorate since we have shown that restoration of blood flow aids structural recovery (Li and Murphy, 2008; Murphy et al., 2008).

However, during a chronic recovery period, there is the potential that future perturbations to an already stressed system, even minor, such as brief interruptions in blood flow or spreading depolarizations (Risher et al., 2010), may be enough to reintroduce damage to dendrites which had previously recovered under moderate or deep hypothermia. For example, following focal ischemia, repeated occurrence of spreading depression (SD) in the penumbra was coincident with brief instances of blebbing, and while dendrites recovered between individual cases of SD, terminal blebbing eventually occurred, presumably from the accumulated stress of repetitive cases of energy failure (Risher et al., 2010). Such a case is contrary to the idea of ischemic preconditioning, that an initial incidence of sublethal ischemia would make the system more resistant to future cases of ischemic insult (Gidday, 2006). A potential reason why Risher and colleagues observed repeated bouts of blebbing (Rischer et al., 2010) could be that SDs occurred before a preconditioning effect could take place since it may take up to days to come into effect (Gidday, 2006). The effect of preconditioning has not been studied at the level of neuron structure, but if this were to be the case, a second incidence of global or focal ischemia would be predicted to have a longer latency to blebbing onset, faster or greater recovery from blebbing during reperfusion, or the prevention of ischemia-induced blebbing altogether.

4.9 Transient or no dendritic blebbing may be due to incomplete ischemia

Animals included in the final analysis showed a reduction of blood flow as seen by 2P imaging of TR-dextran labeled plasma during the occlusion period (Figure 3). In our hands, we

find that reduced blood flow is sufficient to denote a successful occlusion and we further confirmed this by the occurrence of ischemic depolarization and a ~90% suppression of spontaneous EEG power (Figure 1D). In previous studies (Li and Murphy, 2008; Murphy et al., 2008), we found these events follow occlusion and precede the onset of dendritic blebbing. Indeed, in this study, this series of events was replicated in our four experimental groups (NORMO, HYPO, DEEP, and NORMO short). However, in spite of our efforts to ensure ischemia, there were a few animals which only blebbed transiently or minimally during the occlusion period (HYPO, n=1; DEEP, n=2). While these cases did occur under moderate and deep hypothermia, this still came as a surprise since other animals in the same group followed a typical pattern of blebbing and we have previously demonstrated that such structural alterations persist for much longer periods (Zhang et al, 2005; Enright et al, 2007; Zhang et al, 2007; Li and Murphy, 2008; Murphy et al., 2008).

Because we only monitored blood flow in the same small area of the brain (~200 x 200 μm) where dendrites were visualised, we were not able to obtain global measures of blood flow. A better approach would be to use laser speckle contrast imaging (Dunn et al., 2001) or laser Doppler flowmetry (Dirnagl et al., 1989) to confirm global reductions in cerebral blood flow following CCAO as what has been done previously by us (Murphy et al, 2008) and others (Terashima et al., 1998; Yoshioka et al., 2011b; Yoshioka et al., 2011a). Thus, there is the possibility that while we observed good reductions in blood flow in the imaged area near the cortical surface (<70 μm), areas outside of this window may not have experienced severe enough reductions in flow. A previous study in this lab has shown that blebbing may not occur if dendrites were within ~80 μm of a flowing vessel (Zhang et al., 2007). Thus, unimaged surrounding vessels or those deep in the cortex may have had sufficient blood flow to maintain

dendrites structurally intact in spite of blood flow being arrested in the imaged area.

Compensatory cerebrovascular effects (Morita et al., 1997; Schaffer et al., 2006) may have also minimised acute dendrite damage. For example, in the event of interrupted blood supply to a brain territory, an autoregulatory process compensates through vasodilation of nearby arteries (Morita et al., 1997). Also, in response to focal ischemia, blood flow is redistributed to areas in need (Schaffer et al., 2006). The results of past studies have shown that blood flow needs to be reduced by ~90% of baseline levels before dendrites become beaded (Zhang et al., 2005) and if such cerebrovascular compensatory mechanisms were to occur because of incomplete ischemia, it is likely that the lack of more extensive blebbing observed during occlusion was due to the fact that global flow rates did not reach this threshold.

4.10 Anesthetic effects

In this study, anesthetized mice were used throughout the course of the experiment. This is the same approach which we and others have used for *in vivo* 2P imaging (Stosiek et al., 2003; Zhang et al., 2005; Zhang et al., 2007; Brown et al., 2008; Li and Murphy, 2008; Murphy et al., 2008; Brown et al., 2009; Liu and Murphy, 2009). A wide range of anesthetics restrain animals, thereby stabilising the brain and minimising movement artefacts for acute and chronic imaging of cells and their subcellcular components (Yuste and Konnerth, 2005). While anesthetized animals are commonly employed for *in vivo* imaging, anesthetics introduce a confounding variable in experiments since they are known to be neuroprotective (Kawaguchi et al., 2005) and have widespread effects on cerebral metabolism (Choi et al., 2002) and cortical activity (Hentschke et al., 2005). As an advance into the field, some labs have now implemented awake *in vivo* imaging in behaviourally trained rodents to investigate neuronal dynamics (Helmchen et al., 2001; Dombeck et al., 2007).

4.11 Future directions

What needs to be considered is whether hypothermia treatment would also affect dendrite recovery in other stroke models. Because of the important relationship between blood flow and dendrite structure (Zhang et al., 2005; Enright et al., 2007; Li and Murphy, 2008; Murphy et al., 2008) and because we show here that dendrites blebbed even in the presence of moderate and deep hypothermia during occlusion but recovered with reperfusion (Figures 4 and 5A), we predict that similar hypothermic treatment would result in improved structural recovery following transient MCAO where reperfusion is restored (Corbett et al., 2000; Li and Murphy, 2008). In the case where focal ischemia does not restore blood flow such as in permanent MCAO (Clark et al., 2008; Clark et al., 2009) and RB-photothrombosis (Zhang et al., 2005; Enright et al., 2007), we expect hypothermia treatment to be ineffective. Hemorrhagic stroke is a second, less studied, but equally devastating form of stroke (MacLellan et al., 2009). Microvascular hemorrhages cause dendrites to bleb (unpublished observations, Dr. Chris Schaffer) and it would be interesting to determine if hypothermia would be effective in this case. Thus far, hypothermia does not appear to be as potent in treating hemorrhagic stroke (MacLellan et al., 2009). Contrary to focal and global ischemia where acute and chronic intraischemic, delayed intraischemic, and postischemic hypothermia are effective in improving outcomes (Xue et al., 1992; Dietrich et al., 1993; Nurse and Corbett, 1994; Colbourne et al., 2000; Kawai et al., 2000; Hashimoto et al., 2008; MacLellan et al., 2009), application of hypothermia appears to be effective only when induced relatively late (~12 hours) following injury, is prolonged, and combined with a slow rate of re-warming (MacLellan et al., 2004; MacLellan et al., 2009). Hence, such hypothermic administration can determine if cooling would be effective in recovery of dendritic blebbing in hemorrhages.

In assessing neuroprotective effectiveness, the ultimate goal is to determine whether a treatment or intervention is able to restore function after stroke (Corbett and Nurse, 1998). Thus, studies should take a comprehensive approach in combining anatomical, neurophysiological, and behavioural assessment (Corbett and Nurse, 1998). Future studies need to relate the morphological changes described here to functional assessment with functional imaging, electrophysiology, and/or behavioural tests at chronic endpoints to verify any persistent effects of hypothermia on dendrite structure.

4.12 Conclusion

It has been long held that neuronal structure is tied with function (Fiala et al., 2002; Alvarez and Sabatini, 2007) and thus, investigations into structural changes to dendrites can provide insight into physiological processes and provide a potential target for treatment of neurologic injury. In this context, acute structural damage to dendrites should be considered in the scheme of other detrimental ischemic processes (Dirnagl et al., 1999; Traystman, 2003; Doyle et al., 2008).

In summary, we found ischemia-induced dendritic blebbing could not be prevented even with deep hypothermia treatment and this structural alteration may be an obligate effect of ischemia. Thus, mechanisms involved in blebbing are likely still intact during hypothermia, however their kinetics may be altered as we have demonstrated a temperature dependency for dendritic blebbing onset latency. Although the effect of ischemia on dendrite damage was not prevented, deep hypothermia treatment led to more consistent recovery of structure with reperfusion. Since we do report a modest temperature dependence on structural recovery, we advise that future work *in vivo* or *in vitro* should control for temperature effects since this may influence cellular and functional endpoints. Future experiments evaluating the effect of

hypothermia on dendrite structure in other models of stroke and at other times of administration would provide additional insights. An integrative approach by combining chronic imaging with functional and behavioural assessment would uncover chronic and persistent effects of structural recovery in the context of hypothermic neuroprotection.

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