The role of mitochondrial restructuring in neuronal calcium homeostasis and excitotoxicity

Patrick Ryan Houlihan

University of Iowa

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THE ROLE OF MITOCHONDRIAL RESTRUCTURING IN NEURONAL
CALCIUM HOMEOSTASIS AND EXCITOTOXICITY

by

Patrick Ryan Houlihan

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacology
in the Graduate College of
The University of Iowa

May 2013

Thesis Supervisor: Associate Professor Yuriy Usachev
ABSTRACT

Mitochondrial Ca\(^{2+}\) buffering is an important physiological modulator of neuronal signaling and bioenergetics, but this propensity toward Ca\(^{2+}\) regulation proves pathological during excitotoxic insult. Specifically, excessive mitochondrial Ca\(^{2+}\) uptake is a key component of glutamate toxicity within the penumbra surrounding the ischemic core following stroke. This mitochondrial toxicity and Ca\(^{2+}\) dyshomeostasis may be visualized in real time as delayed calcium deregulation (DCD). DCD is a predictor of neuronal, excitotoxic death, and is composed of three phases: 1) an initial response; 2) a latent period of elevated, but stable cytosolic Ca\(^{2+}\); and 3) failure of mitochondrial Ca\(^{2+}\) retention, termed deregulation. The duration of the latent period is an index of neuronal resistance.

Mitochondria are dynamic organelles that rapidly and reversibly undergo fission and fusion (MFF). MFF is tightly regulated by the phosphoregulation of fission inducing Drp1 at serine 656. Drp1-S656 phosphorelation is mediated by PKA/AKAP1, and it is dephosphorylated by PP2A/Bβ2. Phosphorylation of Drp1-S656 inactivates this contractile GTPase resulting in inhibition of mitochondrial fission and a shift toward elongated mitochondria. This PKA/AKAP1 dependent Drp1-S656 phosphorylation has proven to be neuroprotective. Likewise, attenuation of PP2A/Bβ2 signaling enhances neuronal survival during ischemia and excitotoxic insult.

Based on the mitochondrial buffering role in excitotoxicity and MFF modulation of neuronal survival, we began investigating the role of Ca\(^{2+}\) buffering as a function of MFF during glutamate toxicity. Noted above, resistance to excitotoxicity is visualized by the duration of the DCD latent period. Overexpression of AKAP1 in cultured hippocampal neurons greatly prolonged DCD latency in a PKA dependent manner, while Bβ2 ablation prolonged DCD latency by hours. Pharmacological modulation of PKA required PDE4 inhibition to reproduce the AKAP1 observations. Preliminary experiments
to study the effect of Bβ2 overexpression on matrix Ca\textsuperscript{2+} load suggests possible mechanism of MFF regulated of matrix Ca\textsuperscript{2+} accumulation. Using mtPericam DRG neurons as a model system for individual mitochondrial Ca\textsuperscript{2+} recording, we discovered impaired extrusion kinetics in mitochondria fragmented by both Drp1 and Bβ2 overexpression. Ca\textsuperscript{2+} uptake was comparable to that of control. Extreme elongation of mitochondria via dominant negative Drp1-K38A enhanced recovery.

Understanding these observations, however, requires knowledge of the mitochondrial Ca\textsuperscript{2+} buffering mechanism. Mitochondrial uptake candidates include MCU and ccdc109b. Our neuronal characterization of MCU confirms a role in mitochondrial Ca\textsuperscript{2+} buffering, but not a requirement; other components must be involved. Ccdc109b remains an inconclusive candidate, but may be an important regulator of MCU. Mitochondrial efflux transporters include Letm1 and NCLX. Though Letm1 observations are hindered by control artifact, preliminary evidence supports a role in extrusion. The role of NCLX is complicated by possible tissue specificity. Functional expression experiments utilizing Na\textsuperscript{+} free Li\textsuperscript{+} external solution suggests absence of NCLX in hippocampal neurons; DRG neurons were capable of Li\textsuperscript{+} exchange. The above observations confirm the significance of mitochondrial Ca\textsuperscript{2+} extrusion in neuronal survival. Understanding the mechanisms and regulation of mitochondrial Ca\textsuperscript{2+} transport has the potential to provide novel therapeutic targets in pathologies of excitotoxic etiology.

Abstract Approved: _______________________________________

Thesis Supervisor

______________________________
Title and Department

______________________________
Date
THE ROLE OF MITOCHONDRIAL RESTRUCTURING IN NEURONAL CALCIUM HOMEOSTASIS AND EXCITOTOXICITY

by

Patrick Ryan Houlihan

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacology in the Graduate College of The University of Iowa

May 2013

Thesis Supervisor: Associate Professor Yuriy Usachev
CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Patrick Ryan Houlihan

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Pharmacology at the May 2013 graduation.

Thesis Committee:
Yuriy Usachev, Thesis Supervisor

Steven Green

Gloria Lee

Durga Mohapatra

Stefan Strack
For my family
ACKNOWLEDGEMENTS

I would like to begin by thanking Dr. Yuriy Usachev for the opportunity to study the role of modulation of mitochondrial architecture on neuronal Ca\(^{2+}\) homeostasis. Dr. Usachev gave me the freedom to work independently, the chance to learn and develop a number of techniques, and guidance for both my research and my career. I am thankful for my thesis committee members Dr. Steven Green, Dr. Gloria Lee, Dr. Durga Mohapatra, and Dr. Stefan Strack. My thesis committee helped me focus the scope of my research and provided valuable perspective and evaluation of my experimental and control strategies. The pharmacology department was also of incredible help throughout my graduate studies. Lisa Ringen, Linda Buckner, Kate Bolton and Sue Griffin all assisted me in organizing meeting, streamlining communication with collaborators, and managing my grant application and funding. This work was supported by the NIH grant, an AHA pre-doctoral fellowship Grant 10PRE2660008, and the Pharmacological Sciences Training Grant.

My utmost gratitude goes out to Dr. ManSu Kim, Dr. Katrin Schnizler, Dr. Leonid Shutov and Dr. Jason Ulrich for their help and training throughout my graduate studies. Specifically, Dr. Kim taught me dissociated DRG and hippocampal culture and Ca\(^{2+}\) imaging. Dr. Schnizler aided me with fluorescent imaging and confocal microscopy, Dr. Shutov taught me whole-cell and perforated patch-clamp electrophysiology technique (used for other projects), and Dr. Jason Ulrich for teaching me biochemical and molecular biology techniques. Undergraduate research assistants Kubat Rahatbek and Yelena Shabelnik contributed to preliminary Neuro2A MCU/ccdc109b experiments and analysis. Recently, Dr. Zhihong Lin has joined the Usachev Lab and has been essential the characterization of MCU and ccdc109b function.

Outside of the Usachev lab, the Strack lab has been of tremendous help. The majority of overexpression, mutant and shRNA constructs used throughout my thesis
work (as well as the Bβ2 knockout mice) were generated by the Strack laboratory. I regularly turn toward Strack lab members to discuss the optimization of protocols and to learn new techniques. Specifically, I would like to thank Dr. Stefan Strack, Dr. Ronald Merrill, Tom Cribbs, Dr. Audrey Dickey and Andrew Slupe. The Mohapatra lab has played an important role during my graduate training, as well. Sharing weekly work-in-progress meetings with the Mohapatra lab provides unique perspective and discussions regarding my research, critical advice, and exposure to new techniques not performed in the Usachev Lab. Specifically, I would like to thank Dr. Durga Mohapatra and Dr. Andrew Slupe for teaching and aiding me with biochemical and PCR techniques. Duane Hall of the Mark Anderson lab has been a great help with my characterization of MCU and ccdc109b by providing shRNA constructs had confirming shRNA efficacy.

I would also like to extend much thanks to our collaborators. Dr. Stan McKnight at the University of Washington has generously provided us with AKAP1 knockout mice. Additionally, Dr. Brian Andrews at the National Institutes of Health invited me out to his laboratory to learn hippocampal culture cryofreezing and EPMA. Dr. Andrews has also generously provided the Usachev lab with cryofreezing apparatus, compatible coverslips, and his time/expertise with this unique protocol.
MITCHONDRIONAL Ca\textsuperscript{2+} BUFFERING IS AN IMPORTANT PHYSIOLOGICAL MODULATOR OF NEURONAL SIGNALING AND BIOENERGETICS, BUT THIS PROPENSITY TOWARD Ca\textsuperscript{2+} REGULATION PROVES PATHOLOGICAL DURING EXCITOTOXIC INSULT. SPECIFICALLY, EXCESSIVE MITCHONDRIAL Ca\textsuperscript{2+} UPTAKE IS A KEY COMPONENT OF GLUTAMATE TOXICITY WITHIN THE PENUMBRA SURROUNDING THE ISCHEMIC CORE FOLLOWING STROKE. THIS MITCHONDRIAL TOXICITY AND Ca\textsuperscript{2+} DYSHOMEOSTASIS MAY BE VISUALIZED IN REAL TIME AS DELAYED CALCIUM DREGULATION (DCD). DCD IS A PREDICTOR OF NEURONAL, EXCITOTOXIC DEATH, AND IS COMPOSED OF THREE PHASES: 1) AN INITIAL RESPONSE; 2) A LATENT PERIOD OF ELEVATED, BUT STABLE CYTOSOLIC Ca\textsuperscript{2+}; AND 3) FAILURE OF MITCHONDRIAL Ca\textsuperscript{2+} RETENTION, TERMED DREGULATION. THE DURATION OF THE LATENT PERIOD IS AN INDEX OF NEURONAL RESISTANCE.

MITCHONDRIA ARE DYNAMIC ORGANELLES THAT RAPIDLY AND REVERSIBLY UNDERGO FISSION AND FUSION (MFF). MFF IS TIGHTLY REGULATED BY THE PHOSPHOREGULATION OF FISSION INDUCING Drp1 AT SERINE 656. Drp1-S656 PHOSPHORELATION IS MEDIATED BY PKA/AKAP1, AND IT IS DEPHOSPHORYLATED BY PP2A/B\beta2. PHOSPHORYLATION OF Drp1-S656 INACTIVATES THIS CONTRACTILE GTPase RESULTING IN INHIBITION OF MITCHONDRIAL FISSION AND A SHIFT TOWARD ELONGATED MITCHONDRIA. THIS PKA/AKAP1 DEPENDENT Drp1-S656 PHOSPHORYLATION HAS PROVEN TO BE NEUROPROTECTIVE. LIKewise, ATTENUATION OF PP2A/B\beta2 SIGNALING ENHANCES NEURONAL SURVIVAL DURING ISCHEMIA AND EXCITOTOXIC INSULT.

BASED ON THE MITCHONDRIAL BUFFERING ROLE IN EXCITOTOXICITY AND MFF MODULATION OF NEURONAL SURVIVAL, WE BEGAN INVESTIGATING THE ROLE OF Ca\textsuperscript{2+} BUFFERING AS A FUNCTION OF MFF DURING GLUTAMATE TOXICITY. NOTED ABOVE, RESISTANCE TO EXCITOTICITY IS VISUALIZED BY THE DURATION OF THE DCD LATENT PERIOD. OVEREXPRESSION OF AKAP1 IN CULTURED HIPPOCAMPAL NEURONS GREATLY PROLONGED DCD LATENCY IN A PKA DEPENDENT MANNER, WHILE B\beta2 ABLATION PROLONGED DCD LATENCY BY HOURS. PHARMACOLOGICAL MODULATION OF PKA REQUIRED PDE4 INHIBITION TO REPRODUCE THE AKAP1 OBSERVATIONS. PRELIMINARY EXPERIMENTS
studying the effect of Bβ2 overexpression on matrix Ca^{2+} load suggests possible mechanism of MFF regulated of matrix Ca^{2+} accumulation. Using mtPericam DRG neurons as a model system for individual mitochondrial Ca^{2+} recording, we discovered impaired extrusion kinetics in mitochondria fragmented by both Drp1 and Bβ2 overexpression. Ca^{2+} uptake was comparable to that of control. Extreme elongation of mitochondria via dominant negative Drp1-K38A enhanced recovery.

Understanding these observations, however, requires knowledge of the mitochondrial Ca^{2+} buffering mechanism. Mitochondrial uptake candidates include MCU and ccdc109b. Our neuronal characterization of MCU confirms a role in mitochondrial Ca^{2+} buffering, but not a requirement; other components must be involved. Ccdc109b remains an inconclusive candidate, but may be an important regulator of MCU. Mitochondrial efflux transporters include Letm1 and NCLX. Though Letm1 observations are hindered by control artifact, preliminary evidence supports a role in extrusion. The role of NCLX is complicated by possible tissue specificity. Functional expression experiments utilizing Na^{+} free Li^{+} external solution suggests absence of NCLX in hippocampal neurons; DRG neurons were capable of Li^{+} exchange. The above observations confirm the significance of mitochondrial Ca^{2+} extrusion in neuronal survival. Understanding the mechanisms and regulation of mitochondrial Ca^{2+} transport has the potential to provide novel therapeutic targets in pathologies of excitotoxic etiology.
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<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>AKAP1</td>
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</tr>
<tr>
<td>AKAR3</td>
<td>A-kinase activity reporter 3</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
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<td>adenosine triphosphate</td>
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<td>AUC</td>
<td>area under the curve</td>
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<td>BAD</td>
<td>Bcl-xL/Bcl-2-associated death promoter</td>
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<td>Cer</td>
<td>cerebellum</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cort</td>
<td>cerebral cortex</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
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<td>CVD</td>
<td>cardiovascular disease</td>
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<td>cytochrome C</td>
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<td>D/N</td>
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<td>diacylglycerol</td>
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<td>inner mitochondrial membrane potential</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EPMA</td>
<td>electron pulse X-ray microanalysis</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated protein kinase 1/2</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide P-(trifluoromethoxy) phenylhydrozone</td>
</tr>
<tr>
<td>Fis1</td>
<td>fission protein 1</td>
</tr>
<tr>
<td>FIV</td>
<td>fetal immunodeficiency virus</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP/EGFP</td>
<td>green fluorescent protein/enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
</tbody>
</table>
GPCR  G-protein coupled receptor
Grp75  mortalin, a member of the heat shock protein 70 family
GTP  guanosine tryphosphate
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HH Buffer  Hank HEPES buffer
Hippo  hippocampus
HRP  horseradish peroxidase
HS  horse serum
ICUE3  cAMP FRET biosensor
IMM  inner mitochondrial membrane
IP3  inositol triphosphate
IP3R  inositol triphosphate receptor
KO  knockout
LC/MS/MS  liquid chromatography/mass spectroscopy/mass spectroscopy
Lettm1  leucine zipper-EF-hand containing transmembrane protein 1
MCU  mitochondrial calcium uniporter
MEM  modified eagle medium
MFF  mitochondrial fission and fusion
Mff  mitochondrial fission factor
Mfn1/2  mitofusin 1/2
MICU1  mitochondrial calcium uptake 1
MiD49  mitochondrial dynamics protein 49
MiD51/MIEF1  mitochondrial dynamics protein 50/ mitochondrial elongation factor 1
MPT  mitochondrial permeability transition
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mRFP</td>
<td>monomeric red fluorescent protein</td>
</tr>
<tr>
<td>mRyR</td>
<td>mitochondrial ryanodine receptor</td>
</tr>
<tr>
<td>mtEGFP</td>
<td>mitochondria targeted enhanced green fluorescent protein</td>
</tr>
<tr>
<td>NB-A</td>
<td>Neurobasal-A media</td>
</tr>
<tr>
<td>NCKX</td>
<td>sodium calcium potassium exchanger</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium calcium exchanger</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartic acid receptor</td>
</tr>
<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>optic atrophy 1</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenyl cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PAC1R</td>
<td>PACAP type 1 receptor</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP activated protein kinase A</td>
</tr>
<tr>
<td>PKI</td>
<td>PKA peptide inhibitor</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PP2B</td>
<td>protein phosphatase 2B (calcineurin)</td>
</tr>
<tr>
<td>PPM</td>
<td>metal-dependent protein phosphatase family</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PPP</td>
<td>phosphoprotein phosphotase family</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>qPCR</td>
<td>qualitative real-time reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RaM</td>
<td>mitochondrial Ca(^{2+}) rapid uptake mode</td>
</tr>
<tr>
<td>Rho123</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>RI/RII</td>
<td>PKA regulatory subunits I and II</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>Ru360</td>
<td>ruthenium 360</td>
</tr>
<tr>
<td>RuR</td>
<td>ruthenium red</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>Stem</td>
<td>brainstem</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TG</td>
<td>trigeminal ganglia</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>TPR</td>
<td>tetratricopeptide repeat</td>
</tr>
<tr>
<td>TrkB</td>
<td>tyrosine receptor kinase B</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>WHS</td>
<td>Wolf Hirschhorn syndrome</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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CHAPTER I
INTRODUCTION

The nervous system is composed of separate peripheral (PNS) and central (CNS) components. The peripheral nervous system provides one's perception and interaction with the environment and relays said information to and from the CNS. Information from the environment is received via specialized neurons of the dorsal root ganglion (DRG) and trigeminal ganglia (TG). This information includes thermal, tactile and chemical stimuli. The CNS is a much more complex system. All of the information received from the periphery is processed and analyzed by the CNS. Based on peripheral input, the CNS determines and signals/enacts a response or behavior; and the CNS performs the higher functions of complex cognition, learning and memory (Berne et al., 2004). Because these systems are composed of sophisticated networks of highly active neurons, excitability, metabolic stability and susceptibility to excitotoxicity are critical characteristics of neurons that, under pathological conditions, produce catastrophic consequences.

Stroke

Excessive calcium influx and prolonged cellular retention of Ca\textsuperscript{2+} result in neuronal dysfunction and toxicity (Rasmussen and Barrett, 1984). Calcium dyshomeostasis can arise from a number of sources including pathological stimulation, epileptic neuronal activity, concussive injury and ischemic stroke. When analyzed separate of other cardiovascular diseases (CVDs), stroke ranks fourth among all causes of death. In the United States the prevalence of ischemic stroke averages 795,000 incidences per year with almost 17% mortality (based on 2005-2008 statistics). Long term consequences of stroke include hemiparesis (and lesser degrees of physical impairment), aphasia, depression and dependence on assisted living facilities resulting in severely diminished quality of life and $34.4 billion of direct and indirect national annual costs (Roger et al., 2012).
Ischemic foci

Ischemic stroke is the consequence of loss of blood flow to all or portions of the brain due to thrombotic impairment of flow, hemorrhaging, or perioperative consequences. This impaired blood flow results in hypoxia and glucose deprivation in the deprived tissue leading to rapid depolarization of neurons and ion-dyshomeostasis. Neurons within the focus of circulation arrest are susceptible to necrotic death and release of cytosolic content into surrounding tissues. Necrotic neuronal loss within the ischemic foci is a rapid consequence occurring within 5-10 minutes (in the absence of significant collateral perfusion); this necrosis comprises the first phase of neuronal death peri- and post-ischemic stroke (Astrup et al., 1981; Olsen et al., 1983). Due to its rapid and unpredictable nature, pharmaceutical intervention is not a practical solution.

Ischemic penumbra

As described above, the ischemic core undergoes necrosis and rapid depolarization due to depletion in oxidative phosphorylation and ATP leading to attenuated Na+/K+ ATPase function. This sudden failure of neuronal membrane potential and integrity produces a massive release of glutamate and potassium. Though it is stored in synaptic vesicles and is a primary component of neuronal signaling, glutamate is synthesized from glutamine in surrounding support cells termed astrocytes. Ischemic insult also liberates this excitatory neurotransmitter from astrocytes (and eliminates reuptake) contributing to a wave of extracellular glutamate reaching hundreds of micromolar concentrations (Grewer et al., 2008; Kostandy, 2012). These byproducts of hypoxia permeate “low risk tissue” surrounding the foci (Huang et al., 1997). The wave of toxic glutamate activates both synaptic and non-synaptic glutamate receptors including calcium permeable NMDA receptors and Ca2+ permeable AMPA receptor subtypes. The resulting depolarization exacerbates the NMDA dependent calcium influx via activation of voltage gated calcium channels termed “peri-infarct depolarization” (Benveniste et al., 1988; Martin et al.,
Over a prolonged second phase, this depolarization and toxic glutamate signaling drives calcium overload (Dijkhuizen et al., 1999; Hartings et al., 2003; Won et al., 2002). Elevated cytosolic calcium activates pro-apoptotic enzymes (proteases and phospholipases), drives ROS production and impairs mitochondrial function leading to a release of apoptotic factors (Hansen, 1984; Jekabsons and Nicholls, 2004; Kostiuk, 1997; Molz et al., 2008; Stys, 2004; Zhang and Bhavnani, 2006). The resulting extended area of brain damage is termed the ischemic penumbra and represents the majority of neuronal loss. Neuronal degeneration within the ischemic penumbra is a delayed process that may be subject to therapeutic intervention (Dyker and Lees, 1998).

**Mitochondrial calcium buffering**

Under physiological resting conditions, the cytosolic calcium concentration is held remarkably low (50 nM) by active Ca$^{2+}$ efflux, calcium binding proteins and calcium sequestering in intracellular stores (Meldolesi et al., 1992; Shmigol et al., 1994; Tillotson and Gorman, 1983; Verkhratsky and Petersen, 1998). Mitochondria are capable of storing large quantities of calcium and serve as cytosolic calcium sinks. Calcium uptake into the mitochondrial matrix is dependent on the mitochondrial membrane potential ($\Delta \Psi_{\text{mt}}$) as its driving force. At an estimated +150 mV, the $\Delta \Psi_{\text{mt}}$ is able to maintain a 100 fold gradient across the inner mitochondrial membrane or IMM (50 nM in the cytosol to 1-5 μM in the matrix). Ca$^{2+}$ enters the mitochondrial matrix through a uniporter imbedded in the IMM following the stoichiometry of 2Ca$^{2+}$ exchanged for 2H$^+$ per molecule of ATP hydrolyzed (Gunter et al., 2000; Kapus et al., 1991).

**Mitochondrial Calcium Uptake**

Until recent insights, the identity of the components responsible for mitochondrial calcium uptake (termed the mitochondrial calcium uniporter, or MCU) has been entirely speculative. Despite the absence of molecular identity, the kinetics of this theoretical calcium transporter
has been extensively characterized. It is unanimously agreed that MCU is a Ca\(^{2+}\) selective transporter with significantly less permeability to other divalent cations, including Sr\(^{2+}\), Mn\(^{2+}\), Ba\(^{2+}\) (Gunter et al., 2000). Affinity for calcium, however, is more controversial. Using isolated mitochondria, mitoplasts, and \textit{in situ} measurements, investigators have observed affinities ranging from 2 nM to 20 µM; currently, the field supports a relatively low affinity range of 10-20 µM (Kirichok et al., 2004; Moreau et al., 2006; Putney and Thomas, 2006). 10-20 µM cytosolic calcium concentrations are only achieved during Ca\(^{2+}\) transients (a large release of calcium from internal stores or a large influx of calcium observed in excitable cells, including neurons, cardiomyocytes and skeletal muscle cells). Thus, mitochondrial calcium buffering is a critical component in the cellular calcium response, and is responsible for shaping the Ca\(^{2+}\) transient both quantitatively and temporally (Putney and Thomas, 2006; Santo-Domingo and Demaurex, 2010). This micromolar affinity, however, doesn’t support observed high affinity responses. Two high affinity hypotheses have been proposed. One area of mitochondria calcium buffering research supports a microdomain hypothesis for which mitochondria exist in close juxtaposition, or even contact, with points of calcium entry (voltage gated calcium channels and NMDA receptors) of plasma membrane association and coupling sites of calcium release from the endo- and sarcoplasmic reticulum (Gunter and Gunter, 2001; Moreau et al., 2006; Putney and Thomas, 2006; Szanda et al., 2006). The alternative hypothesis supports the existence of a second kinetic mode or the existence of a higher affinity channel. Termed the mitochondrial calcium rapid uptake mode, or RaM, kinetic studies in isolated mitochondria and mitoplasts suggests high affinity, rapid calcium uptake followed by Ca\(^{2+}\) dependent deactivation (Gunter et al., 2000; Gunter and Gunter, 2001; Putney and Thomas, 2006; Ryu et al., 2010). Both modes of MCU conductance are sensitive to the calcium transport blocker ruthenium red (RuR), as well as the more mitochondrial selective Ru360. Treatment with the prometabolic biogenic polyamine spermine significantly enhanced uptake (Chaffee et al., 1979; Gunter et al., 2000; Gunter and Gunter, 2001). Determined by these initial biophysical studies, MCU conductance conforms to second order kinetics; that is, MCU conductance requires
ligand binding at an activation site. It is proposed that Ca$^{2+}$ is the required ligand, and it is this Ca$^{2+}$ dependent gating that establishes the affinity of MCU conductance (Dash et al., 2009; Gunter et al., 2000; Gunter and Gunter, 2001).

MCU and MICU1 identity

In the fall of 2010, a research group led by Vamsi Mootha published an investigative study utilizing in silico genomics and RNA interference coupled with a luciferase based mitochondrial calcium reporter to determine the molecular identities of the elusive MCU proteins. This initial report identified the gene calcium-binding atopy-related autoantigen 1, CBARA1. Based on its role in calcium buffering, CBARA1 was re-termed mitochondria calcium uptake 1, MICU1. MICU1 is an IMM associated protein containing two calcium binding EF hand motifs. In HeLa cells, MICU1 knockdown attenuated mitochondrial calcium uptake following both histamine and thapsigargin induced ER Ca$^{2+}$ release and impaired cytosolic Ca$^{2+}$ transient-metabolism coupling (Drago et al., 2011; Perocchi et al., 2010). MICU1 is not a pore forming protein as an individual gene product or as a subunit of a multimeric channel. Therefore, MICU1 has been proposed to be a calcium sensing regulatory subunit of MCU. The mechanism and consequence by which MICU1 regulates MCU is yet to be determined; based on the initial RNAi studies, MICU1 appeared to be essential for MCU function (Alam et al., 2012; Collins and Meyer, 2010; Hajnoczky and Csordas, 2010; Perocchi et al., 2010).

The following year, Mootha’s group, used MICU1 to determine the molecular identity of MCU. During this same time, Rizzuto’s group, utilized genomics and RNAi to independently identify MCU. In August 2011 the two groups reported on the gene product ccdc109a (coiled-coil domain containing 109a) as a possible MCU pore forming candidate (Baughman et al., 2011; Bick et al., 2012; De Stefani et al., 2011). Sequence analysis of this 40kDa protein revealed two transmembrane spanning domains and an acidic residue rich loop representing a hypothetical cation selection motif. With regard to the cation selection loop, a putative DIME
motif strongly suggests cation selectivity and mutation of DIME to QIMQ (D260Q,E263Q) completely eliminates calcium, but retains localization to the IMM. Over-expression of this mutation reveals an even more interesting result. As commonsense would dictate, two transmembrane domains and a cation selection loop are not sufficient to form a functional channel. Multimerization of MCU proteins is an expected requirement, and the QIMQ mutation supports this view. In the absence of an RNAi/overexpression replacement strategy, simple MCU-D260Q,E263Q overexpression results in attenuated mitochondrial calcium uptake (De Stefani et al., 2011; Raffaello et al., 2012). This observation suggests that MCU-D260Q,E263Q is acting as a dominant negative construct. MCU-D260Q,E263Q retains its ability to localize to the IMM and multimerize with endogenously expressed and normally functional MCU subunits to form an inactive uniporter. The MICU1 requirement observed by Mootha’s group, suggests that MCU-D260Q,E263Q may also be capable of scavenging this hypothetically necessary MCU accessory subunit from functional, endogenous MCU. Biochemical determination of MCU’s multimerization and stoichiometry remains an important aspect of its characterization that has yet to be performed. As was predicted, MCU is also sensitive to RuR and Ru360 inhibition. RuR sensitivity was attenuated following serine 259 to alanine point mutation within the selectivity loop, but the mutation maintained normal expression, localization and calcium conductance (Baughman et al., 2011). MCU sensitivity to Mg\(^{2+}\) and inorganic phosphate (Pi) has been observed, as well. Kinetic analysis of the newly identified MCU is limited and preliminary. As was observed prior to its identification, MCU conforms to second order kinetics; that is ligand binding (in this case, Ca\(^{2+}\) binding) is required for channel gating. Affinity analysis is yet to be performed; Dash et al., predicts low Ca\(^{2+}\) affinity as was observed for pre-2011 characterization (Pradhan et al., 2011).

**CCDC109b, a MCU isoform**

A number of other calcium uptake mechanisms have been proposed, but with much less enthusiastic support from the mitochondrial calcium buffering research community as that
given to MCU. Sharing significant sequence homology and containing a mitochondrial localization sequence, the gene product ccdc109b represents a potential MCU isoform; however, sequence homology analysis displays divergence within the cation selection loop. According to NCBI sequence information, the ccdc109b residue corresponding to glutamate 256 is replaced with valine. The consequence of this substitution is unclear. Based on the QIMQ mutation observations described above, ccdc109b may act as an endogenous dominant negative whose expression regulates MCU function.

**Uncoupling proteins**

Members of the uncoupling protein (UCP) family have been entertained as possible MCU components. UCP members are historically known as proton and chloride conduction channels localized to the inner mitochondrial membrane. Conductance of protons across the IMM uncouples the proton gradient established/maintained by the electron transport chain from oxidative phosphorylation. This metabolic uncoupling has a number of physiological functions. Uncoupling produces heat which maintains non-shiver thermogenesis (Chan et al., 2006; Smorodchenko et al., 2009). Inhibition of oxidative phosphorylation reduces the production of reactive oxygen species. Under pathophysiological conditions, ROS upregulates UCPs and enhances proton conductance and cytoprotection (Dejean et al., 2004; Liu et al., 2006; Paradis et al., 2003; Ryu et al., 2004). Critical of mitochondrial calcium buffering, UCP proton conductance also modulates the mitochondrial membrane potential, and thus the driving force of calcium uptake (Mattson and Liu, 2003; Paradis et al., 2003; Sun and Zemel, 2004). The majority of research supports UCP function as a negative regulator of mitochondrial calcium uptake. However, Graier et al., argues a regularly protested theory of UCPs as candidates for MCUs. Using overexpression and RNAi (but oddly not the UCP inhibitor genipin), Gaier et al. have described in a series of manuscripts UCP 2 and -3 as MCU components required for mitochondrial calcium sequestration (Graier et al., 2007; Graier et al., 2008; Mehta and Li, 2009; Trenker et al., 2007; Turner et al., 2010). Inappropriate controls and models have
provided plenty of fodder for contest (Brookes et al., 2008). Despite its unlikely role as a uniporter, UCPs’ ability to modulate mitochondrial calcium buffering via proton leak and regulation of ΔΨₘ⁰ make UCPs potential targets for neuroprotection (Graier et al., 2008; Paradis et al., 2003; Sun and Zemel, 2004). Of the UCP family, UCP2, -4 and -5 (UCP4/5 are largely neuron specific) are currently being studied as targets in ischemic neuronal damage and neuro-degeneration in Alzheimer’s disease and dementia (Alan et al., 2009; Hoang et al., 2012; Mattson and Liu, 2003).

**Letm1 as MCU candidate**

Deletion of chromosome 4p16.3 gives rise to mental and developmental defects including epileptiform seizure termed Wolf-Hirschhorn syndrome (WHS). Analysis of the haploinsufficient consequences of this chromosomal truncation revealed deletion of the novel gene ‘leucine-zipper, EF-hand containing transmembrane protein 1,’ or Letm1, in almost all WHS patients (Endele et al., 1999; Schlickum et al., 2004). Initial analysis of Letm1 function presented evidence for a mitochondrial localized protein that appeared essential for K⁺ homeostasis and regulation of mitochondrial swelling (Froschauer et al., 2005). Attenuation of Letm1 expression (and its yeast homologue) produced mitochondrial matrix cation retention (primarily K⁺), mitochondrial swelling, Drp1 dependent mitochondrial fission and decrease in membrane potential (Dimmer et al., 2008; Hasegawa and van der Bliek, 2007; Nowikovsky et al., 2004; Tamai et al., 2008). These phenotypes were rescued via the H⁺/K⁺ ionophore nigericin, and suggests Letm1 to be a H⁺/K⁺ exchanger (Froschauer et al., 2005). In addition to the above K⁺ homeostasis effects, RNAi knockdown reduced the release of neurotransmitter from *Drosophila* synapses (a calcium dependent process) and submicromolar mitochondrial calcium uptake. This high affinity calcium uptake characteristic adds Letm1 to the list of candidate MCU proteins (Jiang et al., 2009; McQuibban et al., 2010; Waldeck-Weiermair et al., 2011). Permeabilization of *Drosophila* S2 cells with digitonin and addition of elevated concentrations of Ca²⁺ and H⁺ confirmed a Letm1 role in mitochondrial calcium sequestration,
as well as proton regulation (Demaurex and Poburko, 2009). Rather than a uniporter, Letm1 is proposed to be a Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger responsible for the higher affinity mode of calcium buffering. The stoichiometry is yet to be determined, but based on biophysical calculations, this stoichiometry needs to be 1:3::Ca\textsuperscript{2+}:H\textsuperscript{+} to account for this higher affinity and to achieve a driving force comparable to that observed with the proposed MCU (Nowikovsky et al., 2012).

**Mitochondrial ryanodine receptors**

Lastly, a prominent player in calcium store history has been given new interest as a possible candidate for MCU. Ryanodine receptor (RyR) expression has been exclusively studied in the ER and the majority of research regarding mitochondrial calcium sequestration is limited to ER-mitochondrial calcium coupling. However, Sheu et al described a cardiac mitochondrial channel of 600 kDa and RyR1 immunoreactivity. Electron micrographs stained with RyR1 immunogold labeling revealed IMM cristae localization that has been contested (Altschafl et al., 2007; Beutner et al., 2001; Hoppe, 2010). Despite some controversy, purification of the mitochondrial ryanodine receptor (mRyR) and current recordings in reconstituted lipid bilayer support mRyR as a rapid calcium conducting channel (~225 picoseimens) with nanomolar affinity and a bell shaped calcium dependence; this in vitro analysis likely overestimates mRyR function (Ryu et al., 2010; Ryu et al., 2011; Salnikov et al., 2009). Pharmacological characterization of mRyR revealed inhibition by ryanodine, RuR and Mg\textsuperscript{2+} and activation by impera toxin A. Cardiac mRyR has been proposed to be a central component of excitation-metabolism coupling, yet mRyR expression and function remain unknown (Beutner et al., 2001; Beutner et al., 2005; Ryu et al., 2010).

**Mitochondrial calcium efflux**

In parallel to Ca\textsuperscript{2+} uptake, free inorganic phosphate (P\textsubscript{i}) enters mitochondria following the IMM-matrix pH gradient. Inside the matrix, Ca\textsuperscript{2+} and P\textsubscript{i} complex (as well as other organic counter ions) to form an apatite salt to the effect that free matrix calcium is maintained between
1 and 5 μM. Extrusion of calcium from the mitochondrial matrix is accomplished by the actions of proposed exchanger proteins that will be discussed shortly. Ionic exchange drives Ca\(^{2+}\) into the cytosol at a much slower rate than Ca\(^{2+}\) uptake, thus completing the buffering cycle (Bielawski and Lehninger, 1966; Nicholls and Chalmers, 2004). The mitochondrial contribution to cytosolic calcium buffering is illustrated in Fig.1.1, which shows a depolarization-induced [Ca\(^{2+}\)]\(_i\) response in a dorsal root ganglion (DRG) neuron with characteristic plateau phase of sustained elevated [Ca\(^{2+}\)]\(_i\), lasting for several minutes. Following removal of the mitochondrial buffering component via antimycin treatment, the transient amplitude is increased and the plateau is absent. In the absence of mitochondrial calcium buffering, Ca\(^{2+}\) extrusion by the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger predominates (Miller, 1991; Werth and Thayer, 1994). From this example, it appears that the role of mitochondrial buffering is to establish a peak calcium ceiling, while maintaining a mild elevation of cytosolic Ca\(^{2+}\) that outlasts the stimuli (Kim and Matsuoka, 2008). Similar processes have been described in central neurons (Nicholls et al., 2003; White and Reynolds, 1996). Therefore, it can be concluded that mitochondria actively shape calcium transients by regulating the peak amplitude, as well as determining the duration of elevated [Ca\(^{2+}\)]\(_i\). This prolonged mitochondria-dependent calcium signaling has been shown to be required for activation of the calcium-calcineurin mediated transcription factor family NFAT and is proposed to be important in short term synaptic plasticity (Kim and Usachev, 2009; Medvedeva et al., 2008). Mitochondrial Ca\(^{2+}\) cycling is necessary for calcium homeostasis; disruption of the mitochondrial calcium extrusion component produces cytotoxic consequences that will be described shortly (Nicolau et al., 2009; Nicolau et al., 2010; Scanlon et al., 2000).

**NCX and NCLX**

Like mitochondrial calcium uptake, the calcium extrusion field is emerging with many candidate ion exchangers and ligand gated efflux mechanisms. Early biophysical characterization of mitochondrial calcium extrusion in adrenal chromaffin cells,
cardiomyocytes, and cerebellar granular cells suggested a role for sodium-calcium exchange due to observed sodium dependence (Babcock et al., 1997; Czyz and Kiedrowski, 2003; Noack and Greeff, 1975). Biochemical analysis of purified mitochondria and immunoreactivity of electron micrographs showed expression of NCX1-3 in mitochondria; however, localization appears to be primarily at the OMM (Gobbi et al., 2007). Of the NCX family exchangers, NCX3 has the most support. Though IMM localization evidence is lacking, NCX3 functional analysis provides a strong argument for its role in mitochondrial calcium buffering; primarily, the putative mitochondrial NCX channel inhibitor CGP-37157 inhibits NCX3 (Czyz and Kiedrowski, 2003). Additionally, cleavage of NCX3 as a consequence of Ca^{2+} dependent μ-calpain attenuates mitochondrial calcium extrusion and retains matrix calcium. Calcium dependent NCX3 degradation is blocked by the calpain inhibitor calpeptin, which restores mitochondrial calcium extrusion. NCX3’s role in mitochondrial calcium efflux is controversial within the research community due to disputed localization and tissue specificity (Brustovetsky et al., 2010; Castaldo et al., 2009; Czyz and Kiedrowski, 2003; Kar et al., 2009; Kim and Matsuoka, 2008).

In 2004 the Sekler research group of Israel described a new member of the NCX family termed NCLX, also known as NCKX6 (Lytton, 2007). Structurally dissimilar to all other members of the exchanger family, NCLX possesses a unique physiological characteristic of lithium-calcium exchange; that is, in place of sodium, NCLX is capable of lithium transport (Palty et al., 2006; Palty et al., 2004). NCLX sodium-calcium exchange rate is comparable to that of NCX1. Mitochondrial purification produces an enrichment of NCLX immunoreactivity. A role for NCLX in mitochondrial calcium extrusion has been supported with overexpression studies showing enhanced calcium efflux, while knockdown attenuates extrusion. NCLX activity is also inhibited via Zn^{2+} and CGP-37157 (Kim et al., 2012; Palty and Sekler, 2012; Palty et al., 2010).
Letm1 and DCC as matrix Ca$^{2+}$ export machinery

Alternatively, proton-calcium exchange may contribute to Ca$^{2+}$ efflux. The complementing proton and calcium gradients would support proton-calcium exchange; early biophysical recordings showed proton influx reciprocal to calcium efflux. The MCU candidate Letm1 may be this putative proton-calcium exchanger. Under conditions of mitochondrial calcium load and enhanced metabolism, reversal of Letm1 has potential to occur, as has been observed for other ion exchange proteins. Lastly, ligand-gated calcium extrusion may be a component of mitochondrial calcium efflux (McQuibban et al., 2010; Tamai et al., 2008). Ligand-gated mitochondrial calcium export is an extremely limited field of study with one candidate protein, the DAG (1,2-sn-diacylglycerol) activated cation channel, DCC. DAG is a product of phospholipase C (PLC) metabolism of inositol phospholipids and is an important signaling molecule in all cell types (Smrcka et al., 2012). This DAG sensitive calcium efflux becomes increasingly interesting considering the histamine model used in the majority of literature that is trying to tease apart the mitochondrial calcium buffering mechanism (Chinopoulos et al., 2005; Ryu et al., 2010). Histamine, acting through the H1 receptor, produces the desired cytosolic Ca$^{2+}$ elevation through PLC metabolism of inositol phospholipids, the liberation of inositol 3-phosphate (IP3), and ER calcium release through the IP3-receptors. H1 dependent PLC activation also produces DAG; therefore, this histamine model may favor some contribution of mitochondrial calcium extrusion by DCCs and suggests a complex buffering system that may resolve distinction between the method, source, magnitude and duration of calcium responses (Liu et al., 2008; Notcovich et al., 2010; Tseng and Wei, 2012). A summary diagram of mitochondrial calcium buffering and regulation is presented in Fig.1.2.

Excitotoxicity and DCD

Mitochondria become functionally impaired in their attempt to buffer the pathological Ca$^{2+}$ concentrations observed within neurons of the ischemic penumbra. This dysfunction is manifested as permeabilization of the IMM (termed mitochondrial permeability transition, or
MPT), complete loss of the mitochondrial membrane potential ($\Delta \Psi_{mt}$), increase in reactive oxygen species (ROS), and release of cytochrome C (CytC). CytC is known to activate caspase-dependent apoptosis (Cheung et al., 2007; Gazaryan and Brown, 2007; Greenwood et al., 2007; Hansen, 1984; Jacquard et al., 2006; Pivovarova et al., 2004; Shalbueva et al., 2006; Zieminska et al., 2006). Prior to opening of the permeability transition pore (PTP) and caspase release, calcium dyshomeostasis reaches a threshold. One of the early events following excitotoxic glutamate stimulation is delayed calcium deregulation (DCD). This process is characterized by three distinct phases (Fig.1.3): 1) an initial and physiologically normal glutamate $\text{Ca}^{2+}$ response, 2) an elevated, steady-state buffering phase (or latent period) and 3) a marked loss of buffering capacity, termed delayed calcium deregulation, which is associated with mitochondrial depolarization and release of mitochondrial content (Nicholls and Budd, 2000). The chronology of this pathophysiology is unclear; it is to be determined whether DCD is a product of MPT or a precursor. Based on recoverability of DCD, current research argues that DCD occurs prior to and independent of MPT.

Transient opening of the PTP has been described in non-neuronal systems as a protective mechanism capable of reducing matrix $\text{Ca}^{2+}$ without releasing pro-apoptotic factors. The actual mechanism of DCD mitochondrial depolarization, calcium release, and the role of MPT remain unknown. One can speculated that excitotoxic stress will elevate ROS production via prolonged depolarization, consumption of ATP supply and upregulation of oxidative phosphorylation (Beal, 1996; Bindokas et al., 1996; Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995; Sengpiel et al., 1998). These elevated ROS concentrations may activate UCPs that purge the proton gradient. The resulting mitochondrial depolarization could abolish the driving force for $\text{Ca}^{2+}$ sequestration (Gogvadze et al., 2000). Importantly, what is known is the toxicity of mitochondrial matrix calcium. Elimination of mitochondrial calcium buffering via the protonophore FCCP attenuates neuronal vulnerability to excitotoxic stress, highlighting DCD as a crucial process in
excitotoxicity and as a promising target for therapeutic interventions (Pivovarova et al., 2008; Stout et al., 1998).

**Mitochondrial fission and fusion**

Mitochondria are dynamic organelles that rapidly and reversibly undergo fission and fusion (termed mitochondrial fission and fusion). These fission and fusion processes have been confirmed using mitochondria targeted photoactivatable fluorescent proteins (Youle and Karbowski, 2005). The description of a cell as having either fragmented or elongated mitochondria describes the net effect of these apposing processes, or the state of the mitochondrial fission and fusion equilibrium. Fission and fusion equilibrium reflects the developmental and situational state of the cell and results in a functional mitochondria reticulum or isolated, individual mitochondria (Elgass et al., 2013). This dynamic fragmentation and fusion play important roles throughout cellular physiology. Mitochondrial fragmentation is a necessary process in cellular mitosis important in the distribution of mitochondria to symmetric daughter cells. Fission is also used as a quality control mechanism; unlike its symmetric role in mitosis, asymmetric fragmentation of mitochondria allows the cell to concentrate unwanted/damaged mitochondrial components into mitochondrial fragments that are then targeted for autophagy, a process termed mitophagy. Mitochondrial fragmentation is also an early and required step of cellular apoptosis. Conversely, mitochondrial fusion allows mitochondria to share solutes, metabolites and proteins that optimize bioenergetics.

**Mitochondrial Fission**

Both fission and fusion require a number of evolutionarily conserved proteins. Proteins identified in the process of fission in yeast and mammalian cells include the dynamin-related GTPase Drp1 and the outer mitochondrial membrane (OMM) associated proteins Fis1, MFF, MiD49, and MiD51/MIEF1 (Chen and Chan, 2005; McBride et al., 2006; Palmer et al., 2011; Youle and Karbowski, 2005; Zhao et al., 2011). Like other dynamin GTPases, Drp1 is a
contractile protein that undergoes its scission inducing conformational change upon hydrolysis of GTP. Additionally, Drp1 recruits other Drp1 proteins and forming a large contractile ring, or spiral collar around the mitochondria. Upon contraction, the aperture of the ring complex decreases until the OMM fuses producing two separate mitochondria. Drp1 is recruited to the OMM via a number of membrane bound proteins including MFF, Mid49, and MiD51MI1F1 recruit Drp1 to the OMM (Zhao et al., 2012; Zhao et al., 2011). Fis1 is a small protein localized to the OMM via its C-terminus. Its N-terminus comprises most of the molecule and extends into the cytosol. This N-terminus contains multiple tetratricopeptide repeats (TPR) important for the recruitment of Drp1 and, subsequently, mitochondrial fission. Yoon et al. (2003) describes Fis1 as the limiting factor of the fission apparatus; however, more recent investigation limits Fis1’s importance to yeast biology and recruitment of the Drp1 yeast orthologue Dmn1 (James et al., 2003; Lee et al., 2007; Yoon et al., 2003; Yu et al., 2005; Zhao et al., 2012). The human orthologue of Fis1 (hFis1) has not been shown to recruit Drp1 to the OMM (Otera and Mihara, 2011a). In 2008, Gandre-Babbe and van der Bliek described an OMM associated protein mitochondrial fission factor, or MFF. MFF, recruits Drp1 to the OMM and promotes fragmentation. RNAi knockdown of MFF significantly elongates mitochondria, whereas overexpression of MFF promoted fragmentation via increase Drp1 recruitment (Otera et al., 2010). Conversely, mitochondrial elongation factor 1 (MIEF1) similarly recruits Drp1 to the OMM, yet Drp1 association with MIEF1 inhibits mitochondria fission, thus promoting fusion (Zhao et al., 2011).

Mitochondrial fusion

Distinct from fission, mitochondrial fusion is performed by the following proteins: mitofusin 1 and 2 (Mfn 1 and 2) and optic atrophy 1 (Opa1). With function conceptually similar to the SNARE proteins, Mfn 1 and 2 are both GTPases capable of tethering adjacent mitochondria. Bringing the two OMMs together under the tension of conformational changes as a result of GTP hydrolysis, the OMMs of the two mitochondria fuse (Papanicolaou et al.,
IMM fusion is performed by the GTPase Opa1 in functional association with Mfn 1 and 2. The mechanism by which Opa1 performs IMM fusion and coordinates with OMM Mfn1/2 is not well understood, but is proposed to occur via independent mechanisms (Zhang and Chan, 2007). An alternative role for Opa1 is its sequestration of cytochrome C into cristae of the IMM, which suggests an anti-apoptotic role (Chen and Chan, 2005).

**Modulation of mitochondrial morphology**

To explain the observed maintenance of and shifts in MFF equilibrium, cellular regulation of fission and fusion is expected. At least four types of post translational modifications have been proposed to regulate the MFF machinery: phosphorylation, sumoylation, s-nitrosylation, O-glycosylation, and ubiquitylation (Gawlowski et al., 2012; Knott et al., 2008). Phosphorylation is a readily reversible process capable of tightly regulating protein activity and provides a target for pharmacological manipulation (Lopez-Neblina and Toledo-Pereyra, 2006). Phosphoregulation of mitochondrial fission and fusion is the focus of this study.

**Drp1 phosphorylation via PKA/AKAP1**

The most common form of post-translational modification is phosphorylation (Hunter, 2012). Phosphorylation modulates enzymatic activity, protein localization, susceptibility to degradation via the proteasome, and protein-protein interactions. This posttranslational modification is catalyzed by members of the kinase superfamily (Dissmeyer and Schnittger, 2011; Lopez-Neblina and Toledo-Pereyra, 2006). Phospho-signaling achieves its complexity and selectivity via substrate specificity. Kinases are capable of phosphorylating either serine/threonine or tyrosine amino acid residues; recognition of the effector substrate is accomplished via kinase binding of specific and conserved sequences. One of the most amply studied kinases is the cAMP-dependent protein kinase A, PKA. PKA is a serine/threonine kinase made up of four subunits. The PKA holoenzyme is composed of two regulatory, or R, subunits (either RI or RII) and two catalytic, or C, subunits. The catalytic subunits must be
liberated from the PKA holoenzyme to carry-out their kinase function; this dissociative PKA activation occurs via association with the second messenger cyclic-adenosine monophosphate, cAMP (Pidoux and Tasken, 2010). Diffuse PKA signaling does not sufficiently explain all PKA signaling pathways; PKA compartmentalization is expected. Scaffolding proteins exist that localize PKA to specific cellular compartments and organelles (Alto et al., 2002; Cooper, 2005; Feliciello et al., 2001; Perkins et al., 2001).

A-kinase anchoring proteins (AKAPs) are scaffold proteins that associate PKA within proximity of its effector targets (Feliciello et al., 2001). Many AKAP proteins have been characterized, including the OMM targeted S-AKAP84/121 (Chen et al., 1997). The C-term encodes a PKA-regulatory subunit binding site. Most AKAP molecules bind only the RII subunit; AKAP121 is unique in that it recruits both RII and RI subunits to the OMM (RII bind AKAP121 with nanomolar affinity, while RI binds with 10-100 fold lower affinity). This dual specificity resulted in the AKAP121 nomenclature being changed once again to D-AKAP1, or AKAP1 (Affaitati et al., 2003; Chaudhry et al., 2002; Feliciello et al., 1998; Huang et al., 1999; Means et al., 2011).

AKAP1 association is a required component for PKA regulation of metabolism, mitochondrial morphology, and cell survival; Drp1 activity accurately reciprocates these mitochondria- PKA functions (Carlucci et al., 2008a; Carlucci et al., 2008b). PKA negatively regulates Drp1 via phosphorylation at serine 656. This specific serine residue disrupts the conformation of the Drp1 variable domain (VD). Allosteric modulation of the Drp1 VD is proposed to inhibit the Drp1 protein-protein interaction required for the formation of the contractile, filamentous Drp1 ring (Strack and Cribbs, 2012). This PKA dependent inhibition of Drp1 activity is largely dependent on AKAP1 expression and PKA localization to the OMM. Inhibition of Drp1 via PKA phosphorylation shifts the MFF equilibrium toward fusion. Cribbs and Strack showed that this inhibition of mitochondrial fission has cyto-protective effects; specifically, PC12 cells transfected with a Drp1-S656D phosphomimetic construct enhanced
staurosporine and etoposide resistance (Cribbs and Strack, 2007). Mutation of Drp1 serine 656 to a non-phosphorylatable alanine produced constitutively active Drp1 (Merrill et al., 2011).

**Phosphodiesterase and cAMP signaling**

PKA and substrate localization is just a component of cAMP signaling complexity. Both cAMP production and degradation are subject to compartmentalization. Adenylyl cyclase (AC), the enzyme that performs the cyclic esterification of adenosine triphosphate (AMP) producing 3-5-cyclic adenosine monophosphate (cAMP), is not limited in its localization; AC exists in a classical membrane bound form, as well as a soluble form, which provides cAMP production at the plasma membrane and sub-plasmalemmal compartments (Pierre et al., 2009; Zippin et al., 2001). The lifespan and reach of cAMP is also under regulation. Phosphodiesterases (PDEs) are a family of cyclic-nucleotide diesterases that catalyze the degradation of cAMP and cGMP (cyclic-guanosine monophosphate) to non-reactive AMP and GMP (Cooper, 2005). Concentrated PDE activity at the plasma membrane isolate a microdomain cAMP response at the effectors located at the plasma membrane and creates a PDE firewall impeding sub-plasmalemmal signaling. Like PKA, PDE localization within subcellular compartments, include organelle localization, has been observed (Baillie and Houslay, 2005; Mies et al., 2007; Willoughby et al., 2006). Despite the specificity implied by its nomenclature, AKAP scaffold function is not exclusive to PKA, association with other kinases and phosphatases has been reported, as well as PDE recruitment; PDE-AKAP1 association is confidently theorized and is proposed to contribute to PKA regulation of MFF and mitochondrial mediated cell-survival (Asirvatham et al., 2004).

**Drp1 dephosphorylation via PP2A/Bβ2**

Reciprocal to kinase function, protein phosphatases dephosphorylate serine/threonine and tyrosine residues. Currently, human genome sequence analysis has predicted 500+ individual kinases and thousands of phosphoprotein substrates. The magnitude of protein phosphatase
gene number does not reach kinase gene complexity; as an example only 30-40 serine/threonine phosphatases have been described or predicted. The Ser/Thr phosphatase superfamily is organized into two families, the PPM family and the PPP family. PPM comprises Mg$^{2+}$ dependent protein phosphatases and the PPP family includes PP1, PP2A/B and PP4-7. Limiting in gene number, protein phosphatases accomplish specificity and complexity via subunit/holoenzyme combinations (Shi, 2009a, b). PP2A, one of the most prominent phosphatases accounting for 1% of total cellular protein, exists as a holoenzyme composed of a scaffolding A subunit, a catalytic C subunit and a regulatory B subunit. Subunit isoform combinations produce dozens of possible PP2A holoenzymes selective in their substrate specificity, localization/compartamentalization of phosphate activity, and temporal regulation via susceptibility to degradation (Kurimchak and Grana, 2012; Oberg et al., 2012; Shi, 2009b; Voronkov et al., 2011).

Synonymous with its regulatory designation, the B subunit dictates PP2A activity. The regulatory B subunit is divided into four families distinct in sequence and structure: B, B’, and B’’ (Shi, 2009a; Strack et al., 2002). The B family regulatory subunit is of particular interest to OMM phospho-proteins. Bβ is an isoform of the B family and is expressed as two splice variants, Bβ1 and Bβ2. Bβ2 is an N-terminal variant of Bβ containing a mitochondrial localization sequence. Unlike Bβ1 which lacks the mitochondrial localization sequence, Bβ2 localizes PP2A activity to the OMM (Dagda et al., 2005; Dagda et al., 2003). Under cellular stress (including glutamate toxicity), autodephosphorylation of three specific Bβ2 serines (Ser20-Ser22) promotes translocation of the PP2A holoenzyme from the cytosol to the OMM. Localization of PP2A to the OMM by its regulatory subunit Bβ2 is pro-apoptotic. In the context of excitotoxicity, Bβ2 knockdown protects hippocampal neurons from the stress of prolonged glutamate exposure (Dagda et al., 2008). This proposed involvement was confirmed with PP2A/Bβ2 dependent dephosphorylation of Drp1 at serine 656 (Merrill et al., 2012). Dephosphorylated Drp1 is the active form of this contractile enzyme, which shifts MFF equilibrium toward fragmentation (Cribbs and Strack, 2007; Dickey and Strack, 2011).
Drp1 dephosphorylation via PP2B (calcineurin)

PP2B (also termed calcineurin or CaN) is a unique member of the PPP protein phosphatase family that is activated via elevated cytosolic Ca\(^{2+}\). CaN is composed of a catalytic CaNA subunit and a regulatory CaNB subunit. CaNA contains an auto-inhibitory domain that sterically blocks access to the catalytic site. Binding of Ca\(^{2+}\) bound calmodulin (CaM) displaces the auto-inhibitory domain and exposes the catalytic domain. The catalytic domain performs dephosphorylation of serine/threonine phospho-proteins. Similar to CaM, CaNB contains four Ca\(^{2+}\) binding domains that suggest a more structural and allosteric role than activating role due to significantly lower Ca\(^{2+}\) affinity than CaM. Calcineurin is predominantly expressed in the nervous system and represents the antithesis of PKA function; specific to this study, CaN activity is observed during ischemia and excitotoxicity, and inhibition via FK506 and/or cyclosporine A (CsA) promotes cell-survival (Shibasaki et al., 2002).

Phosphoregulation of Drp1 is not limited to PP2A/Bβ2; Cribbs and Strack, 2007 showed direct dephosphorylation of Drp1 serine 656 by calcineurin. As discussed with PP2A/Bβ2, calcineurin dependent dephosphorylation of Drp1 serine 656 activates Drp1 promoting localization of Drp1 to the OMM and enhanced mitochondrial fission. These effects were blocked using the CaN inhibitors FK506 and CsA (Cereghetti et al., 2008; Cribbs and Strack, 2007). Discussed above, PKA is targeted to the OMM via recruitment by AKAP1; an interaction between CaN and AKAP1 has been proposed and reported, creating a phosphoregulatory complex (Abrenica et al., 2009). A model describing phosphoregulation of mitochondrial fission and fusion is shown in Fig.1.4.
**Dissertation research focus**

My dissertation work characterizes the phosphoregulation of mitochondrial fission and fusion as a potential target for therapeutic intervention of ischemic penumbra-like glutamate toxicity in central neurons. In Chapter 2, I determined the effect of reversible-phosphoregulation at the OMM via PKA/AKAP1 and PP2A/Bβ2 on an early predictor of excitotoxic neural death, termed delayed calcium deregulation (DCD). These experiments were performed using over-expression, RNA interference (knockdown), point mutation and knockout of Bβ2 to determine the pathological effect of OMM reversible-phosphorylation. Pharmacological manipulation was then performed using both exogenous and endogenous regulators of PKA signaling. I have also begun characterizing the spatiotemporal characteristics of DCD, mitochondrial fragmentation and recovery from excitotoxicity. In Chapter 3 I examine the mechanism by which targeted phosphorylation at the OMM modulates susceptibility to excitotoxicity. Using transiently transfected DRG neurons as a model system and cytosolic/mitochondrial matrix calcium recording, I analyzed the kinetics of mitochondrial calcium uptake and recovery in relationship to OMM phosphorylation and mitochondrial morphology. Finally in Chapter 4, I examine mitochondrial calcium buffering components in the context of neuronal calcium signaling.
Figure 1.1. $[\text{Ca}^{2+}]_i$ responses in DRG neurons are shaped by mitochondria. A. Depolarization or capsaicin-induced calcium influx produce a distinct cytosolic calcium response visualized by Fura2 $\text{Ca}^{2+}$ indicator recording. B. Inhibition of mitochondrial calcium buffering via antimycin (1 $\mu$M) pretreatment results in the increase of peak cytosolic calcium response and elimination of the prolonged elevated $[\text{Ca}^{2+}]_i$ plateau.
Figure 1.2. Calcium plays a complex regulatory role in neuronal physiology. The maintenance of neuronal membrane potential and excitability has a tremendous energy demand. (a) $\text{Ca}^{2+}$ serves as a second messenger that signals increased energy to the mitochondrial respiratory system. Calcium directly upregulates the function of the TCA cycle dehydrogenases and the ATP synthase, which increases ATP production, proton shuttling, $\Delta \psi_{\text{mt}}$ polarization, and ROS generation. (b) While the OMM is readily permeable to $\text{Ca}^{2+}$ via the proposed function of the VDAC, the IMM is impermeable. (c) Calcium conductance into the mitochondrial matrix is proposed to be mediated by a putative $\text{Ca}^{2+}$ uniporter. The molecular identity of this uniporter is unknown but several candidates have been identified: MCU, ccdc109b and mRyR. MCU is currently described as the most likely candidate. Ccdc109b is an MCU isoform of unknown function; mRyR is a hypothetical IMM localized ryanodine receptor that displayed high calcium affinity in in vitro studies. (d) UCPs have also been proposed to shuttle calcium into the matrix, however the majority of the research community favor UCPs as ROS regulated proton ($\text{H}^+$) leak channels. (e) Letm1 is a possible $\text{H}^+$/Ca$^{2+}$ exchanger that may function in either Ca$^{2+}$ uptake or efflux across the IMM. (f) Other calcium efflux mechanisms include various Na$^+$/Ca$^{2+}$ exchangers (NCX3 and the lithium capable NCLX) and 1,2-sn-diacylglycerol (DAG) activated calcium channels (DCCs). Figure was inspired by Ryu et al., 2010.
Figure 1.3. Excitotoxicity in the ischemic penumbra. A. Neuronal depolarization and necrosis within the ischemic core releases a wave of cellular content into surrounding tissues, termed the ischemic penumbra (indicated by the green arrows). This wave of neuronal (and astrocytic) metabolites contains toxic concentrations of the excitatory neurotransmitter glutamate. Glutamate toxicity in the penumbra results in progressive neuronal damage over a period of hours to days. The area of the penumbra is outlined in yellow surrounding the red ischemic core. B. Glutamate induced excitotoxicity in hippocampal neurons is visualized by the phenomenon delayed calcium deregulation (DCD). This predictor of post-ischemic apoptosis is characterized by three distinct phases: 1) an initial glutamate response; 2) a latent period during which cytosolic calcium is maintained at a relatively low concentration via mitochondrial Ca\textsuperscript{2+} uptake; and 3) mitochondrial failure resulting in complete release of total mitochondrial content termed calcium deregulation. Ischemic penumbra illustration from Rosso et al., 2008.
Figure 1.4. Phosphoregulation of mitochondrial fission and fusion. Mitochondrial fission and fusion (MFF) is a rapid and dynamic process mediated by specific fission and fusion machinery. Mitochondrial fission is catalyzed by the constricting GTPase Drp1, which is recruited to the OMM via a number of anchoring proteins (MFF is depicted above, but others include mitochondrial fission factor and mitochondrial elongation factor 1). Mitochondrial fragmentation is catalyzed by OPA1 at the IMM and Mfn1/2 at the OMM. MFF is regulated via reversible phosphorylation. Specifically, PKA recruited to the OMM via the PKA scaffolding protein AKAP1 phosphorylates Drp1 at serine 656. This phosphorylation inhibits Drp1 function and promotes mitochondrial fusion. PP2A recruited to the OMM via its mitochondrion targeted regulatory subunit Bβ2 dephosphorylates Drp1 at the same serine 656. Drp1 dephosphorylation reactivates fission function and promotes fragmentation. The provided images of MFF machinery phosphomaniulation were performed in PC-12 cells transiently transfected using Lipofectamine 2000.
CHAPTER II
PHOSPHOREGULATION OF HIPPOCAMPAL NEURON
SUSCEPTIBILITY TO GLUTAMATE TOXICITY

Abstract

Mitochondria quickly and efficiently buffer Ca\(^{2+}\) entering neurons during electrical activity. However, excessive exposure to glutamate during ischemia leads to mitochondrial calcium overload and deterioration of Ca\(^{2+}\) homeostasis, termed delayed Ca\(^{2+}\) deregulation (DCD) (Fig. 2.1). DCD is thought to be an early predictor of neuronal death, and agents that can interfere with or prevent DCD would represent a novel therapeutic approach to treating the sequelae of stroke. Mitochondria are also dynamic organelles that rapidly and reversibly undergo fission and fusion; this is termed mitochondrial fission and fusion, or MFF. MFF is regulated via reversible phosphorylation. Phosphorylation of MFF machinery at the outer mitochondrial membrane (OMM) by protein kinase A (PKA) and A-kinase anchoring protein 1 (AKAP1) promotes mitochondrial fusion, whereas dephosphorylation by protein phosphatase 2A (PP2A) and its regulatory subunit B\(\beta\)2 promotes fission. Increased PKA activity at the OMM significantly improves neuronal survival during toxic glutamate exposure, suggesting a functional link between MFF and mitochondrial calcium buffering.

Focusing on the development of DCD, we investigated the role of OMM reversible phosphorylation on susceptibility to glutamate toxicity. As predicted from neuronal survival studies performed by the Strack research group, increased PKA activity at the OMM recruited by AKAP1 overexpression robustly prolonged DCD latency (Merrill et al., 2011). Utilizing the PKA inhibitor H89 or an AKAP1-I310P,L316P (\(\Delta\)PKA) mutant with ablated PKA association abolished the protective effect of AKAP1, yielding DCD latent period similar to that of control. Pharmacological manipulation of
PKA activity revealed a significant regulatory role of PDEs. Adenylyl cyclase activators forskolin and PACAP38 produced prolonged DCD latent period durations only in the presence of PDE4 inhibitors rolipram and BDNF. Conversely, PP2A activity at the OMM via its regulatory subunit Bβ2 confirmed a negative regulatory role. Bβ2 knockout mouse hippocampal neurons are strongly resistant to DCD. Overexpression of Bβ2 in cultured rat hippocampal neurons also robustly elevated mitochondrial calcium load following transient glutamate stimulation. Treatment with cyclosporine A and FK506 failed to prolong DCD latency; this result argues against a role for both PP2B (calcineurin) and mitochondrial permeability transition (MPT) in the regulation and mechanism of DCD.

Introduction

During stroke, neurons within the ischemic core experience a deprivation of oxygen and glucose (Astrup et al., 1981; Olsen et al., 1983). Detrimental to cellular metabolism, hypoxia and hypoglycemia lead to depletion in ATP. Neurons are energetically demanding cells, primarily due to the maintenance of plasmalemmal membrane potential and neuronal excitability via the Na+/K+ ATPase; thus, perturbation of metabolism leads to attenuation of Na+/K+ ATPase function and neuronal depolarization. In the absence of sufficient collateral circulation, necrotic death of neurons and glia occur. This massive depolarization and necrosis produces an expanding wave of toxic glutamate that spreads into surrounding tissue termed the penumbra (Huang et al., 1997; Kostandy, 2012; Rosso et al., 2009). The resulting excitotoxicity accounts for the majority of neuronal death following stroke; this sequelae is a delayed process that poses a potential target in post-stroke therapeutic intervention (Hansen, 1984; Jekabsons and Nicholls, 2004; Kostiuk, 1997; Molz et al., 2008; Stys, 2004; Zhang and Bhavnani, 2006).

Delayed calcium deregulation (DCD) is an early component of glutamate induced excitotoxicity and a predictor of neuronal death within the ischemic penumbra. DCD
contains three phases (Fig. 2.1): 1) an initial glutamate response; 2) a prolonged latent period, during which mitochondria continue to buffer the toxic influx of calcium; and 3) a loss of mitochondrial integrity resulting in complete efflux of matrix calcium into the cytosol (Nicholls and Budd, 2000). The duration of the second phase latent period is an index of neuronal resistance to excitotoxicity. The third phase represents mitochondrial decoupling and failure that leads to increased production of reactive oxygen species, formation of the mitochondrial permeability transition pore (PTP), release of cytochrome C, and activation of the apoptotic pathway (Schild et al., 2001).

Mitochondria are dynamic organelles that rapidly and reversibly undergo fission and fusion, termed MFF. Mitochondrial fission machinery is driven by the constricting GTPase dynamin related protein 1 (Drp1) (Kageyama et al., 2011). Drp1 recruitment and function at the OMM are phosphoregulated processes; Serine 656 is phosphorylated via cAMP-dependent protein kinase A (PKA) recruited to the OMM by AKAP1, while dephosphorylation is performed by two candidate protein phosphatases, PP2A and PP2B (Cereghetti et al., 2008; Cribbs and Strack, 2007; Merrill et al., 2012). PP2A is recruited to the OMM and within proximity of Drp1 by the regulatory subunit Bβ2; The Bβ splice variant Bβ1 lacks the OMM localization sequence present in Bβ2 (Dagda et al., 2005; Dagda et al., 2003). PP2B (or calcineurin, CaN) localization to the OMM via AKAP1 association has been proposed (Abrenica et al., 2009).

Complexity of PKA signaling is not limited to selective localization by scaffolds. Compartmentalization of cAMP is accomplished via localization of the cAMP producing enzyme adenyl cyclase (AC). AC is classically expressed at the plasma membrane, though sub-plasmalemmal (soluble) AC expression has been proposed to generate microdomains within the cytosol and in proximity to organelles (Pierre et al., 2009; Zippin et al., 2001). This proximal cAMP production is further regulated by phosphodiesterase (PDE) hydrolysis of cAMP to nonfunctional AMP. PDEs, like PKA, may be localized to select compartments of the cell; PDE concentration at the plasma
membrane creates a signaling firewall limiting cAMP to plasmalemmal microdomains, while proposed recruitment of PDEs via AKAP scaffolding proteins targets this regulatory protein at the site of PKA activity (Cooper, 2005).

Though pharmaceutical manipulation of adenylyl cyclase and PDE activity is important in the development of post-stroke therapeutic intervention, manipulation of endogenous modulators of AC and PDEs poses a more attractive, less invasive approach and helps dissect the natural neuroprotective mechanism in place during and following ischemia. Discovered in 1989 as a peptide isolated from hypothalamic fractions, pituitary adenylyl cyclase activating polypeptide (PACAP) is a widely expressed signaling molecule important in modulation and cytoprotection of multiple cell types in many tissues of the body (Ohtaki et al., 2008). In both the PNS and CNS, PACAP plays an important role in protection against injury, including axonal lesions, concussive injury and ischemia (Dejda et al., 2011; Nakamachi et al., 2010; Reglodi et al., 2012). During such neuronal insults, both PACAP and its receptor PAC1R display elevated expression. PAC1R is a GPCR capable of both Gs and Gq coupling (Nakamachi et al., 2012). Activation of Gs coupled GPCR results in the dissociation of the Gαs subunit and activation of adenylyl cyclase (Tamas et al., 2012). Therefore, PACAP signaling has the potential for activation of neuroprotective PKA signaling (Kienlen Campard et al., 1997).

PACAP may present a potential therapeutic avenue for the treatment of ischemic injury, but preservation of the resulting Gs-AC effects would increase the efficacy of PACAP’s neuroprotective function. Inhibition of phosphodiesterase mediated cAMP hydrolysis maintains and prolongs PKA activity. PDE4 is one of the most commonly studied and understood PDEs in the CNS (Bollen and Prickaerts, 2012; Castro et al., 2010; Vincent et al., 2012). This cAMP selective phosphodiesterase is negatively modulated via the neurotrophin brain-derived neurotrophic factor, BDNF. BDNF signals through the TrkB receptor. Activation of TrkB signals the phosphorylation and activation
of ERK1/2. EKR1/2 dependent phosphorylation of PDE4 inhibits cAMP hydrolysis (Gao et al., 2003; Schmidt et al., 2010).

**Experimental procedures**

**cDNA and shRNA FIV vectors**

Overexpression of AKAP1 wild-type was performed using rat AKAP1 N0 splice variant (aa 1-524) with c-terminal GFP fusion. AKAP1-GFP was cloned into an FIV (feline immunodeficiency virus) pVETL vector and incorporated into FIV via the University of Iowa Viral Gene Vector Core. Attenuation of PKA-AKAP1 was performed using overexpression of AKAP1 I310P,L316P double point mutation, termed ΔPKA. ΔPKA is fused with GFP at the C-terminus. The Bβ1 and Bβ2 splice variants of the PP2A Bβ regulatory subunit were expressed using pVETL and reported via GFP fusion. Detailed generation of these constructs has been previously reported (Cribbs and Strack, 2007; Dagda et al., 2008; Merrill et al., 2011). Control neurons were transduced with mitochondrial targeted EGFP (EGFP fused with the Cox8 mitochondrial matrix localization sequence) similarly expressed using the pVETL vector in FIV.

**Dissociated hippocampal neuron culture**

Primary culture of rat hippocampal neurons was performed as previously described (Lu et al., 2008; Usachev et al., 2000). Briefly, P0-2 neonatal Sprague-Dawley rat pups (Charles Rivers, Massachusetts) were euthanized via decapitation. The hippocampi were removed and the extracellular matrix proteins were digested via trypsin incubation diluted in Neurobasal-A® (Gibco) media supplemented with glutamine and penicillin/streptomycin (complete NB-A). The digested hippocampi were then dissociated using fire polished Pasteur pipettes of reduced diameter and plated on poly-O-ornithine and laminin coated glass coverslips with 5% horse serum. After four hours, the
media was replaced with complete NB-A media supplemented with B-27 (Sigma Aldrich). Neonatal (P0-1) C57BL/6J mouse hippocampal culture was similarly performed with optimization of seeding density from mice P0-1. Neurons were cultured for 16-18DIV. At 14DIV NB-A/B27 was replaced with MEM (Gibco) media supplemented with 10% horse serum, pen/strep and insulin. This media change acclimates the culture to the osmolarity of the recording buffer (310 mOsM) and allows glia growth to resume.

**FIV gene expression**

At 10DIV, the neuronal culture volume was reduced by collecting 1200µl of media. The collected media was supplemented with fresh NB-A complete and B27 and saved as conditioned media. Concentrated FIV stock of the desired construct was then diluted to an optimized concentration (final dilutions were 1/500 to 1/2000) and added to the neuron culture. Following 6 to 8 hours of incubation at 37°C and 5% CO₂, the total transduction media was replaced with conditioned media (Dickey and Strack, 2011; Medvedeva et al., 2008).

**Glutamate toxicity and DCD**

DCD induction was be performed using a prolong glutamate protocol. A baseline will be established with a 2 minute perfusion of HH buffer of the following composition: NaCl [140 mM], KCl [5 mM], CaCl₂ [1.3 mM], MgCl₂ [0.5 mM], MgSO₄ [0.4 mM], KH₂PO₄ [0.4 mM], Na₂HPO₄ [0.6 mM], NaHCO₃ [3 mM], HEPES [10 mM], D-glucose [10 mM] and at 310 mOsM and pH = 7.4. The perfusion was then switched to a solution containing 100 µM glutamate, 10 µM glycine and 200 nM TTX (Castilho et al., 1998; Ward et al., 2007). The neurons were stimulated for up to four hours to monitor the occurrence of DCD.
Fura-FF cytosolic calcium imaging

Cytosolic Ca\(^{2+}\) imaging was performed similarly to the protocol described in (Schnizler et al., 2008). Cultures 16-18 DIV were incubated at 22°C for 30 min in 2 µM Fura-FF/AM (Invitrogen) and 0.01% pluronic acid in 2 mL HH buffer. The coverslip was then secured in a flow-through chamber fed HH buffer via a gravity perfusion system and mounted on an IX-71 epifluorescence microscope (Olympus). Fura-FF fluorescence was sequentially excited at 340 nm and 380 nm via a Polychrome IV monochromator (T.I.L.L. Photonics) with either a 20X lens or a 40X oil immersion objective lens (Olympus). Fluorescent emission at 510 (80) nm was collected at 0.2 Hz sampling frequency by a Photonics IMAGO CCD camera coupled to TillVisION live acquisition software. The background subtracted ratio (340nm/380nm) was then converted to \([\text{Ca}^{2+}]_i\) after dye calibration using the following equation:

\[ [\text{Ca}^{2+}]_i = K_d (R_{\text{max}} - R) S_{f380}/S_{b380}, \]

where \(K_d = 5.5 \mu\text{M}\) and \(S_{f380}/S_{b380} = F_{\text{min} 380}\text{nm}/F_{\text{max} 380}\text{nm}\) (Nelson et al., 2007).

Mitochondrial membrane potential imaging

Time-lapse imaging of mitochondrial membrane potential (\(\Delta\Psi_{\text{mt}}\)) was performed using rhodamine 123 (Rho123) simultaneously with Fura-FF imaging (due to the fluorescent properties of Rho123, GFP tagged proteins and mtEGFP are not compatible). For the final 10 minutes of Fura-FF loading, 8µM Rho123 was incorporated into the loading buffer. 8µM Rho123 loads the mitochondrial matrix to the point of self-quenching. In self-quenching mode, depolarization liberates Rho123 from the mitochondrial matrix resulting in an increase in fluorescent intensity. \(\Delta\Psi_{\text{mt}}\) imaging was performed using the epifluorescence rig described for Fura-FF. Excitation was performed at 475nm; emission was collected at 530nm (44nm bandpass) with a 0.2Hz sampling frequency. Confirmation of quenching-mode was observed using 1µM FCCP mitochondrial uncoupling; this confirmation also establishes a maximum depolarization control (Duchen, 1992; Mattson et al., 1993; Prehn et al., 1994; Vergun et al., 1999).
**Pharmacological manipulation of phosphorylation at the OMM**

Inhibition of PKA activity was performed via 5µM H89 (Lochner and Moolman, 2006). The neuron culture was pretreated with H89 10min prior to glutamate application and H89 was present throughout the DCD protocol. Non-receptor adenylyl cyclase activation and cAMP production was performed via 10µM Forskolin (Greengard et al., 1991). Cultures were pretreated for 1 hour prior to the start of the DCD protocol. Co-application of PDE inhibitors and the afore-mentioned Forskolin protocol included: PDE4 inhibited via 1µM rolipram; PDE6, 8, 10, and 11 inhibited via 10µM dipyridamole; and PDE7 inhibited via 1µM BRL-50481 (Alaamery et al., 2010; Fukawa et al., 1982; Moon and Lerner, 2003). PACAP38 application was utilized at 10nM and 100nM (Dejda et al., 2011). Similar to the Forskolin protocol, cultures where pretreated for 1 hour prior to glutamate application. BDNF treatment utilized 200ng/ml supplemented with <0.1% bovine serum albumen (Gao et al., 2003; Schmidt et al., 2010). BDNF was co-administered with Forskolin as a 1 hour pretreatment. Inhibition of MPT was achieved via 1 hour pretreatment with 2µM Cyclosporine A (CsA) (McGee and Baines, 2012). PP2B (calcineurin) inhibition was performed using 5µM CsA and 1µM FK506 (Dileonardi et al., 2012; Liu et al., 1991; O'Keefe et al., 1992).

**Analysis of DCD latent period duration**

The incidence of DCD was determined and represented as a percentage of all stimulated neurons within each population. For those neurons that produced DCD, latent period duration was measured as follows: the raw trace recording (converted to [Ca^{2+}]_i as described above) was smoothed using a running average protocol (Sigma Plot 9 software); the first derivative to the smoothed trace was then calculated and plotted as a function of time; the peak amplitudes corresponding to peak glutamate response slope and peak DCD slope were determined; the duration of time in minutes between the two
peak slope amplitudes was calculated as the latent period duration (Fig2.1E). The amplitude of the initial response was also recorded.

Mitochondrial uncoupling and total matrix Ca\(^{2+}\)

Cultured hippocampal neurons were imaged using the Fura-FF 40X oil emersion protocol described above. Fura-FF loaded neurons were imaged for a one minute baseline recording. The culture was then stimulated with 100 μM glutamate (with 10 μM glycine and 200 nM TTX) for 30 sec. The neurons are then allowed to recover for 10 minutes; this initial stimulation is the control stimulation. Following recovery, the neurons were stimulated a second time (100 μM glutamate for 30 sec) to load the mitochondria. This second stimulation was immediately followed by treatment with 5 μM FCCP in Ca\(^{2+}\) free HH buffer supplemented with 100 mM EGTA (Fig2.8) (Benz and McLaughlin, 1983). Subtraction of the control stimulation corrects for extracellular Ca\(^{2+}\) influx. The resulting FCCP-mitochondrial Ca\(^{2+}\) response was calculated via integration including the entirety and limit of the FCCP treatment period (reported as the area under the curve, AUC).

Statistical analysis

The latent period values, initial response amplitudes and FCCP AUCs for each neuron population are represented as a mean ± SEM. Statistical analysis was performed using either Student’s t-test (when comparing two variables) or one way analysis of varience (one way ANOVA…when comparing 3 or more variables) with the Bonferroni post-test comparing all columns. All statistics were performed at 95% confidence.
Results

Characterization of delayed calcium deregulation in cultured neonatal rat hippocampal neurons

Following ischemic insult, depolarized and necrotic neurons and astrocytes within the foci release copious amounts of the excitatory neurotransmitter glutamate into surrounding tissues, termed the penumbra. Within the penumbra, neurons undergo glutamate induced excitotoxicity, mitochondrial damage and reperfusion injury (Dyker and Lees, 1998). Delayed calcium deregulation (DCD) is an established measure of excitotoxicity and predictor of apoptosis in central neurons (in cerebral cortical neurons and hippocampal neurons) exposed to toxic concentrations of glutamate. This glutamate wave activates both synaptic and non-synaptic glutamate receptors, including Ca^{2+} conducting NMDA receptors. Global NMDAR activation results in a prolonged influx of calcium throughout the processes and soma of the neurons (Nicholls and Budd, 2000). To study the effects of mitochondrial morphology and OMM phosphorylation on neuronal glutamate toxicity, I first established a protocol to study DCD (Fig.2.1).

At 16-18DIV, cultured hippocampal neurons were loaded with the low affinity calcium indicator Fura-FF. Following a brief baseline recording the culture was treated with an excitotoxic solution containing 100 μM glutamate, 10 μM glycine and 200 nM TTX. As predicted, the neurons presented a three phase calcium response: 1) an initial glutamate response; 2) a prolonged latent period; and 3) a massive release of matrix calcium into the cytosol, DCD (Fig.2.1). Latent period duration was calculated from the initial response peak slope to the DCD peak slope recording time, ΔT (Fig.2.1D,E). Since I recorded the latent period duration, it was important to determine the spaciotemporal characterization of DCD in the soma versus the processes (Stanika et al., 2009). I recorded the incidence and kinetics of DCD within the soma and proximal dendrites. Illustrated in Fig.2.1A,B, DCD within the dendrites precedes that of the soma. Though it
is noticeably delayed DCD within the soma represents a summation and average of the dendritic input.

The relationship between DCD and IMM depolarization has been an unexplored area of research. As of yet, the order of DCD and IMM depolarization has remained unclear. Using the mitochondrial membrane potential ($\Delta \Psi_{mt}$) indicator rhodamine 123 (Rho123) in combination with Fura-FF, I was able to simultaneously measure cytosolic $[\text{Ca}^{2+}]_i$ changes and mitochondrial depolarization during the course of DCD. Overlaying the Fura-FF and Rho123 traces reveals $\Delta \Psi_{mt}$ depolarization accurately correlating with the initial cytosolic $\text{Ca}^{2+}$ influx in response to glutamate (Fig.2.1C). This simultaneous mitochondrial depolarization is likely due to the rapid calcium influx and uptake by mitochondria. The $\Delta \Psi_{mt}$ steadily depolarizes during the latent period. Minutes prior to DCD (as observed via cytosolic Fura-FF measurements), the $\Delta \Psi_{mt}$ rapidly and robustly depolarizes. A plateau is observed in correlation with the observed $[\text{Ca}^{2+}]_i$ plateau following DCD. Application of the protonophore FCCP produces further depolarization, indicating that DCD-mediated depolarization is partial or restricted to a subset of mitochondria.

Mitochondrial $\text{Ca}^{2+}$ deregulation was previously considered a point of no return in a neuron’s response to excitotoxic insults (Trump et al., 1989). Simply prolonging the recording period post-DCD revealed otherwise. Termination of the toxic glutamate solution and replacement with normal HH buffer allows some neurons to recover to the basal $[\text{Ca}^{2+}]_i$ levels. $[\text{Ca}^{2+}]_i$ quickly recovery to baseline within minutes; calcium extrusion via exchangers (NCX and NCKX) and ATP dependent calcium pumps (PMCA) at the plasma membrane are most likely responsible for this observation (Siesjo and Bengtsson, 1989). Mitochondrial repolarization is a much slower process. The $\Delta \Psi_{mt}$ is capable of complete recovery, but the process takes approximately an hour.
AKAP1/PKA mediated phosphorylation at the OMM increases neuronal resistance to DCD

PKA is classically known as a pro-survival kinase. Activation of PKA signaling is sufficient to reduce cell death for a number of toxic models, including staurosporin, etopiside, and high glutamate exposure (Cribbs and Strack, 2007; Merrill et al., 2011). Work by the Strack group revealed a requirement for the A kinase anchoring protein AKAP1 in this PKA induced resistance. The mechanism by which AKAP1 achieves neuroprotection involves inhibition of Drp1-mediated mitochondrial fission by phosphorylation of Drp1 at S656 (Merrill et al., 2011). Using FIV infection, rat hippocampal neurons were transduced with an AKAP1 overexpression vector. Compared to control neurons transduced with an EGFP control, AKAP1 overexpressing neurons produced greatly prolonged latent period durations. Control neurons underwent DCD within 20 minute of the glutamate application; AKAP1 neurons took upwards of 2 hours to produce DCD (Fig. 2.2).

Recruitment via AKAP1 is not exclusive to PKA; a number of other kinases the Tyr kinase Src and the phosphatases PTPD1, PP1 and CaN also associate with this OMM localized scaffold protein (Abrenica et al., 2009; Bridges et al., 2006; Cardone et al., 2004). To confirm that PKA was required for this AKAP1 DCD effect, we first attempted to inhibit PKA activity pharmacologically. Treating AKAP1 transduced neurons with the cell permeant PKA inhibitor H89 (5µM) 10 minutes prior to glutamate treatment reduced the latent period effect of AKAP1 overexpression (Lochner and Moolman, 2006). Specific recruitment of PKA via AKAP1 was further tested using an AKAP1 double point mutation (I310P,L316P), termed ΔPKA, that ablates AKAP1-PKA (RI,RII) association, but retains AKAP1 OMM localization and recruitment of other regulatory proteins (Merrill et al., 2011). ΔPKA expressed neurons produced latent period durations comparable to that of control neurons (Fig. 2.2).
Pharmacological manipulation of cAMP signaling and PKA activity

Simple overexpression of AKAP1 robustly improved neuronal resistance to glutamate toxicity without any modulation of global cAMP signaling or PKA activity. It is unclear to what extent basal PKA activity is recruited to the OMM with AKAP1 overexpression; therefore, it may be possible to reproduce the AKAP1 effect via global activation of PKA via the non-receptor adenyl cyclase (AC) activator forskolin (Greengard et al., 1991). Following one hour of pretreatment with 10 μM forskolin, cultured rat hippocampal neurons produced DCD latency comparable to that of vehicle control treated neurons (10 μm forskolin was present throughout glutamate application…Fig.2.3).

Due to compartmentalization of cAMP and PKA activity, forskolin mediated activation of AC may not be sufficient to activate PKA at the OMM. Phosphodiesterases (PDEs) are regulatory enzymes capable of catalyzing the hydrolysis of cAMP and cGMP. Increased expression and activity of PDEs close to the source of cAMP or site of PKA effectors could greatly attenuate the Forskolin affect. One of the most studied PDEs in neurophysiology is PDE4. PDE4 specifically hydrolyzes cAMP to AMP and is inhibited by rolipram and has been reported to associate with AKAP1 (Asirvatham et al., 2004; Moon and Lerner, 2003). Following the failure of forskolin, we then performed a one hour pretreatment with both 10 μM forskolin and 1 μM rolipram. This dual treatment successfully prolonged the latent period duration (both forskolin and rolipram were present throughout the glutamate application). Rolipram alone was not capable of delaying calcium deregulation. The hippocampal neuron proteome is not limited to PDE4. To determine the roles of other PDEs we performed Forskolin treatment and the DCD protocol with dipyridamole (which inhibits PDE6, 8, 10, and 11) and BRL-50481 (which inhibits PDE7) (Alaamery et al., 2010; Fukawa et al., 1982). Dipyridamole and BRL-50481 did not significantly increase the DCD latency (Fig.2.3).
Endogenous modulators of cAMP signaling and PKA activity increase neuronal resistance to glutamate toxicity

Pituitary adenylyl cyclase activating polypeptide (PACAP) is an endogenous modulator of AC activity via activation of a Gs coupled GPCR, PAC1. PACAP and PAC1 expression is elevated during cerebral ischemia. This increased PACAP signaling activity is proposed to be a neuroprotective mechanism. To enhance this protective quality, we investigated PDE4 as a possible drug target to prolong and amplify PACAP’s beneficial properties. In place of Forskolin, we similarly pretreated cultured neurons with either 10nM or 100nM PACAP38 (the exogenously stable form of PACAP) for one hour (Dejda et al., 2011). Following the same DCD protocol, both concentrations of PACAP38 failed to improve latency beyond control. However, co-application of PACAP38 and rolipram significantly improved DCD latency, although the effect was more modest compared to the effect of forskolin+rolipram (Fig. 2.4).

PDE4 inhibition has been proposed to occur through BDNF signaling. Brain-derived neurotrophic factor (BDNF) binds to the TrkB receptor, which starts a kinase signaling cascade leading to the activation of ERK1/2. Phosphorylation of PDE4 by ERK1/2 inhibits its esterase activity (Gao et al., 2003; Schmidt et al., 2010). Similar to rolipram, pretreating neurons with BDNF had no beneficial effect on DCD latency; however, co-application of forskolin and BDNF robustly prolonged the latent period duration by hours. Future studies will determine whether co-application of PACAP38 and BDNF significantly improves resistance to excitotoxicity (Fig. 2.5).

Inhibition of phosphatase activity at the OMM improves neuron resistance to excitotoxicity

Phosphatase activity at the OMM has two primary candidates, PP2A and PP2B. Protein phosphatase 2 A (PP2A) is a ubiquitous phosphatase accounting for 1% of cellular protein content. It exists as a holoenzyme composed of a scaffolding A subunit,
a catalytic C subunit, and a regulatory B subunit (Shi, 2009a; Slupe et al., 2011). Characterized by Strack et al., the Bβ regulatory subunit isoform exists as two splice variants. Bβ2 contains a putative OMM localization sequence, and thus targets PP2A activity to mitochondria. Bβ1 lacks this targeting sequence. Inhibition of Bβ2 dependent PP2A signaling via RNAi significantly increased hippocampal neuron survival following glutamate and rotenone challenge and OGD (Dagda et al., 2008). PP2A/Bβ2 antagonizes the action of PKA/AKAP1 at the OMM by dephosphorylating Drp1 at S656 (Merrill et al., 2012). To determine the role of PP2A/Bβ2 in neuronal resistance to excitotoxicity, we prepared neonatal hippocampal cultures from Bβ2 knockout mice (Bβ2−/−). Without any further pharmacological or genetic manipulation, the Bβ2−/− neurons produced robustly prolonged DCD latency (averaging approximately 2 hours); aged matched mice of the same C57Bl/6J lineage produced latent period durations averaging 5 minutes (Fig. 2.6).

PP2B (also known as calcineurin) is a serine/threonine phosphatase regulated by calmodulin and Ca2+. Historically characterized as a pro-apoptotic phosphatase, inhibition of calcineurin via FK506 and cyclosporine A (CsA) protects cerebral cortical neurons during an ischemic insult (Saganova et al., 2012; Yousuf et al., 2011). Within the penumbra, elevated [Ca2+]i poses a potential positive regulator of this pro-apoptotic phosphatase. To determine the effects of calcineurin during our penumbra-like DCD protocol, we pretreated neurons with 5µM CsA and 1 µM FK506. FK506 and CsA treated neurons developed DCD at the same rate as vehicle treated neurons. CsA not only inhibits calcineurin, CsA sufficiently inhibits the hypothetical MTP signaling component cyclophilin D (McGee and Baines, 2012). To rule out a role of the permeability transition pore (PTP) as a mechanism for DCD, neurons were treated with 2 µM CsA and subjected to our DCD protocol. PTP inhibition failed to delay DCD. This observation did not come as a surprise; incomplete depolarization, reversibility, and normal DCD latency with upwards of 5 µM CsA support PTP independent DCD (Fig 2.7).
Increase PP2A activity at the OMM via Bβ2 overexpression increases mitochondrial calcium uptake

Currently we know that increased phosphorylation at the OMM via PKA/AKAP1 significantly increases neuronal resistance to DCD. Global modulation of AC and PDE4 activity via Forskolin, PACAP, rolipram and BDNF (all of which promote sustained activation of PKA) similarly prolong latent period durations. Alternatively, inhibition of PP2A activity at the OMM via Bβ2 knockout prolonged latent period duration, indicating a negative role for PP2A. Though the experiments have yet to be performed, it is anticipated that Bβ2 overexpression would sensitize neurons to glutamate toxicity (similar to the higher 10 μM dose of H89). Stout et al. have shown that inhibition of mitochondrial calcium buffering via FCCP uncoupling improves neuronal survival during a toxic glutamate insult (Pivovarova et al., 2008; Stout et al., 1998). Based on these results and others, the role of mitochondrial matrix calcium as a pro-apoptotic/necrotic mechanism is well accepted. Therefore, we hypothesize that reversible phosphorylation at the OMM regulates mitochondrial buffering mechanisms resulting in greater matrix calcium in neurons susceptible to our DCD protocol. To test this idea cultured hippocampal neurons were infected with an FIV construct overexpressing either control Bβ1 or the OMM targeted Bβ2. Fura-FF loaded neurons were stimulated with 100µM glutamate for 30 second to load the mitochondria with calcium. Immediately following the glutamate application, the neurons were treated with 5µM FCCP in Ca^{2+} free recording media supplemented with 100µM EGTA. FCCP causes complete depolarization of the inner mitochondrial membrane potential. In the absence of this electrochemical gradient, total mitochondrial matrix calcium (both free Ca^{2+} and bound apatite calcium) is released into the cytosol. The resulting Fura-FF response (in the absence of external calcium) is an index of the total mitochondrial calcium load (Fig.2.8A,B). Neurons overexpressing Bβ2 produce four fold larger calcium load
measurements as Bβ1 expressing neurons. This suggests either enhanced calcium uptake, or impaired calcium extrusion (Fig 2.8C).

**Discussion**

The ischemic penumbra presents a large area of potential neuronal loss following stroke. Despite the looming degeneration, the slow progression of glutamate excitotoxicity, reperfusion injury, and apoptosis provides an optimistic window for therapeutic intervention (Huang et al., 1997). The role of mitochondria in the pathology of excitotoxicity made an about-face in 1998 when Stout et al. published a seminal paper entitled, “Glutamate-induced neuron death requires mitochondrial calcium uptake.” Prior to this publication, mitochondrial calcium buffering was believed to be a neuroprotective mechanism alleviating the cytosol from toxic concentrations of Ca$^{2+}$. Stout et al. revealed mitochondria as an essential component of excitotoxicity and as a potential therapeutic target (Stout et al., 1998). Our preliminary investigation begins with the characterization of mitochondrial calcium buffering and the regulation of glutamate toxicity. To measure neuronal susceptibility to glutamate toxicity, we focused on an early component termed delayed calcium deregulation, DCD. DCD is the pathophysiological representation of mitochondrial calcium overload and failure (Nicholls and Budd, 2000). Previous studies have demonstrated a role for PKA as a pro-survival kinase. Here we demonstrate that localization of PKA to the OMM via the scaffolding protein AKAP1 significantly prolongs DCD latency, while PP2A/Bβ2 activity at the OMM has a reciprocal affect. Pharmacological manipulation of adenylyl cyclase activity and preservation of cAMP signaling via PDE4 inhibition reproduced our PKA/AKAP1 results.

**Delayed calcium deregulation**

The interplay of delayed calcium deregulation (DCD), mitochondrial permeability transition (MPT), and induction of apoptosis remains an issue of intense debate. Early
work that failed to continue cytosolic calcium measurements following deregulation synonymously discussed DCD and MPT as processes that only differed in nomenclature (Stanika et al., 2009). Our characterization of DCD revealed a reversible phenomenon capable of complete recovery. This observation, however, is based on cytosolic calcium measurements and does not illustrate the failure of individual mitochondrial function. The Andrews research group proposes a minimum mitochondrial failure threshold required for the initiation of apoptosis; that is, a minimum of 35% failed mitochondria is required for the irreversible induction of apoptosis (Pivovarova and Andrews, 2010). Analysis of \( \Delta \Psi_{\text{mt}} \) by imaging Rho123 fluorescence conveyed a close relationship between cytosolic \( \text{Ca}^{2+} \) and IMM depolarization during the initial response phase of DCD. A modest, but steady depolarization continued throughout the latent period. The latent period is a critical phase that represents a poorly understood, slow process of mitochondrial failure. Based on what is known in the current field, the latent period represents a gradual development of mitochondrial uncoupling. Further investigation determining the progression of \( \text{Ca}^{2+} \) dependent reactive oxygen species (ROS) production and modulation of uncoupling proteins (UCPs) is critical for understanding DCD latency (Alan et al., 2009; Hoang et al., 2012; Mattson and Liu, 2003). Another important observation we made during our characterization was the incomplete depolarization following DCD; FCCP application was able to further depolarize the \( \Delta \Psi_{\text{mt}} \). This incomplete depolarization coupled with reversibility and insensitivity to CsA argues against a role for MPT in the development of DCD. However, MPT is a poorly understood process and irreversibility is a point of controversy. Investigators have proposed two mechanisms of MPT, transient MPT (tMPT) and persistent MPT (pMTP). While pMPT is proposed to function as the historically described MPT, tMPT represents a brief formation of the PTP as a cytoprotective mechanism to alleviate mitochondrial calcium load (Jou, 2011). Further investigation using additional indicators of PTP formation (including Cyt C liberation and Smac/Diablo release) is required to definitively determine a role for MPT.
in DCD. The current PTP research field also suggests two possible modes of MPT: transient and persistant; therefore, MPT may still be a component of reversible PTP (Rasola and Bernardi, 2011).

**PKA/AKAP1 and PP2A/Bβ2: phosphoregulation of mitochondrial physiology**

The outer mitochondrial membrane (OMM) is a platform for cell signaling. Kinases and phosphatases recruited to the OMM play important roles in the regulation of multiple cellular processes; specific to this study, reversible phosphorylation at the OMM regulates mitochondrial architecture and Bcl-2 family mediated apoptosis and MPT. PKA has been shown to phosphorylate and inhibit the Bcl-2 family member BAD. BAD is a positive regulator of PTP (Harada et al., 1999). MPT is an unlikely component of DCD; however, PTP formation is a poorly understood process that is difficult to definitively measure. BAD phosphorylation remains an unexplored candidate component in our PKA/AKAP1 observations.

Discussed in Chapter I, mitochondria are dynamic organelles that rapidly and reversibly undergo fission and fusion, MFF. The Strack research group has published a number of studies on the phosphoregulation of the MFF machinery via PKA/AKAP1 and PP2A/Bβ2 (Cribbs and Strack, 2007; Dickey and Strack, 2011; Merrill et al., 2011; Merrill et al., 2012). Increased PKA activity at the OMM promotes mitochondrial fusion; Recruitment of the constitutively active PP2A to the OMM via its regulatory Bβ2 promotes mitochondria fragmentation (Dagda et al., 2008; Merrill et al., 2012). This manipulation of mitochondrial architecture has a number of neuronal consequences including modulation of synapse number and dendritic arborization, changes in $\Delta \Psi_m$ and modulation of neuronal susceptibility to cytotoxins (staurosporin and etopiside) and models of ischemia (oxygen/glucose deprivation and glutamate toxicity) (Cribbs and
Strack, 2007; Dagda et al., 2008; Dickey and Strack, 2011). The latter consequence is significant to our investigation.

Previous studies determining the role of OMM phosphorylation and MFF regulation on neuronal toxicity have all focused on the end-point measurement, cell death. To begin deducing the mechanism by which PKA and mitochondrial fusion protect neurons against excitotoxic insults, we have adopted the model of delayed calcium deregulation (DCD). DCD is a representation of Ca\(^{2+}\) dependent mitochondrial failure, and the DCD latent period duration is an index of mitochondrial integrity and decay. Promotion of PKA signaling at the OMM, either by overexpression of AKAP1 or enhancing cAMP signaling via AC activation (forskolin and PACAP) and PDE4 inhibition (rolipram and BDNF), maintains mitochondrial integrity and function for extended periods of time. Promoting mitochondrial fragmentation has the opposite effect. Based on our matrix Ca\(^{2+}\) hypothesis, OMM PKA activity and fusion either reduces mitochondrial calcium uptake or enhances mitochondrial calcium extrusion or changes the threshold for calcium overload. Preliminary work in our lab using FCCP mediated ΔΨ\(_{mt}\) depolarization and matrix calcium unloading has produced interesting results. Hippocampal neurons overexpressing Bβ2 (promoting mitochondrial fragmentation), produced much greater FCCP responses than control Bβ1 expressing cells. Therefore, dephosphorylation and mitochondrial fragmentation result in greater matrix calcium loads. Such a result indicates changes in mitochondrial calcium buffering kinetics. Mitochondrial physiology is complicated; teasing apart the mechanism by which phosphoregulation at the OMM will be difficult. Fig.2.9 summarizes our current model of phosphoregulation of MFF and excitotoxicity. Based on my data and published literature activation of PKA/AKAP1 at the OMM requires PAC1/adenylyl cyclase/cAMP signaling and simultaneous inhibition of PDE4. In our model PDE4 activity is antagonized via BDNF/TrkB-dependent ERK1/2 phosphorylation of PDE4. Increased and maintained PKA activity at the OMM phosphorylates Drp1 inhibiting its function, promoting
mitochondrial fusion, reducing mitochondrial calcium load and increasing neuronal resistance to glutamate toxicity. PP2A, localized to the OMM by Bβ2, dephosphorylates and activates Drp1 inducing mitochondrial fragmentation, increased total matrix calcium, and sensitivity to excitotoxicity.
Figure 2.1. Characterization and analysis of delayed calcium deregulation. **A,B.** Glutamate toxicity is not limited to the synapses. Extrasynaptic NMDA receptors along the dendrites and soma plasma membrane contribute to DCD. Dendritic DCD precedes the soma, but with comparable initial and deregulation response amplitudes. Given difficulties associated with monitoring low-level FuraFF signal from dendrites and similarities between the dendritic and somatic DCD, we chose to record DCD in the soma for the majority of the studies. **C.** Simultaneous Rhodamine123 ΔΨ<sub>mt</sub> recording illustrates the temporal relationship between DCD progression and depolarization. Following Ca<sup>2+</sup> deregulation, the extent of ΔΨ<sub>mt</sub> depolarization was assessed with 5µM FCCP treatment in Ca<sup>2+</sup>-free HH buffer. **D,E.** Latent period duration is an index of neuronal susceptibility to glutamate toxicity. To calculate the latent period duration, we determined and plotted the Fura-FF signal derivative. The peak derivative values correspond to the rapid slope observed during the initial response and Ca<sup>2+</sup> deregulation. The duration between these two identifiers is recorded as the latent period.
Figure 2.2. AKAP1 overexpression in cultured hippocampal neurons prolongs the latent period duration. This resistance to glutamate toxicity requires PKA activity at the OMM. 

**A.** On average, prolonged exposure to 100µM glutamate produced DCD within 20 min in naive hippocampal neurons. **A.** Overexpression of AKAP1 delays the onset of DCD by hours. **C.** The role of PKA recruitment as a required component of the AKAP1 effect was confirmed both pharmacologically via the PKA inhibitor H89 (2, 5 and 10µM) and genetically using a AKAP1-I310P,L316P mutant (ΔPKA), which ablates PKA binding. Latent period duration was calculated as previously describe. Results are presented as mean ± SEM. Statistics were performed using one way ANOVA with Bonferroni post test. * P < 0.05; ** P < 0.01, with 95% confidence.
Figure 2.3. Forskolin and rolipram pretreatment prolongs latent period duration. A. 1 hour pretreatment with Forskolin (10µM) and the PDE4 inhibitor rolipram (1µM) delays DCD by several hours. B,C. These observations were not reproduced with co-administration of PDE 6, 7, 8, 10 or 11 inhibitors (using 1µM BRL 50481 and 10µM dipyridamole). Each treatment population is presented as mean ± SEM. Statistics were performed using one way ANOVA with Bonferroni post-test. * P < 0.05 with 95% confidence.
Figure 2.4. PACAP-38 is an endogenous regulator of adenylyl cyclase activity. Binding to the Gs coupled PAC1 receptor, PACAP-38 induces cAMP production and PKA activity. 10nM and 100nM PACAP-38 alone failed to prolong DCD latency (data not shown). 100nM PACAP-38 and 1µM rolipram modestly prolonged DCD latency. Each treatment population is presented as mean ± SEM. Statistics performed using Student’s t-test. ** P<0.01 at 95% confidence.
Figure 2.5. Endogenous inhibition of PDE4 may be achieved via brain derived neurotrophic factor (BDNF). BDNF signals through the TrkB receptor, which leads to ERK1/2 activation and, consequently, phosphorylation and inhibition of PDE4. 1 hour pretreatment was performed with 1µM Forskolin and 200ng/mL BDNF. This BDNF mixture was present throughout glutamate toxicity. Latent period duration was recorded as mean ± SEM. Statistics were performed using one way ANOVA and Bonferroni post-test. * P<0.05 at 95% confidence.
Figure 2.6. Loss of Bβ2 (OMM PP2A) robustly prolongs latent period duration during continuous glutamate exposure. Genetic ablation of Bβ2 (knockout mouse) significantly delays latent period duration in cultured mouse hippocampal neurons exposed to a toxic glutamate insult. Control neurons (wild type C57Bl/6J) were significantly more sensitive to toxic glutamate exposure. Latency duration was calculated and presented as mean ± SEM. Statistics were performed using Student’s t-test. * P<0.05 at 95% confidence.
Figure 2.7. Cyclophilin D is proposed to be a positive regulator of mitochondrial permeability transition (MPT). The immunosuppressant Cyclosporin A (CsA) has been reported to inhibit MPT via cyclophilin D inhibition. 1 hour pretreatment with 2µM CsA prior to glutamate toxicity (and during DCD protocol) had no effect on DCD latency duration. PP2B (calcineurin) is an alternative protein phosphatase shown to dephosphorylate Drp1-S656p. Inhibition of calcineurin via 5µM CsA with 1µM FK506 had no effect on the latent period (CsA/FK506). Latency duration was calculated as mean ± SEM. Statistical analysis was performed using one way ANOVA at 95% confidence.
Figure 2.8. Bβ2 overexpression increases total mitochondrial calcium load. A. Total mitochondrial calcium load was measured using a loading stimulus (100µM Glu) immediately followed by 5µM FCCP (mitochondrial protonophore) in Ca²⁺-free recording buffer. B. The difference (green trace) between the Glu/FCCP response (red trace) and the Glu/control response (black trace) was used as an index of total mitochondrial Ca²⁺ load. C. Overexpression of Bβ2 (promoting mitochondrial fragmentation) greatly increases mitochondrial calcium load (AUC = area under the curve). AUC was presented as mean ± SEM. Statistics performed using Student’s t-test. * P<0.05 at 95% confidence. Experiments performed by Yuriy Usachev.
Figure 2.9. Model of DCD regulation by phosphorylation and mitochondrial restructuring. Recruitment to the OMM by AKAP positions PKA within proximity of its effector Drp1. Activation of OMM localized PKA requires PAC1/adenyl cyclase/cAMP signaling and simultaneous inactivation of PDE4 via TrkB/ERK1/2-dependent phosphorylation of PDE4. The resulting PKA-dependent phosphorylation of Drp1 inhibits mitochondrial fission, reduces mitochondrial Ca$^{2+}$ load and significantly enhances neuronal resistance to calcium deregulation (DCD). Conversely, the protein phosphatase PP2A is recruited to the OMM by its regulatory subunit Bβ2. Increased PP2A activity at the OMM dephosphorylates and activates Drp1, promotes mitochondrial fission, and increased mitochondrial calcium load. Ablation of PP2A signaling at the OMM improves promotes mitochondrial fusion and excitotoxic resistance comparable to increased PKA/AKAP1 signaling.
CHAPTER III

MODULATION OF MITOCHONDRIAL CALCIUM BUFFERING VIA REVERSIBLE PHOSPHORYLATION AT THE OMM AND MANIPULATION OF MITOCHONDRIAL MORPHOLOGY

Abstract

Mitochondrial fission and fusion (MFF) is a rapid dynamic process tightly regulated by phosphoregulation of the key fission component dynamin related protein1 (Drp1). Phosphorylation of Drp1-serine 656 by PKA (targeted to the OMM via AKAP1) inhibits its GTPase and fission functions and promotes mitochondrial elongation. Drp1-S656p can be dephosphorylated by either PP2A (targeted to the OMM via its regulatory subunit Bβ2) or the calcium activated phosphotase PP2B (calcineurin). This dephosphorylation activates Drp1 function and induced mitochondrial fragmentation. Work by the Strack research group has demonstrated modulation of neuronal sensitivity to ischemia and neurotoxicity by MFF and phosphoregulation of Drp1. Both direct induction of mitochondrial fusion and promotion of PKA activity at the OMM enhanced neuronal resistance. Induction of mitochondrial fragmentation via Drp1 overexpression or PP2A/Bβ2 activity at the OMM increased neuronal sensitivity. These observations complement our DCD data described in Chapter II. In Chapter II, we observed enhanced resistance to calcium deregulation during glutamate toxicity in hippocampal neurons genetically and pharmacologically manipulated to promote PKA activity at the OMM. PP2A/Bβ2 activity at the OMM sensitizes neurons to DCD and increases mitochondrial calcium load. These results suggest MFF dependent impairment of mitochondrial calcium buffering.

To determine the effect of OMM reversible phosphorylation (specifically Drp1-S656 phosphoregulation) and MFF on mitochondrial calcium buffering we used transiently transfected dorsal root ganglion (DRG) neurons. DRG neurons were co-
transfected with the matrix Ca\(^{2+}\) indicator mtPericam and genetic modulators of Drp1 activity and OMM phosphorylation. Induction of mitochondrial fusion via dominant negative Drp1-K38A and Drp1-replacement with the phosphometic Drp1-S656D enhanced mitochondrial calcium recovery of individual mitochondria in neurites. AKAP1 overexpression produced a modest result comparable to control. Induction of mitochondrial fission via Drp1 overexpression and increased PP2A activity at the OMM via Bβ2 overexpression significantly impaired mitochondrial Ca\(^{2+}\) extrusion. This observation complements our previous observation of elevated mitochondrial calcium load with similar Bβ2 overexpression in transduced hippocampal neurons. The toxicity of the constitutively active Drp1-S656A in DRG neurons in my hands may complicate our selection process resulting in a severely underestimated effect on mitochondrial calcium recovery kinetics.

**Introduction**

Mitochondria are dynamic organelles that rapidly and reversibly undergo fission and fusion, termed MFF. MFF is an essential characteristic of mitochondrial and cellular physiology. Mitochondrial fission is a critical component of mitosis, mitophagy and neuronal differentiation, and fusion allows optimization of cellular respiration via the sharing of solutes, metabolites, proteins, and mitochondrial DNA (Anne Stetler et al., 2013; Otera and Mihara, 2011b). Current research suggests additional pathological roles for MFF. An early indicator of apoptosis is the fragmentation and swelling of mitochondria. Inhibition of the fission machinery (dynamin related protein 1, Drp1) has anti-apoptotic properties. Drp1 inactivity and cellular survival occurs endogenously, as well. Certain cancers have been reported to express non-functional Drp1 mutations that resist apoptosis (Thomas and Jacobson, 2012).

Strack’s research group has presented pathological consequences of phosphoregulation of MFF. Increased protein phosphatase 2 A (PP2A) activity at the
OMM via targeting of its regulatory subunit Bβ2 promotes both mitochondrial fragmentation and sensitivity to toxic stimuli. As predicted, Bβ2 knockout mice (and Bβ2 RNAi) possess elongated mitochondria and resistance to toxicity (Dagda et al., 2008). Manipulation of protein kinase A (PKA) produces reciprocal results. Targeting of PKA to the OMM via A kinase anchoring protein 1 (AKAP1) promotes mitochondrial fusion and survival (Merrill et al., 2011). Molecular analysis of the Drp1 sequence revealed a PKA phosphorylation site at serine 656 (Cribbs and Strack, 2007). Biochemical experiments using a phosphospecific antibody against a serine 656p epitope demonstrated PKA dependent phosphorylation and dephosphorylation mediated by PP2A and PP2B (calcineurin) (Cribbs and Strack, 2007; Merrill et al., 2012). Strack et al. has also developed Drp1 phosphomutants via point mutagenesis. The non-phosphorylatable Drp1 serine 656 to alanine mutant (Drp1-S656A) produced a constitutively active Drp1 yielding fragmented mitochondria and apoptotic sensitivity. The phosphomimetic Drp1 serine 656 to aspartate mutant (Drp1-S656D) produced inactive Drp1 yielding mitochondrial elongation and neuronal-survival.

To date, the effect of mitochondrial morphology and OMM phosphorylation studies focus on the end point of cell death. These studies provide important correlation between mitochondrial physiology and cytotoxicity, but fail to derive a mechanism of pathogenesis. Mitochondrial buffering is a growing research field. Molecular identification of specific buffering components is underway (Baughman et al., 2011; De Stefani et al., 2011; Gobbi et al., 2007; Nowikovsky et al., 2012; Palty et al., 2010). Our previous data in hippocampal neurons investigated the effect of OMM reversible phosphorylation on delayed calcium deregulation (DCD) kinetics. The same manipulations that promote mitochondrial fusion and neuronal survival maintained mitochondrial integrity represented as a prolonged latent period duration (and thus, resistance to ΔΨ\text{mt} depolarization). Matrix unloading experiments (FCCP uncoupling) showed robustly elevated mitochondrial matrix calcium content in Bβ2 overexpressing
neurons. Described above, Bβ2 overexpression promotes mitochondrial fragmentation via PP2A mediated dephosphorylation of Drp1-S656p. This data implies modulation of mitochondrial buffering kinetics. The following study characterizes mitochondrial calcium buffering kinetics as a function of mitochondrial morphology and phosphoregulation of MFF by directly monitoring Ca\(^{2+}\) fluxes in and out of mitochondria in primary neurons.

**Experimental procedures**

**MFF and OMM phosphorylation cDNA vectors**

Mitochondrial matrix free Ca\(^{2+}\) recordings were performed using the Ca\(^{2+}\) sensitive GFP mutant construct targeted to the mitochondrial matrix via Cox8 localization sequence fusion, mtPericam (in a pcDNA3.1 expression vector) (Medvedeva et al., 2008; Nagai et al., 2001). Control neurons expressed only mtPericam or mtPericam with Bβ1. Drp1 constructs included Drp1 wild type (Drp1WT), dominant negative Drp1 lysine 38 to alanine mutant (Drp1-K38A), phosphomimetic Drp1-S656D, and non-phosphorylatable Drp1-S656A; both phosphomutant constructs are expressed using a bicistronic vector co-expressing shRNA against endogenous Drp1. Regulation of OMM phosphorylation activity was manipulated using AKAP1 overexpression (to promote PKA at the OMM) and Bβ2 overexpression (to promote PP2A at the OMM).

**Dissociated dorsal root ganglion neuron culture**

Primary culture of rat dorsal root ganglion (DRG) neurons was performed as previously described (Kim and Usachev, 2009). Briefly, P0-2 neonatal Sprague-Dawley rat pups (Charles Rivers, Massachusetts) were euthanized via decapitation. The spinal column was then dissected to expose the DRGs. For our electroporation protocol, four pups worth of DRGs were collected and digested in a 2 mg/mL pronase solution of
DMEM media supplemented with HEPES and Pen/Strep. The DRGs were then dissociated into individual sensory neurons via fire polished Pasteur pipettes of decreasing diameter. Following electroporation protocol, the dissociated DRGs were then plated at an optimized seeding density on glass coverslips coated with poly-O-ornithine and laminin. Cultures were maintained in DMEM media supplemented with NaHCO₃, 5% horse serum, 5% fetal bovine serum, insulin and Pen/Strep (DMEM serum). The cultures were incubated at 37°C and 10% CO₂. All experiments were performed 2-3 days post dissection/transfection.

**Transfection procedure**

DRG neurons were transfected with the described plasmids using Lonza nucleofector II device according the manufacturers protocol (Lonza). In brief, dissociated DRG neurons of four rat pups were combined. Mild centrifugation and vacuum aspiration isolated the neuron pellet from the culture media. The neuron pellet was then re-suspended in the ‘rat neuron’ electroporation solution and transferred to the nucleofection cuvette. Nucleofection protocols G-013 or O-003 were used. The transfection solution was then transferred to pre-warmed DMEM serum. Immediately following electroporation, neurons are extremely sensitive; following nucleofection, the transfected neurons were incubated 5-10 minutes prior to re-suspension and plating (post-electroporation quiet time).

**Individual mitochondria mtPericam recording**

Mitochondrial matrix Ca²⁺ imaging with mtPericam was recorded as previously described (Medvedeva et al., 2008). Briefly, mtPericam transfected DRG neurons were mounted for epifluorescent imaging (Olympus IX-71). Using a 40X oil immersion objective, the focal plane was focused on the linearized mitochondria within the DRG neurites. MtPericam recording was performed using fluorescent excitation at 410nm via a
Polychrome IV monochromator (T.I.L.L. Photonics); emission was collected using 510 (80) nm with a Photonic IMAGO CCD camera coupled to TillVisION live acquisition software at 1x1 binning and 2Hz acquisition frequency. Individual neurite mitochondria were selected post-hoc using focus and resolution as selection criteria. The individual mitochondria matrix Ca$^{2+}$ recordings were then normalized using the following equation: 

\[-(F-F_0)/(F_0-F_{\text{background}})\]. Mitochondrial movement during the recording period was determined by analysis of the individual time lapse files (either due to mitochondrial motility or a detached neurites). For incidences of modest mitochondrial movement, the region of interest (ROI) was adjusted. During scenarios of significant movement or detachment of the entire neurite, the data was excluded.

**Field potential stimulation**

Neuronal excitation was performed using a train of action potentials via field potential stimulation. The transfected DRG cultures were mounted on a submerged electrode bath perfusion chamber. For voltage threshold analysis, mtPericam recording was performed with 475nm excitation and collection of emission at 510 (80) nm to reduce potential bleaching at 410nm. Starting at 10V, the potential was increased in 10V increments using a 4 second 5Hz train until an mtPericam response was observed. For the subsequent experimental stimulation, a voltage of threshold + 20V was used for a 10 second 10Hz stimulation train.

**Analysis of mitochondrial matrix calcium kinetics**

As described above, the individual mitochondrial matrix Ca$^{2+}$ recording were normalized. The peak amplitude was determined and collected as an index of mitochondrial calcium uptake. The recovery duration was calculated as duration of time from the incidence of peak mtPericam response to 50% recovery from said amplitude.
Statistical analysis

Peak mtPericam response amplitudes and 50% matrix Ca\(^{2+}\) recovery durations were collected. Correlation and statistical analysis (Student’s \(t\)-test and one way ANOVA for 3 or more groups) showed no significant difference between mitochondria of neuronal sources within the transcription populations. Therefore, individual mitochondria from each transcription population were pooled and represented as mean ± SEM (5-8 mitochondria were recorded per cell). Statistical analysis was performed using either Student’s \(t\)-test (when comparing two variables) or one way analysis of variance (ANOVA…when comparing 3+ variables) with Bonferroni post-test comparing all columns. All statistics were performed at 95% confidence.

Results

Our data exploring the effect of OMM phosphorylation on maintenance of calcium homeostasis and mitochondrial matrix calcium load strongly suggest a neuroprotective mechanism involving the regulation of calcium buffering kinetics. The time constraints of hippocampal culture maturation (requiring 15+ DIV for spine development) present an impractical model for our preliminary study (Murphy and Segal, 1996). The limitations and expense of viral transduction and low efficacy transient transfection make hippocampal neuron culture an unattractive model for our purpose; we decided to investigate mitochondrial calcium buffering using a different cell model. In addition to our interest in neuronal loss within the ischemic penumbra, our lab has a line of research regarding the effects of MFF and reversible phosphorylation at the OMM on sensory neuron physiology and degeneration (Chowdhury et al., 2012). DRG neuron transfection via Nucleofection (Lonza) type electroporation is a high efficiency tool performed immediately following DRG dissociation; this results in sensory neuron cultures with 20%-30% transfection efficiency and an experimental schedule 2-3DIV/post transfection.
The mitochondrial of any cell type (neuronal or otherwise) is not a homogeneous population (Sonnewald et al., 1998; Westergaard et al., 1995). Mitochondrial fission and fusion exists as an equilibrium, not as a state. At any given moment of physiological time, a single neuron contains a spectrum of mitochondrial architecture. The mean or median of mitochondrial morphology is only shifted upon MFF or OMM phosphorylation manipulation; therefore, it is important to measure mitochondrial calcium kinetics in individual mitochondria to prevent dilution of a significant population of organelles that differ from the mode. The DRG soma is a three dimensional volume of significant depth, with regard to microscopy. This three dimensional space is full of mitochondria that may not be aligned within a select focal plane. Even when confocal microscopy was performed to isolate a single plane of somal mitochondria, digital selection of individual, entire mitochondria is an impossible task. To measure individual mitochondria, we focused on the organelle content of the DRG neurites. Neurites are the neuronal processes that extend from the soma of cultured DRG neurons. The neurite extend in close proximity to the plane of the cover glass, and their internal diameter normally accommodates the short axis of an individual mitochondria. This organization results in a linearization of individual mitochondria within a single plane of focus; such organization is optimal for our investigation (Fig.3.1).

**Effects of mitochondrial morphology on calcium buffering kinetics of individual mitochondria**

Initial experiments focused on manipulation of mitochondrial morphology independent of OMM phosphorylation. Promotion of mitochondrial fission was accomplished via transient transfection with wild type dynamin related protein 1 (Drp1-WT). Expression of a Drp1-K38A mutant produced mitochondrial fusion. Drp1-K38A is a lysine to alanine point mutation that attenuates Drp1 GTPase function; this mutation retains Drp1 multimerization characteristics. The preserved protein-protein interactions
aggregates inactive Drp1-K38A with endogenous Drp1 resulting in a dominant negative effect. According to quantitative studies shown by Dickey et al., Drp1-K38A transfection is the most robust exogenous modulator of mitochondrial fusion. Fig.3.2a presents representative images of DRG neurite mitochondria visualized via mtPericam (410nm) co-expression with either Drp1-WT or Drp1-K38A (control neurons were transfected with mtPericam alone). Mitochondrial morphology was further analysed using the ImageJ macro developed by the Strack research group (Cribbs and Strack, 2009).

Fig.3.2b summarizes the correlation between aspect ratio (AR) and form factor (FF). Aspect ratio is the ratio of the long axis and the short axis, and is a measure of elliptical roundness; an AR value close to 1 is rounded, while values greater than 1 indicated an elongated ellipse or mitochondria. FF is a measure of mitochondrial perimeter relative to its area; greater FF values indicate larger and branched mitochondria. As observed in Fig.3.2b Drp1 WT overexpression produces reduced AR/FF values, whereas Drp1 K38A and Drp1 S656D expression produce much larger AR/FF values in DRG neurons.

Fig.3.2c summarizes the linear mitochondrial length in micrometers. Our observations reproduce those of our collaborator performed in cultured rat hippocampal neurons (Dickey and Strack, 2011).

Mitochondrial targeted ratiometric-Pericam (mtPericam) is a GFP derived Ca\(^{2+}\) sensitive construct engineered to respond to Ca\(^{2+}\) with relatively low affinity (Kd~1.7µM)(Nagai et al., 2001). Originally designed to behave as a ratiometric indicator, mtPericam is excited around 410nm and 480nm; these two excitations emit around 510-535nm fluorescence. Chelation of free Ca\(^{2+}\) reduces emission following 410nm excitation, while fluorescent intensity is enhanced for 480nm excitation (the isosbestic point of this ratiometric Ca\(^{2+}\) spectrum is 470nm excitation). Further investigation revealed pH sensitivity analogous to YFP for the 480nm excitation wavelength, only; therefore, we solely utilized 410nm excitation as our Ca\(^{2+}\) indicator (Nagai et al., 2001). Fusion of a Cox8 localization sequence targets mtPericam to the mitochondrial matrix.
Using this matrix targeted Ca\textsuperscript{2+} indicator, we determined the effect of MFF on mitochondrial calcium buffering kinetics. Neuronal stimulation was performed using extracellular field stimulation (10 Hz for 10 seconds). Selecting individual neurite mitochondria, we were able to visualize the uptake and recovery of mitochondrial calcium (Fig.3.1). Peak mtPericam response appeared to be comparable among the three groups of cells; recovery, however, was greatly affected. Mitochondria from DRG neurons overexpressing Drp1-WT showed significantly impaired Ca\textsuperscript{2+} recovery. Control neuron mitochondria achieved 50% recovery within 30 sec of peak amplitude, while 50% recovery time doubled for the Drp1-WT population. Even more intriguing, mitochondrial elongation (via Drp1-K38A expression) enhances recovery; mitochondrial matrix Ca\textsuperscript{2+} achieved 50% recovery within 15 second for the Drp1-K38A population (Fig.3.3A,B).

These data suggest that mitochondrial Ca\textsuperscript{2+} uptake is insensitive to changes in morphology; recovery, however, is significantly impaired with fragmentation. Combined with our data supporting increased matrix calcium load in Bβ2 transduced hippocampal neurons (Fig.2.9), these data argue impaired matrix Ca\textsuperscript{2+} efflux and retention.

As noted above, mitochondria within a single cell is a heterogeneous population. It is not assumed that neurite mitochondria accurately convey the physiology of somal mitochondria. To determine how accurately our neurite data represent global mitochondrial physiology, we measured average mtPericam response and kinetics within the DRG soma. Selecting the entire soma (excluding the nucleus), we successfully reproduced the recovery observations described for neurite mitochondria (Fig.3.3C).

**Drp1 phosphoregulation modulates mitochondrial calcium buffering kinetics**

AKAP1 overexpression robustly prolonged DCD latency through a PKA dependent mechanism. Our current data suggests mitochondrial elongation improves matrix Ca\textsuperscript{2+} efflux kinetics, while fragmented mitochondria accumulate Ca\textsuperscript{2+} in the
matrix due to impaired extrusion. The Strack group has reported elongation of mitochondria via AKAP1/PKA expression (Fig.3.2). Based on these results, we hypothesize that AKAP1 overexpression delays the accumulation of calcium during glutamate toxicity due to mitochondrial elongation and enhanced \( \text{Ca}^{2+} \) buffering. To support this hypothesis, DRG neurons were co-transfected with mtPericam and an AKAP1 overexpressing construct. Though AKAP1 overexpression produces notable mitochondrial elongation (Fig.3.2), matrix \( \text{Ca}^{2+} \) recovery kinetics were comparable to that of control (Fig3.4).

Drp1 serine 656 has been identified as a PKA phosphorylation target important in the regulation of MFF. Phosphorylated Drp1-S656p is inactive and incapable of catalyzing mitochondrial fission. To determine the effect of Drp1 phosphorylation specifically, DRG neurons were co-transfected with mtPericam and either non-phosphorylatable Drp1-S656A or phosphomimetic Drp1-S656D replacement constructs. Drp1-S656A showed very striking effects on mitochondrial morphology; mitochondria appeared very fragmented and swollen in Drp1-S656A transfected neurons. Drp1-S656D transfection yielded elongated mitochondria (Fig.3.2). Matrix \( \text{Ca}^{2+} \) recordings of Drp1-S656A transfected DRG neurons did not perform as expected. Despite significant fragmentation illustrated in Fig.3.2, matrix \( \text{Ca}^{2+} \) efflux only showed a modest trend toward slowed extrusion when compared to control. As indicated by mitochondrial swelling, the effects of Drp1-S656A expression may be too cytotoxic. Consequently, it is possible that neurons expressing high levels of Drp1-S656A are selectively lost, leading to an underrepresentation of neurons with fragmented mitochondria. Drp1-S656D transfected DRG neurons presented a trend towards enhanced efflux kinetics, but this trend is not significant compared to control (Fig.3.4).
PP2A/Bβ2 reduces calcium efflux rate from mitochondria

PP2A is a promising regulator of mitochondrial physiology. Localized to the OMM by its regulatory subunit Bβ2, PP2A is a candidate phosphatase for the dephosphorylation of Drp1-S656p. Bβ2 overexpression increases sensitivity to cytotoxins, induces mitochondrial fragmentation, and increases mitochondrial calcium matrix load (Fig.2.8). Knocking out, or knocking down, Bβ2 promotes mitochondrial elongation, increases neuronal resistance to glutamate toxicity and prolongs DCD latency. MtPericam analysis of matrix Ca$^{2+}$ kinetics supports these early findings. Comparable to our previous experiments, Bβ2 expression did not affect the peak matrix Ca$^{2+}$ response when compared to control (for these experiments control neurons were transfected with the Bβ splice variant Bβ1 that does not localize to the OMM). As was predicted from the Drp1-WT results, Bβ2 significantly impaired mitochondrial matrix Ca$^{2+}$ recovery (Fig.3.5). Bβ2 perturbation of mitochondrial calcium efflux was somewhat less affective than Drp1-WT overexpression. 50% recovery duration was extended to a mean around 40 seconds; Bβ1 control DRG mitochondria recovered to 50% within 20 seconds (Fig.3.4B,C).

Discussion

Reversible phosphorylation at the OMM has been shown to regulate mitochondrial morphology and resistance to cytotoxicity (Cribbs and Strack, 2007; Dagda et al., 2008) and matrix calcium load (Fig.2.8). It is likely that these phenomena are linked. Our previous studies have confirmed a role for PKA/AKAP1 and PP2A/Bβ2 in the modulation of neuronal susceptibility to delayed Ca$^{2+}$ deregulation, DCD. We have yet to determine the phosphorylation target or the requirement of MFF as a component of this Ca$^{2+}$ sensitive mechanism. This important regulation of mitochondrial morphology is critical to understanding these excitotoxicity observations. However, based on work by Strack et al., characterization of the morphological consequences and neuronal survival
of OMM phosphorylation has confirmed Drp1 serine 656 as a target for PKA/AKAP1 phosphorylation (Merrill et al., 2011). Phosphorylation at this specific serine residue imbues Drp1 inhibition and induction of mitochondrial fusion. Dephosphorylation via PP2A/Bβ2 activates Drp1 and promotes fragmentation. To investigate a role for phosphoregulation of MFF in the modulation of mitochondrial calcium handling, we first explored the effect of mitochondrial morphology on matrix Ca\(^{2+}\) uptake and release kinetics.

**Modulation of mitochondrial calcium efflux by MFF**

Due to the long maturation time hippocampal neurons and lack of FIV compatible cDNA and shRNA constructs, our matrix Ca\(^{2+}\) kinetics experiments were performed using transiently transfected dorsal root ganglion (DRG) neurons. Manipulation of mitochondrial architecture was accomplished using constructs established by Strack et al. Drp1-WT overexpression produced mitochondrial fragmentation and Drp1-K38A dominant negative promoted elongation, comparable to Dickey and Strack, 2011. Analysis of mitochondrial Ca\(^{2+}\) handling kinetics displayed impaired recovery kinetics with Drp1-WT overexpression and enhanced rate with the dominant negative action of Drp1-K38A as compared to control. Mitochondrial calcium influx appeared to be unchanged. Visualization of free matrix Ca\(^{2+}\) was performed using mtPericam. Caveats of mtPericam imaging include relatively low Ca\(^{2+}\) affinity and exclusion of bound calcium visualization. The latter criticism of mtPericam imaging impairs our interpretation of mitochondrial calcium efflux. What we have described as matrix Ca\(^{2+}\) recovery is a combination of two distinct processes. Two fates of free matrix Ca\(^{2+}\) exist; this divalent cation may be shuttled into the cytosol via a speculated exchanger mechanism supported by pharmacology or it is sequestered within the matrix as a calcium salt (Chalmers and Nicholls, 2003). In terms of excitotoxicity, total matrix calcium (including bound Ca\(^{2+}\)) is proposed to be a component of mitochondrial failure;
our hypothesis supports the efflux mechanism (Pivovarova et al., 2004). Further experimentation is required to determine which mechanism is responsible.

**OMM and Drp1 reversible phosphorylation can regulate mitochondrial calcium handling**

Direct manipulation of MFF machinery significantly shaped mitochondrial calcium handling. Previously, we have shown that AKAP1 recruitment of PKA to the OMM prolongs DCD latency via an unknown mechanism. PKA/AKAP1 also phosphorylates Drp1 at serine 656 inhibiting its GTPase and mitochondrial fission functions. Overexpression of AKAP1 in DRG neurons produced mitochondrial elongation. Analysis of matrix calcium within these individual mitochondria revealed Ca\(^{2+}\) recovery kinetics comparable to that of control. Failure to enhance recovery may be due to a number of variables. Basal PKA activity within DRG neurons may be different from that of hippocampal neurons, and AKAP1 overexpression in DRG neurons has a modest affect in comparison. In Dickey and Strack, 2011, AKAP1 overexpression induced fusion was not as drastic as the dominant negative consequences of Drp1-K38A expression; thus, the effect of AKAP1 expression on buffering kinetics is expectedly modest in comparison. Additionally, characterization of the role of AKAP1 on mitochondrial morphology in DRG neurons has yet to be performed.

Analysis of non-phosphorylatable and phosphomimetic Drp1 point mutants (Drp1-S656A and Drp1-S656D, respectively) directly investigated the effect of Drp1 phosphorylation on mitochondrial calcium handling. We expected the constitutively active Drp1-S656A mutant to produce much more exaggerated impairment of matrix free Ca\(^{2+}\) recovery than Drp1-WT overexpression. Drp1-S656A did produce drastic mitochondrial fragmentation and swelling as illustrated in Fig.3.2; however, free matrix Ca\(^{2+}\) recovery kinetics was only modestly and insignificantly increased, as compared to control. Discussed briefly in the results, constitutively active Drp1 may have cytotoxic
effects, and mitochondrial swelling and reduced mtPericam transfection efficiency are key indicators of that (Cheung et al., 2007). Also, due to the fluorescent spectra of mtPericam and availability of construct, we used non-fluor tagged Drp1 constructs. Therefore, our selection of DRG neurons relies on a high rate of co-transfection efficiency. The proposed cytotoxic consequences of Drp1-S656A expression could likely dilute its actual physiological effects via increasing our selection of mtPericam only transfected neurons. The phosphomimetic Drp1-S656D produced more encouraging results. Drp1-S656D expression in DRG neurons resulting in mitochondrial elongation, as was previously reported (Dickey and Strack, 2011). Though Drp1-S656D did not significantly enhance free matrix calcium recovery to the same effect as Drp1-K38A; a noticeable trend was observed.

OMM phosphatase dependent modulation of sensitivity to glutamate toxicity in hippocampal neurons strongly favored PP2A/Bβ2 as the likely candidate. Multiple publications from Strack et al. characterized PP2A dependent Drp1 dephosphorylation and Bβ2 expression mediated mitochondrial fragmentation (Dickey and Strack, 2011; Merrill et al., 2012). Transient transfection of Bβ2 in DRG neurons produced mitochondrial fragmentation comparable to results previously reported (Dagda et al., 2008). Like Drp1-WT overexpression, mitochondria from Bβ2 transfected neurons displayed mtPericam response amplitudes comparable to that of Bβ1 control neurons, but significantly impaired recovery kinetics. Currently, PP2A/Bβ2 represents our greatest understanding of OMM phosphoregulation, mitochondrial architecture, buffering kinetics and excitotoxicity. In short, increased PP2A activity at the OMM promotes mitochondrial fragmentation and impaired free matrix calcium recovery. Mitochondrial fragmentation mediated by direct manipulation reproduced this observation. Preliminary work in hippocampal neurons using FCCP presented a much greater index of mitochondrial Ca²⁺ load as compared to control. Together, these data suggest a fragmentation dependent degeneration of the mitochondrial calcium efflux mechanism resulting in the retention of
matrix calcium (free and bound). This tendency to elevated Ca load severely limits the ability of neurons to weather excitotoxic insults. The exact mechanism by which mitochondrial fragmentation modulates calcium efflux and the translation of matrix calcium load to DCD sensitivity is yet to be determined. The Strack research group has described a decrease in $\Delta \Psi_{\text{mt}}$ as a function of mitochondrial fragmentation (Dickey and Strack, 2011). This reduced inner membrane potential may alter possible electrogenic properties of the proposed mitochondrial calcium efflux components (Palty et al., 2006). Additionally, mitochondrial fragmentation may limit the density of mitochondrial Ca$^{2+}$ exporters. Larger mitochondria will have a larger mitochondrial reticulum associated with a shared pool of efflux components, while fragmented mitochondria may have insufficient expression of Ca$^{2+}$ exporters. In conclusion, our data suggest that mitochondrial fragmentation slows and fusion increases the rate of Ca$^{2+}$ efflux from neuronal mitochondria (Fig.3.6). However, the exact mechanism by which Drp1 phosphorylation in controlling mitochondrial Ca$^{2+}$ transport remains to be determined.
Figure 3.1. Mitochondrial free matrix Ca\(^{2+}\) imaging protocol. 

A. Dissociated rat DRG neurons were transfected with the mitochondrial matrix Ca\(^{2+}\) reporter mtPericamR. Using epifluorescence microscopy with 410 nm excitation and 510 (80) nm emission, mtPericam was recorded with a high speed CCD camera. Image shows distribution of mtPericam fluorescence using a false color intensity scale.

B. Mitochondrial Ca\(^{2+}\) elevations were induced by using extracellular field stimulation at 10 Hz for 10 s. Changes in mtPericam fluorescence (F) are shown by the green trace. The corresponding Ca\(^{2+}\) changes in the matrix were quantified using the formula: 

\[-(F - F_{\text{baseline}})/F_{\text{baseline}} - F_{\text{background}} \times 100\%\].

(black trace).

C. Selection of individual mitochondria along the DRG neurites.

D. The amplitude and 50% recovery time of each mitochondrial Ca\(^{2+}\) transient were calculated.
Figure 3.2. Genetic manipulation of MFF and Drp1 phosphorylation. A. Cultured rat DRG neurons were co-transfected with mtPericam and Drp1-WT, Drp1-K38A (dominant negative), AKAP1, Drp1-S656D (phosphomimetic), or Drp1-S656A (non-phosphorylatable) and imaged using the mitochondrial matrix targeted Ca$^{2+}$ indicator mtPericam. B. Morphological analysis reported anticipated AR/FF values, where the greater the value the more fused and elongated the mitochondria. C. Mitochondrial length analysis similarly demonstrates mitochondrial fragmentation with Drp1 WT and Drp1 S656A expression. Mitochondrial morphology was comparable to that described in Dickey and Strack, 2011. Scale bars represent 10µm. Summary data describes average mitochondrial length from n=6+ neurons per transfection population. Mitochondrial length is presented in micrometers as mean ± SEM. Statistics were performed using one way ANOVA with Bonferroni post-test. * P<0.05 with 95% confidence.
Figure 3.3. Mitochondrial fragmentation impairs matrix Ca$^{2+}$ recovery. Mitochondrial fragmentation was induced with Drp1-WT over expression in cultured DRG neurons. Inhibition of mitochondrial fission via Drp1-K38A dominant negative induced mitochondrial elongation. A, B. Individual mitochondrial Ca$^{2+}$ recording was performed using high resolution mtPericam imaging within the DRG neurites. The neurons were stimulated with a 10Hz action potential train for 10 seconds. Recovery duration was calculated as the 50% recovery from peak amplitude. Peak amplitudes were similar among the various MFF neuronal populations C. We recorded average soma mitochondrial Ca$^{2+}$ kinetics and obtained data similar to those from individual neurite mitochondria. Recovery duration in seconds is presented as mean ± SEM. Statistics were performed using one way ANOVA with Bonferroni post-test. * P<0.05, *** P<0.005 with 95% confidence.
Figure 3.4. Phosphoregulation of Drp1 activity modulates mitochondrial calcium recovery. A,B. Cultured DRG neurons were co-transfected with mtPericam and phosphomutant Drp1 constructs (Drp1-S656A or Drp1-S656D) or the PKA scaffold AKAP1. In Dickey and Strack, 2011, the investigators confirmed robust mitochondrial fusion with Drp1-K38A, the phosphomimetic Drp1-S656D and AKAP1 were much less effective fusion promoters. Matrix Ca\(^{2+}\) recovery kinetics correspond with these morphological observations. C. Non-phosphorylatable Drp1-S656A is constitutively active. 50% recovery duration is presented as mean ± SEM. Statistical analysis performed using one way ANOVA with Bonferroni post-test. * P<0.05, *** P<0.005 at 85% confidence.
Figure 3.5. Increased PP2A activity at the OMM via Bβ2 overexpression impairs mitochondrial matrix Ca\(^{2+}\) recovery.  

**A,B.** Representative images of mitochondria within neurites of DRG neurons co-transfected with mtPericam and either Bβ1 (control) or Bβ2.  

**C,D.** Increased PP2A activity at the OMM (via Bβ2 over-expression) induced mitochondria fragmentation and significantly impaired mitochondrial calcium recovery. 50% recovery was calculated as previously described and was presented as mean ± SEM. Statistics were performed using Student’s *t*-test. * P<0.05 at 95% confidence.
Figure 3.6. MFF phosphoregulation and mitochondrial calcium recovery summary model. Direct manipulation of Drp1 expression and function (Drp1 overexpression and Drp1-K38A, respectively) profoundly modulate matrix Ca^{2+} recovery. Manipulation of Drp1 phosphoregulation (including phospho-mutants) had more modest effects.
CHAPTER IV
MOLECULAR CHARACTERIZATION OF MITOCHONDRIAL
CALCIUM TRANSPORT IN NEURONS

Abstract

The molecular identity of the components of mitochondrial Ca\textsuperscript{2+} transport in neurons is not established. Much progress has been made in recent years identifying several candidates for both mitochondrial Ca\textsuperscript{2+} uptake and extrusion. Mitochondrial Ca\textsuperscript{2+} uptake has been proposed to occur via 2\textsuperscript{nd} order kinetics through a Ca\textsuperscript{2+} uniporter. Initial identification of the non-pore forming mitochondrial calcium uptake 1 (MICU1) made possible the discovery of the pore forming ccdc109a, termed MCU (mitochondrial calcium uniporter). This proposed calcium uniporter (or uniporter subunit) is largely regarded as the putative mitochondrial calcium uptake mechanism. Other uptake candidates include the ccdc109a (MCU) paralogue ccdc109b, mitochondrial ryanodine receptors (mRyR), and leucine-zipper, EF-hand containing transmembrane protein 1 (Leml). Mitochondrial calcium efflux is hindered by the same lack of understanding. Efflux kinetics and requirements support Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Current exchange candidates include NCX3, NCLX and Leml. Additionally, the ligand-gated DAG-activated cation channel (DCC) may be a component of matrix calcium efflux.

In our study of mitochondrial calcium uptake, we focus on the characterization of MCU, ccdc109b and Leml in DRG neurons and Neuro2A cells. As previously reported, MCU localizes to the mitochondria. Using a spectrum of stimuli strength, we confirmed a role for MCU in neuronal calcium buffering. MCU overexpression significantly reduced the amplitude of cytosolic calcium responses and prolonged the DRG calcium transient plateau duration; both observations indicated increase mitochondrial calcium uptake. RNAi knockdown of MCU complemented these observations with increased cytosolic calcium response amplitude and reduced matrix calcium uptake. The dominant negative
MCU-QIMQ mutant produced a more pronounced response supporting MCU dependent mitochondrial calcium uptake. However, the observations were not of complete attenuation suggesting that MCU is not the only uptake mechanism. The MCU paralogue ccdc109b is another likely candidate for mediating mitochondrial Ca\(^{2+}\). RT-PCR analysis reveals possible splice variation, but cytosolic and mitochondrial calcium kinetics remain difficult to interpret and require further investigation.

Though we began studying Letm1 as an uptake candidate, the literature and exchange mechanism present Letm1 as a stronger candidate for mitochondrial calcium extrusion. After confirming mitochondrial localization in DRG neurons, we characterized Letm1 RNAi knockdown using a protocol similar to the MCU and ccdc109b studies by applying Ca\(^{2+}\) load of incremental amplitude. Letm1 knockdown had no effect on cytosolic Ca\(^{2+}\) response amplitude, but the duration of mitochondrial-dependent \([\text{Ca}^{2+}]_i\) plateau was significantly reduced. Matrix calcium influx increased. Though these observations support a role for Letm1 in matrix Ca\(^{2+}\) efflux, the transfection controls produced significant response. Lastly, NCLX is an attractive extrusion candidate. To determine functional expression of NCLX, we monitored mitochondrial Ca\(^{2+}\) kinetics with Li\(^+\) substituted Na\(^+\) free recording buffer. Hippocampal neurons were sensitive to the substitution; the Li\(^+\) treatment completely blocked Ca\(^{2+}\) efflux from mitochondria. In contrast, in DRG neurons Na\(^+\) replacement with Li\(^+\) did not slow Ca\(^{2+}\) removal from the mitochondria.

**Introduction**

The biophysical characterization of mitochondrial calcium buffering comprises a half century’s worth of research performed in the absence of molecularly identified components. Mitochondrial calcium uptake has been described as a rapid process with high Ca\(^{2+}\) selectivity, second order kinetics, but low affinity requiring the micromolar cytosolic Ca\(^{2+}\) concentrations achieved during neuronal calcium transients and observed
within juxtaposition of the plasma membrane and endo/sarcoplasmic reticulum (Moreau et al., 2006; Putney and Thomas, 2006; Santo-Domingo and Demaurex, 2010; Szanda et al., 2006). This process is driven by the electrochemical gradient generated by the electron transport dependent proton shuttling across the IMM (Kapus et al., 1991). Matrix calcium concentrations may significantly surpass millimolar values while maintaining a relatively low concentration of bivalent free ionic calcium; the majority of matrix calcium is quickly sequestered as a phosphate bound calcium salt (Chalmers and Nicholls, 2003). Pharmacologically, mitochondrial calcium uptake is inhibited by the calcium channel blocker ruthenium red (RuR), as well as a more selective Ru360 (Kumfu et al., 2012).

Genomic analysis and RNA interference have identified a number of mitochondrial calcium uniporter (MCU) candidates. Knockdown of the gene calcium-binding atopy-related autoantigen 1 (CBARA1) attenuated mitochondrial calcium uptake in response to histamine induced Ca$^{2+}$ release from inositol 1,4,5-triphosphate (IP$_3$) sensitive Ca$^{2+}$ stores. Based on this observation, CBARA1 was re-termed mitochondrial uptake 1, MICU1 (Perocchi et al., 2010). Structurally, MICU1 is an unlikely uniporter; however, MICU1’s requirement in mitochondrial calcium uptake, localization to the IMM and its two Ca$^{2+}$ binding EF hand motifs paint it as a regulatory accessory subunit of the putative MCU. RNAi/histamine analysis and MICU1 association were then used as selection criteria, which identified coiled-coil domain containing 109a, ccdc109a. RNAi knockdown of ccdc109a significantly reduced mitochondrial calcium uptake induced by Ca$^{2+}$ release from IP$_3$-sensitive Ca$^{2+}$ stores. Ccdc109a is a two transmembrane domain containing, IMM protein with a proposed cation selection loop (Baughman et al., 2011; De Stefani et al., 2011). The stoichiometry of ccdc109a is yet to be determined; based on the simplicity of its structure, a multimer is expected. Due to current characterization, ccdc109a has been given the moniker of mitochondrial calcium uniporter, MCU. An isomer of MCU has been identified, ccdc109b. Sequence alignment has shown
divergence within the putative Ca\(^{2+}\) binding loop of ccdc109b, as well as the N- and C-termini; apart from sequence analysis, ccdc109b physiology is largely unexplored.

Many other MCU candidates have been proposed. Prior to the identification of MICU1, the MCU focus was placed on leucine-zipper, EF-hand containing transmembrane protein 1, Letm1. Letm1 was identified as a required component of epileptiform seizures in Wolf-Hirshhorn syndrome; however, Letm1 is an exchanger of controversial ion selectivity and unknown stoichiometry. Early characterization supported H\(^{+}/K^{+}\) exchange (Froschauer et al., 2005). Those that argue Letm1 mediates H\(^{+}/Ca^{2+}\) exchange have hypothesized a role in mitochondrial calcium uptake. The kinetics do not support the biophysical characterization describing mitochondria calcium uptake; Letm1 may still be a cog in the overall mitochondrial calcium uptake machine (Demaurex and Poburko, 2009; Nowikovsky et al., 2012). Other MCU candidates include the uncoupling proteins (UCPs) and IMM localized ryanodine receptors (mRyR). UCP candidacy is largely supported by a single research group. Their known proton leak function is counterintuitive to an MCU role; however, UCPs are important in ROS mediated mitochondrial uncoupling, which could be a critical component of calcium deregulation. mRyR is a much more attractive MCU candidate (Brookes et al., 2008; Paradis et al., 2003; Sun and Zemel, 2004). This IMM localized Ca\(^{2+}\) channel is inhibited by both ryanodine and ruthenium red. Biophysical analysis of mRyR has been limited to \textit{in vitro} studies in reconstituted membranes, but initial results suggest rapid, high affinity Ca\(^{2+}\) conduction (Ryu et al., 2011).

Like MCU, mitochondrial calcium efflux has been subject to biophysical characterization of ambiguous molecular players. This efflux is best observed in DRG neurons. [Ca\(^{2+}\)]\(_i\) transients in these neurons display an exaggerated plateau phase mid-recovery. This prolonged [Ca\(^{2+}\)]\(_i\) elevation is mitochondria dependent and attenuated via application of the mitochondria Na\(^{+}/Ca^{2+}\) exchange inhibitor CGP-37157. Indicated by CGP-37157 sensitivity, mitochondrial Ca\(^{2+}\) efflux is Na\(^{+}\) dependent (Czyz and
Based on this observed Na$^+$ dependence, NCLX and NCX3 are proposed components of calcium efflux. NCLX is unique among NCX and NCKX family members both in its structure and ability to transport Li$^+$. IMM localization of NCLX is well supported (Palty et al., 2006; Palty et al., 2004). NCX3 IMM localization is a subject of controversy. Additionally, NCX3 expression is tissue specific; molecules involved in mitochondrial calcium buffering may not be specifically ubiquitous throughout the anatomy (Gobbi et al., 2007). Other proposed efflux mechanisms include a reversal of the aforementioned Letm1 and DAG activated cation channels, DCC (Smrcka et al., 2012). Much progress has been made in the field of mitochondrial calcium buffering, but the actual identity of components, mechanism of action, and function in cellular physiology and pathology are still undetermined.

**Experimental procedures**

**MCU candidate cDNA and shRNA**

Mitochondrial calcium uniporter candidate cDNA expression constructs included: mouse MCU-turboGFP, human MCU-mRFP, human MCU-QIMQ-mCherry and mouse ccdc109b-mRFP (OriGENE); mouse NCLX-GFP, and mouse Letm1-GFP (Open Biosystems). Control empty vectors include pLKO (Open Biosystems) and pC-RS-scramble (OriGENE). Mitochondrial matrix calcium is reported via mtPericam in pcDNA3.1 expression. Neuro2A mouse cell line was transfected with TRPV1 for allowing a simple tool to induce Ca$^{2+}$ influx.

RNA intereferance constructs included: IR-MCU #04, -61, -62, -63, -69, -p3HA and –pmCherry (Mission); IR-ccdc109b #F1, -F2, -F3, and -F5 (Open Biosystems); IR-Letm1 #70 (Open Biosystems; IR-MICU # (Open Biosystems).
Analysis of RNAi efficacy

Confirmation of RNAi constructs was performed via western blot. Established MCU antibodies are not available; therefore, MCU-tGFP overexpression and knockdown was detected using anti-tGFP immunoreactivity. PC-12 cells were grown in RPMI buffer supplemented with 5% FBS, 10% horse serum and pen/strep on rat-tail collagen coated, plasma treated tissue culture plastic dishes. The cultures were co-transfected with 2µg MCU-tGFP and 2µg of the above noted IR-MCU constructs. Transfection was performed using an optimized Lipofectamine 2000 protocol similar to that described for Neuro2A cell culture described below. At 3 days post transfection, the cells were imaged using confocal microscopy (ex 488nm and em 525 (50) nm) with a 40X water immersion lens to confirm transfection efficiency and estimate knockdown. The PC-12 cells were then harvested and incubated in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 5mM EGTA, 5mM EDTA, 1% Triton, and 1X Halt protease inhibitor cocktail) for 30 minutes. SDS-PAGE was performed on the resulting lysate. Rabbit anti-tGFP was used against MCU-tGFP (secondary anti-rabbit HRP); mouse anti-Grp75 was used as loading control (secondary anti-mouse HRP). Densitometry analysis was performed generating MCU-tGFP/Grp75 ratios for each construct (ImageJ). One way ANOVA was performed with Bonferroni post-test to confirm significance of knockdown as compared to empty vector control.

Analysis of endogenous expression

Adult mice were anesthetized via isoflurane and euthanized by decapitation. Cerebral cortex, hippocampus, cerebellum, brainstem, spinal cord and DRG tissue samples were collected and preserved in TRIZOL. Total RNA was isolated using the Qaigen RNEasy RNA isolation kit (similar preparation was performed for Neuro2A cells, as well). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the Qaigen One-Step RT-PCR kit. Agarose gel electrophoresis was performed and
visualized via ethidium bromide. Sequence analysis of gel purified amplicons was performed by the University of Iowa DNA sequencing core facility.

Confocal microscopy and mitochondrial localization

Localization of MCU, ccdc109b and Letm1 were confirmed using fluorescent confocal microscopy. Visualization of these putative mitochondrial Ca$^{2+}$ uptake proteins was achieved via overexpression of fluorescent-tagged fusion constructs in dissociated DRG culture (described below). Mitochondria were identified using the far-red mitochondria dye MitoTracker 633 (5µM…excitation 633nm and emission 660nm barrier filter). Following MitoTracker 633 incubation, the neurons were briefly washed to improve background contrast. Live cell imaging of the above prepared cultures was performed using an Olympus BX61WI Fluoview scanning confocal microscope with 488nm, 545nm and 633nm excitation. Confocal Z-depth was chosen to include both neurite and soma mitochondria. Fluorescent overlays were created using ImageJ image alignment to confirm mitochondrial localization.

Primary DRG neuron culture

Primary culture of rat dorsal root ganglion (DRG) neurons was performed as previously described in Chapter III (Kim and Usachev, 2009). Primary culture of dissociated adult mouse DRG neurons was performed as previously described (Schnizler et al., 2008). Briefly, 4-8 week old adult C57Bl/6J mice (Jackson Labs, Maine) were anesthetized with 20% isoflurane and euthanized via decapitation. The spinal column was then dissected to expose the DRGs. For our electroporation protocol, DRG from three adult mice were collected and digested with 2 mg/mL pronase and 1 mg/mL collagenase in a solution of DMEM media supplemented with HEPES and Pen/Strep. The DRG were then dissociated into individual sensory neurons via fire polished Pasteur pipettes of decreasing diameter. Following electroporation protocol, the dissociated DRGs were then
plated at an optimized seeding density on glass coverslips coated with poly-O-ornithine and laminin. Cultures were maintained in DMEM media supplemented with NaHCO₃, 5% horse serum, 5% fetal bovine serum, insulin, NGF and Pen/Strep (DMEM serum). The cultures were incubated at 37°C and 10% CO₂. All experiments were performed 2-3 dissection/days post transfection.

**Transfection of DRG neurons**

Nucleofection type electroporation for rat DRG culture was performed as was described in Chapter III and according the manufacturers protocol (Lonza). Electroporation of adult mouse DRG culture has been optimized. Briefly, dissociated DRG neurons of three adult mice (4-8 weeks of age) were combined. Mild centrifugation and vacuum aspiration isolated the neuron pellet from the dissection media. The neuron pellet was then re-suspended in ‘adult mouse neuron’ electroporation solution and transferred to the nucleofection cuvette. Nucleofection protocols G-013 or O-003 were used (due to increased sensitivity, mouse DRG transfection favored the milder O-003 protocol). The transfection solution was then transferred to pre-warmed DMEM serum. Immediately following electroporation, the transfected neurons were incubated 5-10 minutes prior to re-suspension and plating.

**Maintenance of Neuro2A culture**

The Neuro2A cell line is derived from a spontaneous neuroblastoma from white mouse. Neuro2A cultures were grown on plastic, plasma treated 10cm cell culture dishes. For Ca²⁺ measurements, Neuro2A cells were grown on rat-tail collagen coated glass coverslips. Both populations of cells were maintained at 5% CO₂ and 37°C in high glucose DMEM culture media supplemented with 10% fetal bovine serum, 1X GlutaMAX and penicillin/streptomycin. Media was replaced by 50% twice per week. Passage was performed at ~80% confluency (determined by the initial appearance of
multiple cell layers). The cells were displaced from their substrate with 0.05% Trypsin/EDTA, washed and re-plated at optimized seeding density.

**Lipofectamine transfection of Neuro2A cells**

Neuro2A cultures were transfected at >50% confluency. Due to pen/strep toxicity with Lipofectamine 2000 transfection solution, the culture media was collected as conditioned media and replaced with antibiotic free culture media (half total volume, 800µL). For cultures on 2.5 cm coverslips, a transfection mixture of 800µL low serum OptiMEM solution with 2µg mtPericam Ca²⁺ reporter, 2µg TRPV1, 2µg of an experimental or control construct, and 4µL Lipofectamine 2000 transfection solution was prepared. The 800µL transfection mixture was then added to the antibiotic free 800µL culture and the cultures were incubated for 4-6 hours. Complete media replacement was performed with conditioned media supplemented 50% with fresh media.

**Fura2-based monitoring of cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ)**

For cytosolic Ca²⁺ imaging DRG neurons were loaded using the cell-permeant form of Fura2, Fura2-AM (Schnizler et al., 2008). Mouse and rat DRG cultures 2-3 DIV and Neuro2A cells 2-3 days post-transfection were be incubated at 22°C for 30 min in 4 µM Fura2/AM (Invitrogen) and 0.01% pluronic acid in 2 mL HH buffer. The coverslip was then secured in a flow-through chamber fed HH buffer via a gravity perfusion system and mounted on an IX-71 epifluorescence microscope (Olympus). Fura2 fluorescence was sequentially excited at 340 nm and 380 nm via a Polychrome IV monochromator (T.I.L.L. Photonics) and either a 20X dry lens or a 40Xoil immersion objective (Olympus). Fluorescent emission at 510 (80) nm was collected at 0.5 Hz sampling frequency by a Photonics IMAGO CCD camera coupled to TillVisION live acquisition software. The ratio (R) of background subtracted F340/F380 was converted to [Ca²⁺]ᵢ after dye calibration using the following equation: 

$$[Ca^{2+}]_i = K_d(R_{max}-R)/R_{min} - R) S_{f380}/S_{b380},$$

where $K_d = 5.5 \mu M$ and $S_{f380}/S_{b380} = F_{\text{max}} 380 \text{ nm}/F_{\text{max}} 380$
nm (Nelson et al., 2007). Absolute calcium conversion was not performed for Neur2A cells due to required calibration that has yet to be performed. Therefore all Neuro2A data is presented as the fluorescent ration 340nm/380nm.

**mtPericam soma recording**

Measurements of mitochondrial matrix Ca\(^{2+}\) changes were performed as described in Chapter III (Medvedeva et al., 2008). Briefly, mtPericam transfected DRG neurons and Neuro2A cell cultures were mounted for epifluorescent imaging (Olympus IX-71) using a 40X oil immersion objective. MtPericam recording was performed using fluorescent excitation at 410nm via a Polychrome IV monochomator (T.I.L.L. Photonics); emission was collected using 510 (80) nm with a Photonic IMAGO CCD camera coupled to TillVisION live acquisition software at 2x2 binning and 0.5Hz acquisition frequency. The mitochondrial Ca\(^{2+}\) concentration was quantified using the following equation: 

\[-(F-F_0)/(F_0-F_{\text{background}})\]

where F is the real time fluorescent intensity and F\(_0\) is the fluorescent intensity at baseline recording.

**DRG neuron stimulation protocol**

Cytosolic (Fura2) and mitochondrial (mtPericam) Ca\(^{2+}\) elevations in DRG neurons were elicited by applying depolarization of incremental amplitude using various concentrations of high K\(^+\) in extracellular solution (10mM, 15mM, 20mM, 30mM and 50mM KCl). Each concentration was applied for 30 seconds. Following stimulation, the culture was washed allowing adequate cytosolic and matrix Ca\(^{2+}\) recovery (requiring 10-15 minutes between stimulations).

**Neuro2A stimulation protocol**

Neuro2A cells were generated from pre-differentiated neuroblastoma cells lacking the plasma membrane composition for depolarization mediated excitability and Ca\(^{2+}\)
influx. Therefore, we transfected our Neuro2A cells with the Ca\(^{2+}\) conducting TRPV1. TRPV1 is gated by the exogenous chemical irritant capsaicin. Similar to our high K\(^{+}\) protocol, four concentrations of capsaicin were prepared with HH buffer (20nM, 50nM, 200nM, and 1µM capsaicin) to provide Ca\(^{2+}\) load of increasing magnitude. Capsaicin solutions were applied for 30 seconds, and sequential capsaicin applications were separated by 10-15 minutes allowing for complete Ca\(^{2+}\) recovery prior to subsequent capsaicin application.

**Analysis of cytosolic and mitochondrial matrix Ca\(^{2+}\) kinetics**

Average somal cytosolic and mitochondrial matrix Ca\(^{2+}\) recordings were plotted as a function of time in minutes. The peak amplitude (baseline subtracted amplitude) was determined and collected as an index of mitochondrial calcium uptake. The recovery duration was calculated as duration of time from the incidence of peak cytosolic Ca\(^{2+}\) (Fura2) to 90% recovery and peak mtPericam response to 50% recovery from said amplitudes. These amplitude values and recovery durations were calculated and presented as mean ± SEM.

**Statistical analysis**

Statistical analysis was performed using either Student’s \(t\)-test (with two comparisons) or one way analysis of variance (ANOVA…with multiple comparisons) with the Bonferroni post-test comparing all columns. All statistics were performed at 95% confidence.

**Results**

As was discussed in Chapter III, hippocampal neuron culture is limited in its practicality with regard to maturation time and transcription/viral transduction availability and efficacy. Therefore, molecular characterization of mitochondrial Ca\(^{2+}\)
transport components was performed in DRG neurons and mouse neuroblastoma cell line Neuro2A transfected with various expression constructs targeting MCU, ccdc109b or Letm1. Given limited availability of antibodies against the listed proteins, localization and western blot analysis of these mitochondrial calcium uptake and release candidates were performed using tGFP or mRFP fusion constructs of the corresponding proteins.

**MCU and ccdc109b localization and expression**

The majority of mitochondrial calcium buffering investigation is limited to the hepatic and cardiovascular systems. Characterization of MCU and ccdc109b expression in the nervous system is non-existent. To determine the presence and degree to which MCU and ccdc109b are transcribed, we isolated total RNA from six structures of the central and peripheral nervous systems from an adult C57Bl/6J mouse (8 weeks of age): cerebral cortex, hippocampus, cerebellum, brainstem, spinal cord, and dorsal root ganglion. RT-PCR was performed using two sets of primers per gene of interest; one set of primers was targeted at the 5’-half of the transcript and the other set against the 3’-half. MCU expression appeared consistent and comparable across all CNS and PNS tissues for both primer sets; GAPDH transcription was determined as control (Fig.4.1E). Ccdc109b displayed a much more complicated expression pattern. One of the first striking characteristics of ccdc109b RT-PCR is the presence of multiple bands. Preliminary sequencing analysis of a select amplicon that did not correspond to the predicted nucleotide length strongly supported the existence of post-transcriptional splice variation. As compared to the GAPDH control, both the banding pattern and intensity (thus the degree to which the gene is expressed) were not comparable among tissue sources. Specifically, ccdc109b transcription within the DRGs (PNS) was starkly different from that of the CNS tissues (Fig.4.1F). Subcellular localization of MCU and ccdc109b was performed using transient expression of MCU-tGFP and ccdc109b-mRFP in dissociated DRG neuron culture; mitochondria were visualized via the far-red
fluorescing MitoTracker 633 (Hallap et al., 2005). Co-localization of MCU, ccdc109b and mitochondria was observed within the DRG neurites and soma via confocal microscopy. Both MCU and ccdc109b clearly localized to the mitochondria (Fig.4.1A,B).

**Effects of MCU and ccdc109b overexpression on Ca^{2+} buffering**

To begin our investigation into the physiological roles of MCU and ccdc109b, we chose a model of overexpression in dissociated DRG neuron culture. The DRG cytosolic calcium transient ([Ca^{2+}]_i) is composed of a rapid peak response and an incomplete recovery followed by a prolonged elevated [Ca^{2+}]_i plateau that surpasses the duration of the stimuli. Rapid mitochondrial Ca^{2+} uptake establishes a cytosolic ceiling, while slow, prolonged Ca^{2+} efflux from the matrix is responsible for the distinct DRG [Ca^{2+}]_i plateau phase. The duration of the mitochondrial calcium load is the summation of the rate of mitochondrial Ca^{2+} extrusion and the total matrix Ca load. Therefore, to investigate the effect of MCU and ccdc109b on mitochondrial calcium buffering, we calculated the peak amplitude and the plateau recovery duration using the high affinity calcium indicator Fura2. Our depolarizing stimuli included a spectrum of high K^+ solutions (modified HH buffer): 10 mM, 15 mM, 20 mM, 30 mM and 50 mM K^+. Peak [Ca^{2+}]_i was consistently reduced in MCU overexpressing neurons for all five stimuli. Ccdc109b overexpression did not significantly affect [Ca^{2+}]_i response. Plateau duration was markedly prolonged for 50 mM K^+ responses in DRG neurons transfected with MCU, but not ccdc109b (Fig.4.2B,D). Milder stimuli showed no effect on recovery, 50 mM K^+ stimulation produced significantly prolonged plateau durations for MCU overexpressing DRG neurons as compared to both control and ccdc109b (Fig.4.2).

Further confirmation of the above observations was performed using the model of Neuro2A cells. Neuro2A stimulation via high K^+ depolarization did not reproduce any [Ca^{2+}]_i elevation in the majority of cells tested. In order to establish a reliable method for stimulating Neuro2A cells, these cells were transfected with the Ca^{2+} conducting receptor
TRPV1. Stimulation of TRPV1 transfected Neuro2A cells were performed with 30 second applications of 20 nM, 50 nM, 200 nM and 1 µM capsaicin/HH buffer solutions. 

$[Ca^{2+}]_i$ was measured using Fura2 imaging; the peak amplitude was calculated. The 20nM, 50nM and 200nM capsaicin stimuli reproduced the DRG MCU overexpression data. Overexpression of ccdc109b appeared to have a much greater buffering effect at the lower 20 nM and 50 nM capsaicin stimuli than what was observed in DRG neurons (Fig.4.3).

Direct analysis of mitochondrial calcium buffering kinetics was performed using mtPericam matrix $Ca^{2+}$ reporting. Similar to the single mitochondrial calcium recording protocol used in our MFF studies, mtPericam was co-transfected with ccdc109b-mRFP in DRG neurons (MCU-mRFP is a recent acquisition; we have yet to perform these experiments with MCU in DRGs). Using the same stimulation protocol as with Fura2 imaging, we observed both increased response incidence and amplitude for the lower K$^+$10 and 15 stimuli in cells transfected with ccdc109b (similar to experiments using MCU are underway). A trend towards reduced mitochondrial matrix response amplitude was observed for the 30mM and 50mM K$^+$ applications. Mitochondrial calcium recovery duration was reduced for the 50mM K$^+$ stimulation (Fig.4.4). Elevated mitochondrial $Ca^{2+}$ response with ccdc109b was observed with 20 nM capsaicin in Neuro2A cells. MCU mitochondrial calcium kinetics in Neuro2A cells is difficult to interpret and requires additional experiments (Fig.4.5).

**The effect of MCU knockdown on $Ca^{2+}$ signaling**

Ccdc109a (MCU) research is at its very beginnings, especially with neuronal physiology. Tools are limited and lack confirmation. We have acquired a number of potential shRNA constructs targeting MCU: IR-MCU #04, -62, -63, -69, -p3HA, and – pmCherry. To determine the efficacy of our shRNA panel, we chose western blot analysis of MCU-tGFP knockdown (with Grp75 as the loading control). Lipofectamine
2000 mediated co-transfection of MCU-tGFP and shRNA construct, or controls, was performed in PC-12 cells. Analysis of the transfected PC-12 lysates presented three strong candidate shRNAs: IR-MCU #69, -p3HA and pmCherry. 80-90% knockdown was achieved as compared to empty vector control (Fig.4.6).

IR-MCU #pmCherry is a bicistronic construct expressing the anti-mouse MCU shRNA sequence described in (Raffaello et al., 2012), with an mCherry reporter. Adult mouse DRG neurons were co-transfected with mtPericam and either IR-MCU #pmCherry or empty vector (Fig.4.7). Cytosolic calcium response was greatly elevated for 30 mM and 50 mM K+ invoked transients. This observation may be interpreted as reduced mitochondrial calcium buffering after silencing MCU. Direct analysis of mitochondrial Ca^{2+} changes supports our [Ca^{2+}]_i observations. Silencing MCU reduced the incidence of a mitochondrial Ca^{2+} response at mild KCl depolarization (Fig.4.8). Amplitude analysis revealed reduced mitochondrial calcium uptake after silencing MCU for small Ca^{2+} loads. My findings that silencing MCU preferentially reduced mitochondrial Ca^{2+} uptake for low but not high Ca^{2+} loads suggest existence of additional low-affinity Ca^{2+} transporter(s) contributing to mitochondrial Ca^{2+} uptake in neurons. Correlation analysis comparing the [Ca^{2+}]_i response versus mitochondrial calcium uptake reveals a rightward shift suggesting a loss of MCU sensitivity to [Ca^{2+}]_i. (Fig.4.9). These DRG neuron MCU knockdown results were reproduced in Neuro2A cells transfected with an alternative shRNA construct, IR-MCU#69 (Fig4.10). MCU silencing in Neuro2A cells produced elevated [Ca^{2+}]_i for 20nM and 50nM capsaicin stimuli. Analysis of mitochondrial Ca^{2+} uptake using mtPericam fluorescent imaging revealed significantly reduced matrix Ca^{2+} response amplitude. These results further support a role for MCU in mitochondrial Ca^{2+} transport and suggest the existence of additional low-affinity Ca^{2+} transport mechanisms.
Characterization of dominant negative MCU

De Stefani et al., 2011 reported an MCU double point mutation (D260Q,E263Q) that is proposed to function as a dominant negative. Again, MCU is expected to form a functional multimer of MCU-subunits. Expression of non-conducting MCU-QIMQ results in the formation of non-functional heteromers with endogenous MCU subunits. Theoretically, MCU-QIMQ will act as a dominant negative construct against ccdc109b, as well; thus, MCU-QIMQ should approximate the effect of MCU/ccdc109b double knockdown, and possibly with greater efficacy. Neuro2A cell cultures were transfected with MCU-QIMQ-mCherry, mtPericam and TRPV1 (control cells were transfected with pLKO empty vector in place of MCU dominant negative). At 2-3 days post transfection cytosolic and mtPericam Ca\textsuperscript{2+} changes were characterized using stimulation with incremental capsaicin concentrations described previously. Peak [Ca\textsuperscript{2+}]\textsubscript{i} response amplitude was significantly increased in the MCU-QIMQ transfected cells for the 200nM and 1µM capsaicin stimuli, as compared to control (Fig.4.11). This cytosolic index of impaired mitochondrial calcium buffering was confirmed via mtPericam imaging. For the 200nM and 1µM capsaicin stimuli, peak mitochondrial calcium uptake (estimated by the peak amplitude of the mtPericam response) was significantly attenuated. As predicted, this attenuation of mitochondrial calcium buffering was much greater than that achieved with shRNA knockdown of MCU, but the impaired uptake was not complete. At the milder 50nM capsaicin stimulation, MCU-QIMQ had no measureable effect (Fig.4.12).

Role of Letm1 mitochondrial Ca\textsuperscript{2+} transport

Candidates for mitochondrial calcium uptake are not limited to a putative uniporter. Leucine-zipper , EF-hand containing transmembrane protein 1 (Letm1) is a poorly characterized proton/cation exchanger identified in Wolf-Hirshhorn syndrome, WHS. Letm1 haplo-insufficiency is proposed to be a component of the epileptiform seizure mechanism in WHS. The hypothetical seizure pathophysiology supports Letm1 as
a mitochondrial targeted H+/K+ exchanger; however, work in *Drosophila* suggests H+/Ca\(^{2+}\) exchange. Basing their hypothesis on a very specific exchange stoichiometry, Nowikovsky et al. argue a role for Letm1 in high affinity mitochondrial calcium uptake (Demaurex and Poburko, 2009; Nowikovsky et al., 2012).

To begin our Letm1 characterization, we confirmed its localization to the mitochondria. Dissociated DRG neuron culture was transfected with Letm1-GFP. Prior to imaging, the neurons were stained with the mitochondrial membrane potential indicator TMRM\(^+\). Using confocal microscopy, Letm1-GFP co-localized with TMRM\(^+\) within the DRG soma and neurites (Fig4.13A). Analysis of Letm1’s contribution to mitochondrial Ca\(^{2+}\) was performed using the matrix calcium indicator mtPericam and Letm1 knockdown. Cultured DRG neurons were co-transfected with mtPericam and IR-Letm1#70 (initial control transfection was performed using a scramble control). At 2-3DIV/post-transfection, 410nm-mtPericam imaging was performed using an early, modified version of our high K\(^+\) protocol. The DRG neurons were treated with 10mM and 15mM K\(^+\) for 2 minute intervals, 20mM K\(^+\) for a 1 minute interval, and 30mM and 50mM K\(^+\) for 30 second durations. Analysis of cytosolic and mitochondrial Ca\(^{2+}\) response amplitude and recovery duration contradicted a role for Letm1 as MCU. Cytosolic calcium transient amplitude was not affected by Letm1 knockdown (Fig.4.13C); however, recovery duration was significantly attenuated following 50mM K\(^+\) (Fig.4.13D). MtPericam mitochondrial Ca\(^{2+}\) analysis in part supported these observations. Peak matrix Ca\(^{2+}\) response was elevated following both 20mM and 50mM K\(^+\); however, based on recovery data, the scramble control may have a strong physiological effect (Fig.4.14). These data suggest an efflux role for Letm1; however, thorough analysis of vector and scramble control contribution suggest potential artifact. Figure 4.15 summarizes [Ca\(^{2+}\)]\(_i\) analysis for a panel of vector controls in relation to Letm1 knockdown.
NCLX is a tissue specific mediator of mitochondrial calcium efflux

The mechanism and regulation of mitochondrial calcium efflux is of great interest. Our previous work with hippocampal neurons has shown modulation of neuronal sensitivity to excitotoxicity dependent on retention of calcium within the mitochondrial matrix. Preliminary characterization of mitochondrial Ca\(^{2+}\) buffering as a function of mitochondrial architecture and OMM reversible phosphorylation supports impaired matrix calcium recovery with Drp1 dephosphorylation and mitochondrial fragmentation; mitochondria elongation enhances matrix Ca\(^{2+}\) recovery. Together, these data propose regulation of mitochondrial calcium efflux. The molecular identity of the matrix Ca\(^{2+}\) extrusion machinery remains unknown. Letm1 is a candidate and NCX3 localization is a controversial topic (Gobbi et al., 2007). Recent studies identified NCLX as the molecule responsible for mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger in cell lines (Palty et al., 2010). However, expression of NCLX-tGFP in DRG neurons indicates broad distribution of NCLX throughout the cell, in contrast to distinct mitochondria localization of MCU, ccdc109b (Fig.4.1), and Letm1 (Fig.4.13).

A unique characteristic of NCLX cation exchange is the ability to transport Li\(^{+}\) in place of Na\(^{+}\) (Palty et al., 2006; Palty et al., 2004). All other members of the NCX and NCKX family are incapable of such exchange and are rendered non-functional by Na\(^{+}\) replacement with Li\(^{+}\). To determine the contribution of NCLX for mitochondrial calcium extrusion, we first transduced (FIV) rat hippocampal neurons with the matrix targeted calcium reporter mtPericam. Neurons were repeatedly stimulated with 30mM K\(^{+}\) depolarization for 30 seconds (modified HH buffer). Following two control stimulations, the Na\(^{+}\) in the bath solution was replaced with Li\(^{+}\). Given that Li\(^{+}\) passes through voltage-gated Na\(^{+}\) channels, it is expected that repeated K\(^{+}\)30 stimulation in Li\(^{+}\)-containing solution will lead to replacement of intracellular Na\(^{+}\) with Li\(^{+}\). Notably, such replacement blocked Ca\(^{2+}\) efflux from mitochondria in hippocampal neurons (Fig.4.16). The impaired
matrix Ca\(^{2+}\) recovery and toxicity could be due to inhibited Na\(^+\)/Ca\(^{2+}\) exchange at the plasma membrane resulting in a mild increase in \([\text{Ca}^{2+}]_i\) (Fig. 4.16A, black trace). To test this hypothesis, we mimicked the Li\(^+\) induced \([\text{Ca}^{2+}]_i\) elevation using a prolonged 15mM K\(^+\) treatment immediately following the 30 second 30mM K\(^+\) application. Despite the resulting mild increase in \([\text{Ca}^{2+}]_i\), this increase by itself did not inhibit Ca\(^{2+}\) efflux from mitochondria (Fig. 4.16B). These observations suggest that hippocampal neurons do not functionally express mitochondrial NCLX. In contrast, replacing Na\(^+\) with Li\(^+\) using the same approach as described above, did not slow mitochondrial Ca\(^{2+}\) efflux in DRG neurons (Fig. 4.17). As a positive control, replacing Na\(^+\) with choline blocked Ca\(^{2+}\) release from mitochondria. Thus, the described tolerance of the mitochondrial Ca\(^{2+}\) efflux to Li\(^+\) is consistent with NCLX involvement in this transport in DRG neurons.

**Discussion**

Mitochondrial Ca\(^{2+}\) buffering is a critical component in many physiologies. In the nervous system mitochondrial calcium uptake and extrusion regulates dehydrogenase function and cellular respiration, shapes neuronal Ca\(^{2+}\) transients, and modulates neuronal survival during excitotoxic insult. Over the last three decades, biophysical characterization has determined the general mechanism, kinetics and pharmacology of mitochondrial Ca\(^{2+}\) transport; the molecular identities of these Ca\(^{2+}\) transport systems had remained elusive. Recent progress has identified a number of candidate proteins. Mitochondrial calcium uptake candidates include: MICU1, MCU, UCPs, Letm1 and mRyR; current mitochondrial calcium extrusion candidates include: Letm1, NCX3, NCLX, and DCC. Confirmation and characterization of these Ca\(^{2+}\) transport components has the potential to identify therapeutic targets for a number of neurological conditions, including the pharmacological intervention following ischemic stroke.
Mitochondrial calcium uptake candidates

Prior to the start of our investigation, structural and functional characterization narrowed the potential field. Analysis of mitochondrial uptake 1 (MICU1) suggested a more regulatory role. Though MICU1 knockdown attenuated mitochondrial Ca\(^{2+}\) uptake, sequence analysis revealed calcium binding EF-hand motifs and the complete absence of pore forming domains (Perocchi et al., 2010). Uncoupling proteins (UCPs) have also been largely discounted by the majority of the field (Brookes et al., 2008). UCPs are primarily characterized as proton leak channels responsible for the uncoupling of mitochondrial oxidative phosphorylation. Support for UCPs as mitochondrial calcium uniporters was based on poorly controlled experiments. Lastly, mitochondrial ryanodine receptors (mRyRs) are an emerging field based entirely on in vitro biophysical analysis with purified receptors and reconstituted lipid membranes (Ryu et al., 2011). Therefore, we focused our interests on mitochondrial calcium uniporter (MCU) and leucine-zipper, EF-hand containing transmembrane protein 1 (Letm1).

Mitochondrial Ca\(^{2+}\) uniporter (MCU)

Originally identified as coiled-coil domain containing 109a (ccdc109a), MCU has been proposed to be the putative mitochondrial Ca\(^{2+}\) uniporter. Preliminary characterization by the Rizzuto and Mootha research groups support MCU as the primary mitochondrial calcium uptake mechanism in several cell lines (Baughman et al., 2011; De Stefani et al., 2011). In this study, we have confirmed localization of MCU to the mitochondria and robust transcription of MCU in a number of CNS and PNS tissues including cerebral cortex, hippocampus, cerebellum, brainstem, spinal cord, and dorsal root ganglion (DRG). As mentioned above, early characterization of MCU function was based on histamine induced Ca\(^{2+}\) released from IP\(_3\)-sensitive stores as the main source of Ca\(^{2+}\) for mitochondrial Ca\(^{2+}\) uptake. Instead, in our model mitochondrial Ca\(^{2+}\) uptake was induced by various degrees of Ca\(^{2+}\) load achieved via Ca\(^{2+}\) entry through TRPV1.
(Neuro2A cells) or voltage-gated Ca\textsuperscript{2+} channels in DRG neurons. Overexpression of MCU reduced the amplitude of the [Ca\textsuperscript{2+}]\textsubscript{i} response and prolonged the duration of the subsequent [Ca\textsuperscript{2+}]\textsubscript{i} plateau. These observations support the MCU involvement in mitochondrial Ca\textsuperscript{2+} uptake in DRG neurons. At the same time, our mtPericam measurements showed that MCU silencing significantly reduces Ca\textsuperscript{2+} uptake by mitochondria for low, but not high Ca\textsuperscript{2+} load. These data suggest that MCU is responsible for high-affinity Ca\textsuperscript{2+} uptake in mitochondria, and also point to an existence of additional, likely low-affinity mitochondrial Ca\textsuperscript{2+} transport systems.

**MCU isoform ccdc109b**

Genomic analysis of MCU in skin keratinocytes has revealed an MCU paralogue of significant sequence homology. Termed ccdc109b, this MCU isomer contains a substitution of one of the acid residues within the inter-transmembrane loop, as well as reduced homology in the N- and C-termini. Localization analysis, like MCU, illustrates ccdc109b expression at the mitochondria; transcription characterization proved more complicated. Similar to our MCU RT-PCR protocol, we chose primers targeting the 3’-half and the 5’-half. Initial observations noted stark differences in expression that contrast that of MCU. Closer analysis reveals multiple bands, which suggest tissue specific expression of ccdc109b splice variants. Sequence analysis of a non-predicted hippocampal amplicon supports this hypothesis with what appears to be a possible exon deletion. All of this genetic complexity in relation to MCU makes ccdc109b a mysterious beast. We hypothesize that ccdc109b has a regulatory role as an MCU chaperone or a dominant negative in heteromers. Overexpression of ccdc109b in DRG neurons and Neuro2A cells produced unremarkable effects on the shaping of cytosolic Ca\textsuperscript{2+} transients. However, modest trends suggest a biphasic role in mitochondrial calcium uptake. Overexpression of ccdc109b increased the incidence and amplitude of measurable matrix Ca\textsuperscript{2+} uptake with mild stimuli (Fig.4.4); just the same, Calcium uptake induced by K\textsuperscript{+}30
and K⁺50 depolarization trended towards a reduction (Fig.4.8). Additional studies will be required to determine the exact role of ccdc109b in mitochondrial Ca²⁺ transport.

**Mitochondrial calcium efflux candidates**

Mitochondrial calcium efflux is proposed to be primarily sodium dependent and selectively inhibited by CGP-37157 (Baron and Thayer, 1997). Current candidates include the Na⁺/Ca²⁺ exchangers NCX3 and NCLX. Tissue specificity has been observed with NCX3 (specifically regarding the nervous and cardiovascular systems). NCX3 targeting to the IMM remains a controversial issue, with many reports limiting its expression to the OMM (Gobbi et al., 2007). NCLX targeting to the IMM is well accepted (Palty et al., 2010). Alternative efflux mechanisms include Letm1 (which we have already addressed) and the DAG activated cation channel, DCC (Smrcka et al., 2012). DCC has the potential to complicate previous work in the mitochondrial buffering community. Mentioned previously, mitochondrial calcium buffering has been chiefly characterized using a PLC dependent model of ER calcium release. PLC catalyzes the hydrolysis of PIP2 into IP3 and DAG. IP3 activates the Ca²⁺ conducting ER channel IP3R; this mobilization of internal stores has served as a calcium source in the majority of mitochondrial buffering studies. DAG has not been discussed as a contributing variable. DCC research is extremely limited and its role in mitochondrial calcium efflux is speculative.

**NCLX functional tissue specificity**

NCLX is a unique member of the NCX and NCKX families, exclusively capable of transporting Li⁺ in place of Na⁺. Sodium replacement inactivates all other members of the NCX and NCKX families and presents a powerful tool in the identification of NCLX contribution (Palty et al., 2006; Palty et al., 2004). We began our studies with NCLX localization using the same fluor-tagged overexpression and confocal microscopy
protocol used previously for MCU and ccdc109b. NCLX overexpression produced a diffuse, cytosolic signal. A diffuse cytosolic signal from a known membrane targeting protein suggests protease activity liberating the fluorescent tag. Antibodies are available for NCLX (SLC24A6) that may be used; however, electron microscopy confirming localization is well accepted. Due to this diffuse signal, we could not reliably determine the physiological consequences NCLX overexpression.

To determine a functional role for NCLX in neuronal physiology, we performed Na\(^+\) replacement experiments monitoring cytosolic and mitochondrial matrix calcium in DRG and hippocampal neurons. Hippocampal neurons proved to be sensitive to the Na\(^+\)/Li\(^+\) replacement at both the plasma membrane and mitochondria; recovery was attenuated in both compartments. A prolonged, mild depolarization approximately mimicking the persistent cytosolic calcium failed to reproduce the perturbed matrix recovery. Therefore, NCLX is not functionally expressed in hippocampal neurons. DRG neurons proved capable of Li\(^+\) exchange. We hypothesize the initial reduction and gradual correction of the mitochondrial calcium response is due to a delay in Na\(^+\) replacement within the cytosol. Once an equivalent concentration of Li\(^+\) is achieved, kinetically normal NCLX function resumes. Additionally, NCLX Li\(^+\)/Ca\(^{2+}\) exchange kinetics may differ from Na\(^+\)/Ca\(^{2+}\).

In summary, our studies suggest that MCU and its novel isoform ccdc109b contribute to the high-affinity mode of mitochondrial Ca\(^{2+}\) uptake. Our findings also suggest that MCU is not the only molecule mediating Ca\(^{2+}\) influx into mitochondria. Finally, our results indicate that different neuronal populations may utilize distinct Ca\(^{2+}\) efflux mechanisms from mitochondria (e.g., NCLX in DRG neurons, and a system different from NCLX in hippocampal neurons).
Figure 4.1. MCU and ccdc109b localization and expression. **A-D.** MCU and ccdc109b localization as assessed via transient expression of fluorescent tagged constructs: MCU-tGFP and ccdc109b-mRFP. Visualization of the mitochondria was confirmed via MitoTracker Deep Red (633nm) mitochondrial dye. Analysis of colocalization was performed using ImageJ software. **E,F.** MCU (E) and ccdc109b (F) RT-PCR were performed using two sets of primers; Set1 recognizes and amplifies the 3’ half of the full length transcript and Set2 amplified the 5’ half. Loading and expression control were performed using GAPDH amplification. Total RNA was collected from 8 week adult C57Bl/6J mouse. Tissue sources include the cerebellum (Cer), cerebral cortex (Cort), DRGs, hippocampus (Hippo), spinal cord (Spine) and brain stem (Stem).
Figure 4.2. MCU overexpression prolonged the mitochondria-dependent DRG Ca\(^{2+}\) transient plateau. A. Sequence analysis reveals a key amino acid substitution in ccdc109b as compared to MCU. This glutamine to valine substitution is ccdc109b may impair Ca\(^{2+}\) conduction and mitochondrial Ca\(^{2+}\) uptake function. B. MCU-tGFP and ccdc109b-mRFP were co-expressed in DRG neuron culture. At 2-3DIV cytosolic Ca\(^{2+}\) imaging was performed using various levels of depolarization: 10mM, 15mM, 20mM, 30mM and 50mM KCl. C. Peak cytosolic [Ca\(^{2+}\)]\(_i\) amplitude was modestly reduced with MCU overexpression. D. DRG neurons produce a mitochondria-dependent [Ca\(^{2+}\)]\(_i\) plateau phase following incomplete recovery of the [Ca\(^{2+}\)]\(_i\) transient. The duration of the plateau phase reflects both the level of mitochondrial calcium accumulation and the rate of efflux. Control n=14, MCU n=8, and ccdc109b n=7. Peak cytosolic [Ca\(^{2+}\)]\(_i\) and 90% recovery duration are presented at mean ± standard error. Statistical analysis was performed using one way ANOVA with Bonferroni post-test. * P<0.05, ** P<0.01 at 95% confidence.
Figure 4.3. Reduced cytosolic $[\text{Ca}^{2+}]_i$ response in Neuro2A cells overexpressing MCU. 

A. Neuro2A cells were transfected with mtPericam, TRPV1, and MCU-mRFP or ccdc109b-mRFP or empty vector. 

B. The cytosolic $[\text{Ca}^{2+}]_i$ response to 20nM, 50nM, 200nM and 1µM capsaicin was recorded using Fura2 calcium imaging. The fura2 340nm/380nm fluorescence ratio was used as a measure of $[\text{Ca}^{2+}]_i$. Control n=65, MCU n=20, and ccdc109b n=35. Data are presented as mean ± standard error. Statistics were performed using one way ANOVA with Bonferroni post-test. * P<0.05, ** P<0.01, *** P<0.005 at 95% confidence. These experiments were performed by Kubat Rahatbek and Yelena Shabelnick.
Figure 4.4. Ccdc109b increases Ca²⁺ sensitivity of mitochondrial Ca²⁺ uptake and enhances recovery during a large Ca²⁺ response in DRG neurons. A. Sample mtPericam recording of ccdc109b and empty vector transfected rat DRG neurons. B. The response frequency for mild stimuli is increased with ccdc109b overexpression. C. Peak mitochondrial Ca²⁺ elevation was modestly reduced with ccdc109b expression. D. 50nM K⁺ depolarization distinguishes ccdc109b matrix Ca²⁺ recovery duration from control. Ccdc109b matrix recovery appears disproportionate to mitochondrial calcium uptake. Control n=15 and ccdc109b n=9. Percent responders were recorded as the number of neurons producing a measurable matrix Ca²⁺ as a percentage of total neurons in the field. Relative mtPericam fluorescence and recovery duration (in minutes) are presented as mean ± standard error. Statistics were performed using Student’s t-test. * P<0.05 at 95% confidence.
Figure 4.5. MCU and ccdc109b overexpression increase mitochondrial sensitivity to lower concentrations of cytosolic Ca$^{2+}$ in Neuro2A cells. A. The number of Neuro2A cells presenting measureable mitochondrial matrix calcium response to 20nM capsaicin stimulation was greatly increased. B. This observation is observed in the peak matrix Ca$^{2+}$ amplitude. Control n=61, MCU n=24, and ccdc109b n=29. Percentage of responding cells was recorded as the number of Neuro2A producing a measurable matrix Ca$^{2+}$ as a percentage of total neurons in the field. Relative mtPericam fluorescence is presented as mean ± standard error. Statistics were performed using one way ANOVA with Bonferroni post-test. *** P<0.005 at 95% confidence. These experiments were performed by Kubat Rahatbek and Yelena Shabelnik.
Figure 4.6 Evaluation of candidate mouse MCU shRNA constructs. Cultured PC-12 cells were co-transfected with MCU-tGFP and a candidate shRNA or control vector (Empty = pLKO.1; None = MCU-tGFP transfection only). 3 days post transfection, cell lysates were collected. Western blot analysis was performed using anti-rabbit tGFP to detect MCU-tGFP protein level. Grp75 immunoreactivity was performed as loading control. Densitometry analysis (normalized to Grp75) confirms 80-90% knockdown efficiency with IR-MCU#69, IR-MCU#p3HA and IR-MCU#pmCherry. For all transfection populations n=2. Relative expression is presented as mean ± standard error.
Figure 4.7. MCU knockdown increases cytosolic peak calcium response following strong depolarization in cultured mouse DRG neurons. 

A. Sample cytosolic $[\text{Ca}^{2+}]_i$ recording (Fura2) from cultured adult mouse DRG neurons co-transfected with mtPericam and IR-MCU#pmCherry or empty pLKO vector. Fura2 imaging was performed 2-3 post-transfection.

B. Peak cytosolic $[\text{Ca}^{2+}]_i$ was increased in only the MCU knockdown following strong depolarization, 30mM and 50mM K$. C. Cytosolic calcium plateau duration was comparable among the groups. Control n=5 and IR-MCU n=5. Peak cytosolic calcium and 90% recovery duration (minutes) are presented as mean ± standard error. Statistics were performed using one way Student’s t-test. * P<0.05 at 95% confidence.
Figure 4.8. MCU knockdown in adult mouse DRG neurons produced modest results on mitochondrial calcium uptake following moderate depolarization. A. Sample matrix Ca\(^{2+}\) response to a spectrum of depolarizing high K\(^+\) stimuli. Cultured adult mouse DRG neurons were co-transfected as described for Fig.4.7. B. MCU knockdown reduced the number of neurons displaying measurable matrix Ca\(^{2+}\) response. C. IR-MCU#pmCherry produced modest results following K\(^+\)20 and K\(^+\)30 depolarization. D. Matrix Ca\(^{2+}\) recovery was unremarkable (recovery was calculated at 50% peak amplitude). Control n=10 and IR-MCU n=6. Peak free matrix calcium and 50% recovery duration (minutes) are presented as mean ± standard error. Statistics were performed using Student’s t-test. * P<0.05 at 95% confidence.
Figure 4.9. Correlation between cytosolic calcium ([Ca$_{2+}$]$_i$ (nM) and mitochondrial calcium reporter mtPericam ((-f-f$_0$)/(f$_0$-f$_{background}$)). IR-MCU#pmCherry appears to significantly reduce the affinity of mitochondrial Ca$_{2+}$ uptake for cytosolic calcium.
Figure 4.10. MCU knockdown in Neuro2A cells reduces high-affinity mitochondrial Ca\(^{2+}\) uptake. A. Neuro2A cultures were transfected with either MCU targeted shRNA IR-MCU#69 or control pLKO empty vector. Incremental stimulation was performed as previously described using 20nM, 50nM, 200nM and 1µM capsaicin concentrations. 20 and 30nM capsaicin stimuli significantly increased the \([\text{Ca}^{2+}]\) response in Neuro2A cells with silenced MCU expression. B. Mitochondrial Ca\(^{2+}\) uptake was measured via mtPericam, which resulted in reduced mitochondrial Ca\(^{2+}\) response in the MCU knockdown population. \([\text{Ca}^{2+}]\) and mitochondrial calcium responses are presented as mean ± SEM. Statistics were performed using Student’s t-test. * P<0.05, ** P<0.01, *** P<0.005 with 95% confidence. Performed by Zhihong Lin.
Figure 4.11. Increased cytosolic calcium response in Neuro2A cells transfected with MCU-QIMQ following strong stimulation. A. Dominant negative MCU-QIMQ (D260Q,E263Q...Red) colocalizes with mitochondria targeted mtPericam (Green). B. Sample recording of $[Ca^{2+}]_i$ (Fura2) in Neuro2A cells transfected with mitochondrial calcium reporter mtPericam, TRPV1 and either pLKO empty vector or MCU-QIMQ-D/N. Stimulation performed using 20nM, 50nM, 200nM and 1000nM capsaicin. C. Following 200nM and 1000nM capsaicin, Neuro2A cells expressing the dominant negative MCU-QIMQ produced significantly larger cytosolic calcium responses. Control n=46 and MCU-QIMQ n=53. Peak $[Ca^{2+}]_i$ and 90% recovery duration (minutes) are presented as mean ± SEM. Statistics were performed using Student’s $t$-test. * P<0.05 and *** P<0.005 at 95% confidence.
Figure 4.12. Mitochondrial Ca\(^{2+}\) uptake is significantly reduced in Neuro2A cells expressing MCU-D/N (QIMQ). A. Sample mtPericam recording of control and MCU-D/N transfected Neuro2A cells. B. MCU-D/N expression limited the number of responding cells for across all stimuli. C. Peak matrix Ca\(^{2+}\) response was robustly reduced via MCU-D/N expression. Control n=42 and MCU-QIMQ n=32. Peak [Ca\(^{2+}\)]\(_i\) and 90% recovery duration (minutes) are presented as mean ± SEM. Statistics were performed using Student’s t-test. * P<0.05 and *** P<0.005 at 95% confidence.
Figure 4.13. Letm1 knockdown modestly reduces mitochondrial Ca\(^{2+}\) recovery duration following strong depolarization in DRG neurons. A. Neonatal rat DRG cultured neurons were transfected with Letm1-GFP. 24 hours post transfection, the neurons were stained with the mitochondrial Ψ\(_{mt}\) dye TMRM\(^+\) and Letm1 localization was determined via confocal microscopy. B. To characterize the role of Letm1 in [Ca\(^{2+}\)]\(_i\) signaling, DRG neurons were transfected with shRNA targeted for Letm1 (IR-Letm1#70) knockdown and mtPericam. Control neurons were transfected with a scramble control. Incremental depolarization was performed as follows: 10mM K\(^+\) for 2 min, 15mM K\(^+\) for 2 min, 20mM K\(^+\) for 1 min, 30mM K\(^+\) for 30 min, and 50mM K\(^+\) for 30 min. C,D. Letm1 knockdown did not effect the amplitude of the [Ca\(^{2+}\)]\(_i\) response; however, the K\(^+\)50 depolarization appears to be much more transient with Letm1 knockdown. Control n=5-10 and Letm1 = 4-8. Peak cytosolic calcium and 90% recovery duration (minutes) are presented as mean ± standard error. Statistics were performed using Student’s t-test. * P<0.05 and *** P<0.005 at 95% confidence.
Figure 4.14. Letm1 knockdown produced inconsistent matrix Ca$^{2+}$ affects. A. mtPericam imaging of IR-letm1#70 transfect DRG neurons was performed using the incremental high K+ stimuli described in Fig.4.13. Control neurons were transfected with scramble control. B. Letm1 knockdown appears to increase peak matrix calcium for the K$^{+}$20 and K$^{+}$50 stimuli. This would indicate a role in mitochondrial Ca$^{2+}$ extrusion, but further investigation is required. C. Free matrix Ca$^{2+}$ recovery was not affected by Letm1 knockdown. Control n=7 and IR-letm1#70 n=12. Peak mitochondrial matrix Ca$^{2+}$ and 50% recovery duration (minutes) are presented as mean ± SEM. Statistics were performed using Student’s t-test. * P<0.05 at 95% confidence.
Figure 4.15. Letm1 shRNA control vector $[\text{Ca}^{2+}]_i$ artifact. Letm1 shRNA constructs were acquired from OriGENE Technologies and were expressed using the mRFP reporting OriGENE pRFP-C-RS RNAi delivery vectors. Initial pRFP-Scramble control construct produced $[\text{Ca}^{2+}]_i$ responses comparable to that of mtPericam-only and non-transfected control DRG neurons. Further analysis of the empty vector (pRFP-C-RS) and the non-fluor reporting pRS produced $[\text{Ca}^{2+}]_i$ responses of reduced amplitude. The pRFP-C-RS and pRS vector affects makes IR-Letm1#70 interpretation difficult.
Figure 4.16. Hippocampal neurons are resistant to Na\(^+\)/Li\(^+\) substitution. A. To test for functional expression of NCLX, the extracellular solution was replaced with a Na\(^+\) free/Li\(^+\) substitution solution. The neurons were depolarized with 30mM K\(^+\). Both mitochondrial and cytosolic Ca\(^{2+}\) failed to completely recover in with Li\(^+\) external solution. B. To determine if the mitochondrial Ca\(^{2+}\) extrusion attenuation observed with Li\(^+\) substitution was due to the absence of functional NCLX or the persistent cytosolic Ca\(^{2+}\) elevation, a mild and prolonged depolarization (15mM K\(^+\)) was performed to approximate the persistent [Ca\(^{2+}\)]\(_i\) elevation. Free matrix Ca\(^{2+}\) sufficiently recovered.
Figure 4.17. Functional expression and regulation of DRG mitochondrial NCLX. A. Mitochondrial matrix Ca\textsuperscript{2+} responses persisted with rapid recovery following 30mM K\textsuperscript{+} depolarization in Li\textsuperscript{+} substituted recording buffer. Negative control recording was performed using Ca\textsuperscript{2+} free choline buffer, which cannot be transported by NCX and NCKX family members. Loss of mitochondrial matrix Ca\textsuperscript{2+} recovery was modestly restored with Li\textsuperscript{+} substituted recording buffer.
CHAPTER V
DISCUSSION AND FUTURE DIRECTION

In my pre-doctoral thesis work, I have studied the pathophysiology of excitotoxicity as a function of mitochondrial morphology and mitochondrial calcium buffering. I utilized the low affinity Ca\(^{2+}\) indicator FuraFF for cytosolic Ca\(^{2+}\) imaging in cultured hippocampal neurons to characterize the development of calcium dyshomeostasis during glutamate toxicity. This delayed calcium deregulation (DCD) is composed of three phases: 1) an initial glutamate response; 2) a latent period phase; and 3) complete release of mitochondrial calcium content. The duration of the latent period is an index of neuronal sensitivity to excitotoxicity. I found that phosphoregulation at the OMM modulates latent period duration; PKA/AKAP1 dependent phosphorylation at the OMM prolonged DCD latency, while PP2A/B\(\beta\)2 sensitized neurons to DCD. PP2A/B\(\beta\)2 activity also enhanced mitochondrial calcium load. Retention of matrix calcium may be a consequence of enhance uptake or impaired extrusion.

Characterization of mitochondrial Ca\(^{2+}\) transport was performed in cultured DRG neurons. PKA/AKAP1 and PP2A/B\(\beta\)2 regulate mitochondrial fission and fusion via phosphoregulation of the fission machinery component Drp1 at serine 656. Direct manipulation of MFF by Drp1 overexpression (fragmentation) and Drp1-K38A dominant negative (elongation) modulated matrix Ca\(^{2+}\) recovery; fragmentation significantly impaired mitochondrial calcium recovery and elongation enhanced it. OMM phosphoregulation produced more moderate results. The Strack and McKnight research groups have developed B\(\beta\)2 and AKAP1 knockout mouse lines, respectively. The shortcomings of transfection and transduction can be overcome using these knockout models, producing more reliable results. The follow-up studies are underway of using electron microscopy X-ray microanalysis of total mitochondrial Ca content in hippocampal neurons of AKAP1 and B\(\beta\)2 KO mice.
Based on our DCD results and individual mitochondrial calcium buffering kinetics data, mitochondrial uptake and release are critical components of neuronal pathology and physiology. The molecular identities of the buffering components remain elusive. Several candidates have been identified for both mitochondrial Ca\(^{2+}\) uptake and extrusion. Of the proposed influx components, mitochondrial calcium uniporter (MCU) is the most attractive candidate. Previous work that identified MCU as the putative mitochondrial uniporter utilized mobilization of ER calcium stores. We have characterized the role of MCU in DRG neurons and Neuro2A cells during a transient calcium response evoked by high K\(^+\) depolarization and TRPV1/capsaicin, respectively. Though overexpression clearly supports a role for MCU in mitochondrial calcium uptake, MCU RNAi knockdown and matrix calcium imaging suggest that MCU is not the only mitochondrial Ca\(^{2+}\) uptake mechanism. Ccdc109b sequence analysis revealed divergence from MCU that suggest a possible MCU regulatory role. Real time PCR analysis of ccdc109b transcripts using multiple combinations of primers also support tissue specific splice variation that further complicate ccdc109b physiology.

**Phosphoregulation of DCD sensitivity and MFF**

Regulation of PKA and PP2A activity at the OMM has proven to be a powerful modulator of neuronal sensitivity to excitotoxicity. Strack et al. have described phosphoregulation of mitochondrial fission and fusion via the same signaling components (Cribbs and Strack, 2007; Dagda et al., 2008). Briefly, manipulation of AKAP1 expression at the OMM promotes PKA dependent phosphorylation and inhibition of the fission component Drp1 at serine 656; expression of the PP2A regulatory subunit Bβ2 promotes dephosphorylation of Drp1. Bβ2 knockout mice display mitochondrial elongation in the hippocampus and cortex. AKAP1 knockout mice have also been acquired (Newhall et al., 2006). Preliminary electron microscopy analysis show significant mitochondrial fragmentation in hippocampal neurons. We have yet to
determine the consequence of AKAP1 knockout on neuronal sensitivity, but the hypothesis is rapid onset of Ca$^{2+}$ deregulation. Endpoint/cell death analysis has confirmed neuroprotective roles for both OMM phosphorylation of Drp1 and mitochondrial elongation. Likewise, we must confirm the requirement of Drp1 S656p on delayed calcium deregulation using non-phosphorylatable Drp1-S656A and phosphomimetic Drp1-S656D rescue. Additionally, all of our current results are dependent on chronic manipulation of OMM phosphorylation component and MFF. This chronic modulation can have global/developmental consequences on neuronal physiology. To determine the acute effects of mitochondrial elongation on the progression of calcium dyshomeostasis we will transiently inhibit mitochondrial fragmentation utilizing the Drp1 inhibitor mdivi-1 (Cassidy-Stone et al., 2008; Tanaka and Youle, 2008).

**Mitochondrial calcium load**

We have just begun to determine the effects of OMM phosphorylation on mitochondrial calcium sequestration. Bβ2 overexpression robustly increased matrix calcium load. These experiments rely on FCCP dependent decoupling and low affinity calcium imaging of individual neurons; this approach is time consuming and impractical for our DCD protocol. We have established a collaboration with Brian Andrews at the National Institutes of Health. A powerful specialty of the Andrews lab is electron probe X-ray microanalysis (EPMA); in brief, cultured hippocampal neurons are cryogenically frozen, sectioned and electron microscopy is performed at liquid nitrogen temperatures. The electron beam is focused on individual mitochondria and X-ray composition of the resulting energy dispersion is collected as a spectrum. This spectrum is accurately represents the composition and concentration of all ions within the structure of interest (Pivovarova et al., 1999). Using this technique, we will be able to select key time points during glutamate toxicity and determine the calcium composition of individual mitochondria for an entire field of neurons. We will perform EPMA on wild type, Bβ2
knockout and AKAP1 knockout cultures and control cultures treated with forskolin and rolipram.

**ROS production and decoupling during glutamate toxicity**

Calcium deregulation is preceded by mitochondrial decoupling, represented as rapid $\Delta \Psi_{\text{mt}}$ depolarization. We are able to measure $\Delta \Psi_{\text{mt}}$ using Rhodamine 123 (Rho123); this technique (in combination with Fura-FF imaging) will be performed on Bβ2 and AKAP1 knockout cultures, as well as cultures treated with Forskolin and rolipram. With this protocol we can determine the decline of $\Delta \Psi_{\text{mt}}$ during the DCD latent period as a function of OMM phosphorylation. One possible mechanism for this uncoupling is the generation of reactive oxygen species and regulation of mitochondrial uncoupling proteins. Mitochondrial ROS production (specifically superoxide) can be visualized via MitoSOX. Utilizing a specific dichroic filter set, MitoSOX imaging is compatible with simultaneous Fura-FF imaging. The specific requirement of ROS in the development of DCD may then be investigated using pharmacological scavengers or genetic manipulation of superoxide dismutase (SOD) expression (Atlante et al., 2001; Liang et al., 2009; Zimmerman et al., 2007).

Reactive oxygen species regulate the function of mitochondrial uncoupling proteins (UCPs). During ROS development, activation of UCPs results in the neuroprotective uncoupling of the mitochondrial proton circuit in an attempt to relieve oxidative stress (Echtay, 2007; Liu et al., 2006; Paradis et al., 2003). This uncoupling could also be the mechanism of $\Delta \Psi_{\text{mt}}$ depolarization during glutamate toxicity and the DCD latent period, and would implicate UCPs as a possible therapeutic target. General inhibition of UCPs is performed via guanosine diphosphate (GDP) and some members of the UCP family can be selectively targeted (UCP2 inhibition via genipin). Alternatively, genetic manipulation of UCPs may be performed utilizing RNAi knockdown (Dejean et al., 2004; Hoang et al., 2012; Trenker et al., 2007; Turner et al., 2010).
PKA and cAMP signaling compartmentalization

In our attempt to reproduce the neuroprotective effects of PKA/AKAP1 via pharmacological activation of adenylyl cyclase (AC), we discovered a PDE4 inhibition requirement (rolipram). PDEs hydrolyze the second messenger cAMP into the non-responsive AMP. Localization of PDEs thus regulate cAMP/PKA signaling within specific subcellular compartments. To visualize the regulation of cAMP/PKA signaling at the OMM, we will utilize the FRET based cAMP and PKA reporters ICUE3 and AKAR3, respectively. We have cytosolic, mitochondrial, and plasma membrane targeted ICUE3 and AKAR3 (Allen and Zhang, 2006; del Puerto et al., 2012). Using these tools, we will be able to screen AC activators and PDEs inhibitor in an effort to determine a combination that optimizes cAMP/PKA activity at the OMM.

Mitochondrial permeability transition and DCD

Inhibition of cyclophilin D by cyclosporine A (and thus inhibition of MPT) failed to prolong DCD latency, but this observation is not an absolute indicator of the absence of MPT. MPT, like mitochondrial calcium buffering, is an ill-defined component of mitochondrial physiology. The composition of the permeability transition pore (PTP) is a subject of speculation. In addition to cyclophilin D, the Bcl-2 family member BAD is a proposed positive regulator of PTP formation. PKA mediated phosphorylation of BAD at a number of residues inhibits its hypothetical MPT function (Harada et al., 1999). The PKA/AKAP1 observations may be mediated by BAD regulation and not Drp1 regulation. We will determine the contribution of BAD phosphorylation in PKA/AKAP1 neuroprotection via expression of non-phosphorylatable BAD mutants provided by Green et al. Other methods may be used to visualize MPT. Mobilization of a number of fluorescent-tagged apoptosis mediators (cytochrome C, Smac/Diablo etc.) may be used as a positive marker of PTP formation (De Oliveira et al., 2006).
Acute vs chronic regulation of MFF and mitochondrial calcium buffering kinetics

Genetic manipulation of MFF proved to be a powerful regulator of mitochondrial Ca^{2+} recovery kinetics. As illustrated by Dickey and Strack, 2011, such chronic manipulation of MFF and OMM reversible phosphorylation has many neurophysiological consequences. We will reproduce the Drp1-K38A observations (as well as rescue our Drp1-WT results) using acute inhibition of Drp1 via mdivi-1 (Cassidy-Stone et al., 2008; Tanaka and Youle, 2008). Acute regulation of OMM phosphorylation will also be performed. The effects of forskolin/rolipram and PACAP/BDNF treatment on individual mitochondrial calcium buffering kinetics will be determined. The effects of chronic genetic manipulation of MFF regulation on neuronal physiology may be determined by using simultaneous imaging of [Ca^{2+}]_i. MtPericam is not compatible with most calcium indicators. We will target the 480nm excited inverse (or flash) pericam Ca^{2+} sensitive GFP mutant to the mitochondrial matrix via fusion with a Cox8 localization sequence. Inverse pericam and Fura2 imaging will allow correlation of our observed matrix Ca^{2+} kinetics with the magnitude of the cytosolic calcium response (Nagai et al., 2001). More exact measure of plasma membrane calcium conductance may also be performed using whole cell patch technique and voltage clamp. Additionally, variability in neuronal excitability (and Neuro2A TRPV1 transfection/stimulation) may dilute statistical significance. Simultaneous measures of cytosolic calcium and Ca^{2+} conductance across the plasma membrane will allow correlation and normalization of the mitochondrial calcium kinetics to the concentration of calcium experienced by the mitochondria.
**Bioenergetics and matrix Ca^{2+} recovery**

MFF and reversible phosphorylation at the OMM regulates mitochondrial Ca^{2+} recovery, but the actual mechanism responsible for this observation is unknown. Dickey and Strack, 2011, reported reduced basal $\Delta \Psi_{\text{mt}}$ in hippocampal neurons overexpressing Drp1 or Bβ2; Bβ2 RNAi knockdown and AKAP1 overexpression hyperpolarized $\Delta \Psi_{\text{mt}}$. This bioenergetic differentiation may regulate matrix calcium recovery. $\Delta \Psi_{\text{mt}}$ is an index of metabolism. Increasing metabolism produces hyperpolarization via up-regulation of the metabolic proton circuit. Metabolism may be stimulated utilizing the special amino acid L-carnitine which transports fatty acids (metabolic fuel) into the mitochondria; thus, L-carnitine application produces $\Delta \Psi_{\text{mt}}$ hyperpolarization (Dickey and Strack, 2011; He et al., 2011). We will rescue Drp1 and Bβ2 depolarization via L-carnitine application; single mitochondria mtPericam recording will be performed to determine if the L-carnitine induced hyperpolarization restores matrix calcium recovery of fragmented mitochondria.

**MCU and ccdc109b**

Characterization of mitochondrial calcium uniporter candidates is a new, but important, focus for our lab. Understanding the mechanisms by which mitochondrial morphology and reversible phosphorylation at the OMM modulate neuronal signaling and sensitivity requires the molecular identity of the players involved. The most attractive Ca^{2+} uniporter candidate has been ccdc109a, termed MCU. Overexpression results clearly confirm a role for MCU in the uptake of calcium into the mitochondrial matrix; however, RNAi knockdown of MCU does not produce an absolute consequence. Other components must be involved. The extent of our RNAi analysis has been limited to the human targets shRNA used by the Rizzuto group. Our shRNA western blot confirmation also identified a mouse specific MCU shRNA (IR-MCU#69). With IR-MCU#69, we will also be able to perform human orthologue replacement. That is, the effects if IR-MCU#69 will be rescued with co-transfection with human MCU-mRFP. Complete rescue (or enhanced
mitochondrial uptake) will confirm the evolutionarily conserved role of MCU in mitochondrial calcium uptake, as well as determine possible non-selective effects of our MCU shRNA. Rescue from MCU knockdown will also be attempted using a novel MCU isoform, ccdc109b-mRFP.

Our current progress studying the expression and function of the novel MCU isoform ccdc109b is very preliminary. Overexpression of mouse ccdc109b in rat DRG neurons produced enhanced incidence of mitochondrial calcium uptake following mild depolarization with 10 mM, 15 mM and 20 mM K⁺. In Chapter 4 we have presented results for ccdc109b knockdown in mouse DRG neurons, but the shRNA construct used (IR-ccdc109b#F1) has only been characterized via epiflourescent verification of ccdc109b-mRFP knockdown in PC-12 cell culture. A panel of ccdc109b shRNA constructs developed by the Broad Institute will be carefully verified via knockdown of co-expressed mouse ccdc109b-tGFP and western blot analysis. Additionally, the pLKO vector expressing these shRNAs encodes puromycin resistance. We have designed primers against full length mouse ccdc109b transcript. Further qPCR verification of our shRNA panel will be performed in transiently transfect Neuro2A cells followed by puromycin selection. Due to the significant homology between MCU and ccdc109b, cross selectivity of both the MCU and ccdc109b shRNAs will be determined using overexpression/western blot and endogenous/qPCR analysis. Once we have verified our shRNAs, we will determine the contribution of ccdc109b to mitochondrial calcium uptake. Noted above, MCU knockdown using 90% effective shRNA fails to proportionately attenuate mitochondrial Ca²⁺ buffering. One possible role for ccdc109b is that of an alternative source of mitochondrial Ca²⁺ uptake. Double knockdown of both MCU and ccdc109b will be performed and analyzed in both mouse DRG neurons and Neuro2A cells using our high K⁺ and capsaicin stimuli protocol, respectively.
The inclusion of ccdc109b in the physiology of mitochondrial calcium buffering suggests a complex system. Key amino acid substitution within the proposed selection pore argues against a role in calcium conductance. Our RT-PCR data also presents a number of splice variants (estimating 5 total variants) that differ in identity and degree of expression across multiple tissues of the nervous system. Multiple hypotheses present ccdc109b in a regulatory role of MCU. Based on sequence analysis and predictions in structure, the mitochondrial Ca\textsuperscript{2+} uniporter is expected to exist as a multimer of MCU subunits. Heteromerization of MCU and ccdc109b presents the possibility of a dominant negative effect due to sequestration of conducting MCU with non-conducting ccdc109b.

Determination of MCU/ccdc109b heteromerization will be performed using MCU-tGFP and ccdc109b-FLAG co-expression in Neuro2A or PC-12 cell culture. MCU-tGFP will be precipitated via tGFP antibody immunoreactivity followed by immunoblot using –FLAG antibody. Co-precipitation of tGFP and –FLAG will be evidence of MCU/ccdc109b heteromerization. Once MCU and ccdc109b antibodies have been verified, endogenous heteromerization will be determined from Neuro2A cell culture (De Domenico et al., 2007). Alternatively, ccdc109b may act as a chaperone regulating expression of MCU at the mitochondria. To determine a chaperone role, we will perform cellular fractionation to isolate a mitochondria pure fraction from Neuro2A cell culture transfect with ccdc109b overexpression construct, ccdc109b shRNA or vector control. MCU expression within the mitochondrial fraction will then be calculated via quantitative immunoreactivity, as compared to a mitochondrial expression control. This protocol may be performed with co-expression and detection of MCU-tGFP or verified antibody against endogenous MCU (Palty et al., 2010).

Noted above, this investigation is complicated by ccdc109b splice variation. Multiple splice variants imply multiple functions and our MCU cDNA construct represents only one of these variants. Additionally, ccdc109b shRNA was designed against the full length form and this sequence may be excluded in some of the splice...
variants. Further characterization of ccdc109b will require cloning of all possible splice variants. RT-PCR and amplicon gel filtration will be performed to selectively amplify the proposed splice variant, which will then be placed in appropriate expression vector and characterized using the above molecular biology and physiology protocols.

Mitochondrial Ca\textsuperscript{2+} transporters and Ca\textsuperscript{2+} Deregulation

Our observations characterizing the role of MCU and its paralogue ccdc109 suggest a limited role for both proposed Ca\textsuperscript{2+} transporters. Mild cytosolic Ca\textsuperscript{2+} load (produced using a range of depolarizing stimuli) presented a clear “high Ca\textsuperscript{2+} affinity” role for MCU and ccdc109b. High K\textsuperscript{+} stimuli of 15 mM and 20 mM K\textsuperscript{+} demonstrated a strong effect of MCU and ccdc109b shRNA knockdown. With larger cytosolic Ca\textsuperscript{2+} loads (produced by 30 mM and 50 mM K\textsuperscript{+}), MCU and ccdc109b knockdown did not significantly attenuate mitochondrial Ca\textsuperscript{2+} buffering. The ability to overcome MCU knockdown with elevated cytosolic Ca\textsuperscript{2+} argues against a pathological role and presents MCU as an unlikely therapeutic target in the prevention of excitotoxicity; specifically, we would not expect MCU inhibition or knockdown in hippocampal neurons to prevent delayed calcium deregulation, or even prolong the latent period duration. A lower affinity Ca\textsuperscript{2+} transporter must be present; as discussed in the introduction, many mitochondrial Ca\textsuperscript{2+} transporters have been proposed. Other proposed mitochondrial Ca\textsuperscript{2+} transporters that have yet to be carefully characterized in a neuronal model include the Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger Letm1, mitochondrial ryanodine receptors (mRYR), and most recently, inner mitochondrial membrane localized NMDA receptors. Discussed in Chapter 4 and summarized in Figures 4.13-4.15, our Letm1 experiments produced inconclusive results due to significant vector affects. Therefore, it is unclear as to what role Letm1 plays in neuronal mitochondrial calcium buffering. Letm1 as a mitochondrial Ca\textsuperscript{2+} transporter has previously been described in \textit{Drosophila} as a significant “fast” component of mitochondrial Ca\textsuperscript{2+} buffering, as well as a persistent efflux mechanism. These
experiments were performed using shRNA knockdown of Letm1 and histamine induced store Ca\(^{2+}\) release (Pan et al., 2011). These observations would argue against low affinity role, but the \textit{Drosophila} physiology may not be identically conserved in a mammalian model. Mitochondrial NMDARs and mRyRs are very new candidates within the mitochondrial Ca\(^{2+}\) transport field. The limited studies describing these novel channels utilize isolated mitochondria and reconstituted lipid bilayers to study their function (Altschafl et al., 2007; Korde and Maragos, 2012). The \textit{in situ} affinities and conductance are yet to be described and may present possible low affinity transport mechanisms that may be important in mitochondrial Ca\(^{2+}\) overload.

**Extracellular versus ER calcium source**

The inability to reproduce the histamine/store Ca\(^{2+}\) MCU observations made by the Rizzuto and Mootha research groups is likely due to differences in the calcium source. Work identifying MCU as the putative mitochondrial calcium uniporter were performed utilizing histamine stimulation of ER calcium release in non-neuronal cells. The juxtaposition of mitochondria and the endoplasmic reticulum may be necessary (and thus implicating a source specific role) for MCU. We will investigate this source specificity using a number of stimuli capable of liberating Ca\(^{2+}\) from the ER including bradykinin, ATP and the non-receptor PLC activator m-3M3FBS (Cho and Chaban, 2012; Krjukova et al., 2004; Myers and Larkins, 1989). We will characterize the effect of MCU overexpression and knockdown (as well as ccdc109b) on mitochondrial buffering of ER calcium.

**Mitochondrial calcium efflux candidates**

Mitochondrial calcium efflux candidates include NCX3, NCLX, Letm1, and DCC. We have confirmed Letm1 localization to the mitochondria, but RNAi analysis of the contribution to mitochondrial calcium influx or efflux was inconclusive. Preliminary
confirmation of Letm1 shRNA was performed using co-expression/knockdown of GFP-tagged Letm1 observed via confocal microscopy. Careful verification of Letm1 shRNA knockdown will be performed using both western blot analysis and qPCR. NCLX mitochondrial localization has yet to be confirmed and will require confirmation of antibody immunoreactivity. Detection of functional expression via Li\(^+\)/Na\(^+\) replacement produced interesting results that suggest tissue specific expression of NCLX. Verification of this tissue specificity will be determined using RT-PCR analysis of total RNA collected from various structures of the CNS and PNS. Determination of NCLX localization and tissue specificity will be performed using immunocytochemistry and immunohistochemistry, respectively.

**Mitochondrial calcium efflux regulation**

Individual mitochondria calcium recording in DRG neurons revealed modulation of mitochondrial calcium recovery. Preliminary mitochondrial load analysis reported increased matrix calcium in neurons fragmented via PP2A/Bβ2 signaling. These results suggest impaired extrusion of mitochondrial matrix calcium by an unknown mechanism. Dickey and Strack, 2011, reported decreased ΔΨ\(_{\text{mt}}\) in hippocampal neurons transfected with Bβ2 and Drp1 promoting fragmentation (mediators of elongation increase ΔΨ\(_{\text{mt}}\)). We will characterize the electrogenic regulation of NCLX in DRG neurons via L-carnitine treatment with Li\(^+\)/Na\(^+\) replacement to isolate NCLX function (Palty et al., 2006). We have also begun preliminary analysis of NCLX phosphoregulation. Using the PKC activator PDBu, we observed a mitochondrial calcium buffering transport effect that was complicated by a strong effect on neuronal excitability and Ca\(^{2+}\) conductance across the plasma membrane. We will isolate mitochondrial physiology from the plasma membrane via ionomycin plasmalemmal permeabilization (Tornero et al., 2011; Waldeck-Weiermair et al., 2011). We will also selectively monitor NCLX function via Li\(^+\)/Na\(^+\) replacement. Pharmacological manipulation of phosphatase and kinases may then be performed.
Alternatively, NCLX regulation may be a consequence of NCLX expression. Neuronal NCLX content will be investigated as a function of mitochondrial architecture and OMM phosphorylation. We will determine expression of NCLX within a purified mitochondrial fraction (again, this requires characterization of an NCLX antibody) and transcriptional analysis via qPCR.
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