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Neurodegeneration After Cardiac Arrest: Cell Death Mechanisms And Methods Of Neuroprotection

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Abstract
Brain injury after cardiac arrest is a significant contributor to morbidity and mortality. Selectively vulnerable neuron populations undergo delayed neurodegeneration in the hours to days following reperfusion. Multiple factors influence neuronal death after global brain ischemia, including excitatory synaptic input, mitochondrial dysfunction, oxidative stress, inflammation, and disruption of intracellular Ca2+ homeostasis. In hippocampal CA1 pyramidal neurons, these factors activate cell death via pathologic activity of calpains, the family of Ca2+-dependent proteases, and calpain inhibition protects against neurodegeneration. Therefore, we hypothesized that pathologic calpain activity is necessary and sufficient for neurodegeneration in other selectively vulnerable neuron populations. In this thesis, the mechanism of calpain-mediated neurodegeneration and optimization of current therapeutic approaches were explored using adult rat models of brain ischemia. First, post-ischemic protease activity in mitochondria was characterized, specifically the mechanism of electron transport chain subunit proteolysis. Complex I subunit NDUFB8 and Complex V subunit \( \alpha \) were cleaved in a Ca2+-independent manner in vitro by a mitochondrial cysteine protease. In addition, NDUFB8 proteolysis was detected in hippocampal synaptosomes after global and focal brain ischemia, indicating that proteolytic activity occurs in vivo and could play a role in post-ischemic mitochondrial dysfunction. Second, the role of calpain activity in neurodegeneration of cerebellar Purkinje cells was examined using an RNA interference approach to cause functional knockdown of calpain activity. Surprisingly, knockdown of calpain activity did not significantly alter Purkinje cell neurodegeneration 48 hours after cardiac arrest, suggesting that alternative mechanisms may be involved in post-ischemic neurodegeneration in the cerebellum. Finally, the optimal parameters of post-cardiac arrest therapeutic hypothermia to minimize Purkinje cell loss were explored. Based on the parameters tested, therapeutic hypothermia initiated 0-8 hours after return of spontaneous circulation and maintained for 24 or 48 hours resulted in similarly significant neuroprotection compared to normothermia seven days after cardiac arrest in the adult rat. In conclusion, these results paint a more complex picture of post-ischemic neurodegeneration, which may include contributions of multiple pro-cell death stimuli rather than pathologic calpain activity alone, and highlight the effectiveness of therapeutic hypothermia, which has broad effects on post-cardiac arrest pathology that could contribute to neuroprotection.

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NEURODEGENERATION AFTER CARDIAC ARREST: CELL DEATH MECHANISMS AND METHODS OF NEUROPROTECTION

Michael Gregory Paine

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ABSTRACT

NEURODEGENERATION AFTER CARDIAC ARREST: CELL DEATH MECHANISMS AND METHODS OF NEUROPROTECTION

Michael G. Paine
Robert W. Neumar

Brain injury after cardiac arrest is a significant contributor to morbidity and mortality. Selectively vulnerable neuron populations undergo delayed neurodegeneration in the hours to days following reperfusion. Multiple factors influence neuronal death after global brain ischemia, including excitatory synaptic input, mitochondrial dysfunction, oxidative stress, inflammation, and disruption of intracellular Ca\(^{2+}\) homeostasis. In hippocampal CA1 pyramidal neurons, these factors activate cell death via pathologic activity of calpains, the family of Ca\(^{2+}\)-dependent proteases, and calpain inhibition protects against neurodegeneration. Therefore, we hypothesized that pathologic calpain activity is necessary and sufficient for neurodegeneration in other selectively vulnerable neuron populations. In this thesis, the mechanism of calpain-mediated neurodegeneration and optimization of current therapeutic approaches were explored using adult rat models of brain ischemia. First, post-ischemic protease activity in mitochondria was characterized, specifically the mechanism of electron transport chain subunit proteolysis. Complex I subunit NDUFB8 and Complex V subunit α were cleaved in a Ca\(^{2+}\)-independent manner \textit{in vitro} by a mitochondrial cysteine protease. In addition, NDUFB8 proteolysis was detected in hippocampal synaptosomes after global and focal brain ischemia, indicating that proteolytic activity occurs \textit{in vivo} and could play a role in post-
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INTRODUCTION

1.1. General overview of cardiac arrest

Sudden cardiac arrest is among the most common causes of death in the United States. Estimates of the incidence of out-of-hospital cardiac arrest are in the range of 300,000 adults per year (Nichol et al., 2008). Unfortunately, only 5% to 10% of patients who suffer an out-of-hospital cardiac arrest are successfully resuscitated and survive the incident without intervention beyond standard practices of the intensive care unit (Aufderheide et al., 2011; Stiell et al., 2011). Among survivors, only 50% have a good neurologic outcome, characterized by the ability of the patient to regain normal daily living without assistance (Aufderheide et al., 2011; Stiell et al., 2011). This provides a great opportunity and room for improvement of post-cardiac arrest care to minimize the severity of this debilitating injury. Of those who are resuscitated after out-of-hospital cardiac arrest and admitted to the hospital, brain injury is the most common cause of death (Laver et al., 2004). Therefore, it is imperative to focus on neuroprotection to improve survival and neurologic outcome after cardiac arrest.

1.2. Selectively vulnerable neuron populations

1.2.1. Hippocampus

One intriguing aspect of cardiac arrest-induced brain injury is that not all of the brain responds equally to the temporary global loss of blood flow. Instead, specific neuron subtypes in various brain regions are more susceptible to neurodegeneration. These
groups of neurons that are most sensitive to global ischemic injury are called selectively vulnerable neuron (SVN) populations. The most thoroughly studied SVN population is the CA1 pyramidal neurons in the hippocampus (Larsson et al., 2001). Kirino (1982) first characterized delayed neuronal death after global ischemia. Since then, CA1 pyramidal neuron death has been commonly used as a representative of global ischemic injury. Protection against post-ischemic CA1 pyramidal neuron loss in animal models often correlates with improved performance in learning and memory behavioral tasks (Block and Schwartz, 1998; Milani et al., 1998).

1.2.2. Cerebellum

Next to CA1 pyramidal neurons in the hippocampus, the Purkinje cells of the cerebellum are among the most susceptible to excitotoxic injury after cardiac arrest (Horn and Schlote, 1992, Ng et al., 1989; Welsh et al., 2002). Extensive loss of Purkinje cells in human cerebellum after cardiac arrest has been well documented (Horn and Schlote, 1992, Figure 1.1). However, the pathologic effect of Purkinje cell loss is poorly understood. Limited evidence suggests that loss of Purkinje cells after cardiac arrest could contribute to neurologic dysfunction, including post-hypoxic myoclonus (Welsh et al., 2002; Venkatesan and Frucht, 2006; Sarna et al., 2003). Examination of Purkinje cell death after global ischemia is made more difficult by the caveat that the frequently utilized animal models of transient forebrain ischemia do not injure the cerebellum (Kagstrom et al., 1983). Therefore, analysis of cerebellar neurodegeneration is typically
Figure 1.1. Degeneration and loss of Purkinje cells in human tissue after cardiac arrest. A-C: human cerebellar sections stained with hemotoxylin and eosin from patients who died 5 hours (A), 12 hours (B), and 13 days (C) after ROSC. D: The percentage of degenerating nuclei from the CA1 pyramidal layer of hippocampus, the Purkinje cell layer of cerebellum (yellow), and the frontal neocortex was quantified. Figure adapted from Horn and Schlote, 1992.

performed using a whole-body cardiac arrest injury model (Tai et al., 2007, Welsh et al., 2002).

1.2.3. Other brain regions

Several other SVN populations are less well studied. In the striatum, dorsolateral projection neurons and parvalbumin-positive striatal interneurons, but not neuropeptide Y-positive striatal interneurons, were lost after global ischemia in rats (Larsson et al., 2001). In the thalamus, neurons in the somatosensory nucleus are lost, leading to
impaired thalamocortical circuitry (Shoykhet et al., 2012). In the neocortex, pyramidal neurons in layers III and V are most sensitive to global ischemic insult (Sieber et al., 1995). Each of these regions may contribute to neurologic dysfunction and mortality after cardiac arrest.

1.3. *Factors involved in selective vulnerability*

A multitude of factors appear to influence the relative vulnerability of different neuron populations to transient ischemia and reperfusion. Some of these factors, including the nature of excitatory input to the neuron population, blood flow to the region of the brain, and inflammation at the site of injury, are extrinsic to the neuron populations, while others, such as the magnitude of and response to oxidative stress and Ca$^{2+}$ influx, are intrinsic to the neurons. Although each of these factors will be discussed individually, there is likely to be considerable overlap in their contribution to neuronal death.

1.3.1. *Excitatory input to SVN populations*

One aspect of SVN populations that appears to be consistent is that, compared to other neuron populations, SVN populations possess a greater extent of excitatory synapses, which could be more likely to result in overexcitation under extreme stimulation. CA1 pyramidal neurons of the hippocampus are a part of the basic hippocampal circuit, in which the granule cells of the dentate gyrus project to pyramidal neurons in CA3, which then project to the pyramidal neurons of CA1. CA1 pyramidal neurons project to the subiculum and out of the hippocampus to the entorhinal cortex. All three of the main components of this circuit extend excitatory projections, which allows for overexcitation
in some pathologic circumstances such as seizure and ischemia-reperfusion injury (Griffiths et al., 1982; Deshpande et al., 1987).

Cerebellar Purkinje cells are a critical component of the cerebellar circuit. These neurons possess extensive dendritic arborizations, which receive inputs from two sources. The parallel fiber pathway originates from cerebellar granule cells and many thousand parallel fibers pass through and synapse with the distal dendritic arbors of each Purkinje cell. Alternatively, the climbing fiber pathway originates from the inferior olive and sends a single projection with multiple synapses to an individual Purkinje cell’s proximal dendrites and soma. These dual synaptic inputs provide the basis for the refinement and performance of fine motor control function of the cerebellum. Some evidence suggests that the climbing fiber input from the inferior olive could cause excitotoxic stimulation of the Purkinje cells to lead to their neurodegeneration (Welsh et al., 2002).

1.3.2. Cerebral blood flow

Variations in blood flow to different regions of brain tissue could contribute to the severity of ischemia-reperfusion injury. First, it is important to understand regional cerebral blood flow (CBF) in the baseline context. CBF in anesthetized animals appears to be similar throughout all regions of the brain, estimated in the range of 0.7-0.8 ml min⁻¹ g⁻¹ (Goldman and Sapirstein, 1973; Reivich et al., 1969). In the conscious state, regional CBF is more variable, between 0.8-1 ml min⁻¹ g⁻¹ (Goldman and Sapirstein, 1973). Interestingly, in this study the dorsal hippocampus was at the low end of the range of CBF in both the conscious and anesthetized state (Goldman and Sapirstein, 1973).
addition, neuronal stimulation is known to correlate with an uptick in CBF specifically in
the activated region (Sokoloff, 1961).

Upon reperfusion after transient ischemia, total CBF elevates to hyperemia. However,
there is wide regional variation in this effect (Kagstrom et al., 1983; Lin et al., 1979). In
fact, midbrain regions that are susceptible to ischemia-reperfusion injury, particularly the
hippocampus, achieve a lower-than-baseline CBF upon reperfusion, which can include
areas of no-reflow (Kagstrom et al., 1983). However, in the cerebellum there is very little
change in regional CBF between baseline and post-ischemic reperfusion (Kagstrom et al.,
1983). As reperfusion continues, regional CBF moderates toward baseline levels. The
timecourse of CBF recovery depends upon the duration—and model—of ischemia, with
longer ischemia duration correlating with a longer period until moderation of CBF and
larger areas of no-reflow in some models (Kagstrom et al., 1983; Lin, 1975).

Further evidence supports a role of CBF in the severity of ischemia-reperfusion injury.
Pre-treatment with an activator of the protein kinase C-ε pathway alleviated the post-
reperfusion modulation in hippocampal CBF and protected against CA1 pyramidal
neuron death (Della-Morte et al., 2011). In addition, brief ischemia-reperfusion insults
given just before (ischemic preconditioning) or after (ischemic post-conditioning)
ischemia-reperfusion injury have been demonstrated to moderate the post-reperfusion
CBF response as well as reduce hippocampal injury (Nakamura et al., 2006; Wang et al.,
2008). Therefore, the correlation between recovery of normal hippocampal CBF and the
protection of CA1 pyramidal neurons from post-ischemic neurodegeneration suggests
that ischemia-reperfusion-mediated alterations in CBF could contribute to neuronal injury, particularly in the hippocampus.

1.3.3. Inflammation

In the brain, the inflammatory response to ischemia-reperfusion injury is principally mediated by activated microglia. In the resting state, microglia exist throughout the CNS tissue and monitor the extracellular space for the presence of molecules indicating neural injury, such as intracellular proteins, pro-inflammatory factors, and foreign particles. When microglia encounter such species, they proliferate, migrate to the injury site, and differentiate into phagocytic cells. Within the first 8 hours after global ischemia, neurons express the interleukin-1 receptor to attract glial cell types, including microglia, expressing interleukin 1-β (Sairanen et al., 1997). Activated microglia are detected in SVN populations including hippocampal CA1, peaking between 4-6 days post-injury but lasting up to 2-4 weeks post-injury (Stoll et al., 1998; Orzyłowska et al., 1999).

Inflammation plays a somewhat complex role in the events following ischemia-reperfusion injury. Activated microglia benefit the injured tissue via phagocytosis of cellular debris (Danton et al., 2003). In addition, inflammatory signaling may promote neurogenesis and proliferation of new neurons at the injured region in the hippocampus via cyclooxygenase-2, although inhibition of this enzyme has been shown to be neuroprotective (Sasaki et al., 2003; Nakayama et al., 1998). However, activated microglia promote the release of neurotoxic species, including superoxide, nitric oxide, and their product peroxynitrite, which could damage neurons that may otherwise survive
the ischemic insult (reviewed in Neumar, 2000; Yang et al., 2008). Multiple studies have demonstrated that inhibition of the inflammatory response reduces global ischemic injury severity, and that exacerbation of the inflammatory response increases injury severity, in the hippocampus (Yrjänheikki et al., 1998; Ooboshi et al., 2005; Walker and Rosenberg, 2009; Xuan et al., 2012; Hazelton et al., 2010).

1.3.4. Ca\(^{2+}\)-mediated cell death mechanisms in ischemia-reperfusion

The excitatory inputs to the hippocampal and cerebellar circuits described above consist of glutamatergic synapses. AMPA receptors are glutamate-activated Na\(^+\) channels that generate a depolarizing current when activated. NMDA receptors are Ca\(^{2+}\)-permeable glutamate-activated channels. The Ca\(^{2+}\)-permeability of NMDA receptors is blocked by Mg\(^{2+}\) at resting membrane potential, and the cellular membrane must be depolarized to release this inhibition and allow extracellular Ca\(^{2+}\) entry. Neuronal depolarization activates voltage-gated Na\(^+\) channels in the membrane, and intracellular Ca\(^{2+}\) acts as a second messenger in multiple signaling pathways, including activation of the IP\(_3\) receptor to release Ca\(^{2+}\) from its intracellular stores in the endoplasmic reticulum (ER). If left unchecked, intracellular Ca\(^{2+}\) (Ca\(^{2+}\)_i) elevation acts in a feed-forward mechanism. However, neurons possess multiple strategies to maintain Ca\(^{2+}\)_i homeostasis (reviewed in Dong et al., 2006). The Na\(^+\)/Ca\(^{2+}\) exchanger and the plasma membrane Ca\(^{2+}\) ATPase extrude Ca\(^{2+}\) from the cytosol, the former with a larger capacity and the latter with a higher affinity (reviewed by Guerini et al., 2005). The sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase imports Ca\(^{2+}\)_i from the cytosol to the ER (Dong et al., 2006; Sepúlveda and Mata, 2005). If mitochondria are in a polarized state, they can buffer large
amounts of free Ca\(^{2+}\) via the mitochondrial Ca\(^{2+}\) uniporter and their phosphate pool in the matrix, which can precipitate free Ca\(^{2+}\) (Dong et al., 2006; Kannurpatti et al., 2000). In addition, glial and neuronal glutamate transporters limit the time that AMPA and NMDA receptors are activated by glutamate in the synaptic cleft (reviewed by Camacho and Massieu, 2006).

Under physiologic conditions, these mechanisms are sufficient to control Ca\(^{2+}\)\(_i\) levels such that spikes in Ca\(^{2+}\)\(_i\) activate second messenger pathways without causing an excitotoxic buildup of Ca\(^{2+}\)\(_i\). However, in an energy-depleted environment such as ischemia, Ca\(^{2+}\) extrusion methods become exhausted and Ca\(^{2+}\)\(_i\) levels rise to potentially toxic levels (Silver and Erecinska, 1998). In the case of resuscitation after cardiac arrest, the ischemic tissue is reperfused, allowing cells to produce the energy required to regain Ca\(^{2+}\)\(_i\) homeostasis (Silver and Erecinska, 1998). In a transient ischemia, such as cardiac arrest, the vast majority of neurons and glia are capable of withstanding the brief Ca\(^{2+}\)\(_i\) overload (Dux et al., 1987). However, in SVN populations, a delayed secondary wave of increased Ca\(^{2+}\)\(_i\) occurs in these neurons between 24-48 hours post-ROSC, leading to excitotoxic neurodegeneration (Dux et al., 1987; Neumar et al., 2001). The underlying mechanisms of this secondary Ca\(^{2+}\)\(_i\) wave require further investigation.

Multiple factors may play a role in Ca\(^{2+}\)\(_i\)-induced toxicity. First, Dux et al. (1987) found that mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_m\)) follows a similar spiking pattern to Ca\(^{2+}\)\(_i\) concentration after reperfusion. These spikes in [Ca\(^{2+}\)]\(_m\) can cause depolarization of the mitochondrial membrane, inhibiting ATP production and enhancing ROS generation (Canevari et al., 1999; Piantadosi and Zhang, 1996; Votyakova and Reynolds, 2005;
Friberg et al., 2002). Upon reperfusion, resumption of mitochondrial activity induces a surge in production of reactive oxygen species (ROS), which are capable of damaging lipids, proteins, and DNA (Piantadosi and Zhang, 1996). In vitro studies have demonstrated that increased [Ca\(^{2+}\)]\(_{m}\) impaired electron transport chain (ETC) function and increased ROS release (Votyakova and Reynolds, 2005). Overexpressing enzymes that break down ROS is neuroprotective in animal models of injury (Weisbrot-Lefkowitz et al., 1998; Gu et al., 2004). Alternatively, mild uncoupling of mitochondria by overexpressing endogenous proteins or injecting exogenous uncouplers reduces [Ca\(^{2+}\)]\(_{m}\) accumulation and ROS production and enhances neuronal survival (Korde et al., 2005; Mattiason et al., 2003). Therefore, there appears to be a direct link between post-injury increases in Ca\(^{2+}\)\(_{i}\) concentration, [Ca\(^{2+}\)]\(_{m}\), ROS production, mitochondrial dysfunction, and cell death.

Additionally, if the buffering capacity of mitochondria is overwhelmed, the increase in mitochondrial free Ca\(^{2+}\) causes swelling of the matrix that leads to opening of the mitochondrial permeability transition pore, which alleviates the Ca\(^{2+}\)-induced swelling but also releases pro-cell death factors such as Omi, apoptosis inducing factor (AIF), and cytochrome c (Dong et al., 2006). Third, Ca\(^{2+}\) depletion from the ER can activate a pro-apoptotic ER stress response (Mekahli et al., 2011). Finally, calpains, the family of Ca\(^{2+}\)-dependent proteases, can induce cell death when activated by high Ca\(^{2+}\) via proteolysis of its substrates. The calpain system and its role in excitotoxic neuronal death are described below.
1.4. Role of calpain activity in neurodegeneration after cardiac arrest

Calpains are a family of Ca\textsuperscript{2+}-dependent cysteine proteases that specifically cleave proteins in a limited manner to alter their function. The physiological roles of calpains include cytoskeletal remodeling, cell cycle progression, and long-term potentiation (Goll et al., 2003; Mellgren et al., 2007). Additionally, calpain activity plays a pathologic role. Pathologic calpain activity has been associated with neurodegeneration in numerous models of acute and chronic neurodegeneration, including global and focal ischemia (Neumar et al., 2001; Markgraf et al., 1998), traumatic brain and axonal injury (Schoch et al., 2012; Ma et al., 2012), Alzheimer’s disease (Higuchi et al., 2012; Ferreira and

**Figure 1.2. Domain structure of brain calpain isoforms.** From Bevers and Neumar (2008). Classical calpains 1 and 2 require heterodimerization with calpain small subunit 1 (Capns1) to form an active enzyme, μ-calpain and m-calpain, respectively. Calpain 3 possesses a Ca\textsuperscript{2+}-binding EF-hand domain, but does not require heterodimerization with Capns1 for activity. The C-terminal domains of the atypical calpains 5 and 10 do not appear to possess Ca\textsuperscript{2+}-binding domains, and the activity and Ca\textsuperscript{2+}-dependence of these calpain isoforms is not completely understood.
Bigio, 2011), and Parkinson’s disease (Vosler et al., 2008).

1.4.1. The calpain system in the brain

Of the 15 calpain isoforms discovered, 5 of these (1, 2, 3, 5, and 10) have been detected in brain tissue. The classical calpains 1 and 2 are nearly ubiquitously expressed and are the most thoroughly studied. Each requires heterodimerization with calpain small subunit 1 (Capns1) for activity; heterodimerized forms are referred to as μ- and m-calpain, respectively (Bevers and Neumar, 2008). In a cell-free system, μ-calpain required a range of 3-50 μM free Ca$^{2+}$ for half-maximal activity, while m-calpain required 400-800 μM free Ca$^{2+}$ (Goll et al., 2003). Calpains 1 and 2 possess Ca$^{2+}$-binding EF hand domains in their C-terminal domain IV. Additionally, the active site contains Ca$^{2+}$-binding domains that are thought to be critical for alignment of the active site for proteolytic activity (Suzuki et al., 2004). Calpain 3 is most predominantly expressed in skeletal muscle, and mutations of this gene are implicated in limb-girdle muscular dystrophy type 2A (Richard et al., 1995). Calpains 5 and 10 are termed atypical calpains because they lack a Ca$^{2+}$-binding domain IV at the C-terminus of classical calpains, replaced by a C-terminal domain T (Bevers and Neumar, 2008). Additionally, calpain 10 does not contain the active site Ca$^{2+}$-binding domains of the typical calpain isoforms (Moldoveanu et al., 2002). Calpain activity is regulated by the endogenous inhibitor calpastatin, which possesses a repeated domain structure that allows each calpastatin molecule to inhibit up to four calpain molecules (Maki et al., 1987).
1.4.2. Mechanistic role of calpain activity in global brain ischemia

Specific and sensitive detection of calpain activity has been achieved by utilizing antibodies directed against the exposed calpain cleavage site of calpain substrates such as spectrin and the inositol-1,4,5-triphosphate (IP₃) receptor subtype 1 (Roberts-Lewis et al., 1994; Kopil et al., 2011). Using this approach, post-ischemic calpain activity has been demonstrated in the hippocampal CA1 sector, cerebral cortex, striatum, and Purkinje cells of the cerebellum—all of which are SVN populations—in a timecourse that correlates with neurodegeneration (Neumar et al., 2001; Kopil et al., 2011).

The critical role of calpain activity in the hippocampus in neurodegeneration after global brain ischemia has been demonstrated using a variety of techniques. Multiple calpain inhibitor drugs have proven effective in reducing ischemia-reperfusion injury in animal models, even when given up to 22 hours after reperfusion (Lee et al., 1991; Li et al., 1998; Frederick et al., 2008). One important caveat of these calpain inhibitor compounds is their non-specific activities (Wang and Yuen, 1999). Therefore, genetic manipulations of the calpain system have been used to isolate their role in the mechanisms of neurodegeneration. Overexpression of the specific endogenous inhibitor calpastatin has been used to inhibit calpain activity and prevent neurodegeneration (Cao et al., 2007). Alternatively, RNA interference has been used to block activity of specific calpain isoforms. Recent studies have demonstrated that calpain 1 is the critical isoform causing neurodegeneration in hippocampal CA1 pyramidal neurons in a rat model of global brain ischemia (Bevers et al., 2010), while calpain 2 is the isoform activated in primary hippocampal neurons subjected to NMDA excitotoxicity (Bevers et al., 2009).
It is important to note that most in vivo studies of neurodegeneration after global ischemia have focused on hippocampal CA1 pyramidal neuron loss. Exploration of the role of calpain activity in post-ischemic neurodegeneration in other SVN populations is necessary to determine whether blockade of calpain activity alone would be sufficient as a therapeutic strategy after cardiac arrest.

1.4.3. Timecourse of calpain activity in the brain after global ischemia

Due to the raised Ca\(^{2+}\) levels during and immediately after the ischemic event, calpain activity has been detected as early as 15 minutes after reperfusion (Yokota et al., 1995; Roberts-Lewis et al., 1994; Neumar et al., 2001). However, this period of calpain activity is not sufficient to induce immediate neuronal death, and neurons recover from the ischemic event and degrade the calpain cleavage products. Upon the secondary wave of Ca\(^{2+}\) entry, calpain-cleaved substrates accumulate until neuronal death. The delayed calpain activity in the hippocampus and cortex begins between 24-48 hours after reperfusion, leading to neurodegeneration between 48-72 hours (Neumar et al., 2001; Siman et al., 2005). However, in the cerebellum, this process appears to be accelerated, such that calpain activity in Purkinje cells peaks by 24 hours and neurodegeneration begins between 24-48 hours after reperfusion (Kopil et al., 2011; unpublished observations). Overall, the mechanisms underlying the timing of the secondary Ca\(^{2+}\) overload and delayed calpain activity are poorly understood, but this time period may provide a therapeutic window during which calpain inhibition could be initiated to prevent neuronal death (Frederick et al., 2007).
1.4.4. Localization of pathologic calpain activity

Cytosol
Classical calpains 1 and 2, as well as calpain 3, are expressed in the cytosol (Bevers et al., 2008). These isoforms have access to cytosolic substrates, as well as transmembrane and membrane-associated proteins on the plasma membrane, the ER membrane, and the synapse. As reviewed by Bevers et al. (2008), calpains cleave substrates involved with post-synaptic reorganization, Ca\(^{2+}\) regulation, and signaling cascades. Proteolysis of many of these substrates—including but not limited to p53, p35, collapsin response mediator proteins, IP\(_3\) receptor, Bid, AIF, and caspase 7—converts these molecules towards a pro-cell death function (Bevers et al., 2008). It is likely that convergence of multiple cell death mechanisms via calpain activity could cause calpain-mediated toxicity.

Mitochondria
At the time of their initial discovery and characterization, calpains were thought to have purely cytosolic localization. Recently, calpain 1 was detected in mitochondria (Garcia et al., 2005; Kar et al., 2007). Conflicting studies have reported calpain 1 to be present in the intermembrane space (IMS) (Badugu et al., 2008) or the IMS face of the inner membrane (IM) (Kar et al., 2008). Calpain 2 has been reported to be present in the IMS of rat liver mitochondria (Ozaki et al., 2009). The regulatory subunit Capns1 has also been found in mitochondria (Badugu et al., 2008; Ozaki et al., 2007; Kar et al., 2007). Arrington et al. (2006) found calpain 10 to be specifically localized to the mitochondrial matrix in rabbit and rat renal cortical mitochondria. This group has also demonstrated that
three calpain 10 isoforms are expressed in mitochondria (Giguere et al., 2008). In addition, the calpain inhibitor calpastatin has been detected in mitochondria of bovine pulmonary smooth muscle, suggesting that mitochondrial calpains may be regulated a manner analogous to cytosolic mechanisms (Kar et al., 2007; Kar et al., 2008).

The best-characterized mitochondrial calpain substrate is AIF, which is bound to the IMS face of the IM (Polster et al., 2005). There is evidence that calpain-mediated cleavage of AIF generates a truncated form that translocates to the nucleus to initiate cell death after global brain ischemia (Cao et al., 2007). Polster et al. (2005) demonstrated that μ-calpain cleaves AIF in a Ca^{2+}-dependent manner. Further studies of the ability of mitochondrial μ-calpain to cleave AIF have provided conflicting results (Joshi et al., 2009; Ozaki et al., 2009). Additionally, recent evidence suggests that AIF proteolysis by calpain is not necessary for nuclear translocation and activation of AIF-induced cell death (Wang et al., 2009).

Two independent studies report calpain-mediated cleavage of ETC subunits. Goudenege et al. (2007) detected a calpain-cleaved fragment of ATP synthase/Complex V subunit α (Complex Vα), part of the F_0 complex in the matrix. Arrington et al. (2006) found Ca^{2+}-dependent cleavage of two subunits of Complex I, NADH dehydrogenase (ubiquinone) flavoprotein 2 (NDUFV2) and NADH dehydrogenase (ubiquinone) 1 β subcomplex 8 (NDUFB8) (originally reported as ND6; Capaldi, 2008). In addition, reduced levels of purported calpain 10 substrates NDUFB8 and Complex V subunit β have been reported in models of acute kidney injury (Funk and Schnellmann, 2011).
While multiple studies have detected calpain-mediated proteolytic fragments in mitochondria (Polster et al., 2005; Goudenege et al., 2007; Arrington et al., 2006; Arrington and Schnellman, 2008; Funk and Schnellmann, 2011), only proteolysis of AIF has been detected in brain mitochondria. Therefore, further studies of mitochondrial

Figure 1.3. Hypothetical model of mitochondrial calpain activity after ischemic brain injury. Pathways known to occur post-ischemia are in blue, and hypothesized pathways are in red. Ischemia-reperfusion causes elevated intracellular Ca$^{2+}$, which is absorbed by mitochondria. Elevated [Ca$^{2+}$]$_{m}$ can induce MPT, reduce ATP synthesis, and increase ROS production. Calpains cleave AIF post-ischemia to induce cell death. Calpain-mediated cleavage of ETC subunits in Complex I (NDUFV2 and NDUFB8) and Complex V ($\alpha$ and $\beta$) have been detected in vitro and/or in vivo. As shown, post-ischemic increases in [Ca$^{2+}$]$_{m}$ could lead to activation of mitochondrial calpains to contribute to post-ischemic cell death via cleavage of ETC subunits, resulting in ETC complex dysfunction, reduced ATP synthesis, and increased ROS production.
calpain activity in the brain are needed to elucidate its role in mitochondrial dysfunction and neurodegeneration.

Taken together, these results suggest that mitochondrial calpains may be important mediators of mitochondrial homeostasis, and that alteration of the mitochondrial calpain system could contribute to mitochondrial dysfunction (Figure 1.3). However, further study is needed to characterize mitochondrial calpain activity.

1.5. **Therapeutic hypothermia**

Currently, the only therapy proven in randomized clinical controlled trials to improve survival rate and neurologic outcome after cardiac arrest is therapeutic hypothermia (Bernard et al., 2002; Hypothermia after Cardiac Arrest Study Group, 2002). In these studies, survival rate and neurologic outcome were improved by 30-50%. Presently, the standard operating procedure for therapeutic hypothermia is quite variable among treatment centers, but current guidelines recommend cooling body temperature from 37 °C to 32-33 °C once the patient is resuscitated and stabilized (ideally within 4 hours of the incident) for a period of 12-24 hours, at which time the patient is passively or actively rewarmed to normothermic temperature (Peberdy et al., 2010). Numerous studies utilizing a similar hypothermia regimen have been successful in human patients (Belliard et al., 2007; Castrejon et al., 2009). However, the optimal therapeutic hypothermia regimen has yet to be elucidated. Additional studies in translational models and clinical settings should provide beneficial insight into the maximal therapeutic benefit of hypothermia treatment.
Although the definitive mechanism of neuroprotection is not known, post-ischemic therapeutic hypothermia has multiple effects that could contribute to neuronal survival. First, the basal metabolic rate is reduced to 50-65% of normothermia (reviewed in Polderman, 2009; Erecinska et al., 2003). The inflammatory response is alleviated due to hypothermia treatment, as evidenced by a reduction in activated microglia in the hippocampus (Webster et al., 2009). Free radical production is mitigated after ischemia, although these studies performed therapeutic hypothermia throughout the ischemia-reperfusion period (Globus et al., 1995; Horiguchi et al., 2003). Disruption of Ca$_{2+}$ homeostasis is also attenuated by therapeutic hypothermia (Polderman, 2009). It is likely that a combination of these factors contributes to neuroprotection by therapeutic hypothermia. However, a better understanding of these individual mechanisms could help optimize therapeutic hypothermia treatment and aid in the development of novel therapeutic strategies.

1.6. Summary

Brain injury after cardiac arrest causes significant morbidity and mortality. Multiple mechanisms appear to contribute to neuronal injury and loss. One valuable approach to elucidate neuronal death pathways after cardiac arrest is to examine individual neurodegeneration mechanisms in order to determine the causal pathway(s) of post-ischemic neurodegeneration. Previous studies have indicated that cytosolic calpain activity plays a critical role in post-ischemic neurodegeneration in the CA1 pyramidal neurons of the hippocampus. To expand upon our current understanding, the role of calpain activity in post-ischemic mitochondrial dysfunction is analyzed. Next, the role of
Calpain activity in cerebellar Purkinje cell neurodegeneration is examined. An alternative approach to improve outcome in the near-term is to focus on optimizing existing therapeutic strategies. Therefore, the optimal parameters of therapeutic hypothermia in the cerebellum are explored. The results of these studies provide valuable insight into neurodegeneration mechanisms and could have broad clinical impact, as these cell death pathways are found in a variety of diseases.
PROTEOLYSIS OF ELECTRON TRANSPORT CHAIN SUBUNITS BY AN 
ENDOGENOUS MITOCHONDRIAL CYSTEINE PROTEASE

2.1. Introduction
Neurodegeneration caused by ischemic brain injury is a major contributor to death and
disability (American Heart Association Statistics Committee and Stroke Statistics
Subcommittee, 2011). One important contributor to the mechanisms of post-ischemic
neurodegeneration is mitochondrial dysfunction. During the ischemic event, oxygen and
ATP in brain tissue are exhausted, resulting in depolarization of neuronal membranes
(Silver and Erecinska, 1998). Upon reperfusion, oxidative metabolism restarts, causing
elevated reactive oxygen species (ROS) production and mitochondrial swelling due to
absorption of excess intracellular Ca^{2+} (Dux et al., 1987; Starkov et al., 2004; Nicholls,
2009; Racay et al., 2009; Sims and Muyderman, 2009). Although ion gradients return to
baseline in the penumbra after focal ischemia and in selectively vulnerable neurons after
global ischemia, reduced energy metabolism and increased ROS production perpetuate
(Silver and Erecinska, 1998; Starkov et al., 2004; Piantadosi and Zhang, 1996; Racay et
al., 2009; Friberg et al., 2002). Taken together, these disruptions in mitochondrial
function and mitochondrial Ca^{2+} homeostasis can lead to opening of the mitochondrial
permeability transition pore, causing the release of cell death factors such as apoptosis
inducing factor to induce neurodegeneration (Plesnila et al., 2004; Cao et al., 2003).

A recently discovered potential mechanism of mitochondrial dysfunction is proteolysis of
electron transport chain (ETC) subunits by the calpain family of proteases. Of the 15
known calpain isoforms, calpains 1 and 10 and the endogenous calpain inhibitor
calpastatin have been detected in mitochondria (Garcia et al., 2005; Arrington, et al., 2006; Kar et al., 2007; Giguere et al., 2008). However, only calpain 1 has been demonstrated to be present in brain mitochondria, and further study is required to elucidate the localization and activity of mitochondrial calpains and calpastatin in neurons (Garcia et al., 2005).

Mitochondrial calpain-mediated ETC subunit proteolysis has been reported in vitro and in vivo. Arrington et al. (2006) detected cleavage of two inner membrane subunits of Complex I, NDUFV2 and NDUFB8 (originally reported as ND6—see Capaldi, 2008), causing reduced activity of Complex I in mitochondria from rabbit and rat renal cortex. Because calpain 10 is the only known matrix cysteine protease, this group concluded that this isoform was responsible for proteolysis. In addition, reduced levels of purported calpain 10 substrates NDUFB8 and Complex V subunit β have been reported in models of acute kidney injury (Funk and Schnellmann, 2011). Using an RNA interference approach, Goudenege et al. (2007) detected a calpain 2-mediated proteolytic fragment of ATP synthase/Complex V subunit α (Complex Vα), part of the F₀ complex in the matrix, in Ca⁺⁺ ionophore-treated C₂C₁₂ mouse myotube lipid rafts. However, this result is not supported with any evidence that calpain 2 is localized to mitochondria. The regulation of mitochondrial calpain activity is not completely understood. While calpastatin-mediated inhibition of calpain 1 is well documented, the inhibitory effect of calpastatin on calpain 10 has not been reported, and based on the atypical structure of calpain 10, calpastatin may not inhibit this isoform (Croall and Ersfield, 2007). Taken together, these results suggest that mitochondrial calpains may be important mediators of mitochondrial
homeostasis, and that alteration of the mitochondrial calpain system could contribute to the altered energy metabolism and increased ROS production related to post-ischemic mitochondrial dysfunction. However, further study is needed to characterize mitochondrial calpain activity.

In this study, we set out to test the hypothesis that mitochondrial calpains cleave ETC subunits in a manner consistent with classical calpain activity. We first examined the conditions required to induce and inhibit ETC subunit proteolysis in isolated mitochondria from brain, heart and kidney. In addition, we examined whether ETC subunit proteolysis could be detected after brain ischemia and reperfusion. Our results suggest that an endogenous mitochondrial cysteine protease can cleave ETC subunits \textit{in vitro} and \textit{in vivo}. The characteristics of the protease activity are not consistent with the activity of calpain 1 or 2, but are potentially consistent with the atypical isoform calpain 10.

\textit{2.2. Materials and Methods}

\textit{2.2.1. Fractionation of Rat Forebrain}

Adult male Long Evans rat (Harlan Laboratories) forebrain was extracted and placed in 1g:10 mL MSHE buffer (0.22M mannitol, 70mM sucrose, 0.5mM EGTA, 2mM K-HEPES, pH 7.2) with 0.1% fatty acid-free bovine serum albumin (BSA) (MSHE+BSA) and 1% protease inhibitor cocktail (P2714, Sigma), minced, and homogenized using a Dounce homogenizer. Homogenate was centrifuged at 1,000 g for 10 minutes at 4 °C. The supernatant was taken and centrifuged at 10,000 g for 10 minutes at 4 °C. To obtain
purified cytosol, the supernatant from the second centrifugation was centrifuged at 100,000 g for 1 hour at 4 °C, and the resulting supernatant was collected. To obtain purified mitochondria, the pellet from the second centrifugation was resuspended in MSHE+BSA and layered above a 4x volume of 30% Percoll solution (30% v/v Percoll, 0.22M mannitol, 25mM K-HEPES, 1mM EGTA, 0.1% fatty acid-free BSA) for gradient centrifugation at 100,000 g for 30 min at 4 °C. After this step, the mitochondrial band was removed and washed once each in MSHE+BSA and MSHE buffer with centrifugation at 10,000 g for 10 minutes at 4 °C. Purified mitochondria were resuspended at 10.0mg/mL in mitochondria buffer (125mM KCl, 20mM K-HEPES, 2mM KH₂PO₄, 10μM EGTA, pH 7.2) and stored at -20 °C.

2.2.2. Isolation of mitochondria from rat kidney cortex and heart tissue

Adult male Long Evans rat kidney cortex was excised and minced in 1g:10mL MSHE+BSA plus 1% protease inhibitor cocktail. The minced tissue was homogenized using a Potter-Elvenjem homogenizer at 1600 rpm.

Adult male Long Evans rat heart tissue was minced in 1g:10 mL Buffer A (100mM KCl, 50mM Tris-HCl, 5mM MgCl₂, 1mM EDTA, 1mM ATP, 0.5% fatty acid-free BSA, pH 7.5) and briefly homogenized using a Potter-Elvenjem homogenizer at 1600 rpm to release subsarcolemmal mitochondria. To release interfibrillar mitochondria, the homogenized heart tissue was incubated in 4mg trypsin/g tissue in Buffer A on ice for 2 minutes with stirring. Then, 4mg/g tissue soybean trypsin inhibitor was added, and the sample was re-homogenized at 1600 rpm.
Rat kidney cortex and heart tissue homogenates were treated in the same manner as the forebrain homogenate described above to obtain purified mitochondria.

2.2.3. Generation of freeze-thawed mitochondria and in vitro proteolytic activity experiments

Purified mitochondria were subjected to three cycles of freeze-thaw prior to use. Freeze-thawed mitochondria and reaction samples were pre-incubated for 15 minutes at 37 °C prior to the start of each reaction. All proteolytic activity assays were performed with a minimum of three trials. Mitochondria were incubated at 1.00 mg/mL in mitochondria buffer plus 2mM BME (unless otherwise noted) at 37 °C under the conditions described for each experiment. For examination of pH dependence, the HEPES-based mitochondria buffer was not suitable for a pH 6.0 solution. Therefore, after the first freeze-thaw cycle, mitochondria were split into three aliquots and centrifuged at 10,000 g for 10 minutes at 4 °C. One aliquot of mitochondria was resuspended in the same volume of mitochondria buffer, one aliquot was resuspended in 0.1M potassium phosphate (KPi) pH 6.0, and one aliquot was resuspended in 0.1M KPi pH 7.2. These mitochondria samples were then subjected to two more freeze-thaw cycles for proteolytic activity experiments. Protease inhibitors used in this study include: MDL-28170 (Biomol); calpeptin, Z-FF-FMK, and human calpastatin peptide (EMD Biosciences); CA-074 (Bachem); and CYGAK (a generous gift from Dr. Rick Schnellmann, Medical University of South Carolina).

2.2.4. Western Blot
Western blot was performed as previously described (Bevers et al., 2009). SDS-PAGE was performed using 15-well 4-20% Tris-glycine precast gels (Invitrogen) at 125 V. Separated proteins were transferred to nitrocellulose membrane for 1-hour at 425 mA. Molecular weights were estimated using Precision Plus Protein Kaleidoscope Standards (Bio-Rad). Primary antibodies used include rabbit polyclonal antibodies to calpain 1 (generated by our laboratory), calpains 2 and 10 (Triple Point Biologics), and calpains 3 and 5 (Abcam), rabbit polyclonal anti-VDAC (Affinity Bioreagents), rabbit polyclonal anti-calpain-cleaved α-spectrin (Ab38, provided by Dr. Robert Siman, University of Pennsylvania), mouse monoclonal anti-actin AC-40 (Sigma), mouse monoclonal anti-NDUFB8 (Mitosciences), and mouse monoclonal anti-Complex Vα (Molecular Probes). Horseradish peroxidase-linked goat anti-mouse and anti-rabbit (PerkinElmer) secondary antibodies were used. Western blots were visualized using enhanced chemiluminescence solution (PerkinElmer).

2.2.5. Rat model of transient forebrain ischemia

All animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and were conducted according to the Guide for the Care and Use of Laboratory Animals. Adult male Long Evans rats were subjected to 9-minute transient forebrain ischemia (TFI) as previously described (Neumar et al., 2001; Bevers et al., 2010). After anesthesia induction with isoflurane (4%), rats were then orotracheally intubated and mechanically ventilated with 30% O₂ and 70% N₂O. A surgical plane of anesthesia was maintained with 2.0% isoflurane. Femoral arterial and
venous catheters were placed and temperature was monitored with a needle thermocouple probe placed adjacent to the skull below the temporalis muscle. During the procedure, temperature was maintained between 37.0 and 37.5 °C using a warming pad and an overhead lamp. Electrocardiographic monitoring was performed with limb leads. TFI was initiated by the combination of bilateral carotid occlusion and hypovolemic hypotension to a mean arterial pressure (MAP) of 30 mm Hg. Hypovolemic hypotension was achieved by rapidly withdrawing blood from the femoral arterial catheter and was maintained during the ischemic period by withdrawal or infusion of blood through the femoral venous catheter. Once a MAP of 30 mm Hg was achieved, both carotid arteries were reversibly occluded with surgical aneurysm clips. After 9 minutes, the aneurysm clips were removed and shed blood was reinfused. Rats were maintained on mechanical ventilation with invasive hemodynamic monitoring for 1-hour after reperfusion. During this time, an intraperitoneal telemetric temperature probe (Data Systems International) was surgically implanted. Beginning 1-hour after reperfusion, intravenous catheters were removed, surgical wounds were closed, and rats were weaned from mechanical ventilation and extubated. Post-injury body temperature was regulated at 36.5-38.0 °C using the intraperitoneal probe and software relays connected to a 175-W heating lamp, water misters and a cooling fan (Data Systems International) based on methods previously described (Colbourne et al., 1996; Che et al., 2011). Rats were euthanized 48-hours post-reperfusion and both hippocampi were microdissected and processed for synaptosome isolation as described below. Two naïve (non-instrumented) rats were used as controls.
2.2.6. Rat model of middle cerebral arterial occlusion

Adult male Sprague Dawley rats (Charles River Laboratories) were subjected to 120 minutes focal cerebral ischemia using the filament model as described previously (Luckl et al., 2008). Anesthesia was induced with isoflurane (1.5% to 2.0%) in a mixture of 70% nitrous oxide and 30% oxygen. Each rat was intubated and placed on a mechanical ventilator (SAR-830P Ventilator, CWE, Ardmore, PA, USA). Body temperature was maintained at 37.0±0.5°C during surgery. A laser-Doppler flow (LDF) probe was placed over the thinned skull (4 mm lateral to midline, 1 mm posterior to Bregma) to monitor changes in cerebral blood flow. Through a midline neck incision, the right external and internal carotid arteries were dissected from the surrounding connective tissue. A 0.39 mm silicone-coated nylon filament (Doccol Corporation, Redlands, CA, USA) was inserted through the right common carotid artery into the internal carotid artery to occlude blood flow to the middle cerebral artery (MCA) and reduce blood flow in the MCA territory to 20-30% of baseline. After 120 minutes of occlusion, the suture was withdrawn to allow for cerebral reperfusion. Rats were monitored by LDF for 30 min and then allowed to recover from anesthesia and euthanized either 1 or 24 hours after reperfusion. The ipsilateral and contralateral cortex was microdissected from the brain and processed for synaptosome isolation as described below. Three animals per group were performed for each reperfusion time point.

2.2.7. Isolation of synaptosomes
Microdissected brain regions were homogenized in 1g:10mL MSHE+BSA plus 1% protease inhibitor cocktail using a Dounce homogenizer. Homogenates were centrifuged at 1,000 g for 10 minutes at 4 °C. The supernatants were taken and centrifuged at 10,000 g for 10 minutes at 4 °C. This pellet was resuspended in MSHE+BSA and layered above a 4x volume of 30% Percoll solution for gradient centrifugation at 100,000xg for 30 min at 4 °C. The synaptosome band was extracted and washed once each in MSHE+BSA and MSHE Buffer with centrifugation at 10,000 g for 10 minutes at 4 °C. Purified synaptosomes were resuspended and stored in MSHE Buffer.

2.3. Results

2.3.1. Ca\(^{2+}\)-independent proteolytic activity in isolated mitochondria

First, we examined the conditions required to induce ETC proteolysis in rat forebrain mitochondria. Based on previous studies using non-neuronal tissues, we initially hypothesized that ETC proteolysis was Ca\(^{2+}\)-dependent (Arrington et al., 2006; Goudenege et al., 2007). Freeze-thawed brain mitochondria were incubated at 37 °C for 60 minutes in a range of 10μM-1mM CaCl\(_2\) or 1mM EGTA. Western blot was performed against the ETC subunits Complex V\(\alpha\) and NDUFB8 and the loading control VDAC (Figure 1A, left). Surprisingly, we found that addition of up to 1mM CaCl\(_2\) did not induce proteolysis. However, when 0.1% sodium dodecyl sulfate (SDS) was added to the reactions, we detected proteolysis of Complex V\(\alpha\) from the 50kDa full-length to the 30kDa fragment, as well as proteolysis of Complex I subunit NDUFB8 from the 18kDa full-length to the 15kDa fragment. Under these conditions, inclusion of 1mM EGTA or
10μM-1mM CaCl$_2$ had no effect on ETC proteolysis (Figure 1A, right). While the proteolytic fragments observed were consistent with mitochondrial calpain-mediated proteolysis, the Ca$^{2+}$-independence of the protease was contrary to previous reports (Arrington et al., 2006; Goudenege et al., 2007). Analysis of these reactions generated from freshly isolated forebrain mitochondria provided similar results (data not shown). Therefore, we utilized freeze-thawed mitochondria in all subsequent experiments analyzing ETC proteolysis to allow for efficient storage and use of mitochondria. To determine the time-course of ETC proteolysis, we incubated brain mitochondria in the presence of 0.1% SDS for 0-60 minutes. We detected a gradual decrease in the intact and gradual increase in the fragment of both Complex V$\alpha$ and NDUFB8 (Figure 1B).

**Figure 2.1. Conditions inducing proteolysis of ETC subunits.** (A) Rat brain mitochondria were incubated at 37 °C for 60 minutes in mitochondria buffer and 2mM BME with or without 0.1% SDS in the absence (Non-Treated) or presence of 1mM EGTA or 10μM, 100μM, or 1mM CaCl$_2$. (B) Mitochondria were incubated at 37 °C over a time-course of 60 minutes in buffer alone (Untreated) or in the presence of 2mM BME and 0.1% SDS. Western blots were performed using antibodies against the ETC subunits Complex V$\alpha$ and NDUFB8 and loading control VDAC.
Next, we further examined the conditions inducing ETC proteolysis in rat brain mitochondria to characterize the activity of the protease(s). To determine the minimum concentration of SDS required for ETC proteolysis, brain mitochondria were incubated in a range of 0-2% SDS followed by Western blot against ETC proteolytic substrates (Figure 2A). Proteolysis of Complex Vα occurred only in the presence of 0.1% or 0.2% SDS. Proteolysis of NDUFB8 occurred maximally at 0.02% SDS but also occurred in the presence of 0.1% or 0.2% SDS. Incubation of mitochondria in 2% SDS, or 0.1% SDS without β-mercaptoethanol (BME), did not result in proteolysis of either substrate (Figure 2A).

Because forebrain mitochondria had not been examined in previous studies of ETC proteolysis, we were curious as to whether ETC subunit proteolysis could be induced in a similar manner in mitochondria isolated from other tissues. Mitochondria were isolated from rat kidney cortex and heart, and these freeze-thawed mitochondria were incubated in a range of 0-2% SDS. Western blot was performed against the ETC substrates Complex Vα and NDUFB8. We found that mitochondria from rat kidney cortex demonstrated the same profile of SDS-induced proteolysis as rat brain mitochondria (Figure 2B). However, rat heart mitochondria required an order of magnitude less SDS for proteolysis (Figure 2C). In addition, the proteolytic fragments of both substrates were much less stable in heart mitochondria. Overall, it appears that ETC proteolysis can be induced in mitochondria isolated from different tissues in a Ca\(^{2+}\)-independent manner that requires addition of the ionic detergent SDS and a reducing environment.
Figure 2.2. SDS-induced proteolysis of calpain substrates in brain, heart, and kidney cortex mitochondria. Freeze-thawed mitochondria isolated from adult Long Evans rat brain, heart, and kidney cortex were incubated at 37 °C for 60 minutes in the presence or absence of 2mM BME in a range of 0-2% SDS, followed by Western blot of (A) brain, (B) heart, or (C) kidney cortex samples using antibodies against Complex Vα, NDUFB8, and VDAC.

Since we detected Ca\(^{2+}\)-independent proteolytic activity that could be calpain-mediated, we examined the sequence homology of the rat brain calpain isoforms relative to calpain 1 (Figure 3). We found that, in addition to lacking the Ca\(^{2+}\)-binding EF-hand domains, calpain 10 did not possess sequence homology at the Ca\(^{2+}\)-binding sites in domains II and III of calpain 1, which are involved in alignment of the active site of calpain 1 (yellow highlight) (Moldoveanu et al., 2002). However, the active site residues of calpain 10 were homologous with the other calpain isoforms (green highlight), suggesting that proteolytic activity is possible for this isoform (Figure 3).

| Calpain | Sequence
<table>
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<tr>
<td>calpain-1</td>
<td>--------MAEELITPVYCTGSAQVQKORDKELG-------------LGRHENAIKYL 41</td>
</tr>
<tr>
<td>calpain-2</td>
<td>-------------------MAGIAMKLADREAEG---------------LGRHENAIKYL 31</td>
</tr>
<tr>
<td>calpain-3</td>
<td>MPTVISPTVAPRTAEPSPGVPHPAQKTEAGGGHPGIYSAISNFPIT5VKEKT 60</td>
</tr>
<tr>
<td>calpain-5</td>
<td>-----------------------------------------------MFSCTKADSN 12</td>
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<tr>
<td>calpain-10</td>
<td>-----------------------------------------------</td>
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Figure 2.3. Sequence homology of rat brain calpain isozymes. Rattus norvegicus homologs of calpain 1 (NCBI Protein ID: AAH61880.1), calpain 2 (AAH65306.1), calpain 3 (NP_058813.1), calpain 5 (NP_604456.1), and calpain 10 (NP_113861.1) were aligned using ClustalW 2.1 software (Larkin et al., 2007, http://www.ebi.ac.uk/Tools/msa/clustalw2). Sequence homology relative to calpain 1 is marked by black highlight. Domains of calpain 1 are labeled D\textsubscript{1}-D\textsubscript{4} and are separated by vertical lines. The five active site residues are in bold and highlighted green within the sequence and labeled A\textsubscript{1-5} above the sequence. The Ca\textsuperscript{2+}-binding sites are italicized and highlighted yellow within the sequence and labeled above the sequence C\textsubscript{2A-2B} for domain II and C\textsubscript{3A-3C} for domain III.
2.3.2. pH-dependence of ETC proteolysis

Because classical calpains are known to be most active at neutral pH, we examined the pH-dependence of ETC proteolysis. Rat brain SMPs were resuspended in mitochondria buffer (pH 7.2) or 0.1M KPi, pH 6.0 or 7.2, then incubated for 60 minutes in the absence or presence of 0.1% SDS and 2mM BME (Figure 4). We observed that the rate of proteolysis of both ETC substrates was higher at pH 6.0 than pH 7.2 in 0.1M KPi, and that proteolysis in 0.1M KPi, pH 6.0 was greater than in our HEPES-based mitochondria buffer. This finding is not consistent with the activity of calpains 1, 2, or 3, which are most active between pH 7.0-8.2 (Shevchenko et al. 1998; Goll et al., 2003). However, the optimal pH values for calpains 5 and 10 have not been determined. The lower pH optimum could implicate an alternate cysteine protease family, the cathepsins (Grynbaum and Marks, 1976; Whitaker and Seyer, 1979; Bradley and Whitaker, 1986).

![Figure 2.4. pH-dependence of ETC proteolysis.](image)

*Figure 2.4. pH-dependence of ETC proteolysis.* Rat brain mitochondria were incubated at 37 °C for 15 or 60 minutes in mitochondria buffer (Mito Buffer), 0.1M KPi pH 6.0, or 0.1M KPi pH 7.2 in the absence or presence of 0.1% SDS and 2mM BME. Western blot was performed using antibodies against Complex Vα, NDUFB8, and VDAC.
Figure 2.5. Effect of calpain inhibitors on ETC proteolysis. Mitochondria were incubated at 37 °C for 60 minutes in buffer alone (Untreated) or with 0.1% SDS and 2mM BME in the absence (Control) or presence of (A) pan-calpain inhibitors MDL-28170 (1, 0.1, or 0.01μM), calpeptin (1, 0.1, or 0.01μM), or calpain 10-specific inhibitor CYGAK (10, 1, or 0.1μM); (B) human calpastatin (HCAST) peptide (10μM); or (C) cathepsin B and L inhibitor Z-FF-FMK (10, 1, 0.1, or 0.01μM) or cathepsin B-specific CA-074 (1μM). Western blot was performed using antibodies against Complex Vα, NDUFB8 and VDAC.

2.3.3. Targeted inhibition of ETC proteolysis with calpain and cathepsin inhibitors
Table 2.1. IC50 values of inhibitors used in this study and effective concentrations blocking ETC proteolysis under the assay conditions described in Figure 2.5.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Calpain 1 IC50</th>
<th>Calpain 10 IC50</th>
<th>Cathepsin L IC50</th>
<th>Cathepsin B IC50</th>
<th>Effective [inhibitor] blocking ETC proteolysisa</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDL-28170</td>
<td>10nMb</td>
<td>Unknown</td>
<td>14.2nMc</td>
<td>25nMb</td>
<td>100nM</td>
</tr>
<tr>
<td>Calpeptin</td>
<td>6.7nMd</td>
<td>4nMe</td>
<td>3.3nMd</td>
<td>220nMd</td>
<td>100nM</td>
</tr>
<tr>
<td>CYGAK</td>
<td>&gt;100uMe</td>
<td>100nMe</td>
<td>Unknown</td>
<td>Unknown</td>
<td>1uM</td>
</tr>
<tr>
<td>HCAST-P</td>
<td>0.2nMf</td>
<td>Unknown</td>
<td>6uMf</td>
<td>&gt;50uMf</td>
<td>&gt;10uM</td>
</tr>
<tr>
<td>Z-FF-FMK</td>
<td>No effectg</td>
<td>Unknown</td>
<td>1.66nMh</td>
<td>397nMh</td>
<td>1uM</td>
</tr>
<tr>
<td>CA-074</td>
<td>200uMij</td>
<td>Unknown</td>
<td>172uMj</td>
<td>2.24nMj</td>
<td>&gt;1uM</td>
</tr>
</tbody>
</table>

aBased on results shown in Figure 5.

jValue for calpain 2, calpain 1 not measured.

To explore whether ETC proteolysis is calpain-mediated, we incubated brain SMPs in mitochondria buffer with pan-calpain inhibitors MDL-28170 or calpeptin plus 0.1% SDS (Figure 5A). Inclusion of pan-calpain inhibitors MDL-28170 and calpeptin blocked proteolysis at concentrations consistent with their IC50 to calpains (Figure 5, Table 1; Sasaki et al., 1990; Mehdi, 1991). In addition, we found that the novel calpain 10-specific inhibitor CYGAK also inhibited ETC proteolysis consistent with its IC50 for calpain 10 (Figure 5A, Table 1; Rasbach et al., 2009). However, human calpastatin peptide, a potent inhibitor of calpain isoforms 1 and 2 (Maki et al., 1989; Pfizer et al., 2008), did not inhibit ETC proteolysis (Figure 5B). In addition, cathepsin B and L inhibitor Z-FF-FMK,
but not cathepsin B-specific inhibitor CA-074, blocked ETC proteolysis (Figure 5C, Table 1).

2.3.4. ETC subunit cleavage in rat brain after global and focal ischemia-reperfusion.

Because calpain activity is involved in the mechanisms of neurodegeneration after global and focal ischemia, we hypothesized that mitochondrial calpains could become activated after ischemia to cleave ETC subunits, and that such proteolysis could contribute to the

![Figure 2.6. Examination of ETC subunit proteolysis after global and focal ischemic brain injury.](image)

Synaptosome samples were isolated from naïve or 48-hour post TFI (TFI) Long Evans rat hippocampus (Hipp.) and 1- or 24-hour post-MCAO Sprague Dawley rat contralateral (C) and ipsilateral (I) cortex. Western blots were performed using antibodies against the calpain-cleaved fragment of spectrin (Ab38), Complex Vα, NDUFB8, and loading control VDAC. As a positive control for ETC proteolysis, forebrain mitochondria treated with 0.1%SDS and 2mM BME for 1 hour at 37 °C was used (+ Control).
mitochondrial dysfunction that plays a role in post-ischemic neurodegeneration. Therefore, we examined whether mitochondrial calpain substrates are cleaved post-ischemia in vivo in a time-course similar to that of cytosolic calpain activity. ETC proteolysis was measured in purified synaptosomes because 1) it is possible that the most injured mitochondria may be lost during the isolation of purified mitochondria, and 2) the synaptosome sample is a neuron-specific compartment known to have calpain activity after ischemic brain injury.

When adult rats were subjected to 9-minute transient forebrain ischemia (TFI), we detected the calpain-cleaved fragment of the cytosolic cytoskeletal protein α-spectrin (Ab38) in hippocampal synaptosomes isolated 48 hours after reperfusion, but not in synaptosomes isolated from a naïve rat (Figure 6, lanes 2-3). We detected the cleavage fragment of NDUFB8, but not Complex Vα, in TFI-injured synaptosomes. Next, we subjected adult rats to 2-hour middle cerebral arterial occlusion (MCAO), followed by 1 or 24 hours of reperfusion. Calpain-cleaved spectrin was detected at 1 hour and was greater at 24 hours post-reperfusion in synaptosomes isolated from the ischemic but not contralateral cortex. In these samples, we detected trace cleavage of NDUFB8 at 1 hour but not 24 hours post-reperfusion, and no proteolysis of Complex Vα at either time point (Figure 6, lanes 4-7). These results indicate that ETC proteolysis occurs to a limited extent after transient brain ischemia, but the time-course may be different from that of pathologic calpain activity in the neuronal cytosol.

2.4. Discussion
Our results detecting cleavage fragments of ETC subunits NDUFB8 and Complex Va are consistent with previous studies describing mitochondrial calpain activity (Arrington et al., 2006; Goudenege et al., 2007). We determined that previously described calpain-mediated ETC proteolysis occurs by a Ca$^{2+}$-independent mechanism in rat brain, heart, and kidney mitochondria. Comparison of the effectiveness of different calpain inhibitors provided additional evidence that ETC proteolysis was mediated by a cysteine protease. We found that the pan-calpain inhibitors MDL-28170 and calpeptin as well as the calpain 10-specific inhibitor CYGAK blocked ETC proteolysis, but the human calpastatin peptide inhibitor, which blocks calpain isoforms 1 and 2, did not prevent ETC proteolysis (Figure 5A-B). In developing the CYGAK inhibitor, Rasbach et al. (2009) established that this compound inhibited calpain 10 but not calpain 1. Because calpastatin is only known to inhibit calpain isoforms 1 and 2 (Maki et al., 1987; Ono et al., 2004), it is possible that structural differences in calpain 10 may preclude its inhibition by calpastatin.

Because MDL-28170 and calpeptin also inhibit other cysteine proteases including cathepsins, we tested whether Z-FF-FMK (cathepsin B/L-specific) or CA-074 (cathepsin B-specific) could inhibit ETC proteolysis induced by 0.1% SDS. We found that Z-FF-FMK, blocked ETC proteolysis at a concentration consistent with its IC50 for cathepsins B and L (Figure 5C, Table 1; Parkes et al. 1985; Kang et al. 2009). However, CA-074 did not inhibit ETC proteolysis (Figure 5C, Table 1; Murata et al. 1991).

We observed proteolysis of ETC subunits by an endogenous mitochondrial cysteine protease that required the presence of an ionic detergent (SDS) and a reducing agent (BME) (Figures 1-2). Reducing agents such as BME are routinely used to allow for
cysteine protease activity via reduction of the cysteine residue in the active site of the protease. The role of SDS detergent in the induction of proteolysis is unclear. Although this mechanism is not physiologic, previous *in vitro* studies have utilized SDS to activate the proteasome (Tanaka et al., 1988; Tanaka et al., 1989; Yamada et al., 1998). It is possible that mild SDS treatment disrupts the mitochondrial membrane or the protein substrates in a manner that exposes the cleavage site. This could explain the different SDS concentrations inducing proteolysis of the matrix substrate Complex Vα and the membrane-bound substrate NDUFB8 (Figure 2). Alternatively, mild SDS treatment may alter the conformation of the protease to initiate proteolytic activity. It appears that treatment with a higher concentration of SDS fully denatured either the protease or the substrates, preventing ETC proteolysis. We also observed that incubation of mitochondria at 25 °C rather than 37 °C increased the SDS required for ETC proteolysis (data not shown). This is likely due to the decreased lability of proteins and lipid membranes at lower temperature. It was surprising that an order of magnitude less SDS was required to induce proteolysis in heart mitochondria compared to brain and kidney cortex mitochondria (Figure 2). This result could be due to the level of expression of the protease in each tissue, or it could reflect differences in the structure of mitochondria isolated from each tissue.

Our finding that ETC proteolysis was Ca²⁺-independent was inconsistent with the well-defined, Ca²⁺-dependent activity of classical calpains. In addition to the C-terminal Ca²⁺-binding domain IV, calpains 1, 2, 3, and 5 contain highly conserved Ca²⁺-binding motifs in domains II and III that serve to align the active site of these proteases when in the
Ca\textsuperscript{2+}-bound state (Moldoveanu et al., 2002; Suzuki et al., 2004). Based on sequence homology of these isoforms from the rat, calpains 5 and 10 lack the C-terminal domain IV. In addition, calpain 10 lacks both Ca\textsuperscript{2+}-binding motifs in domains II and III (Figure 3; Moldoveanu et al., 2002). Similar results have been reported regarding calpain isoforms of human origin (Sorimachi and Suzuki, 2001; Suzuki et al., 2004). Studies reporting Ca\textsuperscript{2+}-dependent proteolysis by calpain 10 did not directly examine the isolated enzyme (Marshall et al., 2005; Arrington et al., 2006; Giguere et al., 2008; Rasbach et al., 2009). In fact, proteolytic or autolytic activity of isolated or recombinant calpain 10 was not detected when incubated in 5mM Ca\textsuperscript{2+} under conditions that activated calpains 1 and 2 (Dong and Liu, 2008). Therefore, the Ca\textsuperscript{2+}-dependence of calpain 10 is not certain, and the physiologic mechanism of activation of this protease remains to be discovered.

Interestingly, we found that ETC proteolysis was greater at pH 6.0 than at pH 7.2 (Figure 4). Calpains 1, 2, and 3 are most active at neutral pH, but the optimal pH of calpains 5 and 10 are unknown. Another family of cysteine proteases, cathepsins, is typically more active at lower pH. We are not aware of any published reports of cathepsin protein expression in mitochondria under physiologic conditions. Although Kosenko et al. (2010) provided evidence of cathepsin B and D enzyme activity in hippocampal mitochondria that is increased after chronic treatment with amyloid β-protein, cathepsin release from lysosomes has been reported in Alzheimer’s disease models (Nakamura et al., 1991).

Taken together, our results characterizing ETC subunit proteolysis are most consistent with the reported activity of calpain 10. However, the potential involvement of a different endogenous mitochondrial cysteine protease cannot be ruled out.
Although there are other well-known classes of endogenous mitochondrial proteases, it is unlikely that they are the cause of ETC subunit proteolysis measured in this study. The mitochondrial processing peptidase, mitochondrial intermediate peptidase, and inner membrane peptidase act together to cleave mitochondrial localization sequences from successfully targeted proteins (Koppen and Langer, 2007). The matrix- and intermembrane space-facing ATPase associated with diverse cellular activities proteases, caseinolytic peptidase, and Lon protease are responsible for degradation of damaged or misfolded mitochondrial proteins, including ETC subunits, and are critical for prevention of neurodegeneration under oxidative stress conditions (Haynes et al., 2007; Ugarte et al., 2010; Martinelli and Rugarli, 2010). Omi and the presenilins-associated rhomboid-like protein are two other serine proteases located in the intermembrane space, but their endogenous substrates are unknown (Martinelli and Rugarli, 2010). However, none of these proteases are in the cysteine protease family, and they would not be inhibited by the protease inhibitors used in this study.

The importance of the mitochondrial calpain system pathology has been magnified by numerous recent studies correlating polymorphisms in the calpain 10 gene in the susceptibility to type 2 diabetes mellitus (Horikawa et al., 2000; Horikawa, 2006). We hypothesized that calpain-mediated proteolysis of ETC subunits might play a role in mitochondrial dysfunction caused by global and focal brain ischemia. When adult rats were subjected to TFI and MCAO, we observed calpain-mediated proteolysis of spectrin in synaptosomes in a time-course correlating with neuronal cytosolic Ca\(^{2+}\)-overload and delayed neurodegeneration, consistent with previous results (Saido et al., 1993; Roberts-
Lewis et al., 1994; Neumar et al., 1996; Bartus et al., 1998; Neumar et al., 2001). We did not detect proteolysis of Complex Vα in synaptosomes after TFI or MCAO. However, we could detect low levels of NDUFB8 cleavage fragment 48-hours after TFI and 1-hour but not 24-hours after MCAO (Figure 6). Based on the accumulation of spectrin and NDUFB8 cleavage products, it appears that the time-course of ETC proteolysis after MCAO was different from the time-course of cytosolic Ca^{2+}-overload and calpain activity. Therefore, the mechanism of activation of ETC proteolysis after ischemic brain injury remains to be determined, but it appears to be independent of [Ca^{2+}]_{m}. We cannot speculate on the functional consequence of the low level of NDUFB8 proteolysis observed in our in vivo models. However, our results indicate that this ETC subunit is cleaved under pathologic conditions in vivo.

In summary, our results provide evidence for Ca^{2+}-independent proteolysis of select ETC subunits. ETC proteolysis had characteristics consistent with the activity of calpain 10. In addition, we detected a similar pattern of proteolysis of NDUFB8 in vivo after both focal and global transient brain ischemia. Moving forward, it will be important to definitively identify the protease causing ETC subunit proteolysis and determine its role in mitochondrial dysfunction after ischemia-reperfusion and in other pathologies involving disrupted mitochondrial homeostasis.

2.5. Acknowledgements
This work was supported by National Institutes of Health grants NS039481 and NS069951 (RWN) and American Heart Association grant 10PRE3660037 (MGP). We have no conflicts of interest to report.

We thank Dr. Mary Selak for technical assistance in mitochondrial isolation and for providing mitochondria buffer. CYGAK inhibitor was a gift from Dr. Rick Schnellmann, and Ab38 was provided by Dr. Robert Siman.
3.1. Introduction

Calpains are a family of Ca$^{2+}$-dependent neutral cysteine proteases involved with many critical cellular functions such as cell division and long-term potentiation (reviewed in Goll et al., 2003). Pathologic calpain activity has been associated with neurodegeneration in numerous models of acute and chronic neurodegeneration, including global and focal ischemia (Neumar et al., 2001; Markgraf et al., 1998), traumatic brain and axonal injury (Schoch et al., 2012; Ma et al., 2012), Alzheimer’s disease (Higuchi et al., 2012; Ferreira and Bigio, 2011), and Parkinson’s disease (Vosler et al., 2008).

Previous studies by our laboratory and others have determined that calpain activity is necessary for neurodegeneration of hippocampal CA1 pyramidal neurons after global ischemia (Li et al., 1998; Cao et al., 2007; Bevers et al., 2010). The classical calpains, isoforms 1 and 2, share a regulatory subunit calpain small subunit 1 (capns1), and we have shown targeted knockdown of calpain-1, but not calpain-2, prevents post-ischemic neurodegeneration in the hippocampus (Bevers et al., 2010). The role of calpain activity in cerebellar neurodegeneration after global ischemia has not been explored, in part because models of global ischemia targeting the forebrain do not injure the hippocampus, and therefore a whole-body cardiac arrest model is required. Purkinje cells of the cerebellum are a selectively vulnerable neuron population that degenerates after cardiac arrest.
arrest in human patients and animal models (Horn an Schlote, 1992; Ng et al., 1989; Welsh et al., 2002).

The currently available evidence suggests that Purkinje cells degenerate via a Ca$^{2+}$-mediated pathway (Yamashita et al., 2006; Brasko et al., 1995; Zhao et al., 2008). In a high-[Ca$^{2+}$] environment, calpains cleave a variety of intracellular substrates in a targeted fashion to inactivate the substrate, or in some cases create a gain of function of one or both proteolytic fragments. Many of these substrates are Ca$^{2+}$-regulatory proteins, and their cleavage by calpain is thought to create a feed-forward system of intracellular Ca$^{2+}$ entry, which induces a non-apoptotic cell death (reviewed in Bevers et al., 2008). Therefore, we hypothesized that inhibition of calpain activity would prevent neurodegeneration of Purkinje cells in the cerebellum after cardiac arrest. Because mRNA expression of calpain-2 is much greater than calpain-1 in cerebellar Purkinje cells than in hippocampal CA1 pyramidal neurons (Li et al., 1996), we chose to knock down functional activity of both the calpain-1 and calpain-2 catalytic subunits using an RNA interference approach targeting the common regulatory subunit capns1. We transduced cerebellar Purkinje cells in vivo with adeno-associated viral (AAV) vectors expressing short hairpin RNA (shRNA) corresponding to capns1 or a scramble control sequence and measured the effect of functional calpain knockdown on neurodegeneration 48 hours after cardiac arrest.

3.2. Materials and Methods
3.2.1. Primary hippocampal neuron culture

Primary hippocampal neurons were cultured as described previously (Bevers et al., 2009). Neurons from the hippocampus of E19 Sprague-Dawley rat embryos were plated at a density of 100,000 cells/mL in 24-well plates with glass coverslips (immunofluorescence) or 400,000 cells/mL in 35mm Petri dishes (mRNA or protein lysates) pre-treated with poly-L-lysine in serum-free Neurobasal media with B27 supplement (Gibco). Neurons were incubated at 37 °C in 5% CO₂. At seven days in vitro (DIV), neurons were transduced with 1.0 x 10¹¹ genome copies (GC) of adeno-associated virus (AAV2/9.hU6.shCapns1.CB7.mCherry or AAV2/9.hU6.scr.CB7.mCherry, produced by the University of Pennsylvania Vector Core Facility). All appropriate biosafety procedures were followed in handling the AAV vectors, which are rated BSL-1 and have never been shown to cause human pathology. Neurons were harvested at 21 DIV, or two weeks post-transduction.

3.2.2. mRNA harvest and rtPCR

Total RNA lysates were obtained using Trizol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen), as described previously (Bevers et al., 2009). To generate cDNA, 250ng RNA lysates were incubated in OligoDT primer (Invitrogen) and Superscript II reverse transcriptase (Invitrogen).

3.2.3. Western blot
Western blot was performed as previously described (Bevers et al., 2009). Molecular
weights were estimated using Precision Plus Protein Kaleidoscope Standards (Bio-Rad).
Primary antibodies used were anti calpain small subunit 1 clone m-80a (a generous gift
from Peter Greer), anti-actin clone AC-40 (Sigma), and anti-calpain-cleaved spectrin
clone Ab38 (provided by Robert Siman). Horseradish peroxidase-linked goat anti-mouse
and anti-rabbit (PerkinElmer) secondary antibodies were used. Western blots were
visualized using enhanced chemiluminescence solution (PerkinElmer).

3.2.4. Viral vector injection in rat cerebellum

Viral vector injections were performed on rats between 325-425g. A surgical plane of
anesthesia was maintained using inspired 2% isoflurane with 66% nitrous oxide and 33%
oxygen. A 2.0μL volume of AAV2/9.hU6.shRcapns1.CB7.mCherry or
AAV2/9.hU6.scr.CB7.mCherry viral vector at a concentration of 5.0 x 10^{12} genome
copies (GC)/mL (1.0 x 10^{10} total GC) was injected into the primary fissure of the
cerebellum at the midline (coordinates -11.5mm caudal, 0.0mm lateral to Bregma, depth
of 2.8mm) using a rodent stereotax (ASI Instruments) and microinjector pump (World
Precision Instruments) with a 25μL glass microsyringe (Hamilton). Rats were allowed to
recover for two weeks prior to cardiac arrest to allow for vector expression and protein
knockdown.

3.2.5. Rat model of cardiac arrest
This study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (Philadelphia, PA). Adult male Long Evans rats, 350-450g, were subjected to cardiac arrest as previously described, with some modifications (Che et al., 2010). Briefly, rats were orotracheally intubated and maintained under anesthesia using 2% isoflurane with 66% nitrous oxide and 33% oxygen. Temperature, measured using a needle thermocouple probe placed between the temporalis muscle and the skull, was monitored and maintained between 37.0 and 37.5 °C until the time of cardiac arrest. Asphyxial cardiac arrest was induced by cessation of mechanical ventilation after neuromuscular blockade. After seven minutes asphyxia, mechanical ventilation was resumed, intravenous epinephrine (0.005 mg/kg) and bicarbonate (1.0 mEq/kg) were administered, and external chest compressions were performed at a rate of 350–400 compressions/min until return of spontaneous circulation (ROSC). Inspired oxygen was then restored to achieve a pulse oximetry reading between 94% and 98%. One hour after ROSC, rats were weaned from mechanical ventilation. An intraperitoneal temperature probe was inserted, and rats were transferred to a specialized temperature regulation cubicle for extended temperature monitoring and maintenance in the normothermic range (37°C ± 1°C) for 48 hours after ROSC, at which time rats were transcardially perfused using 4% paraformaldehyde.

3.2.6. Histologic staining with FluoroJade B

Histology was performed similar to Siman et al. (2005). Sagittal 20μm sections of the medial 600μm of the cerebellar vermis were generated using a freezing-sliding
microtome (Microm). Every tenth section, randomized to the first section—a total of five sections per cerebellum—was mounted on glass slides and incubated for 10 minutes in 0.06% potassium permanganate, followed by 20 minutes in 0.004% FluoroJade B(Millipore) in solution of 0.1% acetic acid. Slides were coverslipped with DPX mounting media (Fluka).

3.2.7. Real-time polymerase chain reaction (rtPCR)

Quantitative rtPCR was performed using the fluorescent indicator SYBR Green (Applied Biosystems) and primers unique to the sequence of capns1 (Integrated DNA Technologies) on a MX3000P PCR cycler (Stratagene). The median of triplicate samples was chosen for three samples per condition per trial. The number of cycles to half-maximal SYBR Green fluorescence was corrected for each sample using the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). A total of three trials were performed, and all samples were averaged by treatment group, then normalized to naïve.

3.2.8. Immunofluorescence

Immunocytochemistry of primary hippocampal neurons using primary antibodies anti-MAP2 clone AP14 (a gift from Dr. Virginia Lee, University of Pennsylvania) and anti-capns1 clone m80-a (a gift from Dr. Peter Greer, Queens University) were performed using goat anti-mouse Alexa 568 and goat anti-rabbit Alexa 488 secondary antibodies (Invitrogen), respectively, as described previously (Bevers et al., 2009).
Immunofluorescence of 20μm sagittal sections of the cerebellar vermis was performed using a primary antibody specific to the calpain-cleaved fragment of spectrin (a gift from Dr. Robert Siman, University of Pennsylvania) and secondary antibody goat anti-rabbit Alexa 488, as described (Bevers et al., 2010).

3.2.9. **Semiquantitative analysis of calpain-cleaved spectrin**

We quantified mean fluorescence intensity of transduced and non-transduced regions of the primary fissure from every 10th section of the cerebellar vermis (4-5 sections per cerebellum) from sham- or cardiac arrest-injured rats one hour post-ROSC. Background fluorescence intensity was calculated as the mean fluorescence intensity in Lobule X, and was corrected for each section.

3.2.10. **Statistical analysis**

Comparison of capns1 mRNA and protein expression in primary hippocampal neurons transduced with shR-capns1 or scr AAV vector or naïve control was performed using one-way analysis of variance (ANOVA) with Bonferonni post-hoc analysis for significance (Stata). For calpain-cleaved spectrin immunoreactivity, we compared corrected mean fluorescence intensity in transduced vs. non-transduced primary fissure subjected to cardiac arrest injury or sham using one-way ANOVA with Bonferonni analysis. To compare the quantity of FluoroJade-labeled degenerating Purkinje cells in the primary fissure of shR-capns1 vs. scr-transduced cerebellum, average FluoroJade+ cell counts per section were compared using one-tailed Student’s t Test.
3.3. Results

3.3.1. Design and validation of AAV2/9 vector expressing capns1 shRNA.

In order to determine the role of calpain activity in cerebellar Purkinje cell degeneration after cardiac arrest, we implemented a vector-mediated approach to functionally knock down calpain-1 and calpain-2 catalytic activity. We designed short hairpin RNA targeting the regulatory calpain subunit capns1, as heterodimerization of this regulatory subunit is required for activity of the catalytic isoforms calpain 1 or 2. AAV plasmids were constructed co-expressing shRNA targeting capns1 (shR-capns1) or a scramble control sequence (scramble) driven by the human U6 promoter (hU6) and the red fluorescent marker protein mCherry driven by the chicken β-actin promoter with cytomegalovirus enhancer (CB7) (Figure 1A). These plasmids were packaged into AAV2/9 viral vectors for neuron transduction. Expression and targeted knockdown was validated in primary hippocampal neurons transduced with each vector. Neurons were transduced at 7 days in vitro and two weeks later were harvested for protein or RNA lysates. Expression of capns1 mRNA relative to the housekeeping gene HPRT was compared between naïve
Figure 3.2. Expression of AAV2/9 shR-capns1 vector and target knockdown. Primary hippocampal neurons transduced with 1.5 x 10^{11} GC AAV2/9 viral vector were harvested two weeks later. A: Relative normalized mRNA expression level quantified by rtPCR. *p<0.001. B. Western blot of naïve, scr, or shR-capns1 transduced neuronal lysates for Capns1 and actin. C: Densitometry of Western blot represented in B. *p<0.01. D-K: Immunocytochemistry of Scr-transduced (D-G) or shR-capns1-transduced (H-K) neurons against MAP2 (blue, D,H) and Capns1 (green, E-I), mCherry fluorescence (red, F-J), and merge (G-K). and transduced neurons. As shown in Figure 2B, AAV2/9.U6.shR-capns1.CB7.mCherry
transduction reduced capns1 mRNA levels compared to naïve and AAV2/9.U6.scr.CB7.mCherry transduced neurons (p<0.01). In addition, significant Capns1 protein knockdown in shR-capns1-transduced neurons was verified by Western blot, using an antibody specific to Capns1 and quantified by densitometry relative to the

Figure 3.3. Effect of capns1 knockdown on calpain activity 1-hour post-ROSC. Two weeks after intracerebellar injection of AAV2/9 viral vector expressing scr control (A-C) or shR-capns1 (D-F), rats were subjected to cardiac arrest followed by 1-hour reperfusion. Representative images of region of transduced (T) and non-transduced (N) regions of primary fissure depicting mCherry fluorescence (A,D), calpain-cleaved spectrin immunoreactivity (B,E), and merge (C,F).
loading control actin (Figure 2C-D).

3.3.2. Vector-mediated knockdown of calpain activity after cardiac arrest in rat cerebellum

Next, we tested whether expression of the shR-capns1 vector in vivo would block calpain activity after cardiac arrest. Intracerebellar injections into the primary fissure at the midline were performed with either AAV2/9.U6.shR-capns1.CB7.mCherry or AAV2/9.U6.shR-scr.CB7.mCherry in adult rats. Two weeks post-injection, rats were subjected to 7-minute cardiac arrest followed by cardiopulmonary resuscitation. One hour after ROSC, cerebella were processed for immunofluorescence labeling using an

Figure 3.4. No calpain activity in transduced neurons from sham-injured controls. Two weeks after intracerebellar injection of AAV2/9 vector expressing scr control (A-C) or shR-capns1 (D-F), rats were subjected to cardiac arrest and 1-hour reperfusion. Representative images of transduced regions of primary fissure depicting mCherry fluorescence (A,D), calpain-cleaved spectrin immunoreactivity (B,E) and merge (C,F).
Figure 3.5. **No detectable calpain activity in Lobule X 1-hour after ROSC.** Representative images of calpain-cleaved spectrin immunolabeling in cerebellar sections from rats subjected to sham (A) or cardiac arrest injury and 1-hour reperfusion (B). Calpain-cleaved spectrin immunoreactivity is not detectable in Lobule X, but is clearly detectable in Lobule IX 1-hour post-ROSC.

Figure 3.6. **Capns1 knockdown significantly reduces calpain activity 1-hour after ROSC.** Semiquantitative analysis was performed on calpain-cleaved spectrin immunofluorescence in transduced and non-transduced regions of primary fissure from rats subjected to sham or cardiac arrest injury one hour after ROSC. *p<0.05.

antibody specific to calpain-cleaved spectrin. As expected, we found that Purkinje cells in the primary fissure transduced with shR-capns1 (fluorescing red due to expression of mCherry) had decreased calpain activity compared to the non-transduced region of the primary fissure (Figure 3A-C). Alternatively, expression of scramble vector appeared to have no effect on calpain activity (Figure 3D-F). The intensity of calpain-cleaved spectrin was analyzed semiquantitatively, and as expected, calpain activity in the region of shR-capns1-transduced Purkinje cells was significantly decreased compared to non-transduced and scramble-transduced Purkinje cells (p<0.01) (Figure 3G).

3.3.3. Effect of capns1 knockdown on neurodegeneration after cardiac arrest

To determine the role of calpain activity on Purkinje cell degeneration after cardiac arrest, rats were injected with shR-capns1 or scr AAV2/9 viral vector into the cerebellum.
Two weeks post-injection with AAV2/9 scr or shR-capns1 vector, rats were subjected to cardiac arrest injury. At 48 hours post-ROSC, cerebellar sections were stained with FluoroJade. Figure 3A-C: scr-transduced region of primary fissure stained with FluoroJade (green, A), mCherry fluorescence (red, B), and merge (C). Arrows denote FluoroJade-labeled Purkinje cells absent mCherry co-labeling. Figure 3D-E: representative regions of scr (D) and shR-capns1 (E) primary fissure stained with FluoroJade.

as described above. Two weeks post-injection, rats were subjected to cardiac arrest and allowed to recover for 48 hours after reperfusion. Serial cerebellar sections were stained using FluoroJade, an anionic dye that stains degenerating neurons. Unexpectedly, we found that FluoroJade-labeling cells in the Purkinje layer did not co-label with the mCherry expression marker in the transduced region (Figure 3A-C, scr-transduced
One potential cause of this result would be that the mCherry protein leaked out of the degenerating cells. Therefore, we quantified the number of FluoroJade-labeled Purkinje cells in the transduced region of the cerebellum—the primary fissure at the midline—disregarding co-labeling with mCherry. We detected an average of \(44 \pm 27\) cells in the primary fissure of shR-capns1-transduced cerebellar sections and an average of \(35 \pm 23\) cells in the primary fissure of scr-transduced cerebellar sections. Therefore, we have no evidence that knockdown of calpain activity in cerebellar Purkinje cells blocks neurodegeneration after cardiac arrest.

### 3.4. Discussion

As expected, expression of shRNA targeting the calpain regulatory subunit capns1 resulted in a significant reduction in transcript and protein expression (Figure 1). In addition, targeted knockdown of capns1 significantly reduced calpain activity one-hour post-ROSC (Figure 2). However, our attempt to quantify the effect of capns1 knockdown on neurodegeneration 48 hours after ROSC was confounded by the lack of mCherry signal in degenerating neurons labeling with FluoroJade (Figure 3A-C). This was presumably due to the marker protein leaking out of the degenerating cells. Therefore, we took an alternative approach to quantify the number of degenerating cells in the primary fissure irrespective of mCherry co-labeling to get an indication of the effect of capns1 knockdown (Figure 3D-E). However, the scr-transduced and shR-capns1-transduced primary fissure had a comparable number of degenerating cells, which is not indicative of a neuroprotective effect of capns1 knockdown in Purkinje cells after cardiac arrest.
There are a number of caveats to our result that might have prevented us from detecting a protective effect of calpain inhibition in our model. As stated above, we could not specifically compare populations of Purkinje cells transduced with shR-capns1 vs. scr viral vector, presenting a challenge to discerning significant differences. In addition, the level of calpain knockdown was not quantified in vivo and is unlikely to approach 100%. Although we found robust knockdown in calpain activity 1 hour after cardiac arrest, the residual capns1 could be enough to execute its neurodegenerative function over the course of 48 hours. Finally, our observation contained only a single time point, and human data suggests that post-cardiac arrest neurodegeneration in the cerebellum is a process that occurs over days and weeks (Horn and Schlote, 1992). It is possible that quantification of surviving neurons one to two weeks post-injury could provide a more reliable measure of injury magnitude.

If calpain activity does not play a critical role in Purkinje cell degeneration after cardiac arrest, this would differ from the mechanism of cell death of hippocampal CA1 pyramidal neurons. The time-course of degeneration is slightly accelerated in the cerebellum compared to the hippocampus, with maximal FluoroJade labeling at 48-hours post-injury in the cerebellum and at 72-hours post-injury in the hippocampus (unpublished observations). This phenomenon could be explained by the activation of alternative cell death mechanisms, such as activity of the caspase or cathepsin family of proteases or other cell death signaling events, such as mitochondrial permeability transition or release of apoptosis inducing factor (Ofengeim et al., 2012; Yamashima and Oikawa, 2009; Friberg and Wieloch, 2002; Wang et al., 2009). In addition, Purkinje cells may be more
susceptible to excitotoxic injury due to the magnitude of synaptic input to the Purkinje circuit by the inferior olive (Welsh et al. 2002). Expression of channels that alleviate Ca$^{2+}$ overload, including the glutamate aspartate transporter (GLAST) and excitatory amino acid receptor-4 (EAAT4), has been shown to prevent Purkinje cell death after global ischemia (Yamashita et al., 2006). Further exploration of these mechanisms could yield an interesting contrast with the mechanisms of neurodegeneration in the hippocampus. In conclusion, our results reiterate that it is critical to examine multiple brain regions to develop maximally protective therapeutic strategies in complex injury models such as cardiac arrest.
CEREBELLAR PURKINJE CELL NEURODEGENERATION AFTER CARDIAC ARREST: EFFECT OF THERAPEUTIC HYPOTHERMIA

4.1. Introduction
Cardiac arrest is the most common cause of reversible global brain ischaemia (Nichol et al., 2008). Of those who are resuscitated after out-of-hospital cardiac arrest and admitted to the hospital, brain injury is the most common cause of death, and up to half of patients surviving to hospital discharge display significant neurologic dysfunction (Laver et al., 2004; Aufderheide et al., 2011; Stiell et al., 2011). Currently, induced therapeutic hypothermia (TH) is the only therapy proven in prospective randomized clinical trials to improve survival and neurologic outcome of comatose cardiac arrest survivors (Hypothermia After Cardiac Arrest Study Group, 2002; Bernard et al., 2002). In animal models, the neuroprotective effect of prolonged post-cardiac arrest TH has been demonstrated for hippocampal CA1 pyramidal neurons (Hicks et al., 2000; Katz et al., 2004; Che et al., 2011). However, little is known about its effect on other selectively vulnerable neuron populations.

Next to hippocampal CA1 pyramidal neurons, cerebellar Purkinje cells are among the most vulnerable to injury caused by cardiac arrest (Ng et al., 1989; Horn and Schlote, 1992). Loss of Purkinje cells after cardiac arrest could contribute to neurologic dysfunction, including post-hypoxic myoclonus (Welsh et al., 2002; Sarna and Hawkes, 2003; Venkatesan and Frucht, 2006). As in other selectively vulnerable regions, Purkinje cell neurodegeneration is thought to be caused by excitotoxicity (Brasko et al., 1995;
Additionally, Purkinje cell degeneration is delayed after cardiac arrest, beginning at 2-3 days in animal studies and 4 days in humans (Sato et al., 1990; Horn and Schlote, 1992; Welsh et al., 2002). This delayed neurodegeneration presents a potential therapeutic window after return of spontaneous circulation (ROSC) to protect neurons from ischaemic injury and potentially alleviate neurologic deficits. A previous study reported that permissive post-cardiac arrest hypothermia compared to controlled normothermia resulted in decreased neuronal injury at 72 hours in multiple brain regions including the cerebellum based on semiquantitative analysis (Hickey et al., 2000). To our knowledge, there are no published studies that have quantitatively examined the effect of induced post-cardiac arrest hypothermia on Purkinje cell survival.

A persistent concern related to the implementation of post-cardiac arrest TH is optimizing onset and duration of therapy to maximize neuroprotective effect. Our laboratory recently reported that post-cardiac arrest TH initiated at 0, 1, 4, or 8 hours after cardiac arrest resulted in comparable protection of hippocampal CA1 pyramidal neurons, while TH maintained for 48 hours resulted in significantly greater neuroprotection compared to TH maintained for 24 hours (Che et al., 2011). To examine the effect of post-cardiac arrest TH onset and duration on cerebellar Purkinje cell survival, we now report the analysis of cerebella from these same animals.

4.2. **Materials and methods**

4.2.1. *Rat Model of Asphyxial Cardiac Arrest*
This study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (Philadelphia, PA). Detailed methods and results regarding temperature regulation, haemodynamic parameters, survival, neurologic function, and hippocampal CA1 pyramidal neuron survival have been published (Che et al., 2011). The injury model is described briefly here. Adult male Long Evans rats (325-375g, Harlan Laboratories, Indianapolis, IN) were initially anesthetized with 4% isoflurane, 66% nitrous oxide, and 33% oxygen, and maintained with 2% isoflurane after orotracheal intubation. Mechanical ventilation was performed using a pressure-controlled ventilator (Kent Scientific, Torrington, CT). Temperature was monitored during the peri-arrest period with a needle thermocouple probe placed between the temporalis muscle and the skull, and temperature was maintained between 37.0 and 37.5 °C up to the time of asphyxia. Asphyxial cardiac arrest was induced by cessation of mechanical ventilation after neuromuscular blockade. After ten minutes asphyxia, mechanical ventilation was resumed, intravenous adrenaline (epinephrine, 0.005 mg/kg) and bicarbonate (1.0 mEq/kg) were administered, and external chest compressions were performed at a rate of 350–400 compressions/min until ROSC. Following ROSC, inspired oxygen was restored to maintain a pulse oximetry reading of 94% to 98%. One hour after ROSC, a telemetric temperature probe was inserted into the peritoneum for post-surgical temperature regulation. After weaning from mechanical ventilation, rats were transferred to a specialized temperature regulation cubicle. Immediately after ROSC, rats were block randomized to normothermia (37°C ± 1°C) or TH (33°C ± 1°C) initiated 0, 1, 4, or 8 hours after ROSC and maintained for 24 or 48 hours (eight groups, n=21 per group;
seven-day survivors shown in Table 1). Sham injured rats underwent surgical instrumentation under general anesthesia without asphyxiation or cardiopulmonary resuscitation (n = 6; Table 1).

4.2.2. Immunohistochemistry

Rats surviving seven days were transcardially perfused with 4% paraformaldehyde. Brains were post-fixed and processed as previously described (Che et al., 2012). Sagittal 50μm sections of the cerebellum were generated using a freezing sliding microtome (Microm HM 440E, Walldorf, GER) and stored in cryoprotectant solution (30% glycerol, 30% sucrose in 1M phosphate buffered saline, pH 7.2).

Every tenth section from the medial 2.5mm of the cerebellar vermis was selected with randomization of the first section. Sections were incubated in 30% methanol/0.5% H₂O₂ for 20 minutes to inactivate endogenous peroxidases. Purkinje cells were immunolabeled using an antibody to calbindin (AB1778, Millipore). Secondary antibody, biotinylated goat anti-rabbit (BA-1000, Vector Labs) was applied, followed by incubation in avidin-biotin-horseradish peroxidase solution (ABC Kit PK-6101, Vector Labs). Sections were treated with 0.4mg/mL 2,4-diaminobutyric acid (D5905, Sigma) for 5-8 minutes. Sections were mounted and coverslipped using DPX (44581, Sigma).

4.2.3. Thionin Stain and Quantification of Purkinje Cells

Every tenth section from the medial 2.5mm of the cerebellar vermis was selected with randomization of the first section. Nissl stain was performed on mounted slides according
to Gerfen (2003), with some modifications. Slides were incubated in thionin solution (2.5mg/mL thionin (861340, Sigma), 14.4mg/mL NaOH, 1.2% acetic acid in ddH2O) for 1 minute, then rinsed once with ddH2O and incubated for 2 minutes in ddH2O. Slides were incubated for two minutes in a series of 70%, 80%, 95%, and 100% (twice) ethanol. If additional destaining was required, slides were incubated for two minutes in 95% ethanol/1% acetic acid followed by two two-minute washes with 100% ethanol. Slides were cleared with xylene and coverslipped with DPX.

Quantification of Purkinje cell density in the primary fissure and Lobule X was performed using an approach similar to Lu et al. (2008). Photomontages of light microscopic images from each cerebellar region were generated using Photoshop Elements 7.0 (Adobe). Purkinje cells were identified as large, rounded cell bodies in the Purkinje layer, between the granule and molecular layers. Degenerating Purkinje cell bodies, indicated by a darkened and shrunken morphology, were excluded from counting. Purkinje cells were counted and the length of the cerebellum region analyzed was measured using ImageJ (National Institutes of Health). Total Purkinje cell counts were divided by total length measured to calculate Purkinje cell density for each animal. All values are reported as mean ± standard deviation.

4.2.4. Statistical Analysis

For statistical comparison of group means of Purkinje cell density in the primary fissure, one-way analysis of variance was performed with Bonferonni analysis for significance (Stata). For comparison of group means of Purkinje cell density in Lobule X, an
Table 4.1. Number of seven-day surviving animals analyzed per treatment group.

<table>
<thead>
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<th>Injury</th>
<th>TH Onset (Post-ROSC)</th>
<th>24-Hour TH</th>
<th>48-Hour TH</th>
<th>Total</th>
</tr>
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<td>N/A</td>
<td>6</td>
</tr>
<tr>
<td>CA</td>
<td>Normothermia</td>
<td>N/A</td>
<td>N/A</td>
<td>6</td>
</tr>
<tr>
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<td>10</td>
<td>9</td>
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<tr>
<td>Total</td>
<td></td>
<td>30</td>
<td>25</td>
<td>67</td>
</tr>
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</table>

unpaired, one-tailed Student’s t Test was performed. One outlying value in the Purkinje cell density in the primary fissure of the cardiac arrest injured, normothermic group was identified and excluded according to the Grubbs test. The group mean including this value was 6.0 cells/mm, versus 3.8 cells/mm excluding the outlier.

4.3. Results

4.3.1. Effect of TH on Purkinje cell survival after cardiac arrest

To survey the effect of cardiac arrest on cerebellar Purkinje cell survival, sagittal sections of the cerebellar vermis were labeled immunohistochemically using an antibody to calbindin, which is specific for Purkinje cells in the cerebellum. In sham animals, we detected regularly spaced, rounded cell bodies in the Purkinje layer and extensive dendritic arborizations in the molecular layer, each characteristic of Purkinje cells (Figure 1A, D). In the normothermic post-cardiac arrest group, loss of calbindin-labeled Purkinje cell bodies and processes was apparent throughout most lobules and most consistently in the primary fissure (between Lobules V and VI) (Figure 1B, E). In contrast, we observed no perceptible loss of calbindin-labeled Purkinje cells in Lobule X (Figure 1G-H).
Figure 4.1. Purkinje cell loss after cardiac arrest and protection with TH. Adult rats were subjected to sham injury (A, D, and G) or cardiac arrest and resuscitation followed by normothermia (B, E, and H) or TH (C and F). Seven days post-injury, transverse 50µm sections of the cerebellar vermis were immunolabeled using an antibody to the Purkinje cell-specific protein calbindin. A-C: Representative photomontages of the primary fissure after sham injury (A), cardiac arrest followed by normothermia (B), and cardiac arrest followed by TH initiated immediately after ROSC and maintained 48 hours (C) Scale bar is 250µm. D-F: Single image of the base of the primary fissure corresponding to A-C, respectively. Scale bar is 100µm. G-H: Representative photomontages of Lobule X after sham (G) and cardiac arrest followed by normothermia (H). Scale bar is 250µm.
Next, we examined whether post-cardiac arrest TH had a protective effect on cerebellar Purkinje cell survival. Animals in which TH was initiated immediately post-cardiac arrest and maintained for 48 hours appeared to have moderate neuroprotection based on calbindin immunolabeling (Figure 1C, F). Interestingly, Purkinje cells were not evenly distributed, but instead were composed of alternating regions of surviving cells and empty spaces.

4.3.2. Quantification of Purkinje cell survival after cardiac arrest

To quantify Purkinje cell survival, sagittal sections of the vermis were Nissl stained to label neuronal cell bodies. A representative region of the primary fissure from a sham-injured rat is shown in Figure 2A. Purkinje cells were identified as large, rounded cells in the Purkinje layer. The density of Purkinje cells in the primary fissure was $35.9 \pm 2.4$ cells/mm in sham controls (Figure 2D). In cardiac arrest injured rats treated with normothermia, we observed extensive loss of Purkinje cells (Figure 2B). Purkinje cell density in the primary fissure was $3.8 \pm 1.8$ cells/mm, approximately 11% of sham (Figure 2D; $p < 0.001$). Although considerable Purkinje cell loss occurred throughout the medial-lateral distribution of the vermis, cell death was greatest in mid-vermal sections (data not shown). We also quantified Purkinje cell density in Lobule X using Nissl-stained cerebellar vermis as above. Purkinje cell density was $27.6 \pm 1.3$ cells/mm in sham and $27.3 \pm 1.6$ cells/mm in cardiac arrest injured, normothermia-treated rats ($p=0.37$; Figure 2G-I).
Figure 4.2. Quantification of Purkinje cell density in cerebella from sham-injured vs. cardiac arrest-injured rats treated with normothermia or TH. A-C: representative images of the primary fissure from rats that underwent sham injury (A), cardiac arrest followed by normothermia (B), and cardiac arrest followed by TH initiated immediately after ROSC and maintained for 48 hours (C). Scale bar is 100µm. D: Purkinje cell density in the primary fissure of sham injured rats compared to cardiac arrest followed by normothermia (Normo) or all TH treated combined (All TH), *p<0.001. E: Comparison of 24-hour and 48-hour TH duration, *p<0.001, #p<0.05. F: Comparison of 0, 1, 4, and 8 hour TH onset time, *p<0.001, #p<0.05. G-H: representative images of Lobule X from rats subjected to sham (G) or cardiac arrest injury followed by normothermia (H). Scale bar is 100µm. I: Purkinje cell density of Lobule X in sham injured rats compared to cardiac arrest followed by normothermia, p=0.37, Student’s T-test.
4.3.3. Impact of TH onset time and duration on Purkinje cell survival after cardiac arrest

Next, we examined the effect of post-cardiac arrest TH onset time and duration on the degree of neuroprotection. Rats were block randomized to sham or cardiac arrest injury with normothermia or TH initiated at 0, 1, 4, or 8 hours post-cardiac arrest and maintained for 24 or 48 hours. All seven-day survivors were analyzed (Table 1). Figure 2C shows a representative region of the primary fissure from an animal in which TH was initiated immediately post-cardiac arrest and maintained for 48 hours. Grouped together, hypothermia-treated post-cardiac arrest animals had a Purkinje cell density of 14.0 ± 5.6 cells/mm, nearly 39% of sham injured controls (Figure 2D, p < 0.001 compared to normothermia). When grouped by TH duration, Purkinje cell density was 12.5 ± 4.0 cells/mm with 24 hour TH and 15.5 ± 5.6 cells/mm with 48-hour TH. (p = 0.002 and p < 0.001, respectively compared to normothermia). While the density appeared greater for 48-hour compared to 24-hour duration TH, the difference was not statistically significant (p=0.245). Finally, when grouped by onset time of 0, 1, 4, or 8-hours after ROSC, Purkinje cell density was 16.0 ± 7.7, (p < 0.001 compared to normothermia), 11.9 ± 2.9 (p = 0.03), 13.1 ± 4.6 (p = 0.006), and 14.8 ± 3.3 (p = 0.007) cells/mm, respectively. However, no significant differences between TH onset times were detected.

4.4. Discussion

4.4.1. Post-cardiac arrest TH protects cerebellar Purkinje cells
To our knowledge, we are the first to report that post-cardiac arrest TH protects cerebellar Purkinje cells. Surprisingly, there was no significant difference in Purkinje cell survival between onset times from 0- to 8-hours post-ischaemia or durations of 24 or 48 hours. Our results suggest that there is a critical period between 8 and 24 hours after ROSC when neurodegenerative processes in cerebellar Purkinje cells can be inhibited by TH. However, there are a number of caveats to this conclusion. First, although surviving Purkinje cells possessed a normal nuclear and dendritic morphology in rats treated with TH, we did not measure the physiologic activity or extent of dendritic arborization of surviving Purkinje cells. Second, while TH treatment improved post-cardiac arrest Purkinje cell survival from 11% to 39% of sham-injured controls, there was still significant Purkinje cell loss seven days after cardiac arrest, suggesting the existence of injury pathways that were not responsive to TH as it was applied in this model. Third, although TH was initiated immediately after ROSC in the 0-hour onset group, it took 30 minutes to achieve target temperature of 33 ± 1 °C. Therefore, TH achieved either during CPR or immediately after ROSC could provide additional neuroprotection (Takata et al., 2005). Fourth, our asphyxial cardiac arrest model was designed to be severe (17% seven day survival with normothermia) in order to accurately replicate the human condition and to more easily measure the therapeutic benefits of post-ischaemic TH (Che et al., 2011). In addition, we selected the primary fissure of the vermis for quantification of Purkinje cell survival because it was the most consistently and severely injured region, presenting the greatest possibility of detecting differences between groups. In a more moderate
injury model with less severe Purkinje cell loss, post-ischaemic TH may provide more complete neuroprotection.

4.4.2. Potential mechanisms underlying the insensitivity of Lobule X Purkinje cells to post-ischaemic degeneration

The insensitivity of Lobule X Purkinje cells to cardiac arrest was striking compared to the extensive loss of Purkinje cells in the rest of the cerebellum. To our knowledge, we are the first to report this phenomenon in a global brain ischaemia model. However, the resistance of Lobule X Purkinje cells to degeneration has been reported in several neurodegenerative disease models, including Niemann-Pick disease type C1, genetic models of cerebellar ataxia, and neonatal viral infection (Sarna et al., 2003; Williams et al., 2007; Duffin et al., 2010). Lobule X Purkinje cell resistance to neurodegeneration has been attributed to differential gene expression in this region, particularly heat shock protein 25, zebrin II/Aldolase C, EAAT4, glutamate-aspartate transporter, and phospholipase C β4 (Armstrong et al., 2001; Welsh et al., 2002; Sarna et al., 2006; Yamashita et al., 2006; Duffin et al., 2010). Alternatively, the topography of synaptic connections in this region, referred to as the nodular zone, could also play a critical role (Heinsen and Heinsen, 1983; Ozol et al., 1999). Elucidating the underlying mechanisms of Purkinje cell survival in Lobule X could provide therapeutic strategies to protect Purkinje cells in vulnerable regions of the cerebellum after cardiac arrest.

4.4.3. Comparison of TH-induced neuroprotection in the cerebellum to the hippocampus
In our original study, we analyzed the effect of post-cardiac arrest TH onset and duration on seven day animal survival and loss of hippocampal CA1 pyramidal neurons (Che et al., 2011). We found that animal survival was significantly greater in TH-treated animals with an onset time of 0-, 1-, and 4-hours, but not 8-hours. However, similar to Purkinje cells, we observed comparable protection of hippocampal CA1 pyramidal neurons when TH was initiated 0, 1, 4, or 8 hours after ROSC. CA1 pyramidal neuron survival was enhanced when TH was maintained for 48 hours compared to 24 hours. Although we saw a similar trend of a TH duration effect for Purkinje cell survival, the difference was not statistically significant. One potential explanation for this difference is that CA1 pyramidal neuron degeneration could be more delayed relative to cerebellar Purkinje cells, and therefore might require a longer duration of therapy for optimal protection (Pulsinelli et al., 1982; Kirino et al., 1984; Dux et al., 1987; Welsh et al., 2002).

4.4.4. **Implications for neurologic outcomes after cardiac arrest.**

The clinical implications of Purkinje cell loss after cardiac arrest have yet to be fully elucidated. Posthypoxic myoclonus is mechanistically complex and thought to involve multiple brain regions including the cerebral cortex, basal ganglia, thalamus, hippocampus, brainstem and cerebellum (Venkatesan and Frucht, 2006). The contribution of cerebellar pathology is supported by the work of Welsh et al. (2006), who recapitulated myoclonus behavior in rats by inducing patterned Purkinje cell death using the neurotoxic compound ibogaine. It has been hypothesized that the abnormal excitatory activity responsible for post-hypoxic myoclonus is generated in the brainstem, and that loss of Purkinje cells in a concentrated region could remove the basal inhibition of deep
cerebellar nuclei that project to the brainstem (Welsh et al., 2002; Venkatesan and Frucht, 2006). Although not as well documented in cardiac arrest studies, ataxia is strongly correlated with Purkinje cell loss in number of clinical disorders including with spinocerebellar ataxia, alcoholic cerebellar degeneration, Niemann-Pick disease type C1, and paraneoplastic cerebellar degeneration (Walkley et al., 2004; Yokota et al., 2006; Metha et al., 2009). Additionally, selective Purkinje cell degeneration has been shown to cause ataxia in animal models of spinocerebellar ataxia, Niemann-Pick disease type C1, and chronic ethanol exposure (Tavares et al., 1987; Watase et al., 2002; Aguiar et al., 2006; Lopez et al., 2011; Shakkottai et al., 2011). Currently, a better understanding of the contribution of cerebellar injury on neurologic outcome after cardiac arrest is needed. Moving forward, it will be important to examine the effect of post-cardiac arrest TH on the incidence and severity of neurologic disorders such as post-hypoxic myoclonus and ataxia in animal and human studies.

4.4.5. Conclusions

Our results demonstrate that post-cardiac arrest TH protects selectively vulnerable cerebellar Purkinje cells. Moreover, TH appears to exert its greatest protective effect on Purkinje cell survival between 8 and 24 hours after ROSC. These results underscore the importance of considering multiple brain regions when optimizing the neuroprotective effect of post-cardiac arrest TH. Finally, the impact of post-cardiac arrest TH on cerebellar function in humans warrants more in-depth examination.
4.5. Acknowledgments

We thank Dr. Fred Colbourne for his consultation to establish the automated telemetric temperature regulation system and for providing the Thermoreg software. This work was supported with funding from National Institutes of Health grant R21-NS054654 (RWN) and American Heart Association grant 10PRE3660037 (MGP).

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CONCLUSIONS

5.1. Introduction

Brain injury after cardiac arrest is a significant contributor to mortality and neurologic dysfunction. Multiple factors are thought to influence the pathway of post-ischemic neurodegeneration, including excitatory input, inflammation, \( \text{Ca}^{2+} \) homeostasis, mitochondrial dysfunction, and calpain activity (see Chapter 1.3). Previous studies using genetic manipulation showed that calpain activity causes hippocampal CA1 pyramidal neuron death after global ischemia (Cao et al., 2007; Bevers et al., 2010). Based on these results, the role of calpain activity in mitochondrial dysfunction was examined by characterizing the proteolysis of ETC subunits by an endogenous mitochondrial protease. In addition, the role of calpain activity in post-ischemic neurodegeneration of cerebellar Purkinje cells was explored. Finally, the optimal parameters of TH, which has broad effects on factors related to post-ischemic neurodegeneration, were examined in cerebellar Purkinje cells. The results of each study provide a small contribution to our understanding of neuronal death mechanisms after global brain ischemia.

5.2. Proteolysis of ETC subunits by a mitochondrial cysteine protease—likely calpain 10—occurs after global and focal brain ischemia

Despite significant contributions to the characterization of the protease responsible for proteolytic activity of ETC subunits, the identity of the protease is not entirely clear. Proteolysis of ETC subunits NDUFB8 and Complex V\( \alpha \) could be induced \textit{in vitro} and
was detected after global and focal ischemia *in vivo* (see Chapter 2). As a reducing environment is required for activity and a variety of cysteine protease inhibitor drugs block ETC proteolysis, there is clear evidence that it is a cysteine protease. Based on the literature regarding ETC subunit proteolysis and inhibition by CYGAK, the inhibitor profile of this protease is consistent with calpain 10 (Arrington *et al.*, 2006; Rasbach *et al.*, 2009). The pH optimum, inhibition by cathepsin-specific inhibitor Z-FF-FMK, and Ca\(^{2+}\)-independence of proteolytic activity are not consistent with the definition of calpain proteases. However, calpain 10 is an atypical calpain, which does not share sequence homology with the Ca\(^{2+}\)-binding motifs of classical calpains 1 and 2 (See Figure 2.3). It is possible that calpain 10 could possess a different mechanism of physiologic activation than typical calpain isoforms.

Future studies to identify the mitochondrial protease could be performed utilizing a protease probe strategy (Greenbaum *et al.*, 2000). Greenbaum *et al.* typically utilize biotin-conjugated version of the protease inhibitor E-64c to covalently bind and isolate active cysteine proteases. Unfortunately, mitochondria are extensively biotinylated, so much so that endogenous biotin can be utilized as a mitochondrial marker (Coene *et al.*, 2008). An alternative approach is to use a fluorophore-conjugated version of E-64c (Greenbaum *et al.*, 2002). Although my attempts in collaboration with Dr. D.C. Greenbaum have been met with technical difficulties, this approach has great promise to identify the mitochondrial cysteine proteases activated in our *in vitro* model.

An alternative strategy to determine whether calpain 10 specifically is responsible for ETC proteolysis is to reduce or enhance its expression in a cell culture system and
compare the rate of proteolysis to mitochondria isolated from control cells. Overexpression of calpain 10 has been performed in cell culture, resulting in mitochondrial swelling and autophagy (Arrington et al., 2006). RNA interference of calpain 10 has been performed rabbit renal proximal tubular cells in vivo, resulting in mitochondrial dysfunction, increased mitochondrial fission and decreased fusion, and activation of apoptosis (Covington et al., 2009; Smith et al., 2012). Additionally, a calpain-10 knockout mouse has been generated, but the molecular weight of the eliminated protein is not consistent with the reported size of calpain 10 (Johnson et al., 2004; Arrington et al., 2006).

Characterization of calpain 10 activity is of great importance due to correlation of polymorphisms in the calpain 10 gene with type 2 diabetes mellitus (Horikawa et al., 2000; Weedon et al., 2003). If the mitochondrial protease is proven to be calpain 10, if would be interesting to determine whether ETC subunit proteolysis plays any role in the pathology of diabetes mellitus. Polymorphisms in the calpain 10 gene have been correlated with increased type 2 diabetes risk (Horikawa et al., 2000; Horikawa, 2006). Perhaps these polymorphisms or other mutations of the calpain 10 gene could help to determine the physiologic function of the protease.

5.3. Functional calpain knockdown did not reduce Purkinje cell neurodegeneration 48-hours after cardiac arrest

Calpain-mediated proteolysis, as detected by the calpain-specific cleavage fragment of spectrin, has been detected in models of global ischemia-reperfusion in a timecourse
preceding neurodegeneration in SVN populations including the hippocampus, striatum, frontal cortex, thalamus, and cerebellum (Roberts-Lewis et al., 1994; Neumar et al., 2001; Kopil et al., 2011). However, evidence for a causal role of calpain activity in neurodegeneration after global ischemia was limited in vivo to the CA1 pyramidal neurons of the hippocampus (Cao et al., 2007; Bevers et al., 2010). I sought to determine whether our understanding of calpain-mediated post-ischemic neuronal death could be applied to a different SVN population, the Purkinje cells of the cerebellum. I detected significant calpain-mediated proteolysis in Purkinje cells one hour post-ROSC and successfully reduced cardiac arrest-induced calpain activity in Purkinje cells in vivo utilizing an RNA interference strategy to reduce expression of the calpain regulatory subunit Capns1. However, Capns1 knockdown did not result in reduced Purkinje cell neurodegeneration measured by FluoroJade staining 48 hours post-ROSC. The caveats to this set of experiments have been discussed (see Chapter 3.4).

One critical next step will be to validate that Capns1 knockdown can reduce calpain-mediated post-ischemic neurodegeneration. In parallel with the intracerebellar injections, one hemisphere of the hippocampus was also injected with AAV2/9 vector expressing shRNA targeting capns1 or a scramble control sequence. Further analysis can be performed to examine whether this RNA interference approach reduced calpain activity and neurodegeneration of hippocampal CA1 pyramidal neurons from the same rats. Bevers et al. (2010) established that shRNA-mediated knockdown of calpain 1 blocked calpain activity, reduced neurodegeneration, and improved physiologic function of CA1 pyramidal neurons after transient forebrain ischemia. If Capns1 knockdown in a model of
cardiac arrest provides the same neuroprotection, two possibilities remain to explain the lack of neuroprotection of Purkinje cells of the cerebellum. First, there could be a greater degree of $\text{Ca}^{2+}$ overload in the cerebellum than the hippocampus, such that a manipulation that protects CA1 pyramidal neurons is not sufficient to protect Purkinje cells against high $\text{Ca}^{2+}$-induced mitochondrial dysfunction, ROS production, or ER stress (Dong et al., 2006). The accelerated time-course of delayed calpain activity and neurodegeneration in Purkinje cells relative to CA1 pyramidal neurons provides some evidence that this might be the case (Kopil et al., 2011; Frederick et al., 2008; Neumar et al., 2001).

Alternatively, global ischemia-reperfusion injury may activate other mechanisms of neurodegeneration that are critical to Purkinje cell death. There is evidence indicating that the excitatory synaptic input of the climbing fiber pathway from the inferior olive to Purkinje cell dendritic arborizations is hyperactive in an in vivo model of cardiac arrest, and that removal of this synaptic input protects against Purkinje cell loss (Welsh et al., 2002). Future studies could examine the role of inflammation, oxidative stress, mitochondrial dysfunction, and alternative mechanisms of $\text{Ca}^{2+}$-mediated cell death in Purkinje cell degeneration.

5.4. Therapeutic hypothermia protects cerebellar Purkinje cells after cardiac arrest

Previous evidence indicated that 48-hour duration TH provided greater neuroprotection of hippocampal CA1 pyramidal neurons than 24-hour duration TH, but both were significantly neuroprotective compared to normothermia (Che et al., 2010). However, in
the cerebellum, both durations were equally neuroprotective. In addition, onset of TH between 0-8 hours after cardiac arrest provided similar neuroprotection in both brain regions. One caveat to this result is that rats initially treated with TH reached target temperature 30 minutes after onset time, not immediately. As there is evidence for a therapeutic benefit of TH achieved immediately upon ROSC (Kuboyama et al., 1993; Abella et al., 2004; Takata et al., 2005), it would be interesting to determine the effect of immediate cooling on Purkinje cell survival, as well as whether there is a synergistic effect of immediate and prolonged TH.

5.4.1. Potential extrinsic factors causing differential vulnerability of Lobule X Purkinje cells to global ischemia-reperfusion injury.

The lack of Purkinje cell loss in Lobule X after cardiac arrest provides an excellent comparator to determine the mechanisms responsible for post-ischemic Purkinje cell neurodegeneration. First, it is possible that Lobule X is less vulnerable due to a lack of excitatory stimulus. Welsh et al. (2002) detected increased excitation of the climbing fiber pathway via EEG recording of the hindbrain. This method lacked the sensitivity to determine the localization of this overexcitation. One could insert electrodes into vulnerable and invulnerable lobules of cerebellum for in vivo recording of field excitatory postsynaptic potentials (fEPSP’s) or single unit firing of Purkinje cells after ROSC. While use of this technique is uncommon in the cerebellum, it has been demonstrated in a mouse model (Cheron et al., 2009). If hyperexcitation of Purkinje cells is part of the pathway of neuronal death, it should be possible to predict the regions of degenerating
neurons based on the rate and magnitude of fEPSP’s in the molecular layer as well as the Purkinje cell firing rate.

Second, the migration of activated microglia could be compared between different lobules of the cerebellum. However, one could expect that only cerebellar regions that possess cell death would recruit inflammatory cells to the site of injury. Therefore, while inflammation may take part in a feed-forward pathway of post-ischemic neuronal death, an unaffected region of the brain may provide an insufficient comparator.

5.4.2. Potential intrinsic factors causing differential vulnerability of Lobule X Purkinje cells to global ischemia-reperfusion injury:

Multiple genes are differentially expressed between Lobule X Purkinje cells and other lobules. In addition, striations of differential gene expression occur within lobules that are selectively vulnerable to global ischemia. These genes could be involved in a number of intrinsic factors that play a role in post-ischemic Purkinje cell death. Overexpression or knockdown of each of these genes in selectively vulnerable regions of Purkinje cells could provide insight into their role in ischemic brain injury, as well as the mechanisms that are critical for Purkinje cell neurodegeneration. Laser-capture microdissection or live tissue fluorescence-activated cell sorting (FACS) could be used to isolate Purkinje cells from cerebellar tissue, followed by proteomic or transcriptomic approaches to generate a comprehensive list of differentially expressed genes in Purkinje cells between Lobule X and other cerebellar lobules (Guez-Barber et al., 2012; Kuhn et al., 2012). Of each of the genes currently known to be differentially expressed among Purkinje cells, their potential roles in post-ischemic neurodegeneration are discussed below.
Zebrin II/Aldolase C was the first protein discovered to have a striated expression among cerebellar Purkinje cells (Brochu et al., 1990). Aldolase C is an enzyme in the glycolysis pathway, hydrolyzing fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. It is possible that greater expression of Aldolase C could increase energy metabolism to protect Purkinje cells under stressful conditions. As a non-rate-limiting enzyme in the glycolysis pathway, its role in neuroprotection may be minimal. However, expression of this protein can be used to mark striations within vulnerable lobules, or to separate pools of Purkinje cells via FACS (Welsh et al., 2002; Guez-Barber et al., 2012).

Heat shock protein-25 (HSP-25) was found to have a patterned expression in mouse cerebellar Purkinje cells (Armstrong et al., 2001). HSP-25 is expressed in the cytosol, then translocates to the nucleus upon thermal stimulation (Geum et al., 2002). It interacts with heat-destabilized actin to prevent aggregation, and HSP-25 is thought to maintain the structure of the nucleus upon thermal stress (Geum et al., 2002; Panasenko et al., 2003). Expression of HSP-25 in Purkinje cells is correlated with protection against neuronal death in the lurcher mouse model (Duffin et al., 2010).

EAAT4 is more strongly expressed in Aldolase C-positive Purkinje cells and is localized primarily extrasynaptically on the dendritic plasma membrane (Dehnes et al., 1998; Welsh et al., 2002). Although it is nominally a transporter, it acts as an anionic channel when bound to glutamate (Dehnes et al., 1998). In mice genetically manipulated to knock out expression of the GLAST—an astrocytic membrane protein responsible for clearance of glutamate from the synapse and the extracellular space—Purkinje cells
expressing less EAAT4 preferentially lost due to mild global ischemic injury, in which WT mice did not lose Purkinje cells, suggesting that EAAT4 protects Purkinje cells from glutamatergic excitotoxicity (Yamashita et al., 2006).

Phospholipase C (PLC) β3 and β4 are uniquely and strongly expressed in mouse cerebellum (Sarna et al., 2006). Each isoform is responsible for production of IP₃ and diacylglycerol from phosphatidylinositol 4,5-bisphosphate when activated by a seven-transmembrane receptor (Kim et al., 1997). It is possible that expression of PLC β3 or β4 isoforms could influence Ca²⁺ homeostasis via IP₃ receptor-mediated release of Ca²⁺ from the ER.

5.5. Conclusion

Examination of individual mediators of post-ischemic neurodegeneration have demonstrated the importance of each mechanism in the pathway of neuronal death in SVN populations. However, these factors likely overlap in their contribution to neurodegeneration. This may explain why no treatment targeting any individual factor has been proven in a clinical trial as an effective therapeutic strategy to improve patient survival and neurologic outcome after cardiac arrest. It is also important to note that cardiac arrest is a whole-body injury—while brain injury is a key contributor to patient outcome, it is one among many organ systems injured by global ischemia-reperfusion (Reynolds and Lawner, 2012). Currently, TH, which has broad effects influencing multiple factors involved in ischemia-reperfusion injury, is the only successful clinically approved therapy after cardiac arrest (Bernard et al., 2002; Hypothermia after Cardiac Arrest Study Group, 2002). Although specific targeting of an individual factor involved
in post-ischemic neurodegeneration has not been successful, perhaps a combined therapy targeting multiple mechanisms of neurodegeneration—perhaps in concert with TH—could provide additional therapeutic benefit after cardiac arrest.
APPENDIX 1: List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BME</td>
<td>β–mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSL-1</td>
<td>Biosafety level 1</td>
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<tr>
<td>Ca^{2+}_i</td>
<td>Intracellular ionic calcium</td>
</tr>
<tr>
<td>[Ca^{2+}]_m</td>
<td>Concentration of mitochondrial ionic calcium</td>
</tr>
<tr>
<td>Capns1</td>
<td>Calpain small subunit 1</td>
</tr>
<tr>
<td>CB7</td>
<td>Chicken β–actin promoter with cytomegalovirus enhancer</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
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<tr>
<td>Complex Va</td>
<td>Complex V subunit a</td>
</tr>
<tr>
<td>CPR</td>
<td>Cardiopulmonary resuscitation</td>
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<tr>
<td>DIV</td>
<td>Days <em>in vitro</em></td>
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<tr>
<td>EAAT4</td>
<td>Excitatory amino acid transporter-4</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-associated cell sorting</td>
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<tr>
<td>fEPSP’s</td>
<td>Field excitatory postsynaptic potentials</td>
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<tr>
<td>GC</td>
<td>Genome copies</td>
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<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
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<td>hU6</td>
<td>Human U6 promoter</td>
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<td>IM</td>
<td>Inner membrane of the mitochondrion</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>IMS</td>
<td>Intermembrane space of the mitochondrion</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>KPi</td>
<td>Potassium phosphate</td>
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<tr>
<td>LDF</td>
<td>Laser-doppler flow</td>
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<tr>
<td>LGMD2A</td>
<td>Limb-girdle muscular dystrophy type 2A</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral arterial occlusion</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>NDUFB8</td>
<td>NADH dehydrogenase (ubiquinone) 1 β subcomplex 8</td>
</tr>
<tr>
<td>NDUFV2</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 2</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>ROSC</td>
<td>Return of spontaneous circulation</td>
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<tr>
<td>Scr</td>
<td>Scramble</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfide</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SMP</td>
<td>Sub-mitochondrial particle</td>
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<tr>
<td>SVN</td>
<td>Selectively vulnerable neuron</td>
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<tr>
<td>TFI</td>
<td>Transient forebrain ischemia</td>
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<tr>
<td>TH</td>
<td>Therapeutic hypothermia</td>
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<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
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