Molecular mechanisms and functions of mitochondrial calcium transport in neurons

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Molecular Mechanisms and Functions of Mitochondrial Calcium Transport in Neurons

by

Jacob Eugene Rysted

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

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Thesis Supervisor: Professor Yuriy Usachev
It's great to learn, cause knowledge is power!
   -Schoolhouse Rock
*oh, be joyful* - Trying to
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ABSTRACT

During neuronal activity mitochondria alter cytosolic Ca\textsuperscript{2+} signaling by buffering then releasing Ca\textsuperscript{2+} in the cytosol. This calcium transport by mitochondria affects the amplitude, duration, and spacial profile of the Ca\textsuperscript{2+} signal in the cytosol of neurons. This buffering by mitochondria has been shown to affect a variety of neuronal functions including: neurotransmission, gene expression, cell excitability, and cell death. Recently, researchers discovered that the protein CCDC109A (mitochondrial Ca\textsuperscript{2+} uniporter) was the protein responsible for mitochondrial Ca\textsuperscript{2+} uptake. Using a genetic knockout (KO) mouse model for the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) my research investigated the role of MCU in neuronal function. In cultured central and peripheral neurons, MCU-KO significantly reduced mitochondrial Ca\textsuperscript{2+} uptake while significantly increasing the amplitude of the cytosolic Ca\textsuperscript{2+} signal amplitude. Behaviorally, MCU-KO mice show a small but significant impairment in memory tasks: fear conditioning and Barnes maze. Using a maximal electroshock seizure threshold model of in vivo seizure activity my research found that MCU-KO significantly increases the threshold for maximal seizure activity in mice and significantly reduces seizure severity. In addition to mitochondrial Ca\textsuperscript{2+} uptake, my research also investigated the mechanisms involved in mitochondrial Ca\textsuperscript{2+} extrusion. The protein SLC8B1 (SLC24A6, NCLX) is the putative transporter responsible for the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, mitochondrial calcium extrusion. Using genetic NCLX-KO mice, our research found that in neurons NCLX contributes to cytosolic Ca\textsuperscript{2+} extrusion, but does seem to directly affect mitochondrial Ca\textsuperscript{2+} extrusion.
PUBLIC ABSTRACT

Mitochondria are known as the powerhouse of the cell, as they are the part of the cell that produces energy; however, alongside producing energy, mitochondria are also involved in processes such as Ca\textsuperscript{2+} signaling, reactive-oxygen species production, and programmed cell death. For the nervous system, Ca\textsuperscript{2+} is involved in a variety of processes including electrical excitability, synaptic transmission and plasticity, gene regulation, cell death and survival; mitochondria have the ability to alter how much Ca\textsuperscript{2+} is inside the cell, how long it stays elevated inside the cell, and in which compartments inside the cell it is localized. However, if too much Ca\textsuperscript{2+} enters the mitochondria this can lead to cell death, and this mechanism of cell death is believed to be important in a variety of neuronal pathologies including stroke, Alzheimer's, and Parkinson's diseases among others. Until recently, the mechanisms involved in mitochondrial Ca\textsuperscript{2+} uptake and release have been unknown and the effects of mitochondrial Ca\textsuperscript{2+} alteration in the nervous system of behaving organisms remains unknown. Using mice with genetically deleted components of mitochondrial Ca\textsuperscript{2+} transport, my research has uncovered how these mitochondrial transporters affect not only cellular functions but also the animal behavior. Interestingly, my research found that deleting the mechanism involved in mitochondrial Ca\textsuperscript{2+} uptake made mice more resistant to developing seizures. These findings identify new potential therapeutic targets for the treatment of epilepsy.
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LIST OF ABBREVIATIONS

\([\text{Ca}^{2+}]_{\text{cyt}}\): Cytosolic Ca\(^{2+}\) concentration

\([\text{Ca}^{2+}]_{\text{ext}}\): Extracellular calcium

\([\text{Ca}^{2+}]_{\text{i}}\): Cytosolic Ca\(^{2+}\) transients

\([\text{Ca}^{2+}]_{\text{mt}}\): Mitochondrial matrix Ca\(^{2+}\) concentration

18S: Ribosomal protein 18S

AA: Amino acid

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

ATP: Adenosine triphosphate

ATP: Adenosine triphosphate

Ca\(^{2+}\): Calcium ion

CGP (-37157): 7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one

CNS: Central nervous system

CsA: Cyclosporin A

DMEM: Dulbecco’s modified Eagle’s medium

DMSO: Dimethyl sulfoxide

DRG: Dorsal root ganglia

EMRE: Essential MCU regulator protein

ER: Endoplasmic reticulum

FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

FCCP: Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

FIV: Feline immunodeficiency virus
GABA: γ-amino butyric acid
GFP: Green fluorescence protein
H⁺: Proton
HH Buffer: HEPES-buffered Hank’s balanced salt solution
I/R: Ischemia/reperfusion injury
IMM: Inner mitochondrial membrane
K⁺#: HH buffer with # mM of KCl
K⁺: Potassium ion
KCl: Potassium chloride
Li⁺: Lithium ion
LTD: Long term depression
LTP: Long term potentiation
MCU: Mitochondrial Ca²⁺ uniporter (ccdc109a)
MCUb: Paralog/ isoform of MCU (ccdc109b)
MCUC: Mitochondrial calcium uniporter complex
mPTP: mitochondrial permeability transition pore
IMS: Inter-mitochondrial space
Na⁺: Sodium ion
NADH: Reduced nicotineamide adenine dinucleotide
NCKX: K⁺ dependent Na⁺/Ca²⁺ exchanger
NCLX: Na⁺/Li⁺/Ca²⁺ exchanger
NCX: Na⁺/Ca²⁺ exchanger
OMM: Outer mitochondrial membrane
OXPHOS: Oxidative phosphorylation
PBS: Phosphate buffered saline
$\text{P}_i (\text{PO}_4)$: phosphate
PNS: Peripheral nervous system
ROS: Reactive oxygen species
Ru360: Ruthenium 360
RuR: Ruthenium red
SEM: Standard error of the mean
$\Delta \Psi_{\text{mt}}$: Mitochondrial membrane potential
CHAPTER I:
INTRODUCTION

Mitochondria are known as the powerhouse of the cell because they are responsible for producing usable energy for the cell. However, mitochondria have other functions that include production and management of ROS, lipid metabolism, Ca\textsuperscript{2+} dynamics, etc. In neurons, Ca\textsuperscript{2+} is a critical second messenger that links neuronal activity with processes such as synaptic remodeling, neurotransmission, and cell survival among others. The role of mitochondria in regulating Ca\textsuperscript{2+} dynamics in the cytosol has been studied, but the major molecules responsible were unknown until recently. The effect of mitochondrial Ca\textsuperscript{2+} transport has been hypothesized to play a role in normal animal behavior as well as in neuronal pathologies such as neurodegenerative disorders.

Neurological disorders are a major health concern for many adults living today. With the average life expectancy increasing in many parts of the world, more people are at risk for developing neurological disorders including neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's. As of yet, no therapeutics have been developed that can cure these diseases, while the existing drugs improve symptoms until the diseases progress to an intractable state. As the name suggests neurodegenerative disorders cause a loss of specific populations of neurons. Hence, the research in the area of neurodegenerative diseases has focused on therapeutic strategies to prevent or slow neuronal death and thereby halting disease progression. Although the pathological progression is known in many neurological disorders, not all of the factors responsible for the pathologies are understood. Significant research
efforts have being devoted to identifying critical pathological cascades and events can be targeted and intervened for preventing further neuronal loss.

Ca²⁺ dysregulation in neurons is thought to be a major contributing factor in cell death in many neurological disorders. During disease states, the ability of neurons to regulate intracellular Ca²⁺ is impaired and can lead to Ca²⁺ overload. This excess Ca²⁺ disrupts neuronal function and can lead to activation of pathways responsible for cell necrosis or apoptosis.

One of the main pathways for Ca²⁺ induced cell death is excessive uptake of Ca²⁺ into mitochondria. In many cell types, mitochondria can act as temporary Ca²⁺ sink that buffer [Ca²⁺]ₖᵢ during periods of activity. In neurons, mitochondria buffer [Ca²⁺]ₖᵢ during neuronal activity, effectively changing the way the cytosolic Ca²⁺ signal is interpreted by the neuron. However, during pathological conditions where [Ca²⁺]ₖᵢ levels are excessively high, mitochondrial buffering of [Ca²⁺]ₖᵢ leads to [Ca²⁺]ₘₜ overload causing a cascade of toxic events leading to the death of the neuron.

This thesis work attempts to further understand the role of mitochondrial Ca²⁺ transport in normal neuronal function, as well as its role in pathological conditions. This knowledge will hopefully lead to the development of new therapeutics that target novel mechanisms involved in a plethora of diseases and lead to a greater understanding of how the nervous system functions. Chapter I of this dissertation will focus on reviewing current literature on the role of mitochondria in neuron function and disease as well as general mitochondria-Ca²⁺ interactions. Chapter II will focus on the role of MCU in general mitochondrial functions as well as its role in regulating Ca²⁺ signaling in neurons and neuronal network activity in vitro. Chapter III will describe the role of MCU in whole
animal behavior, focusing not only on the performance in standard behavioral tasks, but also how it MCU deletion affects seizure activity in vivo. Chapter IV will focus on the investigation of mtNCX and the role of NCLX as the possible transporter molecule responsible for [Ca\textsuperscript{2+}]\textsubscript{mt} extrusion in neurons. Finally, chapter V will provide a discussion of the findings of this dissertation as well as the potential implications and caveats of this research and the possible impact that these findings could have on future research.

**Mitochondria Function in Neurons**

Mitochondria are dynamic organelles primarily known for generating energy in the cell in the form of ATP [1, 2]. This function is especially important for the nervous cells that accounts for a disproportionately high amount of total body metabolism based on percentage of total body weight [3]. In terms of amount of ATP used, grey matter uses 33-50 µmol of ATP/g/min. Most of the energy utilized by neurons relates to neuronal activity such as action potential firing, post-synaptic potentials, and synaptic vesicle cycling. Most of the ATP used in these functions is due to Na\textsuperscript{+}-K\textsuperscript{+} ATPase function which is needed to re-establish ion gradients across the plasma membrane [3-5]. The energy for ATP production is due to metabolism of molecules such as carbohydrates, fats, and proteins [6]. Enzymatic breakdown of these molecules leads to the production of reducing agents that are fed into the electron transport chain (ETC), such as the breakdown of glucose in the Kreb’s cycle. Utilizing the energy from the transfer of e\textsuperscript{-} into the ETC, a H\textsuperscript{+} electrochemical gradient (\(\Delta \mu \text{H}^+\)) is created which is utilized by the ATP synthase to bond P\textsubscript{i} to ADP to create ATP, a process known as oxidative phosphorylation (OXPHOS). Some ETC dysfunctions can lead to specific
nervous system pathologies, such as mutations in complex I leading to optic nerve atrophy [7]. Although not usually specific to nervous tissue many OXPHOS disorders show many neurological deficits due to the vulnerability of neurons to energy demands [8]. Aside from the production of ATP, mitochondria play many additional roles important to neuronal function, which will be discussed next.

Another important aspect of mitochondrial function in neurons relates to the dynamic nature of mitochondrial fission and fusion. Although typically depicted as a single bean-like structures, mitochondria can form large connected networks in many cell types [9]. In particular, transporting mitochondria to synapses is essential the proper function of neuronal communication [10, 11]. In neurons mitochondrial fission is driven by a protein called dynamin-related protein 1 (DRP1), while fusion requires both Optic Atrophy Protein 1 (OPA1), a dynamin like GTPase, and Mitofusin proteins 1 and 2 (MFN1/2) [12]. These dynamic processes are also important for removing damaged mitochondria and salvaging dysfunctional mitochondria as well as increasing respiratory capacity [12-14]. Certain disorders of the nervous system are caused by a dysfunction of the fusion/fission machinery. Dominant optic atrophy and Charcot-Marie-Tooth disease (CMT) are diseases that lead to degeneration of the optic nerve or sensory and motor neurodegeneration, respectively, caused by dysfunctional mitochondrial fission and fusion processes while also possible affecting mitochondrial trafficking [13]. Similar mitochondrial fission/fusion dysfunction are found in many neurodegenerative disorders as well [15]. In acute injury, such as ischemia/reperfusion (I/R) injury, neurons can be more or less susceptible to the injury depending on the balance between fission and fusion [12]. Mitochondria fission and fusion is very important in proper neuronal function
as mitochondrial fission is necessary to transport mitochondria to distal neuronal processes. This mitochondrial trafficking has been shown to be necessary for the formation of synapses in neuronal processes as well as for the formation and mobility of axonal growth cones [12, 16, 17]. In order to get mitochondria to areas of increased metabolic demand, mitochondria are shuttled through the neurons using specialized proteins called dyneins and kinesins which attached to the mitochondria via the GTPase Miro. Miro is a Ca$^{2+}$-dependent protein that attaches mitochondria to motor proteins, but releases them upon Ca$^{2+}$ binding [18, 19]. This allows mitochondria to be “dropped off” at locations where there is a Ca$^{2+}$ accumulation [20, 21]. Many functions of LTP are linked to Ca$^{2+}$ influx at the synapse, this same signal can be utilized by mitochondria to produce energy at sites in the neuron that require it. Similarly, disruption of mitochondrial trafficking during development has been shown to cause many nervous system defects [12]. Much of the research in mitochondrial trafficking underlies the importance of having a source of energy at the proper site in the neuron, but mitochondria have other functions important in neuronal function not related directly to energy production.

Aside from ATP production, mitochondrial are also known for their function in processes such as ROS dynamics, redox signaling and lipid synthesis. A by-product of mitochondrial respiration, ROS are generally considered to be a harmful ion species causing detriments if not controlled. Indeed, O$_2^-$ created at complexes I and III, can interact with nitric oxide to produce peroxynitrite (ONOO$^-$) which can damage vital Tyr residues, or Fe$^{2+}$/Cu$^+$ to produce hydroxide (OH$^-$) which can lead to DNA or protein damage [22]. However, this generation of ROS is contained via enzymes like
superoxide dismutase 2 which converts $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ which can further be broken down into harmless products. There are physiological levels of ROS that are important in normal neuronal function. In development, ROS activity is important in the proliferation and differentiation of neural precursor cells, where ROS activity can modulate activity of kinases and transcription factors such as PKC, NF-kB, and NFAT [23]. ROS have also been implicated in neurite outgrowth, affecting microtubule and actin formation. In processes of strengthening synaptic connections, i.e. LTP, inhibition of ROS generation seems to negatively impacts mechanisms important for LTP such as activation of PKC [24]. In animal behavior studies inhibition of ROS impairs amygdala dependent emotional processing of pain behaviors [23, 25]. Similarly, ROS manipulation in the spinal cord inhibits central sensitization of pain processing [26]. However, despite the physiological importance of ROS in normal neuronal function, a lot of research implicates high ROS production in almost all neuronal pathologies [27]. In Alzheimer's, neurons show a significant increase in markers for oxidative stress including protein oxidation and upregulation of oxidative stress genes [27]. Similarly in Parkinson's, damage to mitochondrial function and increased $\text{Ca}^{2+}$ influx in vulnerable cells have suggested to increase oxidative stress and damage [28]. Overall, the production and maintenance of ROS in physiological and pathophysiological states are some of the most complex aspects of mitochondrial function [29].

**Mitochondrial Dysfunction in Neuronal Pathology**

High-fidelity control of mitochondrial function is vitally important in any cell type, but especially in neurons because of their high energy demand. Accordingly,
impairment of mitochondrial function has been implicated in many disorders of the nervous system [15]. Important in the maintenance of a healthy population of mitochondria are various mechanisms involved in “quality control” including chaperone proteins, proteases, and fission/fusion mechanisms. These mechanisms of quality control are especially important for neurons because mitochondria can be transported far away from the soma and therefore have, on average, the longest half-life for mitochondria in any tissue [14, 30, 31]. As the mitochondria become older they become less able to cope with certain aspects of mitochondrial dysfunction such as misfolded proteins and oxidative stress. Breaking down old mitochondria is also more challenging in neurons as the autophagosomes must be transported back to the cell soma in order to completely remove the debris [14]. Several different neurodegenerative diseases have been linked to dysfunctional mitochondrial proteases including hereditary spastic paraplegia, and spinocerebellar ataxia. In neurodegenerative disorders like Parkinson’s, proteins PINK1 and Parkin are related to mitophagy and the removal of dysfunctional mitochondria, while in Alzheimer’s the Oligopeptidase Prep protein has been shown to be important in the maintenance and degradation of the Aβ peptides, though its ability to regulate neurodegeneration are still being investigated [14]. These are just some examples of regulation of mitochondria relating to the health of neurons, but there are many more examples of mitochondrial “quality control” mechanisms involved, directly or indirectly, in neuronal pathologies.

Specific mitochondrial dysfunctions have been shown to be important in the progression of certain neuronal pathologies. However, research is starting to examine how the interactions between different organelles can lead to cellular stress and
damage. Recent research on neurodegenerative disorders have started to investigate the interaction between mitochondria and the ER. Cellular organelles such as the endoplasmic reticulum (ER) have been shown to directly interact with mitochondria which has shown to be important for the function of both organelles. These functions include Ca$^{2+}$ homeostasis, lipid metabolism, and cholesterol metabolism [15]. The proteins that allow mitochondria and the ER to interact are known as mitochondria-ER associated membranes (MAMs). Some neurological disorders are associated with MAMs, such as presenilin-1/2 in genetic Alzheimer’s, or CMT where dysfunctional MFN2 leads to aberrant cross-talk between the two organelles and can lead to increased cell stress [15]. These disorders show dysfunctional processes for both mitochondria and the ER; however, it is still unknown if these dysfunctions cause the disorders or are caused by the disorders.

These are just some of the most important functions of mitochondria and how they relate to neuronal function. This is by no means an exhaustive list of how mitochondria affect neuronal processes; there are many functions that are influenced by mitochondria. It is important to understand how integral mitochondria are to neuronal function aside from just their ability to produce energy for the neuron.

**Mitochondria in Neuronal Ca$^{2+}$ Signaling**

Ca$^{2+}$ is a very important ion and second messenger for the nervous system as it enables coupling of neuronal activity with a variety of neuronal processes such as gene expression, protein modification, neurotransmission among other things [32]. There have been a few studies that investigate the role of mitochondrial Ca$^{2+}$ transport in
neuronal function; however, the ability to test the function of mitochondrial Ca\textsuperscript{2+} transport is hampered by lack of selective drugs compatible with \textit{in vivo} testing.

Mitochondrial Ca\textsuperscript{2+} dynamics are important aspect in cytosolic Ca\textsuperscript{2+} signaling in neurons. A major physiological consequence of mitochondrial Ca\textsuperscript{2+} uptake is the ability to increase mitochondrial metabolism through several mechanisms. Ca\textsuperscript{2+} accumulation in the mitochondrial matrix produces mitochondrial depolarization thereby reducing the H\textsuperscript{+} electrochemical gradient (\(\Delta\mu\textsubscript{H}^{+}\)) and enhancing ETC respiration [33]. Ca\textsuperscript{2+} interacts with many enzymes in the mitochondrial matrix that regulate either ATP synthesis or Krebs cycle function. ATP synthase activity is regulated via mitochondrial matrix Ca\textsuperscript{2+} via Ca\textsuperscript{2+}-dependent activation of phosphorylating enzymes that interact with the F\textsubscript{1}-ATPase subunit [34]. Several enzymes involved in the Kreb cycle either directly bind Ca\textsuperscript{2+} or Ca\textsuperscript{2+}-dependent phosphatases that enhance production of reducing agents [35]. These enzymes are pyruvate dehydrogenase, isocitrate dehydrogenase, and \(\alpha\)-ketoglutarate dehydrogenase. Enhancing enzymatic activity of Kreb cycle proteins increases reduction of NAD\textsuperscript{+} to NADH which is utilized via complex I in the ETC.

Research has been done investigating the role of mitochondria Ca\textsuperscript{2+} transport in the various neuronal processes including synaptic transmission [36]. Early research showed the importance of mitochondria Ca\textsuperscript{2+} transport at the synapse of central neurons affecting short-term facilitation in synaptic transmission [37-39]. Similarly, mitochondria in the presynaptic terminals of motor neurons, from a variety of species, are important in Ca\textsuperscript{2+} dynamics affecting neurotransmission [40-42]. Budd and Nicholls 1996 showed that inhibition of mitochondrial Ca\textsuperscript{2+} uptake inhibited Ca\textsuperscript{2+} influx via glutamate NMDA receptors, suggesting a necessary role for mitochondrial Ca\textsuperscript{2+} uptake.
in regulating NMDA channel conductance [43]. In retinal amacrine cells mitochondrial Ca\(^{2+}\) dynamics is shown to be important in regulating synaptic transmission independent of ER-mitochondrial interactions [44, 45]. However, in retinal bipolar cells mitochondrial Ca\(^{2+}\) uptake plays a little role in Ca\(^{2+}\) clearance at the synapse because most of it being is mediated by plasma membrane Ca\(^{2+}\) transport mechanisms at this synapse [46]. Similarly, rat corneal neurons are not affected by mitochondrial Ca\(^{2+}\) transport [47]. Although, in photoreceptors mitochondrial form what a so-called ellipsoid body, which is suggested to act as a barrier for Ca\(^{2+}\) cross-talk between the outer and inner segments [48]. Using the \(\text{mtNCX}\) inhibitor, CGP-37157, Scanlon et al. 2012 showed no effect of blocking glutamate induced neuronal activity in central neurons [49]. Collectively, these studies have shown that depending on the neuronal type, mitochondrial Ca\(^{2+}\) transport can have a more or less significant impact on the regulation of synaptic function.

In dorsal root ganglion (DRG) sensory neurons that represent the peripheral nervous system (PNS), mitochondrial Ca\(^{2+}\) dynamics play a significant role in regulation of cytosolic Ca\(^{2+}\) signaling. One of the most prominent features of mitochondrial Ca\(^{2+}\) uptake in DRG neurons is the prolonged elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) after a strong neuronal depolarization [50-52]. At the synapse, a majority of the Ca\(^{2+}\) is buffered by the mitochondria and would expect to have the same effect on short term facilitation as has been shown in central neurons [53]. Interestingly, this buffering of [Ca\(^{2+}\)]\(_{\text{cyt}}\) by mitochondria has been shown to be important in regulating synaptic transmission at the first sensory synapse, as well as in activation of Ca\(^{2+}\)-dependent transcription factor NFAT, which in turn, could be involved in nociception [54, 55]. Despite the strong
influence of mitochondrial Ca\(^{2+}\) transport in the peripheral nervous neurons, how this affects sensory function and pain processing has not been explored.

**Mitochondrial Ca\(^{2+}\) Dynamics in Neuronal Pathology**

One of the most studied aspects of mitochondrial Ca\(^{2+}\) uptake on neuronal is the pathological effects of mitochondrial Ca\(^{2+}\) overload. In neurons, large Ca\(^{2+}\) loads, like those produced by prolonged NMDA receptor activation, can lead to a large \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation which is buffered by mitochondria; however, too much Ca\(^{2+}\) accumulation in mitochondrial leads to the collapse of the mitochondrial membrane potential (\(\Delta\Psi_{\text{mt}}\)) and opening of the mitochondrial permeability transition pore (mPTP), eventually causing cell death, a process known as mitochondrial Ca\(^{2+}\)-dependent toxicity [56]. This mechanism is believed to be important in I/R in cardiac and nervous tissue [57]. The immunosuppressive compound cyclosporin A (CsA), which binds to the protein cyclophilin D (CypD), is one of the only known drugs to actively inhibit mPTP opening [58]. The proteins that make up the channel have been studied extensively and over the years specific proteins have been tested in knock out studies. Proteins such as the F\(_{0}\)F\(_{1}\) ATP synthase, VDAC, SPG7, adenine nucleotide carrier (ANT), and the phosphate carrier along with proteins BAX and TSPO have been tested, but still no consensus has been reached about the molecular determinants of mPTP [58].

Mitochondrial Ca\(^{2+}\) dysregulation is hypothesized to be one of the mechanisms responsible for neuronal dysfunction in neurodegenerative diseases. In Alzheimer's, Ca\(^{2+}\) dysregulation is implicated in the dysfunction and degeneration of neurons [59]. One of the main consequences of A\(\beta\) plaque in nervous tissue is the elevation of
[Ca\(^{2+}\)]_{cyt} at rest, which can in turn lead to increased mitochondrial ROS production [28]. One of the alternative hypotheses for Alzheimer's is the disruption of MAMs leading to mishandling Ca\(^{2+}\) shuttling between the two organelles [60, 61].

In Parkinson's disease, the role of voltage-gated Ca\(^{2+}\) channels (VGCCs) have been implicated based on the studies using dihydropyridines, negative allosteric modulators of VGCC, or isradipine, that showed protection against sporadic and genetic models of Parkinson's [28, 62]. For example, in PINK1 KD/KO models of Parkinson's a common characteristic is depolarization of $\Delta\Psi_m$, but this is rescued using VGCC inhibitors. While the VGCC research does not directly implicate mitochondria in neuronal dysfunction, mitochondrial Ca\(^{2+}\) overload and dysfunction is a common end target of excessive Ca\(^{2+}\) influx in neurons via VGCC, NMDA receptors or other channels. Other diseases such as amyotrophic lateral sclerosis (ALS) and Huntington's disease also suggest a role of mitochondrial Ca\(^{2+}\) in neurodegeneration [63, 64]. However, many of these diseases do not show mitochondrial Ca\(^{2+}\) overload as the cause of cell death, but instead an increase in baseline [Ca\(^{2+}\)]\(_{mt}\) leading to increased ROS production. That being said, the ability for mitochondria to uptake Ca\(^{2+}\) and activate OXPHOS mechanisms leading to increased ROS is a hypothesized mechanism of action.

Although a significant body of research has been done investigating the role of mitochondrial Ca\(^{2+}\) transport in neuronal function in vitro, our progress in this area has been hampered by the lack of drugs that specifically target the mitochondrial Ca\(^{2+}\) uptake machinery. Ionophores like FCCP are able to inhibit mitochondria Ca\(^{2+}\) uptake by disrupting the $\Delta\mu H^+$, which affects many other processes as well as acidifies the
matrix [45]. Similarly, inhibitors ruthenium red (RuR) and a more potent, Ru360 are the only known somewhat specific inhibitors of mitochondrial Ca\textsuperscript{2+} uptake; however, the ability to diffuse across the plasma membrane is dependent upon cell-type which makes patch-clamp the best method to deliver them [65, 66]. On the other hand, the mtNCX inhibitor CGP-37157 is also known to inhibit VGCC [50, 67]. Overall the limitations of these drugs make them difficult to use \textit{in vitro} and essentially non-feasible for \textit{in vivo} studies.

**Mitochondria and Seizure Activity**

Epilepsy is a very common (>1% of the population) neurological disorder that is characterized by recurrent episodes of abnormal spontaneous and synchronous neuronal activity in the brain, known as seizures. Epilepsy can form either via genetic predispositions such as mutations in excitation or inhibitory synaptic transmission mechanisms, through neuronal insult/damage, or sporadic development [68]. A key component of seizures is the synchronous network activity which is due to aberrant and excessive intrinsic excitability of neurons, imbalance between excitatory and inhibitory synaptic transmission or both [68-71]. Many seizures start at a single focus and then spread through large areas of the brain developing into a generalized seizure [72].

The mechanisms by which a shift in excitatory synaptic activity leads to epilepsy has been studied in great detail. The hippocampus is a brain region that is one of the most likely to develop epilepsy and so the mechanisms by which this can happen have been elucidated [73]. In temporal lobe epilepsy (TLE), the most common form of epilepsy in adults, mechanisms of epileptogenesis include loss of CA1, CA3, and DG
neurons and a resultant increase in aberrant axonal sprouting and connections of granule cells [73]. The main excitatory neurotransmitter, Glutamate, has been shown to be excessively released during seizure activity, and this excessive Glutamate can also lead to excitotoxic damage and neuronal loss. One of the mechanisms by which epileptic circuits are ingrained in the neuronal networks is through LTP and LTD. Stimulating neuronal networks in *in vitro* and *ex vivo* systems can induce LTP at glutamatergic synapses while inducing LTD at GABAergic synapses. These mechanisms overall enhance the excitatory tone in the neuronal network [73].

Some hypotheses of epileptogenesis/seizure activity involve a role for mitochondrial dysfunction in the pathology. Much of the research regarding mitochondrial involvement in seizure activity focused primarily on metabolic dysfunction and ROS production. Mitochondrial energy production was hypothesized to affect Na\(^+\)-K\(^+\) ATPase activity and thereby regulate plasma membrane polarization. When mitochondrial energy production fails the neuron depolarizes leading to activation of voltage-gated ion channel and increased neuronal activity. However, in tissue from human patients and mouse models with epilepsy there has been no evidence of decreased plasma membrane potential, arguing against this hypothesis [74]. It has been shown that ATP is necessary for presynaptic vesicle mobilization. For ROS, the mechanisms are believed to be due to accumulated neuronal damage. One of the main hypotheses for ROS damage leading to cell death is due to Ca\(^{2+}\) dysregulation and opening of the mPTP [75]. This relates to the excitotoxicity model of epileptogenesis where neuronal loss in the GABAergic interneurons leads to an increase in excitatory output [70].
As discussed above, mitochondrial Ca\(^{2+}\) dynamics have been shown to regulate synaptic transmission and short-term facilitation, which could potentially influence neural network activity [37, 38]. In addition, mitochondrial Ca\(^{2+}\) transport can potentially influence the neural network activity and stability by regulating activity-dependent gene expression and experience-dependent changes in Glutamate- and GABAergic synaptic networks [54, 76-78]. Finally, mitochondrial Ca\(^{2+}\) overload can potentially contribute to the death of GABAergic neurons in epileptogenesis [69].

**Mitochondria Ca\(^{2+}\) Uptake Mechanism**

For decades, scientists have understood the role of mitochondrial Ca\(^{2+}\) uptake in many different tissues and organisms. One of the first aspects of mitochondrial Ca\(^{2+}\) transport was the large capacity for mitochondria to accumulate Ca\(^{2+}\). Based on the chemiosmotic theory of mitochondrial ion regulation there is a ~180 mV electrochemical gradient created by oxidative phosphorylation that drives ions, such as Ca\(^{2+}\), into the mitochondrial matrix when a pathway is available [79]. The charge carried by Ca\(^{2+}\) conductance into the mitochondrial matrix would quickly depolarize mitochondria; however, Ca\(^{2+}\) accumulation is balanced by H\(^{+}\) extrusion in order to counteract accumulation of positive charge. This loss of H\(^{+}\) leads to mitochondrial matrix alkalization and a prolonged depolarization of \(\Delta \Psi_{mt}\) unless the pH gradient (\(\Delta pH\)) is restored. Permeant anions such P\(_i\) allow H\(^{+}\) re-entry into the mitochondrial matrix by utilizing \(H_3PO_4\) transporters on the inner mitochondrial membrane (IMM) [80, 81]. Without a permeant anion to restore \(\Delta pH\) mitochondrial Ca\(^{2+}\) uptake capacity is severely hampered [82]. Although other permeant anions can be used to restore \(\Delta pH\), such as
acetate and β-hydroxybutarate, P_i allows for greater Ca^{2+} accumulation due to formation of Ca_3(PO_4)_2 complexes which does not carry charge. However, brain mitochondria also require adenine nucleotide (such as ADP or ATP) in the mitochondrial matrix as well in order to maintain ΔΨ_{mt} and buffer large Ca^{2+} loads [83].

Before the discovery of the identity of the mitochondrial Ca^{2+} uptake molecules, research had discovered many aspects important for understanding the mechanism involved in mitochondrial Ca^{2+} uptake. Several mechanisms for mitochondrial Ca^{2+} uptake were proposed including a Ca^{2+}/2-H^+ exchanger, which would explain the increase in extramitochondrial pH when Ca^{2+} is taken up. However, experiments using dissipation of Δ\nuH^+ but kept ΔΨ_{mt} through addition of valinomycin did not alter mitochondrial Ca^{2+} uptake which does not support the hypothesis of a Ca^{2+}/2-H^+ exchanger [84] another proposed mechanism was a Ca^{2+}/P_i symport due to observations seen in extramitochondrial P_i during mitochondrial Ca^{2+} uptake. Although, in P_i depleted mitochondria, rate of mitochondrial Ca^{2+} uptake was not affected, although P_i depletion did reduce the amount of accumulated Ca^{2+} [85]. Multiple groups have shown a dependence of ΔΨ_{mt} on the function of mitochondrial Ca^{2+} uptake [79, 86]. Patch-clamp recordings from IMM vesicles identified a Ca^{2+}-selective ion channel that was blocked by the inhibitors of mitochondrial Ca^{2+} uptake Ru360 and RuR, and demonstrated ion selectivity consistent with the known properties of mitochondrial Ca^{2+} uptake [86]. This led to the idea that mitochondrial Ca^{2+} uptake is mediated by a Ca^{2+}-selective and Ca^{2+}-dependent ion channel in the IMM. Activation of mitochondria Ca^{2+} uptake is dependent upon [Ca^{2+}]_{ext} with little effect of [Ca^{2+}]_{mt} as long as ΔΨ_{mt} is not affected [87]. Different tissues were shown to have different activation thresholds for
mitochondrial Ca\textsuperscript{2+} uptake depending on the tissue type [88]. Initial studies investigating mitochondrial Ca\textsuperscript{2+} uptake rate utilized the A23187 ionophore or \textsuperscript{45}Ca\textsuperscript{2+} on isolated mitochondria and discovered variable rate in mitochondrial Ca\textsuperscript{2+} uptake between different tissues [88]. Electrophysiology in mitoplasts from COS-7 cells revealed activation of large Ca\textsuperscript{2+}-selective currents (20-30 pA) that reached half-maximum amplitude at around 20 mM [Ca\textsuperscript{2+}]\textsubscript{ext}. These Ca\textsuperscript{2+} currents were almost completely ablated with RuR addition (200 nM) and were not affected by removal of [Na\textsuperscript{+}]\textsubscript{ext} [86]. Modulators of mitochondrial Ca\textsuperscript{2+} uptake include Mg\textsuperscript{2+}, La\textsuperscript{3+}, and ruthenium based compound RuR, and Ru360. Ru360 is considered to be a more specific inhibitor of the mitochondrial Ca\textsuperscript{2+} uptake, while Mg\textsuperscript{2+} doesn’t significantly affect it and La\textsuperscript{3+} affects most Ca\textsuperscript{2+} permeable transporters [65, 89, 90].

**Mitochondrial Ca\textsuperscript{2+} Uniporter**

Molecular identity of the mitochondrial Ca\textsuperscript{2+} uptake was unknown for decades after the discovery of mitochondrial Ca\textsuperscript{2+} accumulation. A study in 1993 suggested that a 40,000 kD glycoprotein is responsible for mitochondrial Ca\textsuperscript{2+} uptake, based on antibody binding assays [91]. Other candidate molecules proposed to function as mitochondrial Ca\textsuperscript{2+} uniporter over the years included the uncoupling proteins 2 and 3 (UCP2 and UCP3), ryanodine and NMDA receptors [92]. However, it wasn’t until 2011 that two back to back papers were published in Nature that the identity of the molecule responsible for mitochondrial Ca\textsuperscript{2+} uptake was revealed. Baughman et al. 2011 and De Stefani et al. 2011, discovered that a previously known, but undescribed protein called CCDC109A was the identity of the MCU [93, 94]. Initial characterization of the AA
sequence revealed that the uniporter protein was a 40 kD protein with two transmembrane domains connected by a linker sequence. This linker sequence contained a so-called “DIME” motif around the pore of the channel, which is a 23 AA sequence with many negative charges that is suggested to confer Ca$^{2+}$ selectivity [93, 95, 96]. After these initial studies many more have been published further elucidating properties of MCU.

Using *C. Elegans* as a model system and paramagnetic NMR of Mn$^{2+}$ ion binding revealed important information about the pore ion selectivity filter. Specifically, the carboxylate rings on the asparagine (D) and glutamate (E) in the linker region can cooperatively bind Ca$^{2+}$ at the mouth of the pore, which seems to be the most critical aspect of the ion selectivity filter [97]. Notably, some research investigating the ion permeability of MCU suggested that the negatively charged glutamate (E) and the positively charged arginine (R) play a more crucial role in determining ion selectivity [98]. However, this has been called into question by other research [99, 100]. The pore itself is also believed to be the location where the mitochondrial Ca$^{2+}$ uptake inhibitors RuR and Ru360 bind. To this end, Mootha and colleagues showed that S259A mutation in human MCU (hMCU) did not inhibit MCU function but prevented Ru360 binding, while Oxenoid et al. 2016 showed S259R mutation acted similarly to Ru360 inhibition [93, 101]. However, Cao et al. 2017 suggested that Ru360 binds to the aspartate (D261) residue in the DIME sequence [97]. Although the AA sequence affects the Ca$^{2+}$ selectivity of MCU, auxiliary subunits of the complex contribute to many other aspects of MCU function as reviewed below.
Since its discovery, research has shown that MCU is regulated in cells via multiple mechanisms. One of the first aspects of MCU regulation discovered was the finding that Western blot showed the MCU band at ~35 kD even though based on the AA sequence a 40 kD protein would be expected [93, 94]. This is hypothesized to be due to the cleavage of an N-terminal mitochondrial targeting sequence [102]. Even though it is hypothesized to be cleaved after transport, deletion of the N-terminal domain represses MCU activity without altering auxiliary subunit binding [103]. Other forms of regulation include posttranslational modification such as phosphorylation via calmodulin-dependent kinase II (CaMKII), which was shown to enhance mitochondrial Ca$^{2+}$ uptake [104]. In addition, another group showed that α1-adrenergic signaling can lead to phosphorylation via proline-rich tyrosine kinase 2 [105]. Lastly, although not necessarily due to an intracellular signaling pathway, Dong et al. 2017 showed increased ROS production lead to oxidation of cysteine 97 during oxidative stress leads to increased channel activity [106]. Some of these regulation mechanisms are called into question based topology of phosphorylation sites or due to the fact that these sites are on the N-terminus which is suggested to be cleaved after transport. Many researchers have also shown regulation of MCU on the genetic level via transcription factors such as microRNA (miRNA). In cardiac myocytes physiological and pathological conditions can lead to miR-138, miR-25, and miR-1 lead to transcriptional repression of MCU, while in cancers regulate MCU via miR-25 or miR-340 [107-110]. Qui et al. 2013 demonstrated in cortical neurons that large mitochondrial Ca$^{2+}$ loads leads to CaMK activation of immediate early gene Npas4-dependent repression of MCU mRNA [111]. Conversely,
Shanmughapriya et al. 2015 showed Ca\(^{2+}\)-dependent CREB activation and upregulation of MCU gene expression [112].

Recent structural analysis using cryo-electron microscopy provided important insight into the subunit composition of the MCU channel. Since its discovery as a protein with only 2 transmembrane domains the question remained how many subunits were required for proper function of the channel [93]. Initially, Raffaello et al. 2013 showed a tetrameric oligomer based on immunoblotting by over expressing mouse MCU in HeLa cells [98]. However, Oxenoid et al. 2016 using NMR and electron microscopy (EM) reported that MCU form a pentamer in C. elegans [101]. Subsequent studies in different fungi, and zebra fish using, typically, Cryo-EM revealed tetrameric MCU oligomers [113-115]. It seems that depending on the organism, MCU channel formation can be different.

**Mitochondrial Ca\(^{2+}\) Uniporter Paralog/Isoform**

Alongside MCU, the greater MCU complex contains several auxiliary proteins. In mammals, a homologous subunit known as CCDC109B (MCUb) is another pore-forming subunit. Discovered in 2013, MCUb is a protein that shares 50% AA sequence with MCU and is believed to inhibit mitochondrial Ca\(^{2+}\) uptake [98]. Distinct from MCU, MCUb has a E256V mutation in the 1\(^{st}\) transmembrane domain near the pore of the channel; this loss of a negative charge is hypothesized to cause the dominant negative phenotype. However, not all research corroborates this original idea about the MCUb function. Knockdown of MCUb in trypanosomes does not alter mitochondrial Ca\(^{2+}\) uptake, while overexpression enhances and can even rescue mitochondrial Ca\(^{2+}\) uptake.
in the cells with silenced MCU [99, 100]. Unfortunately, to date not many studies have been published regarding MCUb function, even though different tissues express MCU and MCUb in different ratios, which might explain the variability of the rate of mitochondrial Ca$^{2+}$ uptake among various tissues [116, 117]. Other organisms are known to have more than just 2 pore-forming subunits that further regulate mitochondria Ca$^{2+}$ uptake such as enhancing Ca$^{2+}$ conductance [100, 118]. Although pore structure can alter channel kinetics, other subunits that do not form the pore itself can exert great control over the channel activity.

**EMRE**

Originally discovered after characterization of the other MCUC subunits, orphaned mitochondrial protein C22orf32 (EMRE) has been found to play a significant role in regulating MCU function. EMRE is a relatively small (10 kD) protein with 1 transmembrane domain that was found to co-immunoprecipitate with the MCUC [119]. It seems to be necessary for MCUC assembly in metazoans, while organisms outside of metazoa do not seem express any sort EMRE homolog [118, 120]. Interestingly, in metazoans, EMRE expression is required for proper MCU function irrespective of other subunit expression [121]. However, MCU from certain fungi do not express, nor require EMRE to function [122]. Based on electrophysiology in mitoplasts from HeLa cells, the C-terminus for EMRE acts as a Ca$^{2+}$ sensor that inhibits mitochondrial Ca$^{2+}$ uptake when matrix Ca$^{2+}$ levels are elevated [123]. Though this function is called into questions by Tsai et al. 2016, who shows the C-terminus being located in the IMS [120]. EMRE
seems subunit of the MCUC unique to metazoans, but is essential for MCUC function in metazoans.

**MICU Proteins**

The last auxiliary subunits that will be discussed are the “gatekeepers” of the MCUC complex that are probably the most components of the complex in regards to their function. The first subunit of the MCUC discovered is known as CBARA1 (MICU1), which lead to the discovery of MCU [93, 94, 124]. MICU1 is a 54 kD protein with 2 EF-hand domains that is known to specifically bind Ca\(^{2+}\) found in the intermembrane space (IMS) [125, 126]. MICU1-knockdown (KD) inhibits mitochondrial Ca\(^{2+}\) uptake in response to stimulation; however, it also leads to an increase [Ca\(^{2+}\)]\(_{mt}\) at rest [126, 127]. Aside from inhibiting mitochondrial Ca\(^{2+}\) uptake at baseline, MICU1 also controls the \([\text{Ca}^{2+}]_{\text{cyt}}\) requirement to activate mitochondrial Ca\(^{2+}\) uptake. Interestingly, MICU1 loss decreases mitochondrial Ca\(^{2+}\) uptake when the MCUC has been activated [126, 128]. However, some research has shown enhanced mitochondrial Ca\(^{2+}\) uptake in MICU1-KD [129]. Structural insights revealed a MICU1 dimerization while suggesting that 3 dimers oligomerize at the mouth of the MCU pore to block Ca\(^{2+}\) uptake; a distinct change in confirmation occurs when Ca\(^{2+}\) binds to the EF hands [130, 131]. The C-helix near the C-terminus seems to be necessary for Ca\(^{2+}\)-free MICU1 binding to MCU as well as enhancement of Ca\(^{2+}\) conductance through MCU. However, other research has suggested a polybasic region near the N-terminus confers MCU binding ability in MICU1 [129]. Lastly, research shows that MICU1 binding affinity for Ca\(^{2+}\) is around 350 nM while MICU1 alone can prevent Ca\(^{2+}\) uptake in isolated mitochondria up to 300 nM,
compared to 200 nM in MICU1/2 KD [132]. A splice variant of MICU1, called MICU1.1, is expressed in skeletal muscle that increases mitochondrial Ca\(^{2+}\) uptake more than MICU1 itself, even when dimerized with MICU2 [133].

A less studied protein in the MCUC is MICU2, which is another protein that has been shown to regulate MCU activity. When first discovered in 2013, MICU2-KD inhibited mitochondria Ca\(^{2+}\) uptake in isolated mitochondria similar to MICU1-KD [134]. However, subsequent studies showed MICU2-KD enhanced mitochondrial Ca\(^{2+}\) uptake, which was shown to provide further enhancement to MICU1 overexpression [135, 136]. This led investigators to hypothesize that there is a cooperative but opposite effects of the MICU proteins on mitochondrial Ca\(^{2+}\) uptake [117, 136, 137]. The last and least studied component of the MCUC complex is a unique MICU isoform that is expressed only in the nervous system, known as MICU3. Discovered alongside MICU2 as a EF-hand protein, not much research on MICU3 has been done [134]. A recent report shows that MICU3 expression dramatically enhances mitochondrial Ca\(^{2+}\) uptake driven by neuronal activity [138]. With the components of the MCUC fully explored, next section will talk about the discoveries made by observing the effects of MCUC manipulation in vivo.

**In vivo Regulation of MCU**

Since the discovery of the molecular identities and functions of the various components of the MCUC complex, a number of studies examined the effects of MCUC genetic manipulation in animal models or the effect of MCUC mutation in humans. In 2013 the first whole body MCU-KO mouse model was generate and characterized [96].
Interestingly, this mouse model had to be generated on the CD1 background because it was embryonically lethal in C57BL/6J mice [139]. Even in the CD1 mice, there still seems to be some embryonic lethality. The most obvious characteristics of MCU-KO mice was a slight decrease in the body weight compared to WT littermates. Mitochondria isolated from both heart and skeletal muscle showed a near complete block of mitochondrial Ca\(^{2+}\) uptake. An altered metabolism in regards to Ca\(^{2+}\)-dependent O\(_2\) consumption, pyruvate dehydrogenase phosphorylation, and lactate production all in skeletal muscles, but no difference in basal metabolism [96]. Interestingly, *in vitro* MCU-KO protected against mPTP opening; however, MCU KO did not protect against infarct in heart in an *ex vivo* Langendorf model of I/R. What’s more, MCU-KO prevented protection against ischemic injury using CsA to block cyclophilin D, in contrast to CsA produced protection found in WT. This finding goes against the hypothesized role of mitochondrial Ca\(^{2+}\) overload in cell death in I/R [57, 104, 140-142]. In the follow-up studies, mice that expressed dominant negative (DN) MCU (disrupted DIME sequence) showed impaired heart function and metabolism, which could be rescued with addition of ATP [143]. DN-MCU mice did not show any protection in the Langendorf model of I/R. Then, two research groups published data on MCU function on the heart function but instead using an heart-specific inducible MCU-KO (iMCU-KO) in adult mice [144, 145]. Again showing deficits in Ca\(^{2+}\)-induced cellular metabolism and isopreteronol-induced heart stimulation, the effects of iMCU-KO were similar to the effects of full MCU-KO. However, unlike full MCU-KO, these mice were protected against a left coronal artery ligation I/R model compared to WT mice. Lastly, the iMCU-KO mice had reduced ability to sprint compared to WT, only if they had a short (2 min) warm-up run
before sprinting, while iMCU-KO mice could match WT sprinting if the warm-up was extended (30 min) [144]. Similarly, using a neuron specific MCU-KO, Nichols et al. 2018 showed neuroprotection against a left common carotid artery occlusion in vivo model of stroke in mice [146]. In cultured cortical neurons overexpressing MCU made neurons more vulnerable to NMDA-induced excitotoxicity and cell death while KD showed the opposite results [111].

Although the majority of the recent work on MCU role focused on cardiac functions, other aspects of MCU in vivo biology have been studied. For example, overexpression of MCU in adult mice skeletal muscles led to an increase in mRNA in muscle hypertrophy intracellular pathway and an increase in muscle fiber size, while shRNA against MCU had the opposite results [147]. MCU expression is also upregulated in humans that frequently exercise or undergo muscle stimulation [148]. MCU deletion in macrophages inhibits pulmonary fibrosis caused by asbestos exposure in mice [149]. Additional major pathway that involve MCU are wound healing process and metastasis pathways in cancers [110, 150, 151]. In trypanosomes, MCU expression is important in the infection cycle and growth rate of these organisms [99, 100]. The only study investigating the role of MCU in learning and memory was carried out in D. melanogaster. The mushroom body neurons in drosophila are important for odor related learning and memory. MCU-KO in pupae led to an intermediate learning deficit, where association between the odor and unconditioned stimulus was delayed by 3 hrs compared to WT [152]. The impaired memory phenotype corresponded to a decrease in synaptic vesicles at the synapse and an increase in axon length. Surprisingly, this memory impairment was not observed when MCU was deleted in adult drosophila,
suggesting a more developmental role for MCU function. These results corroborate previous research showing that mitochondrial Ca\(^{2+}\) transport is important in development until other mechanisms take over for synaptic facilitation [39, 153].

**In vivo Regulation of Auxiliary Proteins**

The last aspect of MCUC discussed will be the function of other auxiliary subunits *in vivo*. One of the most studied auxiliary subunits of the MCUC complex is MICU1. In mice, loss of MICU1 enables mitochondrial Ca\(^{2+}\) uptake at low (~0.5 µM) [Ca\(^{2+}\)\(_{\text{ext}}\)], but inhibited mitochondrial Ca\(^{2+}\) uptake at high (~16 µM) [Ca\(^{2+}\)\(_{\text{ext}}\)] and an increase in resting [Ca\(^{2+}\)\(_{\text{mt}}\)], similar to other studies investigating MICU1 function [126, 128, 154]. Developmentally, MICU1-KO mice show a decrease in the body size, disruption of cerebellar cellular architecture, and dysfunctional skeletal muscle metabolism. However, as the mice age these deficits disappear and MICU1-KO mice become comparable to age-matched WT mice. Interestingly, this developmental change corresponds to downregulation of EMRE. Moreover, heterozygous deletion of EMRE increases MICU1-KD viability while complete EMRE deletion leads to total embryonic lethality. Hemizygous EMRE expression rescues neurological and mycological deficits including baseline mitochondrial Ca\(^{2+}\) uptake, but not normal mitochondrial Ca\(^{2+}\) uptake [154]. These data suggest a greater role for basal [Ca\(^{2+}\)\(_{\text{mt}}\) in proper development compared to the role of activity-induced mitochondrial Ca\(^{2+}\) uptake. In drosophila, MICU1-KO led to a decreased survival rate, impaired locomotor activity, and defects in neuronal development in the eye [155]. Overexpressing the drosophila Bcl2 homolog, Buffy, rescued the MICU1-KO phenotype, suggesting that MICU1-KO led to an increase
in cell death. Since the discovery of the MCUC, mutations in subunits of MCUC in humans has led to important discoveries regarding the role of mitochondrial Ca\textsuperscript{2+} transport in human physiology. Loss of function mutations in MICU1 in humans lead to muscular dystrophy, learning disabilities, and an extrapyramidal movement disorder [156, 157]. Fibroblasts from these patients revealed an increase in basal [Ca\textsuperscript{2+}]\text{mt}, a decrease in phosphorylation of pyruvate dehydrogenase, decreased pDRP1, and increased mitochondrial Ca\textsuperscript{2+} uptake at low [Ca\textsuperscript{2+}]\text{ext} [156, 158]. A MICU2 null mutation in humans leads to encephalopathy and severe cognitive impairments, while mitochondria function assays show similar results as seen in previous literature [159]. These human studies highlight the importance of mitochondrial Ca\textsuperscript{2+} transport in proper development of organ systems, much of which relates to nervous system development.

**Mitochondrial Ca\textsuperscript{2+} Efflux**

Since the discovery of mitochondrial Ca\textsuperscript{2+} uptake, especially given that mitochondria can accumulate a pathophysiological amount of Ca\textsuperscript{2+}, significant attention has been devoted to investigation of the mechanisms of mitochondrial Ca\textsuperscript{2+} extrusion [82]. The process of Ca\textsuperscript{2+} extrusion from mitochondria is complex and is regulated by many factors. One of the first aspects investigators discovered was that the efflux mechanism was independent from mitochondrial Ca\textsuperscript{2+} uptake pathway given certain characteristics of the efflux pathway. One important factor of mitochondrial Ca\textsuperscript{2+} efflux is the ability to move Ca\textsuperscript{2+} against its electrochemical gradient [88]. Another aspect was that inhibitors of mitochondrial Ca\textsuperscript{2+} uptake did not affect the rate of mitochondrial Ca\textsuperscript{2+} efflux [160]. There are several different mechanisms involved in the regulation of the
mitochondrial Ca\(^{2+}\) efflux pathway, with many of them not directly interacting with the Na\(^+\)/Ca\(^{2+}\) exchanger. The efflux pathway is more active when the permeant anion is acetate compared to P\(_{i}\) [80, 81]. This is hypothesized to be due to P\(_{i}\) sequestering matrix Ca\(^{2+}\) and preventing saturation of Ca\(^{2+}\) efflux pathway. This suggests that the activity of the efflux pathway is dependent upon [Ca\(^{2+}\)]\(_{mt}\). Some of the earliest research investigating mitochondrial Ca\(^{2+}\) efflux noted the effect of Na\(^+\) addition to stimulate mitochondrial Ca\(^{2+}\) efflux [161]. Initial studies showed that most of mammalian tissues exhibit a Na\(^+\)-dependent mitochondrial Ca\(^{2+}\) efflux, with a few exceptions such as liver and kidney [162-164]. However, subsequent studies do suggest the presence of Na\(^+\)-dependent mitochondrial Ca\(^{2+}\) efflux both in liver and kidney [165]. Research in the CNS tissue specifically showed a Na\(^+\)-dependent mtNCX mechanism with little effect from other ions [88]. Another important aspect of mtNCX function was its ability to transfer charge across the IMM. It was first hypothesized that Na\(^+\)/Ca\(^{2+}\) exchange was electroneutral (2 Na\(^+\)/1 Ca\(^{2+}\)) [79]. However, later research suggested a stoichiometry of 3 Na\(^+\)/1 Ca\(^{2+}\), leading to a net positive charge transfer into the mitochondrial matrix [166, 167]. The latter finding implies that activity of the efflux mechanism depends on \(\Delta \Psi_{mt}\), as depolarized mitochondria are expected to inhibit the transfer of the positive charge into the mitochondrial matrix. Even before the discovery of the molecular identity of mtNCX, many mechanistic aspects of mtNCX were elucidated.

In addition to Na\(^+\)/Ca\(^{2+}\) exchanger, mitochondria also express a H\(^+\)/Na\(^+\) exchanger which extrudes Na\(^+\) at a very rapid rate [168]. The H\(^+\)/Na\(^+\) exchanger rate is significantly faster than that of Na\(^+\)/Ca\(^{2+}\) exchanger, which is suggested to be important in keeping the Na\(^+\)/Ca\(^{2+}\) mechanism saturated. The mtNCX mechanism from nervous
tissue mitochondria show sensitivity to either Mg\(^{2+}\) or ADP induced inhibition of Na\(^+\)/Ca\(^{2+}\) [169, 170]. Interestingly, even the age of the animal that the mitochondria are isolated from has been shown to affect the rate of mitochondrial Ca\(^{2+}\) efflux [171]. Some tissue, including nervous, exhibit a Na\(^+\)-independent Ca\(^{2+}\) efflux pathway [79].

**Putative Identity of the mtNCX Mechanism**

One of the initial attempts to discover the identity of the mtNCX came from purification of protein from beef heart mitochondria. Li et al. 1992 isolated a 110 kD protein from beef heart mitochondria that showed Na\(^+\)/Ca\(^{2+}\) exchange activity when expressed in lipid bilayers [172]. However, nearly two decades would pass before any new insight into the identity of the mtNCX mechanism would be discovered. In 2010, research suggested that the previously discovered protein NCKX6, was the identity of the molecule responsible for the mtNCX mechanism [173]. NCKX6 was originally cloned from FLJ22233; NCKX6 was shown to be expressed ubiquitously in mouse tissue and is structurally more closely related to the NCKX family of exchangers [174, 175]. Analysis of the gene itself revealed two splice variants with the full-length (fl) isoform acting as a K\(^+\) independent NCX, while NCKX6fl showed some ability to utilize Li\(^+\) in Ca\(^{2+}\) exchange. Localization using immunocytochemistry in HEK-293 cells showed the short-isoform localized to the plasma membrane, while NCKX6fl located to the ER [174]. Similarly, another group published on NCKX6fl, which they renamed to NCLX, and showed the ability for NCLX to utilize Li\(^+\)/Ca\(^{2+}\) exchange for the efflux of Ca\(^{2+}\) on the PM with no difference in efficiency compared to Na\(^+\)/Ca\(^{2+}\) exchange [176]. Interestingly, Cai and Lytton 2004 show major molecular bands at ~55 kD, while Palty et al. 2004 show
major bands at ~70kD with some minor bands at ~55 kD. The 2010 paper was the first to suggest that NCLX can be located on the mitochondria [173]. Western blot analysis on mitochondria fraction from mouse, and rat tissues as well as HEK-293 showed a strong band at ~55 kD while a weak band was present at ~100 kD. Gold particle EM immunostaining showed NCLX localizes to mitochondria in rat cortex and CHO cells; however, some gold particles were localized outside mitochondria. Overexpression of NCLX significantly increased mitochondrial Ca\(^{2+}\) efflux, whereas NCLX-KD using shRNA inhibited Ca\(^{2+}\) efflux. NCLX-KD also inhibits the ability of mitochondria to utilize Li\(^+\) in Ca\(^{2+}\) exchange. However, unlike previous research, Palty et al. 2010 show no deficit in Li\(^+\)/Ca\(^{2+}\) mitochondrial Ca\(^{2+}\) efflux compared to Na\(^+\)/Ca\(^{2+}\) exchange [173]. Although the Li\(^+\)/Ca\(^{2+}\) exchange rate does not match previous literature, research investigating the AA residues responsible for Li\(^+\)/Ca\(^{2+}\) exchange has been done to characterize the NCLX protein. Specific mutations in the α-repeat domains 1 and 2, the conserved Na\(^+\)/Ca\(^{2+}\) exchange domain in NCX proteins, led to inhibition of either Li\(^+\) or Na\(^+\)/Ca\(^{2+}\) exchange. Specifically Asn149, Pro152, Asp153, Asn467, Ser468, and Gly494 mutations inhibited Na\(^+\)/Ca\(^{2+}\) but without affecting Li\(^+\)/Ca\(^{2+}\) exchange, while Asp471 mutation inhibits Li\(^+\)/Ca\(^{2+}\) but not Na\(^+\)/Ca\(^{2+}\) exchange [177, 178].

**Function of NCLX in vivo**

This research has led to many studies investigating the role of NCLX on mitochondrial Ca\(^{2+}\) efflux and how that affects cellular function. NCLX controls stimulation-induced Ca\(^{2+}\)-dependent NAD(P)H production, and mitochondrial redox state. NCLX overexpression inhibits NAD(P)H production and reduction of mitochondrial
redox state, which corroborates previous research on the role of mitochondrial Ca\(^{2+}\) transport [179]. In B lymphocytes, NCLX-KD prevents mitochondrial polarization and decreased chemotaxis in response to chemokine exposure [180]. NCLX-KD in HL-1 cardiomyocytes reduces the frequency of spontaneous Ca\(^{2+}\) oscillations and affects kinetics of each [Ca\(^{2+}\)], while overexpressing NCLX rescues this phenotype [181]. Inducible NCLX-KO (iNCLX-KO) in the hearts of adult mice lead to many deleterious effects [182]. iNCLX-KO significantly inhibited mitochondrial Ca\(^{2+}\) efflux and increased mitochondrial swelling in response to Ca\(^{2+}\) application in cultured myocytes. iNCLX-KO caused swelling in the heart and lead to death in 87% of iNCLX-KO mice within two weeks of NCLX-KO induction. Lastly, overexpression of NCLX reduced damage/infarct volume in ventricles caused by LCA I/R [182].

Many studies have been done investigating the function of NCLX in the nervous tissues as I will review next. In DRG neurons, NCLX-KD lead to inhibition of mitochondrial Ca\(^{2+}\) efflux in response to capsaicin, as expected; however, it led to a decrease in [Ca\(^{2+}\)]\(_{cyt}\) amplitude [183]. This is in contrast of previous research investigating the effects of mitochondrial Ca\(^{2+}\) efflux in DRG neurons [50]. The authors speculate that decrease in mitochondrial Ca\(^{2+}\) efflux leads to inhibition of MCU function which builds up [Ca\(^{2+}\)]\(_{cyt}\) that leads to TRPV1 inhibition. In glia from the CNS, NCLX-KD lead to a decrease in stimulation evoked mitochondrial Ca\(^{2+}\) efflux, store-operated Ca\(^{2+}\) entry, [Ca\(^{2+}\)]\(_{cyt}\) amplitude, and glutamate release. Interestingly, NCLX-KD decreased astrocyte proliferation and migration caused by wounding in culture [184]. Relevant to the role of nervous system pathology several groups studied the effect of NCLX on Parkinson-like phenotypes in culture. Kostic et al. 2015 showed that the loss of PINK1
function inhibited mitochondrial Ca\(^{2+}\) efflux leading to increased cell death. PKA activation rescued this phenotype by phosphorylating NCLX on S259, restoring mitochondrial Ca\(^{2+}\) efflux [185]. Similarly, using Parkinson's associated protein LRRK2 mutants, Verma et al. 2017 showed phosphomimetic (S259D) of NCLX (enhancing NCLX function) rescued neurite defects seen in diseased neurons [186]. These results show an important function of NCLX on mitochondrial bioenergetics, and Ca\(^{2+}\) handling which affects overall health of neurons, especially in diseased states.

Despite evidence suggesting the identity of the \(m\)NCX mechanism, not all research strictly shows NCLX as the molecule responsible for mitochondrial Ca\(^{2+}\) efflux. Similar to previous research some groups report NCLX being found on the plasma membrane, which is important for function of cells [174, 176, 187]. Importantly, NCLX is not listed in the MitoCarta, the most comprehensive database of mitochondrial proteins [188]. In contrast, all of the components of the MCU complex are listed in MitoCarta. Other NCX proteins have been suggested to be located at mitochondria in adult brain tissue from rats [189]. In 2013, research on SH-SY5Y cells showed that NCX2 or 3-KD, or antibody targeting led to a significant decrease in mitochondrial Ca\(^{2+}\) extrusion [190].

Other mechanisms for mitochondrial Ca\(^{2+}\) efflux include a H\(^{+}\)/Ca\(^{2+}\) exchange mechanism [90, 191]. The H\(^{+}\)/Ca\(^{2+}\) exchange mechanism was initially proposed to be the major mitochondrial Ca\(^{2+}\) efflux mechanism in mammalian liver mitochondria [162]. In fungi, research has shown that the major mechanism for mitochondrial Ca\(^{2+}\) efflux [161, 192, 193]. Although excitable mammalian tissues utilize a \(m\)NCX mechanism, there does exist a mitochondrial H\(^{+}\)/Ca\(^{2+}\) exchanger expressed ubiquitously in mammals. Letm1 was initially characterized as a K\(^{+}\)/H\(^{+}\) antiporter; however, expression
of Letm1 on liposomes showed H\(^+\)/Ca\(^{2+}\) activity [194]. Subsequent research further corroborated Letm1 as a H\(^+\)/Ca\(^{2+}\) exchanger [195, 196]. Although some recent research propose a Letm1 K\(^+\)/H\(^+\) mediated effect on mitochondrial Ca\(^{2+}\) dynamics [197]. Interestingly, deletion of Letm1 in humans has been linked to seizure disorders [198-200].
CHAPTER II:
EFFECT OF MCU DELETION ON Ca^{2+} DYNAMICS AND MITOCHONDRIAL BIOENERGETICS

Introduction

During neuronal activity, mitochondria efficiently buffer cytosolic Ca^{2+} then release it back into the cytosol. This process shapes cytosolic Ca^{2+} signaling, affecting the [Ca^{2+}]_{cyt} and duration of the Ca^{2+} signal. Mitochondrial Ca^{2+} transport has been shown to affect many neuronal processes including neurotransmission, gene expression, and cell death among others. However, even though there were mechanisms proposed for mitochondrial Ca^{2+} uptake the specific molecules responsible were unknown. Then, in 2011, two papers were published back to back in nature that discovered the pore-forming subunit for the mitochondrial Ca^{2+} uniporter complex (MCUC) called CCDC109A (MCU) [93, 94]. Alongside MCU, other subunits of the MCUC have been discovered such as MCU homolog MCUb, MICU1 and MICU2, and EMRE [201]. Even in 2010, the molecule for the mtNCX was discovered as being the molecule NCLX (NCKX6) [173]. However, it is still unknown how these specific molecules affect normal neuronal and mitochondrial functions.

The MCUC as a whole acts as a Ca^{2+} activated, Ca^{2+} permeable channel on the IMM that is believed to be the canonical pathway for mitochondrial Ca^{2+} uptake. MCU is the core, pore-forming subunit of the MCUC that forms a Ca^{2+} permeable channel. Alongside MCU is another pore-forming subunit known as CCDC109B (MCUb), which research has shown to actually inhibit mitochondrial Ca^{2+} uptake [202]. Other Auxillary subunits MICU1 and MICU2 act as “gate keepers” for the channel itself, keeping the channel closed at low [Ca^{2+}]_{cyt} and opening at high [Ca^{2+}]_{cyt} as well as increasing...
channel conductance [137]. Lastly, EMRE is an auxiliary subunit that connects the
MICU subunits to MCU [203]. The function MCU plays in humans is unknown; however,
life studies have shown an important role for MICU1 and MICU2. In humans, loss-of-
function MICU1 or null mutations in MICU2 lead to cognitive and motor deficits, which
is attributed to mishandling mitochondrial Ca\textsuperscript{2+} uptake at rest [157, 159].

Mitochondrial Ca\textsuperscript{2+} transport has been shown to be important for many functions;
however, its role in mitochondria bioenergetics is the most heavily investigated aspect
of it. Ca\textsuperscript{2+} in the mitochondrial matrix interacts with several enzymes in the Kreb cycle
including pyruvate dehydrogenase phosphatase, isocitrate dehydrogenase, and α-
ketoglutarate dehydrogenase increasing production of NADH to be fed into the ETC
[204, 205]. There is also evidence to suggest that Ca\textsuperscript{2+} is able to interact with the ETC
as well [206]. These interactions with the enzymes related to production of NADH and
function of the ATP synthase overall leads to enhancement of ATP synthase for cells.
However, the role of MCU in regulating mitochondrial bioenergetics has not been
tested.

In spite of all of the evidence suggesting a strong role for mitochondrial Ca\textsuperscript{2+}
buffering in neuronal function, knockdown of MCU resulted in a decrease or no change
in stimulation evoked [Ca\textsuperscript{2+}]\text{cyt} amplitude [37, 52, 55, 183, 207, 208]. Additionally, MCU
knockdown created variable inhibition of mitochondrial Ca\textsuperscript{2+} uptake in neurons [208].
Thus, many questions remain on the function MCU plays in handling mitochondrial Ca\textsuperscript{2+}
transport and its effect on cytosolic Ca\textsuperscript{2+} signaling in nervous tissue.

In the present study, we investigated the role MCU has in regulating
mitochondrial and cytosolic Ca\textsuperscript{2+} dynamics as well as the impact it has on mitochondrial
bioenergetics in nervous tissue. Thus using Ca\textsuperscript{2+} imaging and electrophysiology we determined how loss of MCU affected the interplay of Ca\textsuperscript{2+} signaling between the IMM and plasma membrane. Additionally, using fluorescent microscopy we interrogated some bioenergetic processes of mitochondria and the role MCU plays in regulating them.

**Methods**

**Animal Subjects**

All experiments involving the use of mice and the procedures used therein were approved by the University of Iowa Institutional Animal Care and Use Committee and were carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. The MCU knockout (KO) mouse strain was obtained from Dr. Toren Finkel (NIH/NHLBI) and was previously described [209]. Mice were maintained on a CD1 background (Charles River Laboratories, Wilmington, MA). Mice were housed in the Bowen Science Building and Central Vivarium of the University of Iowa animal facilities under a 12 h light: dark cycle with ad libitum access to food and water. For genotyping, genomic DNA was extracted from either tail clips or ear punches and amplified by PCR (forward primer: 5’-GTGCCCTCTGTAGCGTGACGG-3; reverse primer: 5’-ATGACAAGCTTAAAGTCATC-3’) as described by Pan and colleagues [209].
Primary Neuronal Cultures

Primary cultures of dorsal root ganglia (DRG) neurons were prepared from adult (6-10 week-of-age) mice of either MCU^{+/+}, MCU^{+-} or MCU^{-/-} genotype and transfected with one of the three mitochondrial Ca^{2+} indicators (mito-R-GECO1, mito-LAR-GECO1.2 or mito-GEM-GECO) using an Amaza nucleofector as previously described [210]. DRG cultures were grown in a DMEM supplemented with NGF (50 ng/mL), insulin (6 μg/mL), 5% heat-inactivated horse serum (HIHS), 5% fetal bovine serum (FBS) and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) in a 10% CO_{2} incubator at 37°C for 2-3 days before experimentation.

Primary cultures of hippocampal neurons were prepared from neonatal (P0-P1) mice of either MCU^{+/+}, MCU^{+-} or MCU^{-/-} genotype and transfected with one of the four mitochondrial Ca^{2+} indicators (mito-R-GECO1, mito-LAR-GECO1.2, mito-GEM-GECO or mtPericam) using Lipofectamine 2000 using a previously described protocol [211]. The cultures were grown in Neurobasal-A medium supplemented with B-27, 0.5 mM L-glutamine and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) in a 5% CO_{2} incubator at 37°C for 12-14 days before experimentation.

The DRG and hippocampal cultures were prepared from female and male mice. No significant differences were apparent for the results derived from the two sexes; hence the results from both were combined for statistical analysis. Detailed descriptions of procedures for the DRG and hippocampal primary cultures are provided in Method Details.
cDNA Constructs and FIV Lentivirus

The plasmid encoding the mitochondrial Ca\(^{2+}\) indicator mtPericam (pcDNA3-mtPericam) was a gift from Dr. Atsushi Miyawaki (RIKEN, Japan) [212]. The plasmid encoding Flag/DDT-tagged mouse wild-type MCU (WT MCU) was obtained from OriGene (Cat.# MR218926). The mtEGFP plasmid (mitochondria targeted EGFP) was a gift from Dr. Colin Campbell (University of Minnesota) [213]. The plasmid encoding the low-affinity mitochondrial Ca\(^{2+}\) indicator mito-LAR-GECO1.2 (CMV-mito-LAR-GECO1.2) was previously described [211], and the plasmids encoding two other mitochondrial Ca\(^{2+}\) indicators, mito-R-GECO1 and mito-GEM-GECO1, were obtained from Addgene (Cat.# 46021 for CMV-mito-R-GECO1 and Cat.# 32461 for CMV-mito-GEM-GECO1).

For the preparation of the mito-R-GECO1 and mito-LAR-GECO1.2 FIV (feline immunodeficiency virus) lentiviruses, the corresponding cDNA sequences were PCR amplified (forward primer: 5'-GAGGTCTATATAAGCAGAGC-3'; reverse primer: 5'-GACGTCGACGAATTCGAGGCTGATCAGCGGTTTAAAC-3'), cut using EcoRI and NheI, and ligated into the EcoRI and SpeI sites of the lentiviral shuttle vector pFIV3.2-CAG-mcs. The resulting plasmids were termed pFIV3.2-mito-R-GECO1 and pFIV3.2-mito-LAR-GECO1.2, respectively. The corresponding lentiviruses (1x10\(^7\) - 5x10\(^8\) transforming units/ml) were produced by the University of Iowa Viral Vector Core.

Preparation and Transfection of Primary DRG Cultures

DRG neurons were prepared from adult mice and transfected using a method similar to those previously described [210]. Specifically, lumbar, thoracic and cervical DRG were dissected from adult (6-10 wk) mice of the MCU\(^{+/+}\), MCU\(^{+/−}\) or MCU\(^{−/−}\)
genotypes. Isolated DRG were digested using first collagenase A (2 mg/mL for 20 min; Roche) and then Pronase E (1 mg/mL for 10 min; Serva); both were applied in DMEM/HEPES (20 mM; pH 7.4) solution at 37°C. Cells were then washed in DMEM/HEPES (20 mM; pH 7.4) and mechanically dissociated by sequential trituration with increasingly smaller bore-sized fire-polished Pasteur pipettes. Cells were transfected with one of the mitochondrial Ca\(^{2+}\) indicators (mito-R-GECO1, mito-LAR-GECO1.2 or mito-GEM-GECO1) using an Amaxa Nucleofector according to the manufacturer's protocol (program G-013; Mouse Neuron Nucleofector Kit; Amaxa/Lonza). Where indicated, MCU-KO DRG neurons were co-transfected with a wild-type MCU plasmid. Transfected cells were resuspended in a DMEM supplemented with NGF (50 ng/mL), insulin (6 μg/mL), 5% HIHS, 5% FBS and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) and plated onto 25 mm glass coverslips pre-coated with poly-L-ornithine (0.2 mg/mL) and laminin (50 μg/mL) in a 6 well plate. DRG cultures were maintained in a 10% CO\(_2\) incubator at 37°C for 2-3 days before experimentation.

**Preparation and Transfection of Primary Hippocampal Cultures**

Primary cultures of hippocampal neurons were prepared from neonatal (P0-P1) mice of the MCU\(^{+/+}\), MCU\(^{+-}\) and MCU\(^{-/-}\) genotypes and transfected using a protocol similar to those previously described [211]. Specifically, the brain was removed from P0-P1 mice and hippocampi were dissected in ice-chilled Neurobasal A medium supplemented with 20 mM HEPES (pH 7.35) and 0.5 mM L-glutamine, and then digested in trypsin solution (1 mg/mL) for 10 min at 24°C. Cells were washed in fresh
medium, mechanically dissociated by sequential trituration with increasingly smaller bore-sized fire-polished Pasteur pipettes, and plated onto 25 mm glass coverslips, pre-coated with poly-L-ornithine (0.2 mg/mL) and laminin (50 μg/mL) in a 6-well plate. The cultures were grown in Neurobasal-A medium supplemented with B-27, 0.5 mM L-glutamine and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) in a 5% CO2 incubator at 37°C. The medium was replaced by 50% with fresh medium every 3-4 days.

After 6-7 DIV, hippocampal neurons were transfected with one of four mitochondrial Ca\(^{2+}\) indicator plasmids (mito-R-GECO1, mito-LAR-GECO1.2, mito-GEM-GECO or mtPericam) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. For MCU-KO rescue experiments, hippocampal neurons were also co-transfected with a wild-type MCU plasmid. Cells were used for experimentation at DIV 12-14. In some cases, mito-R-GECO1 or mito-LAR-GECO1.2 was expressed in hippocampal neurons by adding the appropriate FIV lentivirus to the culture at DIV 5-7. The results obtained using FIV did not differ from those obtained using Lipofectamine 2000, and therefore were pooled together for final statistical analysis.

Simultaneous Imaging of Mitochondrial and Cytosolic Ca\(^{2+}\) in Neurons

Simultaneous monitoring of the Ca\(^{2+}\) concentration in the cytosol ([Ca\(^{2+}\)\text{cyt}]) and mitochondria ([Ca\(^{2+}\)\text{mt}]) of cultured DRG or hippocampal neurons was performed largely as previously published [211], but with some modifications. Specifically, DRG or hippocampal neurons that had been transfected with either the high-affinity
mitochondrial Ca\(^{2+}\) indicator mito-R-GECO1 \((K_d=0.48 \, \mu M)\) [214] or the lower-affinity, mito-LAR-GECO1.2 \((K_d=12 \, \mu M)\) [211], were loaded with either Fura-2/AM or FuraFF/AM Ca\(^{2+}\) indicators (2 \, \mu M for 30 min). In the case of whole-cell patch-clamp experiments, the cells were loaded with Fura-2 pentapotassium salt via the patch-pipette solution (100 \, \mu M), as described under Patch-Clamp Recording. Cells were then placed into a chamber for flow-through perfusion and mounted on an inverted IX-71 microscope (Olympus, Japan). Cells were perfused with a standard extracellular HEPES-buffered Hank’s salt solution of the following composition: 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl\(_2\), 0.4 mM MgSO\(_4\), 0.5 mM MgCl\(_2\), 0.4 mM KH\(_2\)PO\(_4\), 0.6 mM NaHPO\(_4\), 3 mM NaHCO\(_3\), 10 mM glucose, 10 mM HEPES, pH 7.4, with NaOH (310 mOsm/kg with sucrose).

For imaging, fluorescence was sequentially excited at 340 nm (12 nm bandpass), 380 nm (12 nm bandpass), and 550 nm (12 nm bandpass) using a Polychrome V monochromator (TILL Photonics, Germany) and a 40x oil-immersion objective (NA=1.35, Olympus). Fluorescence emission was separated from excitation using a dual fluorophore beamsplitter FF493/574-Di01 (Semrock; Rochester NY) and signal was collected using a dual band emission filter FF01-512/630 (Semrock) and an IMAGO CCD camera (640x480 pixels; TILL Photonics, Germany). 2x2 binning was used for acquisition (1 pixel ~500 nm). Series of images at 340 nm, 380 nm and 550 nm images were acquired at a rate of 1-10 Hz, depending on the experiment. \([\text{Ca}^{2+}]_{\text{cyt}}\) was calculated from the fluorescence ratio \((R = F_{340}/F_{380})\), using the formula:

\[
[\text{Ca}^{2+}]_{\text{cyt}} = K_d \beta (R_{\text{min}})/(R_{\text{max}}-R).
\]

\(R_{\text{min}}, R_{\text{max}}\) and \(\beta\) were calculated by applying 10 \, \mu M ionomycin in either Ca\(^{2+}\)-free buffer (1 mM EGTA) or Ca\(^{2+}\)-containing (1.3 mM)
HEPES-buffered Hank’s salt solution. A $K_d$ value of 275 nM was used for Fura-2 [215], and a $K_d$ value of 5.5 $\mu$M was used for FuraFF (Molecular Probes Handbook). Changes in $[Ca^{2+}]_{mt}$ were quantified as $\Delta F/F_0 = (F-F_0)/F_0$, where $F$ is current fluorescence intensity ($\lambda_{ex} = 550$ nm) and $F_0$ is the fluorescence intensity at baseline. At each wavelength, fluorescence was corrected for background as measured in an area free of cells.

In some experiments $[Ca^{2+}]_{mt}$ was monitored using other mitochondrial $Ca^{2+}$ indicators, mtPericam [212] or mito-GEM-GECO [214]. These experiments were carried out without $[Ca^{2+}]_{cyt}$ measurements due to spectral overlap with Fura-2 (FuraFF). $[Ca^{2+}]_{mt}$ measurements using mtPericam were performed as previously described [210, 216]. mtPericam fluorescence was excited at 410 nm (12 nm bandpass) via a 40x oil-immersion objective (NA=1.35, Olympus) and collected at 530 nm (50 nm bandpass) every 2 s using the same fluorescent microscope as described above. $[Ca^{2+}]_{mt}$ changes were presented as $-\Delta F/F_0 = -(F-F_0)/F_0$, where $F$ is current fluorescence intensity and $F_0$ is fluorescence intensity at baseline. Fluorescence was corrected for background, as determined in an area that did not contain any cell.

The resting $[Ca^{2+}]_{mt}$ in DRG and hippocampal neurons was measured using the ratiometric dual-emission mitochondrial $Ca^{2+}$ indicator mito-GEM-GECO1 [214] was used. The dye fluorescence was excited at 400 nm (12 nm bandpass) via a 40x oil-immersion objective (NA=1.35, Olympus) and measured at 483 nm (32 nm bandpass) and 530 nm (44 nm bandpass) using the same fluorescent microscope (IX-71, Olympus) as described above. $[Ca^{2+}]_{mt}$ was calculated from the fluorescence ratio ($R = F_{483}/F_{530}$) using the formula: $[Ca^{2+}]_{mt} = (K_d' \cdot n \cdot (R-R_{min})/(R_{max}-R))^{1/n}$, where $K_d'$ is an apparent dissociation constant (340 nM) and $n$ is the Hill coefficient (2.94) [214]. $R_{min}$
and $R_{\text{max}}$ were calculated by applying 10 μM ionomycin in either Ca$^{2+}$-free buffer (1 mM EGTA) or Ca$^{2+}$-containing (1.3 mM) HEPES-buffered Hank’s salt solution. At each wavelength, fluorescence was corrected for background as measured in an area free of cells. For the majority of the cells tested, $R$ was approximating the $R_{\text{min}}$ value at resting conditions. Therefore, resting [Ca$^{2+}$]$_{\text{mt}}$ was set to 0 if $R$ was ≤ ($R_{\text{min}}$ + 2σ), where σ is the standard deviation of $R_{\text{min}}$ determined from the calibration procedure (n=6 cells).

[Ca$^{2+}$]$_{\text{cyt}}$ and [Ca$^{2+}$]$_{\text{mt}}$ data were analyzed using the TILLvisION 4.5 software (TILL Photonics).

**Patch-Clamp Recordings**

The methods used for monitoring whole-cell Ca$^{2+}$ currents and action potential firing were similar to those we previously described [216, 217]. Whole-cell patch-clamp recordings were obtained using a patch-clamp amplifier Axopatch 200B and an analog-to-digital converter Digidata 1322A (Molecular Devices, Union City, CA). Data were collected (filtered at 2 kHz and sampled at 5 kHz) and analyzed using the pClamp 9 or 10 software (Molecular Devices, Union City, CA). Patch pipettes were pulled from borosilicate glass (Narishige; 3-5 mΩ) on a Sutter Instruments P-87 micropipette puller (Novato, CA).

For the recordings of Ca$^{2+}$ currents simultaneously with [Ca$^{2+}$]$_{\text{cyt}}$ and [Ca$^{2+}$]$_{\text{mt}}$, the composition of the patch-pipette solution was: 110 mM CsGluconate, 10 mM CsCl, 15 mM NaGluconate, 3 mM Mg-ATP, 1 mM MgCl$_2$, 10 mM HEPES, 100 μM Fura-2, pH 7.25 adjusted with CsOH (290 mOsm/kg with sucrose). The composition of the extracellular solution was: 115 mM CholineCl, 30 mM TEACl, 1.3 mM CaCl$_2$, 1 mM
MgCl₂, 10 mM glucose, 10 mM HEPES, 1 μM tetrodotoxin (TTX), pH 7.4 adjusted with TEAOH (310 mOsm/kg adjusted with sucrose). Voltage-gated Ca²⁺ currents (I\text{Ca}) were evoked by step depolarizations from -60 to 0 mV for 10, 20, 50, 100, 200, 300, 500 or 1500 ms, as specified in the text. The total electrical charge carried by Ca²⁺ during the depolarization step (i.e., total Ca²⁺ influx) was quantified as ∫I\text{Ca}dt.

For the whole-cell current-clamp recordings, patch pipettes were filled with a solution of the following composition: 125 mM KGluculate, 10 mM KCl, 3 mM Mg-ATP, 1 mM MgCl₂, 10 mM HEPES, 100 μM Fura-2, pH 7.25 adjusted with KOH (290 mOsm/kg with sucrose). The composition of the extracellular recording solution was (mM): 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.4 mM MgSO₄, 0.5 mM MgCl₂, 0.4 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 3 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, pH 7.4 adjusted with NaOH (310 mOsm/kg with sucrose). Action potentials were evoked by current injections as indicated in the figure legends.

Extracellular Field Stimulation

In some experiments, [Ca²⁺]\text{cyt} and [Ca²⁺]\text{int} responses were produced in intact neurons by trains of action potentials using extracellular field stimulation, as previously described [210, 216]. Specifically, field potentials were generated by passing current between two platinum electrodes via a Grass SS stimulator and a stimulus isolation unit (Quincy, MA, USA), and they were monitored using an SDS1052DL digital storage oscilloscope (Siglent Technologies, China/USA). Trains of 1 ms pulses were delivered at 8-10 Hz. In the beginning of each experiment, the stimulus voltage threshold for eliciting a detectable increase in [Ca²⁺]\text{cyt} was determined, and the stimulus voltage for
further experimentation was set 20 V higher. An increase in the stimulus voltage above threshold did not lead to a change in amplitude of the resulting $[Ca^{2+}]_{cyt}$ transients.

**Measurement of Mitochondrial Membrane Potential**

Two methods were used for measuring the mitochondrial membrane potential ($\Delta\Psi_{mt}$) in DRG and hippocampal neurons. For the first method, $\Delta\Psi_{mt}$ was measured in resting neurons using the voltage-sensitive mitochondrial fluorescent indicator tetramethylrhodamine methyl ester (TMRM; Thermo Fisher Scientific) [218]. DRG or hippocampal neurons were loaded with 50 nM TMRM in standard extracellular HEPES-buffered Hank’s salt solution (see Simultaneous $Ca^{2+}$ Imaging section above) for 30-40 min in complete darkness at 24°C, to allow the dye to equilibrate across the plasma and inner mitochondrial membranes) [218]. TMRM fluorescence was measured using an Olympus Fluoview 300 laser-scanning confocal imaging system mounted on a BX61WI microscope equipped with a 60x water-immersion objective (NA 1.0, Olympus). The focus was adjusted such that the image contained the cell nucleus. TMRM fluorescence was excited at 543 nm and measured at 580 nm (40 nm bandpass). The same instrumental settings (e.g., laser power, iris opening size, photomultiplier voltage, gain and zoom) were used for all of the experiments. $\Delta\Psi_{mt}$ was quantified as the ratio of TMRM fluorescence intensity in mitochondria ($F_{mt}$) to that within the cytosol. Since the nucleus is permeable to small molecules such as TMRM and is distinct from the cellular regions occupied by the mitochondria, nuclear fluorescence ($F_{nuc}$) was used to represent TMRM fluorescence within the cytosol. Specifically, $\Delta\Psi_{mt}$ was quantified using
the formula \((F_{mt}-F_{bg})/(F_{nuc}-F_{bg})\), where \(F_{bg}\) is background intensity as determined in an area free of cells.

For the second method, \(\Delta \Psi_{mt}\) was measured simultaneously with \([Ca^{2+}]_{cyt}\) in response to depolarization or stimulation using glutamate or capsaicin. In these experiments, another voltage-sensitive mitochondrial indicator, rhodamine 123 (Rh123) was used in the dequenching mode [218]. Unlike TMRM, Rh123 slowly redistributes across the cellular membranes (i.e., inner mitochondrial and plasma membranes), and therefore is more suitable for dynamic experiments that involve depolarization of the plasma membrane. For these experiments, DRG or hippocampal neurons were loaded with either Fura-2/AM or FuraFF/AM (2 µM) and Rh123 (2.5 µM) for 30 min at room temperature in the dark. Cells were then placed in a chamber for flow-through perfusion and mounted on an inverted IX-71 microscope (Olympus, Japan). Cells were perfused with standard extracellular HEPES-buffered Hank’s salt solution composed of 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.4 mM MgSO₄, 0.5 mM MgCl₂, 0.4 mM KH₂PO₄, 0.6 mM NaHPO₄, 3 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, pH 7.4 adjusted with NaOH (310 mOsm/kg with sucrose). Fluorescence was sequentially excited at 340 nm (12 nm bandpass), 380 nm (12 nm bandpass), and 475 nm (12 nm bandpass) using a Polychrome V monochromator (TILL Photonics, Germany), and the cells were imaged using a 20x objective (NA=0.75, Olympus). Fluorescence was measured at 530 nm (44 nm bandpass) using an IMAGO CCD camera (640x480 pixels; 2x2 binning; TILL Photonics, Germany). A series of 340 nm, 380 nm and 475 nm images was acquired every 2 s. \([Ca^{2+}]_{cyt}\) was calculated from the fluorescence ratio \((R = F_{340}/F_{380})\) using the formula: \([Ca^{2+}]_{cyt} = K_d \beta (R-R_{min})/(R_{max}-R)\) as described above. Changes in \(\Delta \Psi_{mt}\) were
quantified as \( \Delta F/F_0 = (F-F_0)/F_0 \), where \( F \) is current fluorescence intensity (\( \lambda_{\text{ex}} = 475 \text{ nm} \)) and \( F_0 \) is the fluorescence intensity in the cell at rest. Fluorescence was corrected for background at each wavelength, as determined in an area that did not contain any cell. 

\([\text{Ca}^{2+}]_{\text{cyt}}\) and \( \Delta \Psi_{\text{mt}} \) data were analyzed using the TILLvisION 4.5 software (TILL Photonics). Because Rh123 was used in the dequenching mode [218], an increase in Rh123 fluorescence corresponded to mitochondrial depolarization. A positive control was included in each experiment, with the protonophore FCCP (1 \( \mu \text{M} \)) applied at the end to produce strong mitochondrial depolarization.

**Western Blot Analysis**

Nervous tissue from adult (2-3 months of age) mice was collected in lysis buffer (150 mM NaCl, 50 mM Tris/HCl pH 8.0, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM sodium orthovanadate, 1 mM NaF, 0.1% Triton X-100) containing a protease inhibitor cocktail (Sigma) or protease inhibitor tablet (Roche applied Science). Tissues were homogenized using a Pestle mixer (Argos), and centrifuged at 16,000g for 20 min at 4°C. The supernatant was collected and diluted with Laemmli reducing sample buffer and heated at 95°C for 5 min, after which 20 \( \mu \text{g} \) protein/sample was loaded onto an 8% SDS-PAGE gel in Tris-Glycine/SDS running buffer. Proteins in SDS-PAGE gels were transferred to a nitrocellulose membrane using transfer buffer (25 mM Tris-HCl, 190 mM glycine, and 20% methanol dissolved in 1000 mL \( \text{H}_2\text{O} \)). Then, the membranes were incubated with blocking solution composed of 5% skim milk in Tris-buffered saline with tween 20 (TBST: 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) buffer, and then incubated with a primary antibody (anti-MCU,
anti-GRP75 or anti-α-tubulin; 1:1000 dilution) overnight at 4°C. After membranes were washed with TBST, they were incubated with HRP-conjugated goat anti-rabbit (1:1000) or HRP-conjugated goat anti-mouse (1:1000) antibody and signal was developed using the ECL+HRP detection kit (GE Healthcare). The NIH ImageJ software was used to quantify band densities.

For Western blot examination of isolated brain mitochondria, those were prepared as described above (Ca²⁺ Uptake by Isolated Mitochondria), pretreated with Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) and solubilized by incubation in NuPAGE LDS sample buffer (Invitrogen) supplemented with a reducing agent, at 70°C for 15 minutes. Proteins were separated on 12% Bis-Tris Mops gels (Invitrogen) and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Blots were incubated for 1 hr at room temperature in a phosphate-buffered saline (PBS)-based blocking solution (pH 7.2) containing 5% BSA and 0.15% Triton X-100, and then incubated with primary antibody (rabbit anti-MCU or mouse anti-Complex II; 1:1000 dilution) overnight at 4°C. After washing, the blots were incubated with goat anti-mouse or goat anti-rabbit IgG (1:20000) coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Immunocytochemistry**

Immunostaining of cultured DRG and hippocampal neurons was performed as previously described [216]. Specifically, mitochondria were labelled by transfecting DRG
and hippocampal neurons with a plasmid encoding mitochondria targeted EGFP (mtEGFP). Transfected cells (2-3 DIV for DRG and 12-14 DIV for hippocampal cultures) were briefly washed with PBS and fixed using 4% paraformaldehyde (in PBS) solution for 15 min at 24°C. They were then washed (3 x 5 min) with PBS and incubated in blocking buffer (PBS with 5% goat serum and 0.01% Triton-x 100) for 30 min at 24°C. Cells were then stained with a rabbit polyclonal anti-MCU antibody (1:500; Atlas Antibodies; Cat.# HPA016480) overnight at 4°C, and then after being washed (3 x 5 min), were stained with Alexa555-labeled goat anti-rabbit secondary antibody (1:1000; Thermo Fisher Scientific; Cat.#A-21430) for 30 min at 24°C in the dark. Lastly, cells were washed with PBS (3 x 5 min) and the coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotechnology). The cells were imaged using an Olympus BX61WI microscope equipped with a Fluoview 300 laser-scanning confocal imaging system and a 60x oil-immersion objective (NA 1.40; Olympus). For mtEGFP, fluorescence was excited at $\lambda_{\text{ex}}=488$ nm and measured at $\lambda_{\text{em}}=515$ nm (20 nm bandpass); for Alexa555, fluorescence was excited at $\lambda_{\text{ex}}=543$ nm and measured at $\lambda_{\text{em}}=580$ nm (40 nm bandpass). Images were captured and processed using the Fluoview 300 software (Olympus).

**Ca^{2+} Uptake by Isolated Brain Mitochondria**

Percoll-gradient purified brain mitochondria were isolated from MCU-KO and WT mice as previously described [219]. Briefly, brains from 3 mice of each strain were harvested and processed simultaneously. The following solutions were used during the procedure. **Isolation Buffer 1**: 225 mM mannitol, 75 mM sucrose, 10 mM Hepes, pH 7.4
adjusted with KOH, 0.1% BSA, free from fatty acids, and 1 mM EGTA. *Isolation Buffer 2*: 225 mM mannitol, 75 mM sucrose, 10 mM Hepes, pH 7.4 (KOH), 0.1 mM EGTA. *Isolation Buffer 3*: 395 mM sucrose, 0.1 mM EGTA, 10 mM Hepes, pH 7.4 (KOH). *Percoll Buffer*: 320 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.4 (KOH). In addition, 26% Percoll in Percoll Buffer contained a mixture of 5.2 mL Percoll (Sigma) and 14.8 mL Percoll Buffer; 40% Percoll in Percoll Buffer contained 8 mL Percoll and 12 mL Percoll Buffer.

Following the homogenization of brain tissue in 15 mL glass Dounce homogenizer (10 strokes with pestle A, 30 strokes with pestle B) on ice, homogenates were diluted with 30 mL of *Isolation Buffer 1* and centrifuged for 10 minutes at 2,400 rpm in the Beckman Centrifuge Avanti J-26XP, rotor JA-25.50 (700 x g for 10 min). This and all other procedures and centrifugations were performed at 0-2°C. The supernatant was then centrifuged for 10 min at 12,500 rpm (18,900 x g for 10 min). Supernatant was discarded and the pellet was re-suspended in 35 mL of *Isolation Buffer 2* and centrifuged for 10 min at 12,500 rpm (18,900 x g for 10 min). Next, the pellet was re-suspended in 5 mL of *Isolation Buffer 3* and the suspension was layered onto the top of Percoll gradient (26%/40%) in Beckman Ultra-Clear centrifuge tubes. The gradient with sample was centrifuged for 28 min at 15,500 rpm (41,100 x g for 28 min) using a Beckman Ultracentrifuge Optima L100K and bucket rotor SW41Ti. Brain mitochondria were re-suspended in *Isolation Buffer 3* and centrifuged for 20 min at 15,500 rpm (41,100 x g for 20 min) in the same ultracentrifuge and rotor. The pellet was re-suspend in *Isolation Buffer 3* and centrifuged again for 20 min at 15,500 rpm (41,100 x g for 20 min) in the same ultracentrifuge and rotor. The pellet of brain mitochondria was
collected, re-suspended in 0.15 mL of *Isolation Buffer 3*, and stored on ice.

Mitochondrial Ca\(^{2+}\) uptake was measured using a miniature Ca\(^{2+}\)-selective electrode in a 0.3 mL chamber at 37°C during continuous stirring as previously described [219]. A decrease in Ca\(^{2+}\) concentration in the incubation medium indicated mitochondrial Ca\(^{2+}\) uptake. The standard incubation medium was of the following composition: 125 mM KCl, 0.5 mM MgCl\(_2\), 3 mM KH\(_2\)PO\(_4\), 10 mM HEPES, pH 7.4 and 10 µM EGTA. It was additionally supplemented with 3 mM pyruvate, 1 mM malate, 0.1 mM ADP and 1 µM oligomycin as described previously [220] and with 0.1% BSA (free from fatty acids, MP Biochemicals). Ca\(^{2+}\) was added to mitochondria as 100 µM CaCl\(_2\) pulses. Rates of Ca\(^{2+}\) uptake were quantified as nmol Ca\(^{2+}\) per minute per mg of mitochondrial protein.

**Quantification and Statistical Analysis**

All data are presented as mean ± SEM unless indicated otherwise, and are accompanied by the number (n) of cells, animals or replicates throughout the text and in the figure legends. The statistical analysis was performed using Graph Pad Prism 7.0 software. Sample sizes were determined based on previous studies [216, 217, 221, 222]. The following statistical tests were used for analyzing the data: unpaired two-tailed Student’s *t*-test (comparison of 2 groups), one-way ANOVA with either Bonferroni’s or Dunnett’s (as specified in the text) multiple comparison *post hoc* test (comparison of >2 groups), two-way ANOVA with Bonferroni’s multiple comparison *post hoc* test (for multiple group comparisons against two sets of factors, such as time and genotype or
stimulation strength and genotype). For clarity, the specific tests used are indicated in the figure legends. A value of $p<0.05$ was considered statistically significant in all cases.

**Results**

*MCU is expressed on mitochondria ubiquitously in nervous tissue*

First we wanted to test and see that expression profile of MCU in the central and peripheral nervous tissue in the CD-1 mouse strain we used for these studies. Based on Western blot biochemistry we found that MCU is expressed in the cortex, cerebellum, hippocampi, spinal cord, DRG neurons, and trigeminal neurons (Fig. 2.1C) using a previously validated antibody [96]. Level of MCU expression was also determined using Western blot in various nervous tissue and found that MCU expression is relatively lower in peripheral nervous tissue as compared to central (Fig 2.1D). Knockout of MCU in nervous tissue in MCU-/- mice was confirmed in both central and peripheral nervous tissue using Western blot (Fig. 2.1 E).

Although mitochondria are organelles primarily responsible for production of ATP, the expression profiles of mitochondrial populations within a cell may be different depending on the location of the mitochondria [223-225]. Using immunocytochemistry we examined the localization of MCU in both peripheral (Fig 2.1A) and central (Fig. 2.1B) neurons. We found that MCU expression is not uniform across mitochondria found in both DRG and hippocampal neurons, with some mitochondria expressing considerably low levels of MCU compared to others (noticeable in magnified images Fig. 2.1A-B).
MCU deletion in DRG neurons significantly inhibits mitochondrial Ca$^{2+}$ uptake and alters cytosolic Ca$^{2+}$ dynamics

We next investigated the effect of MCU deletion on both mitochondrial dynamics and mitochondrial bioenergetics in mouse DRG neurons. Mitochondrial Ca$^{2+}$ transport has a big impact on the dynamics of cytosolic Ca$^{2+}$ signaling in DRG neurons. The rapid influx and efflux from mitochondria induced by strong stimulation lead to a distinct “plateau” of cytosolic Ca$^{2+}$ in DRG neurons during recovery from stimulation [50, 54, 55, 226].

We examined the effects of stimulus of evoked [Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{mt}$ responses by simultaneously monitoring both parameters ([Ca$^{2+}$]$_{cyt}$ using Fura-2 and [Ca$^{2+}$]$_{mt}$ using mtR-GECO) in patch-clamped WT (Fig. 2.2A) and MCU KO (Fig. 2.2B). Cells were stimulated with step depolarization pulses from -60 to 0 mV with increasing duration (20-300 ms) in order to examine the effect of MCU deletion over a broad range of Ca$^{2+}$ loads. Monitoring [Ca$^{2+}$]$_{cyt}$ simultaneously provided a control for Ca$^{2+}$ load and [Ca$^{2+}$]$_{mt}$ in response to a given stimulation. Recording the voltage-gated Ca$^{2+}$ currents (I$_{Ca}$) enabled quantification of Ca$^{2+}$ entry as $\int I_{Ca} dt$ (i.e., electrical charge carried by Ca$^{2+}$ during stimulation). This enabled us to control for [Ca$^{2+}$]$_{cyt}$ as a function of Ca$^{2+}$ entry as total [Ca$^{2+}$]$_{cyt}$ can be affected by mitochondrial Ca$^{2+}$ buffering.

In WT DRG neurons depolarization for 50 ms was sufficient to induce a noticeable mitochondrial Ca$^{2+}$ uptake response, with [Ca$^{2+}$]$_{mt}$ amplitude increasing as a function of $\int I_{Ca} dt$ (Fig. 2.2A and 2.2C). Though, interestingly, [Ca$^{2+}$]$_{cyt}$ did not increase linearly with $\int I_{Ca} dt$, but instead the slope decreases as [Ca$^{2+}$]$_{cyt}$ increases (Fig. 2.2A and
In MCU-KO DRG neurons, shorter pulses of depolarization (50-100 ms) showed a near complete inhibition of $[\text{Ca}^{2+}]_{\text{mt}}$ (Fig. 2.2B and 2.2C); however, at longer depolarizations (200-300 ms) $[\text{Ca}^{2+}]_{\text{mt}}$ could nearly recover to WT levels. $[\text{Ca}^{2+}]_{\text{cyt}}$ amplitude in MCU-KO DRG neurons was significantly higher than WT at corresponding durations of depolarization, and while $[\text{Ca}^{2+}]_{\text{cyt}}$ did not increase linearly with $\int I_{\text{Ca}}dt$ the slope did not decrease as much as in WT DRG neurons (Fig. 2.2D). We also showed that $I_{\text{Ca}}$ and $\int I_{\text{Ca}}dt$ did not differ between WT and MCU-KO DRG neurons (Fig. 2.2C and 2.2D). Mitochondrial Ca$^{2+}$ uptake dependent upon the amount $[\text{Ca}^{2+}]_{\text{cyt}}$ we decided to quantify certain aspects of $[\text{Ca}^{2+}]_{\text{mt}}$ given the peak amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 2.2E and 2.2F). We found that in WT DRG neurons that peak $[\text{Ca}^{2+}]_{\text{mt}}$ amplitude increased nearly linearly as a function of peak $[\text{Ca}^{2+}]$ amplitude (Fig. 2.2E). Similarly, mitochondrial Ca$^{2+}$ influx rate was increased linearly as a function of peak $[\text{Ca}^{2+}]_{\text{cyt}}$ amplitude in WT DRG neurons (Fig. 2.2F). However, in MCU-KO DRG neurons there was a rightward shift in peak $[\text{Ca}^{2+}]_{\text{mt}}$ amplitude as a function of peak $[\text{Ca}^{2+}]_{\text{cyt}}$ amplitude (Fig. 2.2E). Similarly, MCU-KO in DRG neurons led to a rightward shift in mitochondrial Ca$^{2+}$ uptake rate as a function of peak $[\text{Ca}^{2+}]_{\text{cyt}}$ amplitude (Fig. 2.2F). These data suggest that DRG neurons contain a MCU-independent mitochondrial Ca$^{2+}$ uptake pathway that requires higher elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in order to activate.

Previous literature has shown an important aspect of mitochondrial Ca$^{2+}$ transport in central neuron function; however, not much research has been done looking at how MCU plays a role in regulating cytosolic Ca$^{2+}$ dynamics [37, 208]. Similar experiments in WT hippocampal neurons (Fig. 2.3) revealed that activation of mitochondrial Ca$^{2+}$ uptake required larger $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations than in WT DRG neurons.
(threshold $[\text{Ca}^{2+}]_\text{cyt}$ of ~500-600 nM). In MCU-KO hippocampal neurons, mitochondrial $\text{Ca}^{2+}$ uptake was essentially abolished throughout the range of $\text{Ca}^{2+}$ loads ($|I_{\text{Ca} \cdot dt}|$) tested (Fig. 2.3C, 2.3E and 2.3F). Unlike DRG neurons, MCU-KO in hippocampal neurons did not result in any change of peak $[\text{Ca}^{2+}]_\text{cyt}$ amplitude (Fig. 2.3D), while again other aspects of mitochondrial $\text{Ca}^{2+}$ uptake had a rightward shift in MCU-KO neurons as compared to WT (Fig. 2.3E and 2.3F).

We performed additional experiments on cultured DRG neurons to rule out a possible influence of intracellular dialysis and ion substitution under whole-cell patch-clamp conditions described in Fig. 2.4 $[\text{Ca}^{2+}]_\text{cyt}$ and $[\text{Ca}^{2+}]_\text{mt}$ transients were induced by depolarization pulses of increasing magnitude using elevated extracellular KCl (Fig. 2.4A and 2.4B). Overall, the results in intact DRG neurons were similar to those under patch-clamp conditions: MCU-KO neurons exhibited almost no mitochondrial $\text{Ca}^{2+}$ uptake in response to low-to-mild stimulation, and the amplitude of $[\text{Ca}^{2+}]_\text{cyt}$ responses were significantly larger than in WT neurons. In addition, a prominent MCU-independent mitochondrial $\text{Ca}^{2+}$ uptake could be uncovered by strong stimulation such as that produced by 50 mM KCl (Fig. 2.4C and 2.4D). Although peak $[\text{Ca}^{2+}]_\text{mt}$ amplitude did not differ from WT, time to recover to baseline $[\text{Ca}^{2+}]_\text{mt}$ was significantly faster in MCU KO neurons compared to WT (Fig. 2.4E). Notably, in WT DRG neurons, 30 and 50 mM KCl induced $[\text{Ca}^{2+}]_\text{cyt}$ responses with a characteristic $[\text{Ca}^{2+}]_\text{cyt}$ plateau, which is mediated by $\text{Ca}^{2+}$ extrusion from mitochondria (Fig. 2.4A). This plateau was completely abolished in the MCU-KO DRG neurons, causing a marked shortening (~4-fold) of the $[\text{Ca}^{2+}]_\text{cyt}$ response (Figures 2.4A, 2.4B, and 2.4E). The duration of the corresponding $[\text{Ca}^{2+}]_\text{mt}$ transient was also significantly shorter in MCU-KO DRG neurons than in WT cells.
Transfection of MCU-KO DRG neurons with WT MCU fully rescued the mitochondrial and cytosolic Ca$^{2+}$ dynamics. The $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ responses in DRG neurons from heterozygous mice were intermediate between those seen in the WT and MCU-KO neurons (Fig. 2.4C and 2.4D). Lastly, we found that MCU-KO DRG neurons had an increase in resting $[\text{Ca}^{2+}]_{\text{cyt}}$ levels as compared to WT DRG neurons (Fig. 2.4F).

Activation of glutamate NMDA receptors is known to trigger robust Ca$^{2+}$ influx and mitochondrial Ca$^{2+}$ load in hippocampal neurons [227]. Therefore, we tested whether glutamate stimulation would help to uncover an MCU-independent mitochondrial Ca$^{2+}$ uptake in hippocampal neurons (Figures 2.5A and 2.5B). Because we anticipated large elevations of both $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ in response to glutamate, we used low-affinity cytosolic and mitochondrial Ca$^{2+}$ indicators, Fura-FF ($K_d=5.5$ µM) and mito-LAR-GECO1.2 ($K_d=12$ µM) [211], respectively. Stimulation of WT neurons with 1, 10 and 100 µM glutamate produced concentration-dependent increases in the amplitudes of $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ responses (Figures 2.5A, 2.5C and 2.5D). In MCU-KO hippocampal neurons the amplitude of glutamate-induced $[\text{Ca}^{2+}]_{\text{mt}}$ elevations were significantly lower, yet the application of 100 µM glutamate resulted in prominent mitochondrial Ca$^{2+}$ uptake in both the cell bodies and dendrites (Figures 2.5B, 2.5C). Similar to DRG neurons, MCU-KO hippocampal neurons showed similar peak $[\text{Ca}^{2+}]_{\text{mt}}$ amplitude to 100 µM glutamate; however, duration to $[\text{Ca}^{2+}]_{\text{mt}}$ recovery to baseline was faster as compared to WT (Fig. 2.5A, 2.5B, 2.5C, and 2.5E). Notably, MCU deletion resulted in a dramatic (~4-fold) increase in the amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ responses to 100 µM glutamate (Figures 2.5A, 2.5B, and 2.5D). These results highlight the importance of MCU in mediating Ca$^{2+}$ buffering by mitochondria after strong stimulation. Transfecting
MCU-KO hippocampal neurons with WT MCU completely rescued mitochondrial Ca$^{2+}$ uptake and reduced the amplitudes of the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations (Figures 2.5C and 2.5D). Lastly, we found that MCU-KO hippocampal neurons had slightly elevated resting $[\text{Ca}^{2+}]_{\text{cyt}}$ as compared to WT, but no significant difference in resting $[\text{Ca}^{2+}]_{\text{mit}}$ (Fig. 2.5F and 2.5G).

Overall, these experiments suggest that MCU is a crucial component of mitochondrial Ca$^{2+}$ uptake in neurons, and that it contributes to Ca$^{2+}$ buffering and the shaping of cytosolic Ca$^{2+}$ signals. These experiments also reveal an MCU-independent pathway for Ca$^{2+}$ entry into neuronal mitochondria that is activated only at larger $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations.

**Characterization of the MCU-independent mitochondrial Ca$^{2+}$ uptake pathway in peripheral and central nervous tissue**

Next, we tested whether MCU-independent mitochondrial Ca$^{2+}$ uptake can be elicited in response to action potentials. In these experiments, DRG neurons were stimulated with repeated trains of action potentials using field stimulation. As in WT neurons, mitochondrial Ca$^{2+}$ uptake can be induced by action potentials in both the cell body and neuronal processes of MCU-KO neurons (Figures 2.6A-E), although it required larger $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations for its activation in MCU-KO cells. By analyzing the step-wise $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations as those shown in Fig. 2.6A and 2.6B, we found that the $[\text{Ca}^{2+}]_{\text{cyt}}$ threshold for inducing mitochondrial Ca$^{2+}$ uptake was significantly higher in MCU-KO (778 ± 56 nM) versus WT (298 ± 31 nM) DRG neurons (Figure 2.6E). Notably,
these \([\text{Ca}^{2+}]_{\text{cyt}}\) thresholds were similar to those determined in patch-clamp experiments using depolarization (Figures 2.2A and 2.2B).

We also examined the rates of \(\text{Ca}^{2+}\) accumulation by isolated brain mitochondria from WT and MCU-KO mice (Fig. 2.7A-D). Pulses of \(\text{CaCl}_2\) were applied to the mitochondrial suspension, and the rate of decrease of \([\text{Ca}^{2+}]\) in the external solution was determined using a \(\text{Ca}^{2+}\)-selective electrode. Although MCU deletion dramatically slowed the rate of mitochondrial \(\text{Ca}^{2+}\) uptake, the MCU-KO mitochondria were still able to buffer \(\text{Ca}^{2+}\) added to the medium (Figures 2.7B-D). This residual \(\text{Ca}^{2+}\) uptake was completely blocked by application of the inhibitor of mitochondrial \(\text{Ca}^{2+}\) uptake, Ru360 (3.3 \(\mu\text{M}\)).

Thus, although MCU represents a major pathway for mitochondrial \(\text{Ca}^{2+}\) entry into the matrix, neuronal mitochondria also possess an MCU-independent, Ru360-sensitive mechanism of \(\text{Ca}^{2+}\) uptake.

**Effect of MCU deletion on stimulus-evoked mitochondrial bioenergetics**

Mitochondrial \(\text{Ca}^{2+}\) uptake is an electrogenic transport that depends on the mitochondrial membrane potential (\(\Delta \Psi_{\text{mt}}\)) [228, 229]. Therefore, we examined the effects of MCU deletion on \(\Delta \Psi_{\text{mt}}\). At rest, \(\Delta \Psi_{\text{mt}}\) did not differ between the genotypes in either DRG or hippocampal neurons (Figures 2.8F and 2.9E). Stimulation using 50 mM KCl (30 s) produced large mitochondrial depolarization in both WT hippocampal and DRG neurons, and the amplitude of this depolarization was significantly lower in MCU-KO neurons, even though amplitudes of the corresponding \([\text{Ca}^{2+}]_{\text{cyt}}\) transients were significantly higher (Fig. 2.8 and 2.9). Similar results were found in DRG neurons.
treated with the TRPV1 agonist, capsaicin (1 μM, 30 s; Fig. 2.8A-E). In addition, capsaicin induced a characteristic plateau $[\text{Ca}^{2+}]_{\text{cyt}}$ in WT DRG neurons as previously described [210, 216], and this plateau was abolished in MCU-KO DRG neurons (compare Fig. 2.8A and 2.8B). Thus, MCU deletion results in significantly reduced mitochondrial depolarization in response to neuronal stimulation, and this is likely a result of diminished $\text{Ca}^{2+}$ uptake and a corresponding transfer of positive charge into MCU-KO mitochondria.

**Discussion**

In neurons, cytosolic $\text{Ca}^{2+}$ dynamics was affected by MCU deletion in several respects. First, resting $[\text{Ca}^{2+}]_{\text{cyt}}$ was significantly higher (Figures 2.4F and 2.5F). Second, MCU loss resulted in a dramatic (2-4 fold) increase in the amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients induced by strong stimulation, such as prolonged depolarization or the application of glutamate or capsaicin (Figures 2.2-2.5). Control, patch-clamp experiments showed that the amplitude of $I_{\text{Ca}}$ and the total $\text{Ca}^{2+}$ load ($\int I_{\text{Ca}} \, dt$) were not affected by MCU deletion (Figures 2.2 and 2.3). This deficit in $\text{Ca}^{2+}$ buffering by MCU-lacking mitochondria was fully rescued by WT MCU (Figures 2.4C and D and 2.5C and D). In contrast, MCU deletion resulted in either no or little change in $[\text{Ca}^{2+}]_{\text{cyt}}$ responses in other tissues tested, such as cardiomyocytes or embryonic fibroblasts [144, 145, 209]. Overall, these findings are consistent with the proposed role of mitochondria in buffering cytosolic $\text{Ca}^{2+}$ in neurons [228, 230] and underscore the importance of MCU specifically in mediating this process.

Another prominent effect of MCU deletion was elimination of the $[\text{Ca}^{2+}]_{\text{cyt}}$ plateau and marked shortening of $[\text{Ca}^{2+}]_{\text{cyt}}$ responses produced by strong stimulation in DRG
neurons (Fig. 2.4 and 2.8). The \([\text{Ca}^{2+}]_{\text{c}yt}\) plateau is generated by mitochondria that buffer \(\text{Ca}^{2+}\) during stimulation and then slowly release accumulated \(\text{Ca}^{2+}\) back to the cytosol, which has been implicated in the regulation synaptic transmission and excitation-transcription coupling in sensory neurons [210, 216]. In contrast, WT hippocampal neurons did not develop a \([\text{Ca}^{2+}]_{\text{c}yt}\) plateau despite large and long-lasting elevation of the \([\text{Ca}^{2+}]_{\text{Mt}}\). This difference between peripheral and central neurons in their ability to develop a \([\text{Ca}^{2+}]_{\text{c}yt}\) plateau could be due to differential expression and function of \(\text{Ca}^{2+}\) transporters in mitochondria and plasma membrane, as well as the difference in the surface/volume ratio between DRG and hippocampal neurons.

DRG and hippocampal neurons also differed with respect to \([\text{Ca}^{2+}]_{\text{c}yt}\) threshold for mitochondrial \(\text{Ca}^{2+}\) uptake activation. Indeed, the \([\text{Ca}^{2+}]_{\text{c}yt}\) threshold in WT hippocampal neurons was nearly twice as large (~600 nM) of that in WT DRG neurons (~300 nM; Fig. 2.3E and 2.6C). It was recently proposed that \(\text{Ca}^{2+}\) activation threshold of the MCU complex is determined by the MICU1/MCU ratio [231]. It is possible that a lower \([\text{Ca}^{2+}]_{\text{c}yt}\) threshold in DRG neurons is due to a lower MICU1/MCU ratio in these cells compared to hippocampal neurons. Overall, the low \([\text{Ca}^{2+}]_{\text{c}yt}\) threshold for activating mitochondrial \(\text{Ca}^{2+}\) uptake in neurons is consistent with the high \(\text{Ca}^{2+}\) affinity of the MICU1-MICU2 complex [232], and is comparable to the sub-micromolar \([\text{Ca}^{2+}]_{\text{c}yt}\) threshold in other excitable tissues such as cardiac and skeletal muscle [231].

Although our work demonstrates that MCU is the principal route for \(\text{Ca}^{2+}\) entry into mitochondria in neurons, it also reveals the existence of an additional, MCU-independent, pathway of mitochondrial \(\text{Ca}^{2+}\) uptake. This mitochondrial accumulation of \(\text{Ca}^{2+}\) was observed in both DRG and hippocampal neurons lacking MCU, within the cell
body as well as in individual mitochondria of neuronal processes, and could be unequivocally detected using three different mitochondrial Ca\(^{2+}\) indicators (mito-R-GECO1, mito-LAR-GECO1.2 and mtPericam [Data no shown]) (Fig. 2.2-2.5). Activation of the MCU-independent pathway required larger \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations suggesting it involves a transporter with lower Ca\(^{2+}\) affinity than that of the MCU complex. Comparison of the rates of Ca\(^{2+}\) uptake by mitochondria from WT and MCU-KO brain suggests that MCU mediates 93-95% of Ca\(^{2+}\) accumulation, whereas the MCU-independent component is responsible for the remaining 5-7%.

In spite of its much slower rate of Ca\(^{2+}\) uptake, the MCU-independent pathway was able to produce \([\text{Ca}^{2+}]_{\text{mt}}\) elevations comparable in amplitude to those in WT neurons (Fig 2.2-2.5), at least in the cases of strong stimulation. Observations of these effects using the low-affinity Ca\(^{2+}\) indicator mito-LAR-GECO1.2 (\(K_d=12\ \mu\text{M}\); Fig. 2.4 and 2.5) argues against the possibility that \([\text{Ca}^{2+}]_{\text{mt}}\) elevations in WT neurons are underreported due to the indicator saturation with Ca\(^{2+}\). A possible explanation for this discrepancy lies in the ability of mitochondria to strongly buffer Ca\(^{2+}\) within the matrix by forming Ca\(^{2+}\)-phosphate complexes, primarily as \(\text{Ca}_3(\text{PO}_4)_2\) and \(\text{CaHPO}_4\), [220]. This has been proposed to limit the amplitude of \([\text{Ca}^{2+}]_{\text{mt}}\) to ~5 \(\mu\text{M}\) even for large Ca\(^{2+}\) loads [233, 234]. It is also estimated that depending on the Ca\(^{2+}\) load, the ratio of free:bound Ca\(^{2+}\) in the matrix of brain mitochondria ranges from 1:4000 to 1:150,000 [220].

The existence of a mitochondrial Ca\(^{2+}\) uptake mechanism independent of MCU is likely not unique to neurons. Indeed, an MCU-independent mechanism has been proposed to exist in cardiomyocytes [144, 145], as well as in mitoplasts from HeLa cells [235]. The MCU KO-resistant component was ablated by the mitochondrial Ca\(^{2+}\) uptake
inhibitor Ru360 (Fig 2.7B and 2.7C). It has been shown that the Ser259 residue within the linker region of MCU confers its high sensitivity to Ru360 [93]. This residue is conserved in MCUb that is also expressed in the brain [98, 236, 237]. Although it was proposed that the substitution of electrically neutral Val for negatively charged Glu257 in the MCU/MCUb linker region would prevent MCUb from conducting Ca\(^{2+}\) [98], subsequent structural and functional analysis demonstrated that this was not the case [237]. It is therefore plausible that MCUb mediates the MCU KO-resistant and Ru360-sensitive component of mitochondrial Ca\(^{2+}\) uptake. Other candidates for mediating MCU-independent mitochondrial Ca\(^{2+}\) uptake include NCLX functioning in reverse mode and Letm1 [238, 239].

In summary, MCU plays a large role in mediating mitochondrial Ca\(^{2+}\) uptake in both peripheral and central neurons. Although, we highlight differences in the amount of \([\text{Ca}^{2+}]_{\text{cyt}}\) required to activate mitochondrial Ca\(^{2+}\) extrusion between peripheral and central neurons. We also show that there is another MCU-independent pathway for mitochondrial Ca\(^{2+}\) uptake not previously shown in literature. Lastly, our data show that MCU plays a pivotal role in regulating activity dependent mitochondrial bioenergetics.
Figure 2.1: MCU is expressed ubiquitously throughout the nervous systems

A and B, Immunocytochemistry of cultured DRG (A) and hippocampal (B) neurons transfected with mtEGFP (green) and stained with rabbit polyclonal anti-MCU antibody (red) with colocalization shown in yellow. White dotted rectangles represent magnified region shown below the cell body image.

C, Representative Western blot showing MCU expression in cortex, cerebellum, hippocampus, spinal cord, DRG neurons, trigeminal ganglia (TG) neuron from WT mice with Grp75 acting as a loading control.

D, Quantification of Western blot data shown in (C) normalized to the Grp75 loading controls. Data represent mean ± SEM.

E, Representative Western blots of MCU
expression in cortex, cerebellum, hippocampus, and DRG neuron from WT and MCU-KO mice.
Figure 2.2: Deletion of MCU alters mitochondrial and cytosolic Ca\(^{2+}\) dynamics in DRG neurons

A and B, Examples of simultaneous imaging of \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fura-2; black) and \([\text{Ca}^{2+}]_{\text{mt}}\) (mtR-GECO; red) traces from both WT (A) and MCU KO (B) DRG neurons alongside voltage-clamp whole-cell patch clamp. \([\text{Ca}^{2+}]_{\text{cyt}}\) and \([\text{Ca}^{2+}]_{\text{mt}}\) transients were evoked by step depolarization from -60 to 0 mV for 20, 50, 100, and 300 ms (arrows). The small insets correspond to \(\text{Ca}^{2+}\) currents (\(I_{\text{Ca}}\)) for the same experiments. C and D, Amplitude of \([\text{Ca}^{2+}]_{\text{mt}}\) (\(\Delta[\text{Ca}^{2+}]_{\text{mt}}\)) and \([\text{Ca}^{2+}]_{\text{cyt}}\) (\(\Delta[\text{Ca}^{2+}]_{\text{cyt}}\)) as a function of the total \(\text{Ca}^{2+}\) influx, quantified as the time integral of \(I_{\text{Ca}}\) (\(\int I_{\text{Ca}} \, dt\)). E and F, \(\Delta[\text{Ca}^{2+}]_{\text{mt}}\) and rate of \([\text{Ca}^{2+}]_{\text{mt}}\) elevation (d[\(\text{Ca}^{2+}\)]\(t\)/dt) as a function of the peak \(\Delta[\text{Ca}^{2+}]_{\text{cyt}}\). Data is plotted as mean ± SEM with each point corresponding to a specific duration of step depolarization (10, 20,
50, 100, 200, and 300 ms) for WT (black) and MCU KO (green) DRG neurons. Analyzed using two-way ANOVA with Bonferroni’s post hoc test (C-F). ** p<0.01, *** p<.001.
Figure 2.3: MCU deletion significantly inhibits mitochondrial Ca\(^{2+}\) uptake in hippocampal neurons

A and B, Representative examples of simultaneous imaging of \([Ca^{2+}]_{\text{cyt}}\) (Fura-2; black) and \([Ca^{2+}]_{\text{mt}}\) (mtR-GECO; red) in WT (A) and MCU KO (B) cultured hippocampal neurons. Using whole-cell patch-clamp electrophysiology \([Ca^{2+}]_{\text{cyt}}\) and \([Ca^{2+}]_{\text{mt}}\) transients are evoked using step depolarization of -60 to 0 mV for a specific duration (200, 1500 ms; arrows). Insets represents Ca\(^{2+}\) currents (I\(_{Ca}\)) induced by depolarization induced in (A) and (B). C and D, Peak amplitude of \([Ca^{2+}]_{\text{cyt}}\) (Δ[Ca\(^{2+}\)]\(_{\text{cyt}}\)) and \([Ca^{2+}]_{\text{mt}}\) (Δ[Ca\(^{2+}\)]\(_{\text{mt}}\)) given the I\(_{Ca}\) into the cell, given the total I\(_{Ca}\) defined as the time integral of I\(_{Ca}\) (\(\int I_{Ca} dt\)). Each data point corresponds I\(_{Ca}\) evoked via a step depolarization (-60 to 0 mV) for a specific duration (20, 50, 100, 200, 300, 500, 1500 ms) for WT (black) and MCU KO (green) hippocampal neurons. E and F, Peak Δ[Ca\(^{2+}\)]\(_{\text{mt}}\) as a function of peak Δ[Ca\(^{2+}\)]\(_{\text{cyt}}\) (E) and maximal mitochondrial Ca\(^{2+}\) uptake as a function of peak Δ[Ca\(^{2+}\)]\(_{\text{cyt}}\)
(d[Ca$^{2+}$]/dt). Data represent mean ± SEM, analyzed using two-way ANOVA with Bonferroni’s post hoc test (C-F). ** p<0.01, *** p<0.001
Figure 2.4: Deletion of MCU strongly but not completely inhibits mitochondrial Ca$^{2+}$ uptake and significantly alters cytosolic and mitochondrial Ca$^{2+}$ dynamics

A and B, Representative traces of [Ca$^{2+}$]$_{cyt}$ (Fura-2; black) and [Ca$^{2+}$]$_{mt}$ (mtLAR-GECO; red) from WT (A) and MCU KO (B) DRG neurons. [Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{mt}$ transients were induced by perfusing cultured cells with K$^+$30 and K$^+$50 (arrows) briefly (30 s) then allowed to recover to baseline. C and D, Peak Amplitude of [Ca$^{2+}$]$_{mt}$ ($\Delta$[Ca$^{2+}$]$_{mt}$; C) and [Ca$^{2+}$]$_{cyt}$ ($\Delta$[Ca$^{2+}$]$_{cyt}$; D) as a function of depolarization by application of different KCl
concentrations (K+15, K+20, K+30, K+50) in WT (black), MCU Het (grey), MCU KO (green), and MCU KO rescue (purple). E, Duration for [Ca\textsuperscript{2+}]\textsubscript{cyt} and [Ca\textsuperscript{2+}]\textsubscript{mt} to recover 70% of peak Δ[Ca\textsuperscript{2+}]\textsubscript{cyt} and Δ[Ca\textsuperscript{2+}]\textsubscript{mt} (T\textsubscript{70}) in response to K+50 application in WT (black), MCU KO (green), and MCU KO rescue (red). F, Measurement of [Ca\textsuperscript{2+}]\textsubscript{cyt} in DRG neurons at rest in both WT(grey) and MCU KO (green). G, Measurement of [Ca\textsuperscript{2+}]\textsubscript{mt} at rest in DRG neurons in both WT (grey) and MCU-KO (green). Data is represented as mean ± SEM (C-F), analyzed using two-way ANOVA (C-E) or two-tail student’s t-test (F and G). * p<0.05, ** p<0.01, *** p<0.001
Figure 2.5: Deletion of MCU strongly but not completely inhibits mitochondrial Ca\(^{2+}\) uptake and alters cytosolic Ca\(^{2+}\) dynamics

A and B, Representative traces of simultaneous [Ca\(^{2+}\)]\(_{\text{c}}\) (Fura-FF; black) and [Ca\(^{2+}\)]\(_{\text{m}}\) (mtLAR-GECO; red) imaging in WT (A) and MCU KO (B) cultured hippocampal neurons. Brief (30 s) applications of glutamate (10, 100 µM; arrows) were used to evoke [Ca\(^{2+}\)]\(_{\text{c}}\) and [Ca\(^{2+}\)]\(_{\text{m}}\) transients. White boxes show selected dendritic area on cultured hippocampal neuron where [Ca\(^{2+}\)]\(_{\text{c}}\) and [Ca\(^{2+}\)]\(_{\text{m}}\) are measured from an area with an individual mitochondria. C and D, peak amplitude of [Ca\(^{2+}\)]\(_{\text{m}}\) (Δ[Ca\(^{2+}\)]\(_{\text{m}}\)) and [Ca\(^{2+}\)]\(_{\text{c}}\) (Δ[Ca\(^{2+}\)]\(_{\text{c}}\)) as a function of specific glutamate conc. (1, 10, 100 µM) in WT (black) and MCU KO (green) hippocampal neurons. E, Duration for [Ca\(^{2+}\)]\(_{\text{c}}\) and [Ca\(^{2+}\)]\(_{\text{m}}\) to recover
70% from peak $\Delta [\text{Ca}^{2+}]_{\text{cyt}}$ and $\Delta [\text{Ca}^{2+}]_{\text{mt}}$, respectively, in WT (grey), MCU KO (green), and MCU KO rescue (red) hippocampal neurons. F and G, $[\text{Ca}^{2+}]_{\text{cyt}}$ (F) and $[\text{Ca}^{2+}]_{\text{mt}}$ (G) levels in resting WT (grey) and MCU KO (green) hippocampal neurons. Data represents mean ± SEM, analyzed using two-way ANOVA (C-E) or two-tailed students t-test (F and G). * p<0.05, ** p<0.01, *** p<0.001
Figure 2.6: Characterization of MCU-independent mitochondrial Ca\textsuperscript{2+} uptake pathway in DRG neurons

A and B, Representative traces of [Ca\textsuperscript{2+}]\text{cyt} (Fura-2; black) and [Ca\textsuperscript{2+}]\text{mt} (mtR-GECO; red) in cell bodies of WT (A) and MCU KO (B) DRG neurons. [Ca\textsuperscript{2+}]\text{cyt} transients were evoked by trains of action potentials (APs; 8 Hz x 2-3 s; vertical arrows) using extracellular field stimulation until Δ[Ca\textsuperscript{2+}]\text{mt} and d[Ca\textsuperscript{2+}]\text{mt}/dt significantly increased. C, Mitochondrial Ca\textsuperscript{2+} uptake threshold as a function of [Ca\textsuperscript{2+}]\text{cyt} in WT (black) and MCU KO (green) determined by the d[Ca\textsuperscript{2+}]\text{mt}/dt. D and E, Example traces of [Ca\textsuperscript{2+}]\text{cyt} (Fura-2; black) and [Ca\textsuperscript{2+}]\text{mt} (mtR-GECO; red) from individual mitochondria in neuronal processes (white boxes) in WT (D) and MCU KO (E) DRG neurons. [Ca\textsuperscript{2+}]\text{cyt} and [Ca\textsuperscript{2+}]\text{mt} transients were evoked with trains of action potentials (APs; 8 Hz applied for 1,
2, 4, or 6 s; vertical arrows) using extracellular field stimulation. Data represents mean ± SEM, analyzed using two tailed student’s t-test (C). *** p<0.001
Figure 2.7: Deletion of MCU significantly inhibits mitochondrial Ca\(^{2+}\) rate and amount of Ca\(^{2+}\) influx in isolated brain mitochondria

Monitoring of Ca\(^{2+}\) uptake by isolated brain mitochondria using a Ca\(^{2+}\)-sensitive electrode. A, Calibration curve of Ca\(^{2+}\) in response to addition of 20 µM boluses of Ca\(^{2+}\) (arrows) in a mitochondria-free suspension. B and C, Representative traces of extramitochondrial Ca\(^{2+}\) \([\text{Ca}^{2+}]_{\text{ext}}\) from both WT (B) and MCU KO (C) isolated brain mitochondria in response to a bolus of 100 µM CaCl\(_2\) (arrow) added to the suspension. Blue lines represent mitochondria in respiration buffer while red is mitochondria in respiration buffer with mitochondrial Ca\(^{2+}\) uptake inhibitor Ru360 (3 µM). D, Maximum rate of mitochondrial Ca\(^{2+}\) uptake quantified as nmol of Ca\(^{2+}\) per minute normalized to mg of mitochondria protein. Inset shows Western blot validating MCU KO with complex II of the ETC used as a loading control. Data represents mean ± SEM, analyzed using two-way ANOVA with Bonferroni’s post hoc test. # p<0.05 (relative to MCU KO), *** p<0.001 (relative to WT)
Figure 2.8: Deletion of MCU alters neuronal activity induced mitochondrial bioenergetic processes in DRG neurons

A and B, Representative traces of $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fura-FF; black) and $\Delta\Psi_{\text{mt}}$ (Rh123; red) in WT (A) and MCU KO (B) DRG neurons. $[\text{Ca}^{2+}]_{\text{cyt}}$ transients and $\Delta\Psi_{\text{mt}}$ depolarizations were induced using brief (30 s) perfusions (arrows) of K+50 or capsaicin (1 µM), FCCP (1 µM) was applied at the end of experiments (yellow bar) as a positive control for $\Delta\Psi_{\text{mt}}$ depolarization. C and D, Peak amplitude of $\Delta\Psi_{\text{mt}}$ (C) and $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ (D) in response to neuronal depolarization in WT (grey) and MCU KO (green) in DRG neurons. E, Duration for $[\text{Ca}^{2+}]_{\text{cyt}}$ to recover 80% to baseline from peak $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ ($T_{80}$) in response to brief (30 s) capsaicin (1 µM) perfusion in WT (grey) and MCU KO (green) in DRG neurons. F, basal $\Delta\Psi_{\text{mt}}$ (TMRM$^+$) was measured in resting WT (grey) and MCU KO (green) DRG neurons. Data represents mean ± SEM, analyzed using two tailed student’s t-test (C-F).

*** p<0.001
Figure 2.9: Deletion of MCU impairs mitochondrial bioenergetics in cultured hippocampal neurons

A and B, Representative traces of simultaneous $[Ca^{2+}]_{cyt}$ (Fura-2; black) and $\Delta \Psi_{mt}$ (Rh123; red) imaging in WT (A) and MCU KO (B) cultured hippocampal neurons. $[Ca^{2+}]_{cyt}$ transients and $\Delta \Psi_{mt}$ depolarizations were evoked via brief (30 s) application of KCl at varying conc. (15, 20, 30, and 50 mM; arrows), application of FCCP (1 µM; yellow bar) at experiment conclusion was used as a positive control for $\Delta \Psi_{mt}$. C and D, Peak $\Delta \Psi_{mt}$ and $[Ca^{2+}]_{cyt}$ amplitude ($\Delta [Ca^{2+}]_{cyt}$) as a function of KCl conc. in both WT (black) and MCU KO (green) hippocampal neurons. E, Resting $\Delta \Psi_{mt}$ (TMRM$^+$) in WT (grey) and MCU KO (green) hippocampal neurons at rest. Data represents mean ± SEM, analyzed using two-way ANOVA (C and D) or two-tailed student’s t-test (E). *** p<0.001
CHAPTER III:

EXAMINING BEHAVIORAL PHENOTYPE OF MCU DELETION IN MICE

Introduction

As discussed previously, the role of mitochondrial Ca\(^{2+}\) transport has been shown to be important in many neuronal processes [37, 210, 240]. Many of these processes are linked to specific behaviors such as motor control, pain sensitivity, as well as learning and memory. Even though mitochondrial Ca\(^{2+}\) transport is suggested to play a major role in many neuronal processes, the ability to test the effect of mitochondrial Ca\(^{2+}\) transport has been limited due to lack of availability of drugs to target this function.

Mitochondria dysfunction is believed to play a role in many neuronal pathologies. Mitochondrial Ca\(^{2+}\) overload is hypothesized to be a major mechanism involved in most neurodegenerative disorders [56, 111, 140, 185]. Some research has also suggested a role for mitochondrial dysfunction in the pathology of seizures [200, 241]. As previous research has shown mitochondrial Ca\(^{2+}\) transport has been shown to facilitate neurotransmission. We hypothesized that disruption of mitochondrial Ca\(^{2+}\) transport would disrupt neuronal network activity in a pathological state such as during epileptiform activity.

This chapter will focus on the investigation of behavioral phenotypes in MCU-KO mice. First, aspects of motor function, mechanical and thermal sensitivity, anxiety-like behavior, spatial learning, and fear-related learning. Next MCU-dependent changes in neuronal network activity both in vitro and in vivo will be discussed.
Methods

Animal Subjects

All experiments involving the use of mice and the procedures used therein were approved by the University of Iowa Institutional Animal Care and Use Committee and were carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. The MCU knockout (KO) mouse strain was obtained from Dr. Toren Finkel (NIH/NHLBI) and was previously described [209]. Mice were maintained on a CD-1 background (Charles River Laboratories, Wilmington, MA). Mice were housed in the Bowen Science Building and Central Vivarium of the University of Iowa animal facilities under a 12 h light: dark cycle with ad libitum access to food and water. For genotyping, genomic DNA was extracted from either tail clips or ear punches and amplified by PCR (forward primer: 5’-GTGCCCTCTGATGACGG-3; reverse primer: 5’-ATGACAAGCTTAAAGTCATC-3’) as described by Pan and colleagues [209]. For all the behavioral experiments, adult mice (2-3 months of age) of both sexes were used. Age- and sex-matched MCU-KO and WT littermate mice were randomly assigned to experimental groups. The results obtained from male and female mice were analyzed and presented separately.

For ex-vivo electrophysiological experiments, hippocampal slices were obtained from 2-4 week old WT and MCU-KO mice of both sexes. No significant difference was found between the sexes; therefore the data from both sexes were pooled together for analysis.
Behavioral Tests

For all of the described behavioral assays, adult mice (2-3 months of age) of both sexes were used. Age- and sex-matched MCU-KO and WT littermates (from MCU+/− intercrosses) were randomly assigned to experimental groups. The investigators performing the behavioral measurements were blinded to mouse genotype.

Thermal and Mechanical Sensitivity Tests

Sensitivity to thermal and mechanical stimuli was tested as previously described [222] using the Hargreaves and von Frey filament tests, respectively. Briefly, mice were acclimatized to the behavior testing chambers for 2 hrs per day starting 3 days before testing. For the Hargreaves test, the mice were examined using an IITC Plantar Analgesia Meter (IITC Life Sciences) on a glass table top, at a thermo-neutral temperature. Heat sensitivity was tested by focusing a beam of light on the plantar surface of the hind paw to produce noxious heat and measuring the time required for the stimulus to induce withdrawal of the hind-paw (paw withdrawal latency) using a programmable digital timer. For the von Frey test, mechanical sensitivity was determined by calculating the 50% response threshold to 5 presentations each of 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2 g von Frey filaments (Stoelting).

Open Field Test

Anxiety and spontaneous activity levels were measured using an open field apparatus (PAS-Open Field; SD Instruments, San Diego, CA, USA). Briefly, mice were placed in a 40.5x40.5 cm box that had clear plastic sides (35 cm), a 16x16 photobeam
grid, and sensors under ambient illumination. During the task, mice were allowed to explore the chamber freely for 5 min. Locomotor activity was quantified as the total number of beam breaks over the course of the trial, and anxiety behavior was quantified as the percentage of time spent in the center (30.5x30.5 cm area) versus the periphery.

**Rotarod Test**

Motor coordination was tested using an accelerating rotarod (IITC Life Sciences) task. Mice were trained to stay on 9.5 cm diameter rods suspended above pressure plates. For each trial, rod rotation started at 4 rpm and accelerated to 40 rpm over a 240 s period. A trial ended when the mouse fell off the rod or when the time set for the trial had elapsed. Mice were subjected to the test for 3 consecutive days, with 4 trials per day, and were given at least 10 min for recovery between trials. The apparatus automatically quantified latency to fall, rpm of rod, and distance traveled during the trial.

**Barnes Maze**

Spatial learning was tested using a Barnes maze apparatus (IITC Life Sciences) consisting of a circular platform (93 cm diameter) with 20 equally spaced holes (5 cm diameter) around its edges, and a 20x5x5 cm escape box below one hole. Extra-maze cues provided spatial information to help the mice learn where the escape box was located. Mice underwent a 4-day Barnes maze protocol with 3 training days (4 nonconsecutive 90 s trials per day) followed by a test day (60 s trial). For each training trial, the mice were placed in the center of the platform and allowed to explore for the duration of the trial; if, at the end of the training trial, a mouse had not found the target
hole, it was gently guided into it and allowed to enter the escape box. Mice were allowed to sit in the escape box for ~10 s after each trial before being returned to their home cage. For the test trials, the escape box was removed, and the mice were allowed to explore the platform for 60 s. After each trial the maze and escape box were cleaned to prevent the use of intra-maze cues.

Each trial was recorded using a small video camera (Basler acA1300-30gm; DanioVision) installed above the platform. Mouse movement and time to escape was monitored using EthoVision XT 11 software (Noldus, Leesburg, VA, USA). The MCU-KO mice and their WT littermates were compared with respect to the average time to escape and the time spent in the quadrant with the target hole.

**Fear Conditioning**

Cue and context based learning and memory were tested using a near infra-red video fear conditioning chamber (Med Associates, St. Albans, VT, USA). Mice underwent a 3-day fear conditioning protocol with one training day, a cued recall day, and a context day. On the training day mice were allowed to explore the fear chamber for the first 3 min, then a 20-s tone was played (80 db, 3000 Hz) which cotermminated with a 1-s foot shock. The tone shock pairing was repeated four more times with 140-s interstimulus interval. On the second day, cue-evoked responses were elicited in a novel environment with altered floor, roof, lighting, and odor. After 3 min exploring the novel context a tone (80 db, 3000 Hz) was played for 3 min followed by 4 min without the tone. On the third day, mice were put in the fear conditioning chamber with the original
context for 5 min. Freezing and maximum motion following footshock were scored with VideoFreeze software (Med Associates).

**Preparation and Transfection of Primary Hippocampal Cultures**

Primary cultures of hippocampal neurons were prepared from neonatal (P0-P1) mice of the MCU\(^{+/+}\), MCU\(^{+-}\) and MCU\(^{-/-}\) genotypes and transfected using a protocol similar to those previously described [211]. Specifically, the brain was removed from P0-P1 mice and hippocampi were dissected in ice-chilled Neurobasal A medium supplemented with 20 mM HEPES (pH 7.35) and 0.5 mM L-glutamine, and then digested in trypsin solution (1 mg/mL) for 10 min at 24°C. Cells were washed in fresh medium, mechanically dissociated by sequential trituration with increasingly smaller bore-sized fire-polished Pasteur pipettes, and plated onto 25 mm glass coverslips, pre-coated with poly-L-ornithine (0.2 mg/mL) and laminin (50 μg/mL) in a 6-well plate. The cultures were grown in Neurobasal-A medium supplemented with B-27, 0.5 mM L-glutamine and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) in a 5% CO\(_2\) incubator at 37°C. The medium was replaced by 50% with fresh medium every 3-4 days.

**Imaging of Cytosolic Ca\(^{2+}\) Oscillations in Neurons**

Monitoring of the Ca\(^{2+}\) concentration in the cytosol ([Ca\(^{2+}\)\(_{\text{cyt}}\)) cultured hippocampal neurons was performed largely as previously published [211], but with some modifications. In the case of whole-cell patch-clamp experiments, the cells were loaded with Fura-2 pentapotassium salt via the patch-pipette solution (100 μM), as
described under Patch-Clamp Recording. Cells were then placed into a chamber for flow-through perfusion and mounted on an inverted IX-71 microscope (Olympus, Japan). Cells were perfused with a standard extracellular HEPES-buffered Hank’s salt solution of the following composition: 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.4 mM MgSO₄, 0.5 mM MgCl₂, 0.4 mM KH₂PO₄, 0.6 mM NaHPO₄, 3 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, pH 7.4, with NaOH (310 mOsm/kg with sucrose).

For imaging, fluorescence was sequentially excited at 340 nm (12 nm bandpass), and 380 nm (12 nm bandpass) using a Polychrome V monochromator (TILL Photonics, Germany) and a 40x oil-immersion objective (NA=1.35, Olympus). Fluorescence emission was separated from excitation using a low-pass beamsplitter FF409-Di03 (Semrock; Rochester NY) and signal was collected using a dual band emission filter FF01-510/84 (Semrock) and an IMAGO CCD camera (640x480 pixels; TILL Photonics, Germany). 2x2 binning was used for acquisition (1 pixel ~500 nm). Series of images at 340 nm and 380 nm images were acquired at a rate of 1-10 Hz, depending on the experiment. [Ca²⁺]ₐq was calculated from the fluorescence ratio (R = F₃₄₀/F₃₈₀), using the formula: 

\[ [\text{Ca}^{2+}]_{\text{aq}} = K_D \beta (R - R_{\text{min}})/(R_{\text{max}} - R). \]

Rₘₐₜ, Rₘᵢₜ and β were calculated by applying 10 μM ionomycin in either Ca²⁺-free buffer (1 mM EGTA) or Ca²⁺-containing (1.3 mM) HEPES-buffered Hank’s salt solution. A Kᵩ value of 275 nM was used for Fura-2 [215], and a Kᵩ value of 5.5 μM was used for FuraFF (Molecular Probes Handbook). At each wavelength, fluorescence was corrected for background as measured in an area free of cells.

[Ca²⁺]ₐq data were analyzed using the TILLvisION 4.5 software (TILL Photonics).
**Patch-Clamp Recordings**

The methods used for monitoring whole-cell Ca$^{2+}$ currents and action potential firing were similar to those we previously described [216, 217]. Whole-cell patch-clamp recordings were obtained using a patch-clamp amplifier Axopatch 200B and an analog-to-digital converter Digidata 1322A (Molecular Devices, Union City, CA). Data were collected (filtered at 2 kHz and sampled at 5 kHz) and analyzed using the pClamp 9 or 10 software (Molecular Devices, Union City, CA). Patch pipettes were pulled from borosilicate glass (Narishige; 3-5 mΩ) on a Sutter Instruments P-87 micropipette puller (Novato, CA).

For the whole-cell current-clamp recordings, patch pipettes were filled with a solution of the following composition: 125 mM KGlucionate, 10 mM KCl, 3 mM Mg-ATP, 1 mM MgCl$_2$, 10 mM HEPES, 100 μM Fura-2, pH 7.25 adjusted with KOH (290 mOsm/kg with sucrose). The composition of the extracellular recording solution was (mM): 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl$_2$, 0.4 mM MgSO$_4$, 0.5 mM MgCl$_2$, 0.4 mM KH$_2$PO$_4$, 0.6 mM Na$_2$HPO$_4$, 3 mM NaHCO$_3$, 10 mM glucose, 10 mM HEPES, pH 7.4 adjusted with NaOH (310 mOsm/kg with sucrose). Action potentials were evoked by current injections as indicated in the figure legends.

**Electrophysiological Recordings in Hippocampal Slices**

Procedures for the preparation of acute hippocampal slices and recordings from them were similar to those previously described [217]. In brief, brains of WT and MCU-KO mice (2-4 week old) were dissected and sectioned into 375 μm slices in ice-cold cutting solution (28 mM NaHCO$_3$, 1.25 mM NaH$_2$PO$_4$, 2 mM KCl, 7 mM MgCl$_2$, 0.5 mM
CaCl₂, 7 mM D-glucose, 220 mM sucrose, 1 mM ascorbic acid, and 3 mM sodium pyruvate, saturated with 95% O₂ and 5 CO₂) using either a Leica VT-1000S (Leica Microsystems, Germany) or OTS-5000 (Harvard Apparatus, USA) vibratome. After sectioning, the slices were transferred to oxygenated artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM glucose), and were incubated at 34°C for 30 min and then transferred to room temperature to recover for at least 30 min before recording. Hippocampal slices were used within 6 hrs of the end of the incubation period.

Whole-cell patch-clamp recordings were performed on pyramidal neurons within the CA1 hippocampal region using either a patch-clamp amplifier Axopatch 200B combined with an analog-to-digital converter Digidata 1440A (Molecular devices, Union City CA), or an integrated patch-clamp amplifier unit EPC 10 USB (HEKA Instruments Inc., Holliston, MA). Data were filtered at 2 kHz and acquired at 20 kHz using either the pClamp 10 (Molecular Devices) or Patchmaster 2x73.3 (HEKA Instruments) software. Patch pipettes were pulled from thick-walled borosilicate glass (Narishige; 3-5 mΩ) using a horizontal pipette puller (P-97, Sutter Instruments, Novato, CA). The following intracellular patch pipette solutions were used: 1) For action potential recordings: 125 mM K-gluconate, 10 mM KCl, 1 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, 5 mM MgATP, 0.5 mM MgGTP, pH 7.25 adjusted with KOH (290 mOsm/kg with sucrose); 2) For miniature EPSC (mEPSCs) recordings: 125 mM Cs-gluconate, 10 mM CsCl, 1 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, 5 mM MgATP, 0.5 mM MgGTP, pH 7.25 adjusted with CsOH (290 mOsm/kg with sucrose); 3) For miniature and spontaneous IPSC (mIPSCs and sIPSCs, respectively) recordings: 135 mM KCl, 1 mM MgCl₂, 5 mM
EGTA, 10 mM HEPES, 5 mM MgATP, 0.5 mM MgGTP, pH 7.25 adjusted with KOH (290 mOsm/kg with sucrose). The external ACSF solution used for mEPSC recordings contained 1 µM TTX, 20 µM bicuculline and 50 µM AP5, and that for mIPSC recordings contained 1 µM TTX, 50 µM AP5 and 20 µM CNQX. The external ASCF solution for sIPSCs recordings as well as action potential recordings contained 50 µM AP5 and 20 µM CNQX. As controls, mEPSCs were blocked by adding 20 µM CNQX in the end of experiments; similarly, mIPSCs/sIPSCs were blocked by adding 20 µM bicuculline in the end of recordings.

Patch-clamp data analysis was carried out using the pClamp 10 (Molecular Devices) and IGOR (WaveMetrics) software.

Seizure Induction and Electroencephalogram (EEG) Recording

Seizures were induced by electroshock and monitored as previously described [221]. Adult mice (2-3 months of age) of both sexes were used. Age- and sex-matched MCU-KO and WT littermates (from MCU<sup>+/−</sup> intercrosses) were randomly assigned to experimental groups and the investigators performing the test were blinded to mouse genotype. To enable EEG recordings, mice were anesthetized using isoflurane (~2%), the skull was exposed and the EEG headmount (8201; Pinnacle Technology Inc., Lawrence, KS, USA) was attached using stainless steel machine screws (two 0.1” anterior, two 0.125” posterior, 000-120; Pinnacle Technology). Dental acrylic (Jet Acrylic; Land Dental, Wheeling, IL, USA) was used to further secure the headmount to the skull, after which the incision was sutured, leaving only the EEG port/socket exposed. Mice received meloxicam (0.3 mg/kg i.p. pre-op; 0.05 mg/kg post op in
drinking water) and were allowed to recover for 1 week before experimentation. One day before the experiment, mice were acclimated to a plexiglass container in which they would be housed during the experiment.

EEGs were recorded by attaching a preamplifier (8202-SL; Pinnacle Technology) to the headmount with the EEG leads running through a commutator (#8204; Pinnacle Technology) into an analog conditioning amplifier (Model 440 Instrumentation Amplifier; Brownlee Precision Co., San Jose, CA, USA). EEG signal was amplified (x50,000), filtered (0.3-200 Hz bandpass), and digitized (1 kHz) using an analog-to-digital converter (PCI-6221; National Instruments, Austin, TX, USA), and then acquired using a custom software in MATLAB (Mathworks, Natick, MA, USA).

A modified maximal electroshock-induced seizure (MES) threshold protocol was used to test the susceptibility of MCU-KO mice and their WT littermates to seizure induction. Mice were connected to a Rodent Shocker (Harvard Instruments) via metal alligator clips and stimulated every 2 min by current injections of incrementally increased amplitude (0.2 s; 60 Hz; first injection=1 mA; increment=1 mA) until a maximal tonic hind-limb extension seizure was observed; this was defined as the MES threshold. Seizure induction was monitored both behaviorally and via EEG. If a maximal tonic hind-limb extension seizure could not be induced (as was the case for the majority of MCU-KO mice) the experiments were stopped at the 40 mA electroshock current amplitude. In some cases, mice were subjected to a single MES induction by a 50 mA electroshock current (~5 times the MES threshold for WT mice) for 0.2 s (60 Hz). In all experiments, seizure intensity was measured as the hind limbs extension/flexion (E/F) ratio: the length of time the hind limbs were extended beyond 90° divided by the length
of time the hind limbs were flexed (≤90°). The E/F ratio was analyzed post hoc using video recordings of the experiment.

**Quantification and Statistical Analysis**

All data are presented as mean ± SEM unless indicated otherwise, and are accompanied by the number (n) of cells, animals or replicates throughout the text and in the figure legends. The statistical analysis was performed using Graph Pad Prism 7.0 software. Sample sizes were determined based on previous studies [216, 217, 221, 222]. The following statistical tests were used for analyzing the data: unpaired two-tailed Student’s t-test (comparison of 2 groups), one-way ANOVA with either Bonferroni’s or Dunnett’s (as specified in the text) multiple comparison post hoc test (comparison of >2 groups), two-way ANOVA with Bonferroni’s multiple comparison post hoc test (for multiple group comparisons against two sets of factors, such as time and genotype or stimulation strength and genotype). In addition, the non-parametric Mann-Whitney tests were used for the analysis of mEPSCs and mIPSCs, and Kaplan-Meier analysis with log rank (Manter-Cox) test was used for comparing seizure induction and seizure survival between WT and MCU-KO mice. For clarity, the specific tests used are indicated in the figure legends. A value of $p<0.05$ was considered statistically significant in all cases.
Results

MCU-KO Mice do not Show Some Cognitive Abnormalities, but no Sensory or Motor deficits

Having established that MCU deletion has a prominent anticonvulsant effect, we used a panel of behavioral tests to examine MCU-KO mice of both sexes for neurological abnormalities (Fig3.1-3.3). We found no differences for any of the following between WT and MCU-KO mice: 1) Thermal sensitivity or mechanical sensitivity, as assessed by measuring paw withdrawal latency (Hargreaves test) and mechanical threshold, respectively (Fig. 3.1A and 3.1B); 2) Locomotor activity and exploration/anxiety behavior, as assessed using the open field test (Fig. 3.1C and 3.1D); 3) Baseline motor performance and motor learning, as assessed by the rotarod test (Fig. 3.1E and 3.1F); 4)

Spatial learning, as assessed using the Barnes maze test (Figures 3.2A and 3.2B). The task did not reveal any deficit in spatial learning between WT and MCU KO mice in either males or females. On the Barne’s maze probe day WT and MCU KO mice preferentially stay in the quadrant where the escape hole was located (Fig. 3.2C and 3.2D). Cue and context learning and recall was tested using the fear conditioning task (Fig. 3.3). Male MCU-KO mice showed an overall deficit in acquiring freezing behavior in response to presentation of a tone co terminating with a 1-s footshock (0.75 mV) as compared to WT male mice (Fig. 3.3A). Male MCU-KO mice also showed a deficit in freezing behavior evoked by presentation of conditioned-tone on the second day for the fear conditioning protocol as compared to WT male mice (Fig. 3.3B). Interestingly,
female MCU-KO mice showed no deficit in freezing behavior acquisition on training day (Fig. 3.3D) or tone induced freezing behavior on day 2 as compared to WT female mice (Fig. 3.3E). However, female MCU-KO mice did show reduced freezing behavior on day 3 when introduced back into the original context used during training day as compared to WT female mice (Fig. 3.3F).

Overall these data suggests that MCU-KO does not impair function in most normal behaviors in mice including thermal and mechanical sensitivity, motor function, anxiety-like phenotype, and spatial learning and memory. However, MCU-KO does lead to a slight deficit in fear induced learning and recall.

**MCU Deletion Prevents Epileptiform Activity in Hippocampal Neurons**

Having established the importance of MCU in controlling neuronal Ca\(^{2+}\) dynamics, we next tested the role of MCU in regulating excitability and neural network activity, both of which are Ca\(^{2+}\)-dependent. In these experiments, we used cultured hippocampal neurons as a model system, and monitored induction of epileptiform activity in WT and MCU-KO neurons. Epileptiform activity can be triggered by pro-convulsant agents and is characterized by synchronous electrical discharges and \([\text{Ca}^{2+}]_{\text{cyt}}\) spikes in synaptically connected neuronal networks [242-244]. We found that two pro-convulsant agents, bicuculline (a selective GABA\(_A\) receptor antagonist) and 4-aminopyridine (4-AP; an A-type voltage-gated K\(^+\) channel inhibitor), readily induced periodic bursts of action potentials accompanied by \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations in WT hippocampal neurons (Fig. 3.4G). These \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations were synchronized among the neurons (Fig. 3.4A and 3.4D). In contrast, MCU-KO neurons were resistant to the
induction of epileptiform activity by both bicuculline and 4-AP (Fig. 3.4B, 3.4E and 3.4H).

These observations suggest that MCU regulates neural network activity, and that MCU deletion has a stabilizing effect on neural networks and protects them from hyperexcitability induced by pro-convulsant drugs.

**MCU Deletion Prevents Induction of Generalized Seizures in Mice**

Next, we tested whether the described anticonvulsant effect of MCU deletion is also observed *in vivo*. To this end, we compared the susceptibility of WT and MCU-KO mice to electroshock-induced generalized seizures, an animal model commonly used for the study of seizures [221, 244]. WT mice of both sexes developed maximal tonic hind-limb extension seizures with a threshold of 9 ± 1 mA for both male (n=12) and female (n=9) mice (Fig 3.5). Seizure severity, quantified as the ratio of the duration (s) of extension to flexion (E/F) was 10.9 ± 1.1 for male (n=12) and 8.5 ± 1.1 for female (n=9) mice (Figures 3.5H and 3.5K). Maximal tonic hind-limb extension seizures caused death in 67% of male (n=12) and 89% of female (n=9) WT mice (Fig. 3.5I and 3.5L). In stark contrast, even though a broad range (1-40 mA) of electroshock currents was tested, none of the MCU-KO male mice (n=11) developed maximal tonic hind-limb seizure (Fig. 3.5G), and only 5 of 9 MCU-KO female mice tested developed seizures (Fig. 3.5J). Notably, in the MCU-KO female mice that developed seizure, the threshold for induction was significantly higher (18 ± 1 mA, P<0.001, two-tailed unpaired Student’s *t*-test versus WT), and seizure severity (E/F) was significantly lower than those for their WT counterparts (Fig. 3.5J-L). None of the MCU-KO mice died during testing.
In addition to determining the electroshock threshold, we examined the effects of a maximal electroshock stimulation (50 mA; ~5x the threshold current for WT mice) in WT and MCU-KO male mice. Stimulation with 50 mA induced a maximal tonic hind-limb extension seizure in all WT mice tested (E/F ratio=23.3 ± 7.6; n=5; not shown). In contrast, such a seizure occurred in only 1 of 6 MCU-KO mice tested, and the severity of this seizure was much lower (E/F ratio=6.5) than those observed in WT mice.

**MCU Deletion Increases Inhibitory Synaptic Activity**

Intrinsic neuronal excitability and the balance between excitatory and inhibitory synaptic transmission are critical determinants of neural networks susceptibility to hyperexcitability and seizures. To provide insight into the cellular mechanisms underlying the strong anticonvulsant phenotype of MCU-KO mice we examined how MCU deletion affects neuronal excitability and synaptic activity in acute slices from the hippocampus (Fig. 3.6 and 3.7), a brain region commonly affected by seizures and epilepsy [245]. In the first series of experiments, we used patch-clamp recordings (current clamp) to compare electrical properties of WT and MCU-KO hippocampal pyramidal neurons. There were no significant differences between the genotypes for any of the examined parameters, including input resistance (WT: 254 ± 16 MΩ; MCU-KO: 240±17 MΩ), resting membrane potential (WT: -64.3 ± 1.3 mV; MCU-KO: -64.2 ± 0.6 mV) and action potential threshold (WT: -42 ± 1 mV; MCU-KO: -41 ± 1 mV; n=10 cells for each group; P>0.05; two-tailed unpaired Student’s t-test). We also found no difference between the genotypes with respect to the frequency of action potential firing when characterized as a function of current injection (Figures 3.6A-C).
Next, we tested the effect of MCU deletion on miniature excitatory (glutamatergic) and inhibitory (GABAergic) postsynaptic currents, mEPSCs and mIPSCs, respectively. The frequency and amplitude of mEPSCs were similar between the genotypes (Figures 3.7A-D). Notably, the frequency of mIPSCs was significantly higher in MCU-KO neurons compared to their WT counterparts (Figure 3.7E-G), but IPSC amplitude was not significantly different between genotypes. To further characterize changes in inhibitory synaptic transmission, we also examined spontaneous IPSCs (sIPSCs; no tetrodotoxin added to allow action potential firing). Similar to the effects of MCU deletion on mIPSCs, the frequency, but not the amplitude, of sIPSCs was significantly increased in MCU-KO hippocampal neurons compared to those from WT (Figures 3.7I-L).

Thus, MCU loss leads to a significant increase in the inhibitory synaptic activity in the hippocampus.

Discussion

MCU deletion produced a strong anticonvulsant effect. This effect was observed in both sexes and characterized by either a failure to develop maximal tonic hind-limb seizure (all male and ~45 % of female mice) or, in the mice that developed seizures, by a marked increase in seizure threshold and decrease in seizure severity (Fig. 3.5). These in vivo findings are in good agreement with the effects of MCU deletion on neural network activity in cultured neurons. Indeed, MCU-KO hippocampal neurons were
highly resistant to network destabilization and induction of epileptiform activity by 4-AP and bicuculline (Fig. 3.4).

Relevant to the regulation of network activity, MCU deletion resulted in a significant increase in inhibitory synaptic activity in hippocampal neurons (Fig. 3.7). Although the underlying mechanisms of these synaptic changes in MCU-KO mice are unclear, it is likely that a change in Ca\textsuperscript{2+} dynamics in MCU-KO neurons plays a role. Indeed, many aspects of synapse formation and remodeling are controlled by cytosolic Ca\textsuperscript{2+} including axonal growth and pathfinding, dendritic arborization, receptor trafficking and endocytosis, and activity-dependent regulation of genes involved in pre- and postsynaptic functions [246, 247]. Notably, Ca\textsuperscript{2+}-dependent increase in the number of GABAergic neurons was reported in the spinal cord during development [247]. It is plausible that an elevation in resting [Ca\textsuperscript{2+}\textsubscript{cyt}] and an increase in the amplitude of [Ca\textsuperscript{2+}\textsubscript{cyt}] spikes due to the deficit in Ca\textsuperscript{2+} buffering by mitochondria contributed to the synaptic network remodeling in MCU-KO mice.

Epileptogenesis is often associated with a loss of GABAergic interneurons [245], and a number of antiepileptic drugs act by increasing GABAergic synaptic transmission [248]. The increase in inhibitory synaptic tone may help to explain the strong anticonvulsant phenotype of MCU-KO mice. Another factor crucial to seizure susceptibility is the intrinsic electrical excitability of individual neurons [245]. Although MCU-KO and WT hippocampal neurons show no difference in electrical properties (Fig. 3.7), we cannot rule out differences in excitability between the genotypes in other brain regions involved in seizure generation.
The finding that MCU loss was associated with marked resistance to network hyperactivation and seizures, suggests that this molecule and the larger MCU complex might be effective targets in antiepileptic therapies. Our study revealed small neurological defects in MCU-KO mice (Fig. 3.1-3.3), suggesting that such an approach holds promise for having little side effects. Similarly, MCU deletion has not been associated with an overt cardiac phenotype, except for a reduced tolerance to strenuous workload [119, 144, 145]. Future work will be required to assess the anti-seizure effects of MCU deletion in additional animal models of epilepsy and to delineate the underlying mechanisms, as well as to develop selective pharmacological tools for manipulating MCU activity in vivo.
Figure 3.1: MCU-KO mice do not show sensory, motor, or anxiety deficits

A and B, Summary of thermal (A) and mechanical (B) sensitivity as assessed by Hargreaves (A) and von Frey (B) test in WT (black circles) and MCU-KO (green squares) mice of each sex. Data are presented as mean ± SEM. Animal number for each group is shown on the graph. Two-way ANOVA showed no significant effect of genotype or sex on either paw withdrawal latency or threshold. C and D, Locomotor activity (C) and anxiety/exploratory behavior (D) as assessed using the open field test. The number of beam breaks (C) and percentage of time in the center (D) were measured and compared between WT (black) and MCU KO (green) mice. Data represent mean ± SEM. Animal number for each group is shown on the graph. Two-way ANOVA showed no significant effect of genotype or sex on either locomotor activity or anxiety. E and F, Motor function and learning as assessed in male (E) and female (F)
WT (black circles) and MCU-KO (green squares) mice using the rotarod test. Latency to fall was measured and compared between WT and MCU-KO mice. Data represent mean ± SEM across 4 trials per day per mouse. Animal number for each group is shown on the graph. Two-way ANOVA revealed a significant effect of time (p<0.0001 for male and female), but not genotype on rotarod performance.
Figure 3.2: MCU deletion does not impair spatial learning or recall

A-D. Spatial learning in male (A and B) and female (C and D) WT (black circles) and MCU-KO (green squares) mice as assessed using the Barnes maze test. Primary latency (A, C; time to escape hole) was measured and compared between WT (black) and MCU-KO (green) male (A) and female (C) mice. In addition, percent time spent on the test day in each of the 4 quadrants was compared between WT (black) and MCU-KO (green) male (B) and female (D) mice. Quadrant #3 (shown by gray box) contained the escape hole. Data represent mean ± SEM. Animal number for each group is shown on the graph. Two-way ANOVA showed a significant effect of time/days (p<0.0001, male and female) and quadrant number (p<0.0001, male and female), but not genotype on Barnes maze performance.
Figure 3.3: Deletion of MCU results in impaired fear memory recall

A-F, Cued and contextual learning in male (A-C) and female (D-F) WT (black boxes) and MCU-KO (green boxes) mice were assessed using fear conditioning task. Freezing behavior quantified as the average amount of time immobile (s) each minute of the experiment. A and D, Average time freezing on training day each minute in male (A) and female (D) WT (black) and MCU-KO (green) mice. Small bars above the x-axis represent presentation of a 20-s tone cotermminating with a 1-s footshock (0.75 mV). B
and E, Average amount of time of freezing behavior (s) each minute on the second day of the fear conditioning protocol in both male (B) and female (E) WT (black) and MCU-KO (green) mice. The horizontal bar over the x-axis represents the time at which the tone was continuously presented. C and F, Average amount of freezing behavior (s) each minute on the third day of the fear conditioning protocol in both male (C) and female (F) WT (black) and MCU-KO (green) mice. Data represents mean ± SEM, animal number for each group are shown on graphs. Repeated two-way ANOVA showed a significant difference between WT and MCU-KO males on training day (A) and cue presentation day (B), while post hoc Sidak's multiple comparison test showed differences at min 5 and 6. A significant difference between WT and MCU-KO females on context re-exposure (F). * p<0.05, ** p<0.01
Figure 3.4: MCU deletion prevents generation of epileptiform activity in hippocampal neurons

A and B, Representative traces showing simultaneous recordings of $[\text{Ca}^{2+}]_{\text{cyt}}$ (top) and membrane potential ($V_m$; bottom) in cultured WT (A) and MCU-KO (B) hippocampal neurons in response to application of the pro-convulsants, bicuculline (1 μM) and 4-aminopyridine (4-AP; 2 μM). C and D, Examples of synchronized $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations in 3 WT (C) and 3 MCU-KO (D) hippocampal neurons elicited by various concentrations of
bicuculline (0.2 and 1 µM). Each color represents an individual cell. E, Summary of the bicuculline effects on the frequency of $[\text{Ca}^{2+}]_\text{cyt}$ oscillations (number/5 min) in WT (black; n=10) and MCU-KO (green; n=11) neurons. Data are presented as mean ± SEM; *p<0.05, **p<0.01, two-way ANOVA, with Bonferroni’s post hoc test. F and G, Examples of simultaneous $[\text{Ca}^{2+}]_\text{cyt}$ recordings from 3 WT (F) and 3 MCU-KO (G) hippocampal neurons in response to 0.5 and 2 µM 4-AP. Each color represents an individual cell. H, Summary of the effects of 4-AP on the frequency of $[\text{Ca}^{2+}]_\text{cyt}$ oscillations (number/5 min) in WT (black; n=8) and MCU-KO (green; n=11) neurons. Data are presented as mean ± SEM; *p<0.05, ***p<0.001, two-way ANOVA, with Bonferroni’s post hoc test.
Figure 3.5: MCU deletion prevents induction of generalized seizures in mice

A, Schematic for electroshock-induced seizure experiments involving electroencephalogram (EEG) recordings using implanted electrodes and electroshock stimulation (Stim.). B-F, Representative EEG recordings in WT (B-D) and MCU-KO (E and F) mice in response to 4 mA (B and E) and 10 mA (C, D and F) electroshock currents. For (C), the animal died after seizure indicated as EEG flattening (vertical
dotted line), whereas the animal in (D) survived the seizure. For the MCU-KO mice, no seizure developed and only electroshock artifacts are seeing on the EEG (E and F). (G-L) Summary of electroshock-induced seizure experiments for WT (black) and MCU-KO (green) male (G-I) and female (J-L) mice. (G) and (J) show the percentage of animals that developed maximal tonic hind-limb extension seizure in response to 0.2 s electroshock of incrementally increased magnitude (1 mA increment, applied every 2 min). (H) and (K) summarize seizure severity quantified as the ratio of extension/flexion duration; this was calculated only for the animals that developed maximal seizure. None of MCU-KO male mice (n=11) developed maximal seizure (NA=not applicable; H), and only 5 of 9 MCU-KO female mice did so (K; green). (I) and (L) show survival curves for WT (black) and MCU-KO (green) mice. For (G) and (J), p<0.0001, and for (I) and (L), p<0.001, Mental-Cox and Gehan-Breslow-Wilcoxon tests. For (K), ***p<0.001 two-tailed unpaired Student’s t-test (mean ± SEM). The number of animals were n=12 for WT male, n=11 for MCU-KO male, n=9 for WT female, n=9 for MCU-KO female mice.
Figure 3.6: MCU deletion does not affect intrinsic excitability

A and B, Representative traces of action potential firing by WT (A) and MCU-KO (B) neurons in CA1 region of hippocampal slices in response to 500 ms current injections (current values are shown at left of the traces). C, Summary of action potential number as a function of current injection for WT (black) and MCU-KO (green) hippocampal neurons. Data are presented as mean ± SEM (n=10 cells for each genotype). No significant difference between the genotypes (p=0.68), two-way ANOVA.
Figure 3.7: Deletion of MCU increases inhibitory synaptic activity

A and B, Representative AMPA receptor-mediated miniature EPSC (mEPSC) traces recorded from WT (A) and MCU-KO (B) hippocampal neurons in slices. C and D, Cumulative probability of mEPSC inter-event intervals (C) and amplitudes (D) for WT (black; n=12) and MCU-KO (green; n=9) hippocampal neurons. Insets show scatterplot summaries of cell averages for mEPSC frequency (C) and amplitude (D). No significant
difference was found between the genotypes (n.s.; p>0.05) for either parameter as analyzed using two-tailed Mann Whitney test. E and F, Representative GABA\textsubscript{A} receptor-mediated miniature IPSC (mIPSC) traces recorded from WT (E) and MCU-KO (F) hippocampal neurons in slices. G and H, Cumulative probability of mIPSC inter-event intervals (G) and amplitudes (H) for WT (black; n=9) and MCU-KO (green; n=10) neurons in CA1 hippocampal slices. Insets show scatterplot summaries of cell averages for mIPSC frequency (G) and amplitude (H). **p<0.01, two-tailed Mann Whitney test. I and J, Representative GABA\textsubscript{A} receptor-mediated spontaneous IPSC (sIPSC) traces recorded from WT (I) and MCU-KO (J) hippocampal neurons in slices. K and L, Cumulative probability of sIPSC inter-event intervals (K) and amplitudes (L) for WT (black; n=9) and MCU-KO (green; n=10) neurons in CA1 hippocampal slices. Insets show scatterplot summaries of cell averages for mIPSC frequency (K) and amplitude (L). **p<0.01, two-tailed Mann Whitney test.
CHAPTER IV:
MOLECULES AND MECHANISMS IMPLICATED IN MITOCHONDRIAL Ca^{2+} EFFLUX

Introduction

The ability of mitochondria to shape cytosolic Ca^{2+} signaling is dependent upon the rapid efflux of accumulated Ca^{2+} overcoming Ca^{2+} buffering mechanisms in the cytosol of cells. Similar to MCU, the mechanism for mitochondrial Ca^{2+} efflux was known for decades; however, the molecule responsible for this mechanism remained elusive [249]. Several different mechanisms have been shown in mitochondrial Ca^{2+} efflux depending on the organism and tissue including a Na^{+}/Ca^{2+} exchanger, a H^{+}/Ca^{2+} exchanger, and a Ca^{2+} induced Ca^{2+} release mechanisms similar to the ER [161, 162]. However, the mechanism most prevalent in excitable tissue (i.e., cardiac, muscle, nervous, etc.) is the mitochondrial Na^{+}/Ca^{2+} exchanger [79]. Much of the research interested in the molecule responsible for this transport comes from its ability to regulate mitochondria Ca^{2+} overload in pathological conditions.

In 2010, a research group discovered that knockdown of the protein NCKX6 led to strong inhibition of mitochondrial Ca^{2+} efflux, and subsequently renamed the protein to NCLX [173]. Since then, a number of studies examined the effects of NCLX knockdown and knockout on mitochondria function in a variety of tissues [179-182]. Several groups have shown that regulation of NCLX can lead to neuroprotection in models of Parkinson’s disease, whereas NCLX knockdown showed a significant effect on cytosolic Ca^{2+} signaling in neurons [183, 185, 186]. However, the effects of NCLX deletion on Ca^{2+} dynamics neurons have not been reported.
A unique property of NCLX not shared by other Na\(^+\)/Ca\(^{2+}\) exchangers is its ability to transport Ca\(^{2+}\) even when Na\(^+\) has been substituted by Li\(^+\) [191, 250]. Recent research on NCLX has suggested that Li\(^+\) substitution doesn’t impair Ca\(^{2+}\) exchange activity compared to Na\(^+\) which is how the identity of \(m\)NCX mechanism was discovered [173, 177, 178]. Notably, the original research on the \(m\)NCX using isolated heart mitochondria showed that replacing Na\(^+\) with Li\(^+\) reduced mitochondrial Ca\(^{2+}\) extrusion by \(\sim30\%\) [90].

The goal of this chapter is to examine the effect of NCLX on cytosolic and mitochondrial Ca\(^{2+}\) dynamics by observing both simultaneously in NCLX-KO neurons. The ability for the \(m\)NCX mechanism to utilize Li\(^+\) will be examined in central and peripheral nervous tissue as well.

**Methods**

**Animal Subjects**

All experiments involving the use of mice and the procedures used therein were approved by the University of Iowa Institutional Animal Care and Use Committee and were carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. The NCLX-KO mouse line was acquired from Jackson Laboratory ref# 026242) generated via CRISPR-Cas deletion on the 2\(^{nd}\) exon. Mice were maintained on a C57BL/6J (Jackson Laboratory, Bar Harbor, ME). Mice were housed in the Bowen Science Building and Central Vivarium of the University of Iowa animal facilities under a 12 h light: dark cycle with ad libitum access to food and water. For genotyping, genomic DNA was extracted from either tail clips or ear punches and
amplified by PCR (forward primer: 5’-ATACTGGAGACGCGTCTGGGA-3; reverse primer: 5’-CTGCGGCAGTCCGGATTCCTC-3’).

cDNA Constructs and FIV Lentivirus

The plasmid encoding Flag(DDT)-tagged mouse wild-type NCLX (NCLX-ddk) was obtained from OriGene (Cat.# MR218926). The mtEGFP plasmid (mitochondria targeted EGFP) was a gift from Dr. Colin Campbell (University of Minnesota) [213]. The plasmid encoding the low-affinity mitochondrial Ca\(^{2+}\) indicator mito-LAR-GECO1.2 (CMV-mito-LAR-GECO1.2) was previously described [211], and the plasmids encoding two other mitochondrial Ca\(^{2+}\) indicators, mito-R-GECO1 and mito-GEM-GECO1, were obtained from Addgene (Cat.# 46021 for CMV-mito-R-GECO1 and Cat.# 32461 for CMV-mito-GEM-GECO1).

For the preparation of the mito-R-GECO1 and mito-LAR-GECO1.2 FIV (feline immunodeficiency virus) lentiviruses, the corresponding cDNA sequences were PCR amplified (forward primer: 5’-GAGGTCTATATAAGCAGAGC-3’; reverse primer: 5’-GACGTCGACGAATTCGAGGGCTGATCAGCGGTTTAAAC-3’), cut using EcoRI and NheI, and ligated into the EcoRI and SpeI sites of the lentiviral shuttle vector pFIV3.2-CAG-mcs. The resulting plasmids were termed pFIV3.2-mito-R-GECO1 and pFIV3.2-mito-LAR-GECO1.2, respectively. The corresponding lentiviruses (1x10\(^7\) - 5x10\(^8\) transforming units/ml) were produced by the University of Iowa Viral Vector Core.
Preparation and Transfection of Primary DRG Cultures

DRG neurons were prepared from adult mice and transfected using a method similar to those previously described [210]. Specifically, lumbar, thoracic and cervical DRG were dissected from adult (6-10 wk) mice of the NCLX\(^{+/-}\), or NCLX\(^{-/-}\) genotypes. Isolated DRG were digested using first collagenase A (2 mg/mL for 20 min; Roche) and then Pronase E (1 mg/mL for 10 min; Serva); both were applied in DMEM/HEPES (20 mM; pH 7.4) solution at 37°C. Cells were then washed in DMEM/HEPES (20 mM; pH 7.4) and mechanically dissociated by sequential trituration with increasingly smaller bore-sized fire-polished Pasteur pipettes. Cells were transfected with one of the mitochondrial Ca\(^{2+}\) indicators (mito-R-GECO1, mito-LAR-GECO1.2) using an Amaxa Nucleofector according to the manufacturer’s protocol (program G-013; Mouse Neuron Nucleofector Kit; Amaxa/Lonza). Where indicated, MCU-KO DRG neurons were co-transfected with a wild-type MCU plasmid. Transfected cells were resuspended in a DMEM supplemented with NGF (50 ng/mL), insulin (6 μg/mL), 5% HIHS, 5% FBS and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) and plated onto 25 mm glass coverslips pre-coated with poly-L-ornithine (0.2 mg/mL) and laminin (50 μg/mL) in a 6 well plate. DRG cultures were maintained in a 10% CO\(_2\) incubator at 37°C for 2-3 days before experimentation.

Preparation and Transfection of Primary Hippocampal Cultures

Primary cultures of hippocampal neurons were prepared from neonatal (P0-P1) mice of the NCLX\(^{+/-}\), and NCLX\(^{-/-}\) genotypes and transfected using a protocol similar to those previously described [211]. Specifically, the brain was removed from P0-P1 mice
and hippocampi were dissected in ice-chilled Neurobasal A medium supplemented with 20 mM HEPES (pH 7.35) and 0.5 mM L-glutamine, and then digested in trypsin solution (1 mg/mL) for 10 min at 24°C. Cells were washed in fresh medium, mechanically dissociated by sequential trituration with increasingly smaller bore-sized fire-polished Pasteur pipettes, and plated onto 25 mm glass coverslips, pre-coated with poly-L-ornithine (0.2 mg/mL) and laminin (50 μg/mL) in a 6-well plate. The cultures were grown in Neurobasal-A medium supplemented with B-27, 0.5 mM L-glutamine and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) in a 5% CO₂ incubator at 37°C. The medium was replaced by 50% with fresh medium every 3-4 days.

Hippocampal neurons were transfected with mito-R-GECO1 by adding the appropriate FIV lentivirus to the culture at DIV 5-7.

**Simultaneous Monitoring of Cytosolic and Mitochondrial Ca²⁺ in Neurons**

Simultaneous monitoring of the Ca²⁺ concentration in the cytosol ([Ca²⁺]_{cyt}) and mitochondria ([Ca²⁺]_{mt}) of cultured DRG or hippocampal neurons was performed largely as previously published [211], but with some modifications. Specifically, DRG or hippocampal neurons that had been transfected with either the high-affinity mitochondrial Ca²⁺ indicator mito-R-GECO1 (K_d=0.48 μM) [214] or the lower-affinity, mito-LAR-GECO1.2 (K_d=12 μM) [211], were loaded with either Fura-2/AM or FuraFF/AM Ca²⁺ indicators (2 μM for 30 min). In the case of whole-cell patch-clamp experiments, the cells were loaded with Fura-2 pentapotassium salt via the patch-pipette solution (100 μM), as described under Patch-Clamp Recording. Cells were then placed into a chamber for flow-through perfusion and mounted on an inverted IX-71 microscope.
Cells were perfused with a standard extracellular Modified Hank's salt solution of the following composition: 140 mM NaCl (or 140 LiCl), 5 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4, with KOH (310 mOsm/kg with sucrose).

For imaging, fluorescence was sequentially excited at 340 nm (12 nm bandpass), 380 nm (12 nm bandpass), and 550 nm (12 nm bandpass) using a Polychrome V monochromator (TILL Photonics, Germany) and a 40x oil-immersion objective (NA=1.35, Olympus). Fluorescence emission was separated from excitation using a dual fluorophore beamsplitter FF493/574-Di01 (Semrock; Rochester NY) and signal was collected using a dual band emission filter FF01-512/630 (Semrock) and an IMAGO CCD camera (640x480 pixels; TILL Photonics, Germany). 2x2 binning was used for acquisition (1 pixel ~500 nm). Series of images at 340 nm, 380 nm and 550 nm images were acquired at a rate of 1-10 Hz, depending on the experiment. 

\[ [\text{Ca}^{2+}]_{\text{cyt}} = \frac{K_d \beta (R-R_{\text{min}})}{(R_{\text{max}}-R)} \]

where

- \( R \) is the fluorescence ratio \( \frac{F_{340}}{F_{380}} \),
- \( K_d \) is the dissociation constant for the Fura-2 indicator (275 nM),
- \( \beta \) is a correction factor,
- \( R_{\text{min}} \) and \( R_{\text{max}} \) are reference fluorescence intensities for 10 μM ionomycin in Ca²⁺-free and Ca²⁺-containing buffer, respectively.

Changes in \([\text{Ca}^{2+}]_{\text{mt}}\) were quantified as \( \Delta F/F_0 = \frac{F-F_0}{F_0} \), where \( F \) is current fluorescence intensity \((\lambda_{\text{ex}} = 550 \text{ nm})\) and \( F_0 \) is the fluorescence intensity at baseline. At each wavelength, fluorescence was corrected for background as measured in an area free of cells.

\([\text{Ca}^{2+}]_{\text{cyt}}\) and \([\text{Ca}^{2+}]_{\text{mt}}\) data were analyzed using the TILLvisION 4.5 software (TILL Photonics).
Patch-Clamp Electrophysiology

The methods used for monitoring whole-cell Ca\textsuperscript{2+} currents and action potential firing were similar to those we previously described [216, 217]. Whole-cell patch-clamp recordings were obtained using a patch-clamp amplifier Axopatch 200B and an analog-to-digital converter Digidata 1322A (Molecular Devices, Union City, CA). Data were collected (filtered at 2 kHz and sampled at 5 kHz) and analyzed using the pClamp 9 or 10 software (Molecular Devices, Union City, CA). Patch pipettes were pulled from borosilicate glass (Narishige; 3-5 mΩ) on a Sutter Instruments P-87 micropipette puller (Novato, CA).

For the whole-cell current-clamp recordings, patch pipettes were filled with a solution of the following composition: 95 mM CsGluconate, 30 mM NaCl (LiCl), 3 mM Mg-ATP, 1 mM MgCl\textsubscript{2}, 0.5 mM Na-GTP, 10 mM HEPES, 100 μM Fura-2, pH 7.25 adjusted with CsOH (290 mOsm/kg with sucrose). The composition of the extracellular recording solution was (mM): 110 mM CholineCl, 30 mM TEACl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 1 μM tetrodotoxin, 10 mM glucose, 10 mM HEPES, pH 7.4 adjusted with TEAOH (310 mOsm/kg with sucrose).

Extracellular Field Stimulation

In some experiments, [Ca\textsuperscript{2+}]\textsubscript{cyt} and [Ca\textsuperscript{2+}]\textsubscript{mt} responses were produced in intact neurons by trains of action potentials using extracellular field stimulation, as previously described [210, 216]. Specifically, field potentials were generated by passing current between two platinum electrodes via a Grass SS stimulator and a stimulus isolation unit.
(Quincy, MA, USA), and they were monitored using an SDS1052DL digital storage oscilloscope (Siglent Technologies, China/USA). Trains of 1 ms pulses were delivered at 8-10 Hz. In the beginning of each experiment, the stimulus voltage threshold for eliciting a detectable increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was determined, and the stimulus voltage for further experimentation was set 20 V higher. An increase in the stimulus voltage above threshold did not lead to a change in amplitude of the resulting $[\text{Ca}^{2+}]_{\text{cyt}}$ transients.

**Immunocytochemistry**

Immunostaining of cultured DRG neurons was performed as previously described [216]. Specifically, mitochondria were labelled by transfecting DRG with a plasmid encoding mitochondria targeted EGFP (mtEGFP). Transfected cells (2-3 DIV for DRG were briefly washed with PBS and fixed using 4% paraformaldehyde (in PBS) solution for 15 min at 24°C. They were then washed (3 x 5 min) with PBS and incubated in blocking buffer (PBS with 5% goat serum and 0.01% Triton-x 100) for 30 min at 24°C.

Cells were then stained with a mouse monoclonal anti-flag antibody (1:1000; Origene; Cat.# TA50011) overnight at 4°C, and then after being washed (3 x 5 min), were stained with Alexa555-labeled goat anti-rabbit secondary antibody (1:1000; Thermo Fisher Scientific; Cat.#A-21430) for 30 min at 24°C in the dark. Lastly, cells were washed with PBS (3 x 5 min) and the coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotechnology). The cells were imaged using an Olympus BX61WI microscope equipped with a Fluoview 300 laser-scanning confocal imaging system and a 60x oil-immersion objective (NA 1.40; Olympus). For mtEGFP, fluorescence was excited at $\lambda_{\text{ex}}=488$ nm and measured at $\lambda_{\text{em}}=515$ nm (20 nm bandpass); for Alexa555,
fluorescence was excited at $\lambda_{\text{ex}}=543$ nm and measured at $\lambda_{\text{em}}=580$ nm (40 nm bandpass). Images were captured and processed using the Fluoview 300 software (Olympus).

**Isolation and Imaging of Free Brain Mitochondria $\text{Ca}^{2+}$ Dynamics**

Isolated free brain mitochondria were obtained from adult (6-10 wks) C57BL/6J mice adapted from previously described [251]. Briefly, mice were swiftly anesthetized, decapitated and the brain was quickly removed. Brains were chopped and homogenized using a tissue grinder (E2355, Eberbach; Ann Arbor MI) in ice-chilled mitochondrial isolation buffer ($MIB$; 225 mM mannitol, 75 mM sucrose, 2 mM $K_2$HPO$_4$, 5 mM HEPES, 1 mM EGTA, 0.1% FAF BSA, pH 7.2 w/ KOH). Homogenate is then centrifuged at 1500 g (5 min) using a Sorvall Legend X1R Centrifuge (Thermoscientific) to pellet unlysed cells. The supernatant was then centrifuged at 21,000 g (10 min) using a Coulter-Beckman Avanti J-E Model Centrifuge (Coulter-Beckman) to pellet crude mitochondria. Pelleted mitochondria are resuspended in 3.5 mL of 15% Percoll solution which was then layered on top of 3.7 mL 24% and 1 mL 40% Percoll solutions in $MIB$. Then crude mitochondria were centrifuged at 30,700 g (Coulter-Beckman) for 8 min using slow acceleration and deceleration in order to not disturb the percoll gradients. Non-synaptosomal mitochondria are collected from in a band between the 24% and 40% Percoll layers using a plastic pipette. Mitochondria are then resuspended 1:5 Percoll solution and $MIB$ then centrifuged at 6900 g for 10 min (Coulter-Beckman). The mitochondrial pellet is then resuspended in EGTA- and BSA-free $MIB$ and BCA analysis is performed on a sample.
For imaging, mitochondria were suspended in a respiration buffer (Res; 150 mM KCl, 2 mM K$_2$HPO$_4$, 0.5 mM MgCl$_2$, 5 mM malate, 5 mM glutamate, 0.1 mM ADP, 10 mM HEPES, 10 μM EGTA, and 1 μM Calcium Green 5N) in a 96 well, black/clear, tissue culture treated plate (Falcon) similar to previously described [252]. Calcium Green 5N was excited at 506 nm and fluorescence collected at 532 nm using a SpectraMax M2 Microplate Reader (Molecular Devices).

qRT-PCR

RNA was isolated from adult (6-10 weeks) C57BL/6J mice. Briefly, mice were anesthetized and swiftly decapitated then had their hippocampi, cortex, and DRG neurons dissected out. Tissue was placed in Thizol then grinded up using a homogenizer then an additional 20% of total volume of chloroform was added and samples centrifuged at 13,200 rpm for 5 min. Most supernatant was removed and diluted 1:1 in 70% EtOH. RNA was extracted using a Qiagen RNeasy kit. RNA was converted to cDNA using a Biorad Iscript-cDNA synthesis kit. qRT-PCR was performed while levels of NCLX were measured using the forward primer: ATACTGGAGACGCGCTCAGGA and reverse primer: CTGCGGCAGTCGGATTTC. 18S cDNA was used as a positive control for baseline mRNA expression.

Quantification and Statistical Analysis

All data are presented as mean ± SEM unless indicated otherwise, and are accompanied by the number (n) of cells, animals or replicates throughout the text and in
the figure legends. The statistical analysis was performed using Graph Pad Prism 7.0 software. Sample sizes were determined based on previous studies [216, 217, 221, 222]. The following statistical tests were used for analyzing the data: unpaired two-tailed Student’s t-test (comparison of 2 groups), one-way ANOVA with either Bonferroni’s or Dunnett’s (as specified in the text) multiple comparison post hoc test (comparison of >2 groups), two-way ANOVA with Bonferroni’s multiple comparison post hoc test (for multiple group comparisons against two sets of factors, such as time and genotype or stimulation strength and genotype). For clarity, the specific tests used are indicated in the figure legends. A value of \( p < 0.05 \) was considered statistically significant in all cases.

**Results**

*Confirmation of NCLX deletion in mice and localization of NCLX in neurons*

With research suggesting NCLX-KD leads to alterations in mitochondrial Ca\(^{2+}\) efflux in nervous tissue, we decided to test the effect of NCLX deletion on both cytosolic and mitochondrial Ca\(^{2+}\) dynamics [183, 185, 186]. To this end, we obtained mice from Jackson Laboratory with full NCLX knocked out (KO; Stock#026242) via a CRISPR-Cas 13 bp deletion on the 2\(^{nd}\) exon of the gene leading to an early stop codon during translation.

In order to confirm NCLX deletion in these mice we used qRT-PCR to measure level of NCLX mRNA in the brain. As Fig. 4.1A shows a melt curve of the reaction creating a trace for the fluorescence of the PCR indicator (fast-sybr) as a function of the number of PCR reactions (cycles). NCLX-KO tissue had almost no expression of NCLX.
(0.006) compared to normalized expression in WT mice (Fig. 4.1B). Lastly, we examined the distribution of NCLX in DRG neurons, based on previous literature NCLX should localize to mitochondria. NCLX was visualized by expressing NCLX-flag (stained with α-flag antibody) and mitochondrial targeted GFP (mtGFP). As shown in Fig. 4.1D NCLX-flag and mtGFP do not colocalize, instead NCLX-flag seems to localize to the plasma membrane, which is especially evident in the cell body.

**NCLX deletion does not affect cytosolic and mitochondrial Ca$^{2+}$ transients induced by action potentials in DRG neurons**

First we tested the effect of NCLX-KO on cytosolic and mitochondrial Ca$^{2+}$ dynamics in DRG neurons. $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ elevations in DRG neurons were evoked by trains of action potentials using extracellular field stimulation (8 Hz, variable duration) (Fig. 4.2A and 4.2B). Trains of action potentials, controlling for the peak amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ [data not shown], elicited no differences in the recovery kinetics of either $[\text{Ca}^{2+}]_{\text{mt}}$ (Fig. 4.2C) or $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 4.2D). Recovery kinetics were calculated using monoexponential fitting approximation using the Clampfit 9.0 software. Recovery of $[\text{Ca}^{2+}]_{\text{mt}}$ in NCLX-KO DRG neurons (Fig. 4.2; 46.25 ± 11.2 s) did not differ from WT (35.37 ± 8.24 s; P>0.05, two-tailed unpaired Student’s t-test), similarly $[\text{Ca}^{2+}]_{\text{cyt}}$ recovery (Fig.4.2D) in NCLX-KO (9.88 ± 0.55 s) did not significantly differ from WT (8.92 ± 0.82 s; P>0.05, two-tailed unpaired Student’s t-test). As a positive control we tested the effect of $\text{mtNCX}$ inhibitor CGP-37157 in NCLX-KO DRG neurons (Fig. 4.2E). Importantly, as Fig. 4.2E shows, treatment with CGP-37157 significantly inhibited mitochondrial Ca$^{2+}$ extrusion in NCLX-KO DRG neurons.
**NCLX deletion results in prolongation of cytosolic and mitochondrial Ca\(^{2+}\) transients elicited by large Ca\(^{2+}\) loads using strong stimulation**

Next we tested the ability for NCLX to affect cytosolic and mitochondrial Ca\(^{2+}\) dynamics in response to large Ca\(^{2+}\) loads in DRG neurons. In these experiments, WT and NCLX-KO DRG neurons were stimulated by strong depolarization using 50 mM KCl (30 s; arrow, K\(^{+}\)50) (Fig. 4.3). We found that NCLX-KO significantly increased the duration of \([\text{Ca}^{2+}]_{\text{cyt}}\) recovery (5.3 min ± 0.95) as compared to WT (1.76 min ± 0.24) (Fig. 4.3F). Similarly, NCLX-KO prolonged \([\text{Ca}^{2+}]_{\text{mt}}\) recovery kinetics (5.62 min ± 1.09) as compared to WT (1.79 min ± 0.3) (Fig. 4.3E). Based on previous literature, inhibition of mitochondrial Ca\(^{2+}\) efflux was not surprising; however, the slowing of \([\text{Ca}^{2+}]_{\text{cyt}}\) recovery kinetics by NCLX deletion was unexpected (add references) [50, 173, 182].

In fact, previous literature suggested that inhibition of \(\text{mtNCX}\) should lead to either an acceleration of \([\text{Ca}^{2+}]_{\text{cyt}}\) recovery or no change [50]. In order to additionally verify this in our system, we used application of CGP-37157 (10 µM) on WT DRG neurons to inhibit \(\text{mtNCX}\) and see if we could replicate NCLX-KO phenotype (Fig. 4.3C). Treating DRG neurons with CGP-37157 led to a significant prolongation of \([\text{Ca}^{2+}]_{\text{mt}}\) recovery (7.43 min ± 1.77), but did not significantly change the duration of \([\text{Ca}^{2+}]_{\text{cyt}}\) recovery (1.96 min ± 0.48). Although these results corroborated previous research it still did not explain the Ca\(^{2+}\) dynamics seen in NCLX-KO. We then hypothesized that the NCLX-KO phenotype was due inhibition of Ca\(^{2+}\) efflux via plasma membrane rather than via the IMM. In order to test this, La\(^{3+}\) (1 mM) was applied immediately after K\(^{+}\)50 stimulation (Fig. 4.3D) in WT DRG neurons to inhibit all Ca\(^{2+}\) fluxes across the plasma membrane.
[253, 254]. We found that application of La$^{3+}$ immediately after stimulation led to significant prolongation of both $[\text{Ca}^{2+}]_{\text{mt}}$ (9.01 min ± 1.26) and $[\text{Ca}^{2+}]_{\text{cyt}}$ (10.63 min ± 0.54) recovery kinetics (Fig. 4.3E and 4.3F) similar to what was seen in NCLX-KO DRG neurons.

Finally in order to further ascertain the effect of the NCLX-KO phenotype independent of mitochondrial Ca$^{2+}$ transport, we stimulated DRG neurons pretreated with the protonophore, FCCP (1 µM), which is known to depolarize mitochondria and thereby prevent mitochondrial Ca$^{2+}$ uptake. As control, cells were first stimulated in normal HH buffer (K$^{+}$50, 30 s); then cells were pretreated with 1 µM FCCP prior to the 2$^{\text{nd}}$ stimulation (Fig. 4.4A and 4.4B). Even with low affinity Fura-FF $[\text{Ca}^{2+}]_{\text{cyt}}$ recovery kinetics again showed an inhibition of cytosolic Ca$^{2+}$ recovery in NCLX-KO (3.33 min ± 0.22) as compared to WT (1.58 min ± 0.32) (Fig. 4.4C). Importantly, we found that even in the presence of FCCP, NCLX-KO DRG neurons showed a significant increase in the duration of $[\text{Ca}^{2+}]_{\text{cyt}}$ recovery (51.7 s ± 2.97) as compared to WT (33.37 s ± 1.77) DRG neurons (Fig. 4.4D). Peak $[\text{Ca}^{2+}]_{\text{cyt}}$ amplitude was also significantly increased in NCLX-KO as compared to WT (Fig. 4.4E). These data suggest that the NCLX-KO strongly affects cytosolic Ca$^{2+}$ signaling, further corroborating the idea that NCLX is located on the plasma membrane rather than the IMM.

Overall these data in DRG neurons suggest that NCLX-KO affects cytosolic Ca$^{2+}$ dynamics primarily which influences mitochondrial Ca$^{2+}$ dynamics.
Li⁺ substitution for Na⁺ does not affect mitochondrial Ca²⁺ extrusion in DRG neurons

Cytosolic Ca²⁺ dynamics in DRG neurons has been shown to be greatly affected by mitochondrial Ca²⁺ transport [50, 226]. Research done previously on NCLX in DRG neuron showed a significant effect of NCLX knockdown (KD) on both mitochondrial and cytosolic Ca²⁺ dynamics [255]. An important property of mitochondrial Na⁺/Ca²⁺ exchanger described nearly 50 years ago in 1970 is the ability of the transport to use Li⁺ instead of Na⁺ to drive Ca²⁺ extrusion from isolated mitochondria [191]. The ability to use Li⁺ instead of Na⁺ to drive the transport of Ca²⁺ across the membranes is also shared by NCLX (“L” for lithium ion) [176]. Therefore, testing the sensitivity of [Ca²⁺]cyt and [Ca²⁺]mt to Li⁺ can help elucidating the mechanism of mNCX in neurons and different tissue types even without knowing the molecular identity of the mechanism.

First, we examined the effect of Li⁺ on [Ca²⁺]cyt and [Ca²⁺]mt dynamics in DRG neurons. The procedure for each experiment is described in Fig. 4.5A. Briefly, neurons were depolarized using brief (~15 s) applications of 30 mM KCl (K⁺30) in a standard extracellular buffer containing 140 mM Na⁺. After both [Ca²⁺]cyt and [Ca²⁺]mt recovered to baseline, extracellular Na⁺ was replaced with Li⁺ (i.e., 140 mM NaCl replaced with 140 mM LiCl) for 10 min prior to depolarization. Importantly, voltage-gated Na⁺ channels are highly permeable for Li⁺, and thus Li⁺ is expected to enter the cell upon depolarization [256]. The Li⁺ substitution did not affect [Ca²⁺]mt recovery kinetics. Specifically, the time to recover 70% from maximum [Ca²⁺]mt amplitude (T70) was (1.81 ±0.32 min) for Na⁺-containing extracellular solution and (2.37 ±0.36 min) for Li⁺-containing extracellular solution. The amplitudes of [Ca²⁺]cyt and [Ca²⁺]mt also were not significantly influenced by the replacement of extracellular Na⁺ with Li⁺ [data not shown]. Next we wanted to test
the ability for the known \textsubscript{mt}NCX inhibitor CGP-37157 using a similar protocol as before except bathing DRG neurons in CGP-37157 (10 \textmu M) before (2 min) and after 2\textsuperscript{nd} stimulation. In cultured DRG neurons, 10 \textmu M CGP-37157 increased T\textsubscript{70} to 29.18 ±3.43 min as compared to 1.74 ± 0.35 min in control conditions (Fig. 4.5D).

We hypothesized that one reason Li\textsuperscript{+} was not affecting mitochondrial Ca\textsuperscript{2+} extrusion was because residual Na\textsuperscript{+} present was able to activate the \textsubscript{mt}NCX mechanism. Therefore we used whole-cell patch-clamp method to control for the intracellular ion composition and to fill DRG neurons with 30 mM of either NaCl or LiCl (Fig. 4.6). Even with this precisely controlled ion substitution via whole-cell patch-clamp, Li\textsuperscript{+} substitution did not change the recovery kinetics of either [Ca\textsuperscript{2+}]\textsubscript{mt} (\tau=45.18 ± 10.5 s) or [Ca\textsuperscript{2+}]\textsubscript{cyt} (\tau=6 ± 0.95 s) as compared to Na\textsuperscript{+}-containing intracellular solution: ([Ca\textsuperscript{2+}]\textsubscript{mt}; \tau=42.22 ± 6.62 s; [Ca\textsuperscript{2+}]\textsubscript{cyt}; \tau=6.48 ± 0.96 s) (Fig. 4.6C and 4.6E). Similarly, replacement of intracellular Na\textsuperscript{+} for Li\textsuperscript{+} did not alter peak [Ca\textsuperscript{2+}]\textsubscript{mt} or [Ca\textsuperscript{2+}]\textsubscript{cyt} amplitudes (Fig. 4.6D and 4.7E).

\textbf{Li\textsuperscript{+} substitution for Na\textsuperscript{+} significantly inhibits mitochondrial Ca\textsuperscript{2+} extrusion in hippocampal neurons}

NCLX has been shown to affect function in some central neurons; however, only aspects of cell death have been examined [185, 186]. Next, we examined the effects of Li\textsuperscript{+} on mitochondrial and cytosolic Ca\textsuperscript{2+} dynamics in central neurons, using cultured hippocampal neurons as a model. Replacement of extracellular Na\textsuperscript{+} with Li\textsuperscript{+} significantly inhibited cytosolic Ca\textsuperscript{2+} efflux (Fig.4.7), which was in contrast to the observations in DRG neurons (Fig. 4.6). To control for [Ca\textsuperscript{2+}]\textsubscript{cyt}, a protocol was developed where after stimulation a Ca\textsuperscript{2+}-free extracellular solution (0 Ca\textsuperscript{2+}, 100 \textmu M EGTA) was perfused until
both \([\text{Ca}^{2+}]_{\text{cyt}}\) and \([\text{Ca}^{2+}]_{\text{mt}}\) recovered to baseline (Fig. 4.7B). With this approach, \([\text{Ca}^{2+}]_{\text{cyt}}\) was able to recover to baseline even after replacing extracellular \(\text{Na}^+\) with \(\text{Li}^+\). Importantly, this \(\text{Li}^+\) treatment led to a significant slowing of \([\text{Ca}^{2+}]_{\text{mt}}\) recovery after depolarization. Specifically, \(T_{70}\) of \([\text{Ca}^{2+}]_{\text{mt}}\) recovery significantly increased (12.04 ±1.14 min) as compared to \(\text{Na}^+\) in \(\text{Ca}^{2+}\) Free (4.11 ±0.39 min) (Fig. 4.7D). As a positive control, we found that the \(\text{mtNCX}\) inhibitor CGP-37157 significantly slowed \([\text{Ca}^{2+}]_{\text{mt}}\) recovery: \(T_{70}\) was increased to 23.69 ± 3.75 min in hippocampal neurons treated with CGP-37157 as compared to 2.96 ± 0.5 min in control conditions (Fig. 4.7E). Overall this data shows that \(\text{Li}^+\) substitution significantly inhibits mitochondrial \(\text{Ca}^{2+}\) extrusion in hippocampal neurons.

\textit{Li}^+ \textit{significantly inhibits mitochondrial Ca}^{2+} \textit{extrusion in isolated free brain mitochondria}

In intact neurons mitochondrial \(\text{Ca}^{2+}\) transport can potentially be influenced by \(\text{Ca}^{2+}\) fluxes via plasma and ER membranes, as well as by \(\text{Ca}^{2+}\) buffering in the cytosol. There limitations can be addressed by studying mitochondrial \(\text{Ca}^{2+}\) fluxes in isolated mitochondria. Previous research has shown the ability for the \(\text{mtNCX}\) to substitute \(\text{Li}^+\) for \(\text{Na}^+\) in isolated heart mitochondria [90, 191]. However the \(\text{Li}^+\) sensitivity of \(\text{mtNCX}\)-mediated \(\text{Ca}^{2+}\) transport in isolated brain mitochondria has not been examined. . Taking a reductionist approach, we examined mitochondrial \(\text{Ca}^{2+}\) efflux in isolated mitochondria in order to control for the ability for \(\text{Li}^+\) to diffuse across the plasma and ER membranes (Fig. 4.8). In these experiments, baseline \(\text{Ca}^{2+}\) was measured before a bolus of 100 \(\mu\text{M}\) \(\text{Ca}^{2+}\) was added to the suspension of isolated brain mitochondria. After \([\text{Ca}^{2+}]_{\text{ext}}\) recovered, 10 \(\mu\text{M}\) Ru360 was added to inhibit mitochondrial \(\text{Ca}^{2+}\) uptake, lastly a 20 mM
bolus of alkali ions (Na\(^+\) or Li\(^+\)) was added to induce mitochondrial Ca\(^{2+}\) efflux (Fig. 4.8A). As shown in Fig. 4.8B maximum mitochondrial Ca\(^{2+}\) efflux rate was 33.8 nmol Ca\(^{2+}\) min\(^{-1}\) mg prot\(^{-1}\) (±2.2 SEM) in the 20 mM of Na\(^+\) condition as compared to 12.1 nmol Ca\(^{2+}\) min\(^{-1}\) mg prot\(^{-1}\) (±1.5 SEM) seen when 20 mM Li\(^+\) was added to the mitochondria suspension. Ca\(^{2+}\) release from mitochondria follows a decreasing exponential function where Ca\(^{2+}\) efflux rate nearly ceases around 30 min (Fig. 4.8A). As shown in Fig 4.8C Na\(^+\) induced 94.79% (±2.41 SEM) of total Ca\(^{2+}\) release from mitochondria while Li\(^+\) induced 56.48% (±2.43 SEM) of total Ca\(^{2+}\) to be released by mitochondria after 30 min. Lastly, we tested CGP-37157 in isolated free brain mitochondria. Briefly, similar to previous experiments a 100 μM bolus of Ca\(^{2+}\) was added, then 10 μM Ru360, after that 30 μM of CGP was added to the suspension, and lastly 20 mM of Na\(^+\) or Li\(^+\) was added to induce mitochondrial Ca\(^{2+}\) efflux (Fig. 4.8D). Application of CGP significantly reduced mitochondrial Ca\(^{2+}\) efflux in both Na\(^+\) (2.43 nmol Ca\(^{2+}\) min\(^{-1}\) mg prot\(^{-1}\), ±0.51 SEM) and Li\(^+\) (1.42 nmol Ca\(^{2+}\) min\(^{-1}\) mg prot\(^{-1}\), ±0.22 SEM) conditions (Fig. 4.8E). Overall these data suggest that in contrast to Na\(^+\), Li\(^+\) fails to effectively drive Ca\(^{2+}\) efflux from mitochondria.

**Discussion**

In this chapter, I have examined the ability of the protein NCLX to regulate mitochondrial and cytosolic Ca\(^{2+}\) signaling in DRG neurons, as well as the ability for the mtNCX mechanisms (putatively NCLX) to utilize Li\(^+\) to catalyze mitochondrial Ca\(^{2+}\) extrusion in the nervous tissue. The described findings suggest that in DRG neurons NCLX does not localize to the IMM but instead seems to localize to the PM. Though interestingly even though NCLX-KO does not directly affect mitochondria Ca\(^{2+}\) dynamics
DRG neuron mNCX mechanism is still able to utilize Li$^+$ instead of Na$^+$ to drive Ca$^{2+}$ extrusion from mitochondria. In contrast, central neurons do not seem to have ability to utilize Li$^+$ to drive mitochondrial Ca$^{2+}$ efflux.

Based on NCLX-flag experiments, NCLX does not seem to localize to mitochondria in neurons (Fig. 4.1D), but found on the plasma membrane instead. For physiologically relevant stimulation, NCLX deletion did not cause a significant alteration in either mitochondrial or cytosolic Ca$^{2+}$ dynamics in neurons (Fig. 4.2A-D). As a positive control for NCLX-KO we tested the effect of the specific mNCX inhibitor, CGP-37157 (10 µM), in response to field stimulation (Fig. 4.2 E). As Fig. 4.2E shows, application of CGP significantly inhibited mitochondrial Ca$^{2+}$ efflux in NCLX-KO neurons which does not corroborate previous research [173].

NCLX deletion did show a significant effect on both mitochondrial and cytosolic Ca$^{2+}$ dynamics in DRG neurons in response to large Ca$^{2+}$ loads. Our additional analysis suggests that this effect was driven by an impaired ability of NCLX-KO neurons to clear cytosolic Ca$^{2+}$ after the stimulation (Figs. 4.3 and 4.4). These results did not corroborate previous literature on the effects of mNCX inhibition via CGP-37157, nor does it replicate NCLX-KD results in DRG neurons previously reported [50, 183]. Taken together, these data suggest that in DRG neurons NCLX lies on the PM which is why deletion of NCLX leads to an increase [Ca$^{2+}$]$_{cyt}$ recovery duration which in turn affects [Ca$^{2+}$]$_{mt}$ recovery duration.

We also investigated the ability for the mNCX to function when substituting Li$^+$ for Na$^+$ in nervous tissue. Previous research on Li$^+$ utilization in mitochondrial Ca$^{2+}$ extrusion has only been investigated in cardiac and heterologous systems. Here, using
Ca²⁺ imaging, we saw differences between the peripheral and central nervous tissue in the ability to induce mitochondrial Ca²⁺ efflux using Li⁺. DRG neurons showed no alteration to cytosolic or mitochondrial Ca²⁺ dynamics when bathed in Li⁺ solution. On the contrary, both cytosolic and mitochondrial Ca²⁺ dynamics were affected in cultured hippocampal neurons by Li⁺ substitution with mitochondrial Ca²⁺ efflux being affected irrespective of [Ca²⁺]ₘₚ levels. Similarly, isolated free brain mitochondria showed an inhibition in mitochondrial Ca²⁺ efflux under Li⁺ conditions as compared to Na⁺. Thus, Na⁺ and Li⁺ significantly differ in their abilities to drive Ca²⁺ extrusion from brain mitochondria.

The results showing that Li⁺ has a different effect on mitochondrial Ca²⁺ extrusion between central and peripheral neurons is a novel finding that has not been shown before. Two mechanisms have been proposed for mitochondrial Ca²⁺ extrusion though both have been shown to be Ca²⁺ exchangers [192, 193, 257, 258]. More relevant for excitable tissues is the electrogenic Na⁺/Ca²⁺ (NCX) exchanger which uses the energy from 3 Na⁺ entering the MM to extrude 1 Ca²⁺ out, similar to other mammalian NCXs [162, 175, 259, 260]. Similar to the research shown here in central nervous tissue, research in 1974 showed the ability for Li⁺ to stimulate Ca²⁺ release from mitochondria which was a novel aspect for this NCX mechanism [191]. Although, in 1977, research from the same investigators showed Li⁺ induced mitochondrial Ca²⁺ efflux at a slower rate (4.6 nmol Ca²⁺ min⁻¹ mg prot⁻¹, 15 mM; Fig. 4D) as compared to Na⁺ induced release (14 nmol Ca²⁺ min⁻¹ mg prot⁻¹, 15 mM; Fig. 4A) [90]. However, recent research investigating the mitochondrial efflux mechanism, NCLX, has shown that Li⁺ does not inhibit mitochondrial Ca²⁺ efflux as compared to Na⁺, just as we have shown in DRG.
neurons [173, 177, 178]. Interestingly, we also found that Li\(^+\) was unable to extrude a large portion of the Ca\(^{2+}\) loaded into the isolated free brain mitochondria. This effect has not been reported in any previous research and raise further questions on the effect Li\(^+\) has on \(\text{mtNCX}\) function. Given the heterogeneity of nervous tissue there may be a heterogeneous population of mitochondria with a subset of those that can utilize Li\(^+\) in mitochondrial Ca\(^{2+}\) extrusion. This would help to explain both the rate (the amount of exchangers able to extrude Ca\(^{2+}\)) and the total Ca\(^{2+}\) efflux (only the Li\(^+\) permeable subset could release Ca\(^{2+}\)) from free brain mitochondria. However, isolated mitochondria from more homogenous tissue (cardiac) still show a deficit in the rate of mitochondrial Ca\(^{2+}\) efflux [90].

Overall these data make an argument for two possible different mechanisms between the central and peripheral neurons in regards to \(\text{mtNCX}\). This is an important aspect of \(\text{mtNCX}\) as the protein NCLX was proposed to be the \(\text{mtNCX}\) mechanism based on its ability to utilize Li\(^+\)/Ca\(^{2+}\) exchange.
Figure 4.1: Expression levels of NCLX in NCLX-KO mice and distribution of NCLX in DRG neurons

A. Melt curve generated via qRT-PCR using Fast SYBR Green between WT (black) and NCLX-KO (blue) examining expression levels of NCLX. Fluorescence intensity (a.u.) is a function of NCLX expression quantified as the number of PCR reactions (cycles) that were necessary for an increase in fluorescent intensity.

B. The expression level of NCLX mRNA was normalized to WT levels (black) quantified as ΔΔCt.

C. Immunocytochemistry of cultured primary DRG neurons. Neurons were transfected with both NCLX-FLAG and mitochondrial targeted EGFP (mtEGFP) in order to visualize the organelle. The scale bars (white) on the bottoms of each panel correspond to 20 µm.
Figure 4.2: NCLX deletion does not affect cytosolic or mitochondrial Ca\(^{2+}\) dynamics in response to physiological relevant stimulation

A and B, Representative traces of simultaneous [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fura-2; black) and [Ca\(^{2+}\)]\(_{\text{mt}}\) (mtR-GECO; red) monitoring in WT (A) and NCLX KO (B) mouse DRG neurons. [Ca\(^{2+}\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{mt}}\) transients were evoked by trains of action potentials (8 Hz, 2-3 s; vertical arrows, APs) using extracellular field potential stimulation. C and D, Recovery kinetics measured in seconds for [Ca\(^{2+}\)]\(_{\text{mt}}\) (C) and [Ca\(^{2+}\)]\(_{\text{cyt}}\) (D) in WT (black circles) and
NCLX-KO (blue squares) DRG neurons to recover 70% to baseline from peak amplitude. No significant differences were found between WT and NCLX-KO in regards to $[\text{Ca}^{2+}]_{\text{cyt}}$ or $[\text{Ca}^{2+}]_{\text{mt}}$ recovery kinetics. E, Example trace illustrating the effect of CGP-37157 (10 µM) on $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fura-2; black) and $[\text{Ca}^{2+}]_{\text{mt}}$ (mtR-GECO; red) dynamics in NCLX-KO DRG neurons. CGP-37157 was applied 2 min before stimulation. Data represents mean ± SEM, analyzed using two-tailed Mann Whitney test.
Figure 4.3: Deletion of NCLX affects recovery kinetics of both $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ in response to large Ca$^{2+}$ loads

A-D, Representative traces of simultaneous $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fura-2; black) and $[\text{Ca}^{2+}]_{\text{mt}}$ (mtLAR-GECO1.2; red) in both WT (A, C, and D) and NCLX-KO (B) mouse DRG neurons. Large $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mt}}$ loads were evoked by brief (30 s) perfusion of 50 mM KCl (vertical arrows; K'50). C, Monitoring of cytosolic and mitochondrial Ca$^{2+}$ dynamics in WT DRG neurons during the application of the mtNCX inhibitor CGP-37157 and La$^{3+}$ (1 mM).
(10 µM), which started 2 min before the stimulation. D, Effect of inhibiting plasma membrane Ca\(^{2+}\) efflux on cytosolic and mitochondrial Ca\(^{2+}\) dynamics using La\(^{3+}\) (1 mM) application immediately after stimulation. E and F, Both mitochondrial (E) and cytosolic (F) Ca\(^{2+}\) recovery kinetics in NCLX-KO (blue squares), WT - control (black circles), WT + CGP-37157 (magenta triangles), and WT + La\(^{3+}\) (purple diamonds). Mitochondrial Ca\(^{2+}\) recovery (E) showed a significant difference between control and either application of CGP or La\(^{3+}\), but not NCLX-KO. Similar statistics for cytosolic Ca\(^{2+}\) recovery (F) revealed a significant difference between control and NCLX-KO as well as La\(^{3+}\) but not CGP-37157. Data represents mean ± SEM, analyzed using nonparametric Kruskal-Willis test with post hoc Dunn’s multiple comparison test. *** p<0.005, ** p<0.01, * p<0.05
Figure 4.4: NCLX-deletion affects cytosolic Ca\textsuperscript{2+} dynamics without mitochondrial contribution

A and B, Representative traces of [Ca\textsuperscript{2+}]\textsubscript{cyt} (Fura-FF; multiple colors; each cell is represented by a different color) from multiple DRG neurons in both WT (A) and NCLX-KO (B) genotypes. Large [Ca\textsuperscript{2+}]\textsubscript{cyt} transients were evoked using brief (30 s) perfusion of 50 mM KCl (K\textsuperscript{+}50, arrows). FCCP (1 µM; yellow bar) application began 2 min before the 2\textsuperscript{nd} stimulation. C, [Ca\textsuperscript{2+}]\textsubscript{cyt} recovery kinetics, calculated as previously described (T\textsubscript{70}), in
both WT (black) and NCLX-KO (blue) DRG neurons in response to K\(^{+}\)50. D, \([\text{Ca}^{2+}]_{\text{cyt}}\) recovery kinetics in WT (black) and NCLX-KO (blue) DRG neurons in response to K\(^{+}\)50 stimulation during FCCP (1 µM) application. E, Peak \([\text{Ca}^{2+}]_{\text{cyt}}\) amplitude in WT (black) and NCLX-KO (blue) DRG neurons in response it K\(^{+}\)50 stimulation during application of FCCP (1 µM). Data represents mean ± SEM, analyzed using unpaired two-tailed student’s t-test.
Figure 4.5: Na\(^+\) substitution with Li\(^+\) does not affect mitochondrial Ca\(^{2+}\) extrusion in DRG neurons

A and B, Representative traces of simultaneously recorded [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fura-2; black) and [Ca\(^{2+}\)]\(_{\text{mt}}\) (mtR-GECO; red) in WT DRG neurons. [Ca\(^{2+}\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{mt}}\) transients were induced using brief (15 s) perfusion with 30 mM KCl (black bars, K\(^+\)30). Rate of mitochondrial Ca\(^{2+}\) efflux measured as the duration for [Ca\(^{2+}\)]\(_{\text{mt}}\) to recover 70% to baseline from peak [Ca\(^{2+}\)]\(_{\text{mt}}\) amplitude. A, Protocol for Li\(^+\) substitution in DRG neurons: DRG neurons were stimulated twice, after [Ca\(^{2+}\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{mt}}\) had recovered, Li\(^+\) substitution (cyan bar) began 10 min prior to the 2\(^{nd}\) pair of stimulations. B, Protocol for CGP-37157 (10 \(\mu\)M) application (green bar) in DRG neurons, after the 1\(^{st}\) stimulation CGP-37157 application began 2 min prior to the 2\(^{nd}\) stimulation, then [Ca\(^{2+}\)]\(_{\text{mt}}\) was
allowed to recover. C, Li⁺ substitution (cyan circles) had no effect on mitochondrial Ca²⁺ efflux as compared to Na⁺ control conditions (orange circles). D, Application of CGP-37157 (green circles) significantly inhibited mitochondrial Ca²⁺ efflux as compared to control conditions (black circles). Data represent mean ± SEM, analyzed using two-tailed paired student’s t-test. **** p<0.001
Figure 4.6: Li⁺ substitution via whole-cell patch-clamp does not inhibit mitochondrial Ca²⁺ efflux in DRG neurons

A and B, Representative traces of simultaneous [Ca²⁺]ᵣ (Fura-2; black) and [Ca²⁺]ₑ (mtR-GECO; red) imaging in WT DRG neurons. Internal patch pipette solution contained either 30 mM NaCl (A) or LiCl (B), while cells were stimulated via step depolarization (-60 to 0 mV, 100 ms). C and D, Peak [Ca²⁺]ₑ amplitude (D) and
recovery kinetics (C) in Na\(^+\) (orange) and Li\(^+\) (cyan) conditions showed no difference. E and F, Peak \([\text{Ca}^{2+}]_{\text{cyt}}\) amplitude (F) and recovery kinetics (E) in both Na\(^+\) (orange) and Li\(^+\) (cyan) conditions showed no difference between either condition. Data represents mean ± SEM, analyzed using two-tailed Mann-Whitney U test.
Figure 4.7: Li⁺ substitution for Na⁺ strongly inhibited mitochondrial Ca²⁺ efflux in hippocampal neurons

A-C, Representative traces simultaneous [Ca²⁺]_{cyt} (Fura-2; black) and [Ca²⁺]_{mt} (mtR-GECO; red) imaging in cultured hippocampal neurons. Rate of mitochondrial Ca²⁺ efflux measured as the duration for [Ca²⁺]_{mt} to recover 70% to baseline from peak [Ca²⁺]_{mt} amplitude. A, Initial protocol for Li⁺ substitution experiments, 1st neuron was stimulated via brief (15 s) perfusion of 30 mM KCl (black bar, K⁺30), then Li⁺ (cyan bar) was
substituted several minutes before the 2nd stimulation. B, Updated Li⁺ substitution protocol to control for [Ca²⁺]_{cyt}, protocol was the same except Ca²⁺-free extracellular solution was used 10 s after each stimulation with either Na⁺ (dark blue) or Li⁺ (striped cyan bar) including 2 min preincubation with Li⁺ (cyan bar). C, Example trace of the effect of CGP-37157 (10 µM) on mitochondrial Ca²⁺ efflux. Similar to previous protocols, first stimulation acted as a control while start of CGP-37157 application began 2 min before the 2nd stimulation. D, Li⁺ substitution (cyan circles) significantly inhibited mitochondrial Ca²⁺ extrusion compared to Na⁺ (orange circles). E, CGP-37157 (10 µM) application (green circles) significantly reduced mitochondrial Ca²⁺ efflux compared to control conditions (black circles). Data represents mean ± SEM, analyzed using two-tailed paired student’s t-test. **** p<0.001, *** p<0.005
Figure 4.8: Li⁺ substitution inhibits mitochondrial Ca²⁺ efflux in free brain mitochondria

A, Representative trace of extracellular Ca²⁺ ([Ca²⁺]₆ₓ) in a suspension of isolated brain mitochondria. Experimental protocol: after baseline measurement a bolus of Ca²⁺ (100 µM, black bar) was added to the suspension, after mitochondria took up Ca²⁺ the mitochondrial Ca²⁺ uptake inhibitor, Ru360 (10 µM, black bar), lastly a bolus of Na⁺ (20 µM, black bar)
mM; orange line, arrow) or Li⁺ (20 mM; cyan line, arrow) was added to induce mitochondrial Ca²⁺ efflux. **B**, Rate of mitochondrial Ca²⁺ efflux in isolated brain mitochondria induced via addition of 20 mM Na⁺ (orange circles) or Li⁺ (cyan circles). Efflux rate is nmol of Ca²⁺ per minute normalized to the amount of mitochondrial protein in the suspension. **C**, Total amount of Ca²⁺ extruded from mitochondria (µM) after 30 min as a function of addition of either 20 mM Na⁺ (orange bar) or Li⁺ (cyan bar). **D**, Addition of mitochondrial Ca²⁺ efflux inhibitor CGP-37157 (30 µM; arrow) on both Na⁺ (orange line) and Li⁺ (cyan line) induced Ca²⁺ efflux. **E**, Rate of mitochondrial Ca²⁺ efflux in the presence of CGP-37157 quantified as nmol of Ca²⁺ efflux per minute normalized to amount of mitochondrial protein in the suspension. Data represents mean ± SEM, analyzed using unpaired two-tailed student’s t-test. *** p<0.005, ** p<0.01
CHAPTER V: DISCUSSION AND FUTURE DIRECTIONS

Summary of the Results

Collectively the presented data demonstrate how specific molecules and mechanisms affect Ca\(^{2+}\) signaling in the cytosol and mitochondria. First, these studies examined the effects of MCU deletion on Ca\(^{2+}\) dynamics and mitochondrial bioenergetics in both central and peripheral neurons. Then, the effects of MCU deletion on normal and pathological behavior in mice was tested. Lastly, the molecular mechanisms responsible for mitochondrial Ca\(^{2+}\) efflux were investigated in both peripheral and central nervous tissue.

Biochemistry data showed ubiquitous expression of MCU throughout nervous tissue with slight differences in expression levels (Fig. 2.1C-E), while immunocytochemistry showed a non-uniform distribution of MCU across mitochondria in central and peripheral neurons (Fig. 2.1A and 2.1B). Next, it was shown that MCU is critical in regulating both mitochondrial and cytosolic Ca\(^{2+}\) dynamics. In DRG neurons MCU-KO led to a significant alteration cytosolic Ca\(^{2+}\) dynamics, losing the characteristic “plateau phase” seen in response to large neuronal depolarizations (Fig. 2.2 and Fig. 2.4) [50]. Consequently, [Ca\(^{2+}\)]\(_{\text{cyt}}\) had significantly larger amplitudes, but recovered to baseline [Ca\(^{2+}\)]\(_{\text{cyt}}\) significantly faster in MCU KO neurons than in WT ones. Similarly, mitochondrial Ca\(^{2+}\) uptake was inhibited at low and moderate stimulation, but not completely abolished in response to large neuronal depolarizations. The [Ca\(^{2+}\)]\(_{\text{cyt}}\) threshold to activate mitochondrial Ca\(^{2+}\) uptake was shifted rightward and more than doubled in MCU-KO neurons compared to WT (Fig. 2.4 and Fig. 2.6). Activity- and
Ca$^{2+}$-dependent mitochondrial depolarization was also inhibited in MCU-KO DRG neurons, highlighting the role Ca$^{2+}$ has in regulating mitochondrial bioenergetics (Fig. 2.8).

In cultured hippocampal neurons, MCU-KO also significantly altered cytosolic and mitochondrial Ca$^{2+}$ dynamics. MCU-KO significantly inhibited mitochondrial Ca$^{2+}$ uptake in response to physiologically relevant stimuli; however, large neuronal depolarizations showed some ability to induce mitochondrial Ca$^{2+}$ uptake (Fig. 2.3 and Fig. 2.5). Peak [Ca$^{2+}]_{\text{cyt}}$ amplitude was significantly increased in MCU-KO neurons when large depolarizations were induced, similar to DRG neurons. ΔΨ$_{\text{mt}}$ depolarization was inhibited in response to KCl application compared to WT neurons (Fig. 2.9). Using free brain mitochondria it was found that MCU-KO mitochondria took up significantly less Ca$^{2+}$ at a slower rate compared to WT mitochondria (Fig. 2.7). Importantly, the data also demonstrated existence of an additional, MCU-independent mechanism that mediates mitochondrial Ca$^{2+}$ uptake in neurons.

Overexpressing an MCU construct in MCU-KO DRG or hippocampal neurons was able to rescue the altered mitochondrial and cytosolic Ca$^{2+}$ dynamics seen in MCU-KO neurons. MCU-KO increased resting [Ca$^{2+}]_{\text{cyt}}$ in both DRG and hippocampal neurons, but did not seem to alter resting levels of [Ca$^{2+}]_{\text{mt}}$. The same was the case for baseline ΔΨ$_{\text{mt}}$ (Fig. 2.4, 2.5, 2.8, and 2.9). There was no difference between WT or MCU-KO in regards to plasma membrane Ca$^{2+}$ influx in either DRG or hippocampal neurons (Fig. 2.2 and Fig. 2.3). Overall these data show a significant role of MCU in regulating Ca$^{2+}$ dynamics and mitochondrial bioenergetics in CNS and PNS neurons.
I saw very little differences between WT and MCU-KO male and female mice in regards to normal behavior. There were differences on tasks investigating, thermal and mechanical sensitivity, locomotor control, anxiety-like behavior, spatial memory tasks (Fig. 3.1 and Fig. 3.2). However, I did find differences in a fear conditioning task. Male MCU-KO mice did not acquire learned freezing behavior as readily as WT counterparts while also showing a deficit in cued fear recall, but not contextual fear recall. Conversely, female MCU-KO mice showed a slight deficit in contextual fear recall, while showing no impairments in acquiring freezing behavior or cued fear recall (Fig. 3.3).

Interestingly, we found that MCU-KO significantly protected mice against seizures using a MES model of seizure induction compared to WT mice. WT male and female mice typically had maximal seizures when 9-10 mA of current was injected and around 85-90% of WT mice died due to having a maximal seizure. MCU-KO mice, however, were significantly protected where a maximal seizure could not be elicited in male MCU-KO mice up to 40 mA, while in females half of the females had seizures (19 mA), but the seizure severity was significantly reduced (Fig. 3.5).

In cultured hippocampal neurons, neuronal network excitability was tested by inducing epileptiform activity using convulsants bicuculline (GABA\textsubscript{A} receptor antagonist) and 4-AP (voltage-gated K\textsuperscript{+} channel inhibitor). MCU-KO neurons were significantly resistant to induced epileptiform activity evoked using 4-AP and bicuculline; however, higher concentrations of bicuculline did elicit some epileptiform activity (Fig. 3.4). Using electrophysiology in hippocampal slices we found no difference in intrinsic excitability between WT and MCU-KO hippocampal neurons (Fig. 3.6). We did not find any
difference in frequency or amplitude of mEPSCs, but saw a significant increase in the frequency of mIPSCs with no difference in the amplitude (Fig. 3.7).

Next I investigated the molecules and mechanisms involved in mitochondrial Ca\(^{2+}\) efflux. By overexpressing and staining for NCLX I found that on DRG neurons, NCLX located to the plasma membrane (Fig. 4.1D). No difference was found in mitochondrial or cytosolic Ca\(^{2+}\) recovery kinetics in response to AP trains between WT and NCLX-KO DRG neurons, while mitochondrial Ca\(^{2+}\) recovery was distinctly inhibited using CGP-37157 in NCLX-KO DRG neurons (Fig. 4.2). Interestingly, both cytosolic and mitochondrial Ca\(^{2+}\) dynamics were inhibited in NCLX-KO DRG neurons in response to large neuronal depolarizations. I was able to replicate NCLX-KO phenotype in WT DRG neurons by inhibiting plasma membrane, but not mitochondrial, Ca\(^{2+}\) extrusion (Fig. 4.3). I further confirmed NCLX-KO plasma membrane Ca\(^{2+}\) extrusion deficits by examining cytosolic Ca\(^{2+}\) extrusion in the presence of FCCP (Fig. 4.4).

In cultured WT DRG neurons Na\(^{+}\) substitution with Li\(^{+}\) had no effect on cytosolic and mitochondrial Ca\(^{2+}\) extrusion (Fig. 4.5 and Fig. 4.6). In WT hippocampal neurons Li\(^{+}\) substitution increased mitochondrial Ca\(^{2+}\) recovery kinetics on average, but not completely in all neurons (Fig. 4.7). Taking a reductionist approach we examined mitochondrial Ca\(^{2+}\) efflux rate in isolated brain mitochondria and found that Li\(^{+}\) induced Ca\(^{2+}\) efflux a \(\frac{1}{3}\) as much as 20 mM Na\(^{+}\) (Fig. 4.8).

**Discussion and Future Directions**

The research conducted here explored the role of MCU in neuronal Ca\(^{2+}\) signaling, mitochondrial bioenergetics, and animal behavior. Using Ca\(^{2+}\) imaging, this
work showed that MCU-KO significantly altered Ca$^{2+}$ signaling in both the mitochondria and the cytosol. One function of mitochondrial Ca$^{2+}$ transport studied extensively in neurons is the ability to regulate presynaptic Ca$^{2+}$ clearance. This regulation has been hypothesized to affect neuronal excitability [37, 38, 41, 42, 53]. However, we found that MCU-KO mice had only few mild behavioral deficits compared to WT mice. Aside from alterations in activity dependent Ca$^{2+}$ dynamics, our data show a change in resting [Ca$^{2+}$]$_{cyt}$ but not in resting [Ca$^{2+}$]$_{mt}$, similar to how the MCU-KO mouse line was initially reported [96]. It is possible that these alterations of resting Ca$^{2+}$ in neurons do not affect neuronal function or animal behavior. However, increased resting [Ca$^{2+}$]$_{cyt}$ was reported to alter neurite outgrowth in cultured CNS neurons, but we did not investigate this effect in our MCU-KO mouse model or whether our difference in [Ca$^{2+}$]$_{cyt}$ at rest would affect these aspects of neuron function [16]. Interestingly in MICU1 KO mice many neurological and anatomical deficits were reported including a decrease in body weight and morphological defects in the cerebellum [154]. The authors show that resting [Ca$^{2+}$]$_{mt}$ was elevated and that these mice had impaired stimulation-dependent mitochondrial Ca$^{2+}$ uptake. Behavioral and morphological deficits were eventually resolved as the mice matured which the authors suggest is due to a delay in development. This developmental phenotype was rescued by deleting a copy of EMRE to induce hemizygous expression. Hemizygous EMRE expression in MICU1-KO mice rescued the resting [Ca$^{2+}$]$_{mt}$ phenotype, but did not rescue deficits in mitochondrial Ca$^{2+}$ uptake [154]. This research shows an important role of baseline [Ca$^{2+}$]$_{mt}$ in regulating CNS development, but suggests less important role in activity dependent mitochondrial Ca$^{2+}$ uptake.
These data might argue against a role of stimulation dependent mitochondrial Ca\(^{2+}\) uptake being important in development or regulation of neuronal networks. However, unlike with the MICU1-KO mice, MCU-KO mice are completely lethal on a C57BL/6J background, this is hypothesized to be due to defects in cardiac tissue during development [139]. Similarly, the CD-1 mouse line showed an embryonic lethal phenotype where Het x Het breeder pairings resulted in only 7% MCU-KO mice being produced (compared to expected 25%) [96]. CD-1 mice are known for a more genetically diverse background, which is probably responsible for a better ability for these mice to survive MCU-KO [261]. This of course could complicate understanding the mechanisms behind the behavioral phenotypes. This might also explain why there are not many behavioral phenotypes as other mechanisms compensate almost completely for MCU-KO. If embryonic lethality is caused by cardiac tissue then MCU-KO in only nervous tissue would allow us to investigate MCU function without possible compensatory mechanisms.

Previous research using MCU-shRNA did suggest that possibility of an MCU-independent pathway, but no one had shown such a pathway exists in MCU-KO nervous tissue [262]. Unexpectedly, we observed an alternative, low affinity, MCU-independent mitochondrial Ca\(^{2+}\) uptake pathway. This pathway was only active at large [Ca\(^{2+}\)]\(_{\text{cyt}}\) loads in both DRG and hippocampal neurons. Based on isolated mitochondria experiments, this pathway transports much less Ca\(^{2+}\) at a much slower rate compared to MCU-dependent uptake. Interestingly, the MCU-independent pathway was blocked by Ru360. One possible explanation could be that the reverse mode of the mtNCX mechanism is responsible for the MCU-KO resistant mitochondrial Ca\(^{2+}\) uptake.
Previous research has not shown an effect of RuR or Ru360 on mitochondrial Ca\(^{2+}\) efflux, but these studies did not investigate the effect of these inhibitors on the reverse mode [79, 90]. Experiments on MCU-KO neurons using CGP-37157 did not show an effect on the MCU-independent mitochondrial Ca\(^{2+}\) uptake [data not shown]. However, it is possible that CGP-37157 does not affect the reverse mode of the \(\text{mtNCX}\) similar to how KB-R7943 only inhibits the reverse mode of NCXs [263]. MCUb is another molecule that may be responsible for the MCU-independent pathway as it is hypothesized to reduce mitochondrial Ca\(^{2+}\) uptake in lipid bilayers while still having the AA sequence important for Ru360 binding [202]. Other mechanisms for mitochondrial Ca\(^{2+}\) uptake have been proposed, but based on the descriptions of these mechanisms it is unlikely to be any previously hypothesized mechanisms [201]. A rapid uptake mode (RAM) mechanism is proposed to account for mitochondria Ca\(^{2+}\) uptake when \([\text{Ca}^{2+}]_{\text{cyt}}\) is low (<100 nM), which would not correspond to the high activation threshold of the MCU-independent pathway. Another mechanism proposed is a mitochondrial ryanodine receptor type 1 (mRyR1) previously shown to be present in both cardiac and nervous tissue. However, the mRyR1 is hypothesized to activate at a lower \([\text{Ca}^{2+}]_{\text{cyt}}\) than MCU while also having a higher \(\text{Ca}^{2+}\) conductance [201]. \textit{In vitro} neuronal culture experiments and isolated brain mitochondria experiments show a higher \([\text{Ca}^{2+}]_{\text{cyt}}\) activation threshold and lower conductance compared to the MCU pathway. Lastly, MCU-KO could lead to a compensatory gene expression and the expression of unexpected protein on the mitochondria to try and rescue mitochondrial Ca\(^{2+}\) uptake.

One of the most interesting phenotypes of our MCU-KO mice is the anti-seizure effect in our \textit{in vivo} seizure mouse model which is described by Fig. 5.2. MCU-KO mice
showed an increase in seizure threshold while also a reduction in seizure severity when they did have seizures. Female MCU-KO mice are more susceptible to MES seizure induction, but are significantly more protected compared to WT females. We hypothesize that the female mice showed a decrease in seizure protection due to variations in the estrus cycle [264]. *In vitro* hippocampal vultures and *ex vivo* hippocampal slice experiments showed an increase in inhibitory synaptic tone. Seizure disorders are characterized by neuronal network activity shifting to a more excitable state [73]. We hypothesize that the protection against the MES model of seizure induction is due to the increased inhibitory tone seen in MCU-KO mice. Despite the hypothesized role of mitochondrial Ca$^{2+}$ uptake in numerous neuronal functions, no research has suggested a role in affecting synaptic tone. One possible explanation for this finding is due to synaptic re-specification caused by Ca$^{2+}$ signaling during development [77]. MCU-KO could be leading to changes in Ca$^{2+}$ signaling during development and to an alteration in the development of GABAergic neurons, although the role of mitochondrial Ca$^{2+}$ transport signaling in interneuron development hasn't been explored [265]. In order to test this hypothesis, iMCU-KO or MCU-shRNA can be induced at different time points during development of synapses in culture to see if MCU can alter synapse formation. Conversely, MCU-KO phenotype could be rescued by buffering Ca$^{2+}$ during synapse development by introducing compounds such as EGTA into the neurons. Another possibility is that MCU-KO leads to an increase in the interneuron population as illustrated in Fig 5.2. In order to test this we have acquired GAD67-GFP expressing CD-1 strain where we can count cell number as well as synapse number from GABAergic neurons. MCU-KO in CD-1 lead to developmental
changes in order for the mice to survive MCU deletion. Interestingly, Nestin-Cre MCU-KO allows survival in C57Bl/6J mouse strain while still showing an anticonvulsant phenotype. However, nestin is also found in non-neuronal tissues at various developmental stages, leaving the possibility that MCU loss in other tissues may contribute to the anticonvulsant phenotype [266].

In order to test the therapeutic value of MCU-KO in the treatment of epilepsy, deletion of MCU in mice during adulthood will need to be tested. Without deletion of MCU during development, the inhibitory synaptic tone may not develop. This would corroborate the role mitochondrial Ca\(^{2+}\) transport has on short-term facilitation seen during development but loss soon after [39]. Similarly, deletion of MCU during the larval during adulthood [152]. However, inhibition of mPTP using CypD had the same effect as ketogenic diet in the inhibition of excitability in hippocampal slices [241]. There is also a role of excitotoxic cell death in the progress of epilepsy, which is inhibited in MCU-KO [68, 75, 111, 245]. Even without affecting synaptic tone MCU regulation may play a role in the treatment of seizures, as long as it is applied early in epileptogenesis.

Mitochondrial Ca\(^{2+}\) uptake is hypothesized to be the one of the main mechanisms involved in cell death in I/R and neurodegenerative disorders [7, 15, 27, 267]. However, the MCU-KO CD-1 mice do not show protection against *in vivo* models of I/R while also show a deficit in mPTP regulation via CsA [96, 142]. Interestingly, our lab saw a significant decrease in neuronal cell death in response to a glutamatergic excitotoxic insult (100 µM Glu, 30 min), but we did not see an effect in an *in vivo* ischemia/reperfusion injury caused by middle cerebral artery occlusion. This alteration in mPTP opening is hypothesized to be due to changes in posttranslational modification
of CypD [142]. However, conditional MCU-KO mouse strains do show protection against I/R in both cardiac and nervous tissue [145, 146]. These studies clearly show a role for mitochondria Ca$^{2+}$ overload in cell death and would possibly translate to models of mitochondrial dysfunction in an in vivo neurodegeneration mouse models.

Parkinson's in vivo mouse models in particular use mitochondrial dysfunction in triggering neurodegeneration [28, 62, 186, 268, 269]. Parkinson's disease is a complex disease where both genetic and environmental factors can contribute to disease progression. A lot of research has investigated the properties of VGCCs in vulnerable neuron populations in Parkinson's disease. Expression of Cav1.3 channels in dopaminergic neurons is especially considered to make these neurons vulnerable to Ca$^{2+}$ induced neuronal dysfunction [62]. One of the popular in vivo mouse models of Parkinson's disease uses 6-OHDA (6-hydroxydopamine), a toxic compound that induces dopaminergic cell death. Several research groups have shown that 6-OHDA can lead to increased cytosolic Ca$^{2+}$ uptake and subsequent dysfunction leading to cell death [270, 271].

Similarly, in vivo models of Alzheimer's have shown a potential role for mitochondrial Ca$^{2+}$ overload in disease progression [27, 59, 267, 272]. Based on the Aβ hypothesis of Alzheimer's, introduction of Aβ plaques in culture leads to a significant increase in [Ca$^{2+}$]$_{cyt}$ as well as a decrease in ΔΨ$_{mt}$ in synaptic mitochondria [273]. Consequently, Aβ plagues in vivo leads to an increase in [Ca$^{2+}$]$_{cyt}$ as measured using 2-photon microscopy in the APP23xPS45 (overexpresses human amyloid precursor protein and expresses mutant Presenilin 1) mouse model of Alzheimer's [274, 275].
Based on *in vivo* disease models of neurodegenerative disorders, Ca\(^{2+}\) dysregulation seems to be a very prominent feature in these pathologies. Established strains of MCU-KO have shown a role for mitochondrial Ca\(^{2+}\) uptake in acute models of neuronal injury; however, the role of mitochondrial Ca\(^{2+}\) uptake has not been tested in a slow-progressing neurodegenerative model of neuronal injury. Given the role of mitochondrial Ca\(^{2+}\) uptake in mPTP opening as well as regulating ROS production based on Ca\(^{2+}\)-dependent CAC activity, there is a hypothesized mechanism of action regarding mitochondrial Ca\(^{2+}\) uptake in neuronal dysfunction in neurodegenerative models.

The question of whether or not NCLX is the mechanism responsible for \textit{mt}NCX still remains elusive as our data do not support this claim. In PNS DRG neurons, NCLX-KO had no appreciable effect on mitochondrial Ca\(^{2+}\) efflux, but instead showed a small deficit in plasma membrane Ca\(^{2+}\) extrusion in response to large neuronal depolarizations. It is possible that NCLX-KO leads to developmental changes that compensate for the loss of \textit{mt}NCX. If this true then the data showing inhibition of mitochondrial Ca\(^{2+}\) efflux in NCLX-KO DRG neurons without affecting cytosolic Ca\(^{2+}\) extrusion questions the specificity of the drug to target a specific molecule when inhibiting \textit{mt}NCX. In order to rule out the possibility of compensatory action, we have acquired the inducible NCLX-KO mouse line seen in Luongo et al. 2017 [182]. By either injecting tamoxifen *in vivo* or by transfecting cultured neurons with Cre-plasmids, we will bypass any developmental mechanisms involved in NCLX-KO. We still might not see an effect using the inducible NCLX-KO, there may be alternative explanations for the data we generated.
It is simply possible that NCLX is not the \textit{mt}NCX mechanism in the nervous tissue. Other molecules might be responsible for mitochondrial Ca\textsuperscript{2+} efflux in central nervous tissue. Other known NCXs have been suggested to be located on mitochondria and are primarily expressed in nervous tissue [189, 190, 276, 277]. Some of the NCX proteins have shown the ability to function, albeit less able, when Li\textsuperscript{+} is used instead of Na\textsuperscript{+} [278, 279]. Wood-Kaczamar et al. 2013 showed that knockdown or neutralizing antibodies against NCX2 and NCX3 led to a strong inhibition of mitochondrial Ca\textsuperscript{2+} efflux [190].

Gene analysis from MCU-KO mice showed a decrease in expression of NCKX2, a known Ca\textsuperscript{2+} extrusion mechanism in neurons. Overexpression of NCKX2-flag show a mitochondrial distribution in both cultured hippocampal neurons as well as HeLa cells, with some constructs localizing to the Golgi. However, NCKX2-KO neurons showed no deficit in mitochondrial Ca\textsuperscript{2+} extrusion or cytosolic Ca\textsuperscript{2+} extrusion either in DRG neurons despite findings of previous research [280, 281].

It is difficult to dissect the effect Li\textsuperscript{+} has on cellular processes from functions such as Li\textsuperscript{+}/Ca\textsuperscript{2+} exchange. The biological relevancy of Li\textsuperscript{+} is unknown and yet one of the therapeutic uses for it is for the treatment of bipolar disorder [282, 283]. Li\textsuperscript{+} can affect numerous processes in the nervous system including neurite outgrowth, neuronal repair, or neuronal excitability among other things [284-288]. However, many of the research investigating Li\textsuperscript{+} effect on the nervous system is the effect it has on the intracellular pathways and organelle function in particular, mitochondria. Li\textsuperscript{+} is able to prevent mitochondrial dysfunction in chronic excitotoxicity, stimulate oxidative phosphorylation, and is able to prevent mitochondrial Ca\textsuperscript{2+} overload [289-291].
mechanisms underlying Li\(^+\) effects on mitochondria are unknown and could have an effect on \(\text{mtNCX}\). Of course, it is possible that the reason there are differences between Li\(^+\)/Ca\(^{2+}\) exchange in tissues and heterologous systems is due to other effects Li\(^+\) has on mitochondrial functions not related to \(\text{mtNCX}\) function. It may not be possible to disentangle the effects of Li\(^+\)/Ca\(^{2+}\) exchange from other influential aspects, but it is also possible that Li\(^+\) simply is unable to efficiently substitute for Na\(^+\) in certain tissue types for reasons yet unknown. Future research will help in dissecting the specific roles of NCLX-dependent and independent effects of Li\(^+\) on the nervous system. More importantly, future work will be needed to establish the molecular identity of mtNCX in neurons.

This work provides several key points of knowledge that can be added to the body of knowledge on neuronal function. First, as shown in Fig. 5.1, MCU is the main pathway by which mitochondria take up Ca\(^{2+}\); however, another MCU-independent pathway does exist though it has a lower affinity and influx rate for Ca\(^{2+}\). Second, MCU deletion significantly alters cytosolic and mitochondrial Ca\(^{2+}\) dynamics in both CNS and PNS neurons as well as significantly reduces neuronal depolarization-dependent \(\Delta \Psi_{\text{mt}}\) depolarization. Third, as shown in Fig. 5.2, MCU deletion increases inhibitory synaptic tone and increases resistance to seizure activity, but does not cause any major neurological deficits at baseline. Fourth, based on experiments in NCLX-KO neurons and Li\(^+\) substitution experiments, it is unlikely that NCLX is the identity of the molecule responsible for mitochondrial Ca\(^{2+}\) efflux.
Figure 5.1: Model of Mitochondrial Ca^{2+} uptake and efflux pathways

Shown here is the model of mitochondrial Ca^{2+} transport that we have found to be relevant based on studies conducted in this thesis work. Mitochondrial Ca^{2+} influx is primarily through MCU; however, MCU-KO mice show an MCU-independent pathway that is lower affinity and slower compared to MCU. Although nervous tissue expresses NCLX, experiments using NCLX-KO mice call this into question as well as the ability for the mtNCX mechanism to efficiently transport Li^{+}. 
Figure 5.2: Effect of MCU deletion on Ca\textsuperscript{2+} dynamics and neuronal network activity

MCU-KO neurons showed a significant inhibition of activity-dependent mitochondrial Ca\textsuperscript{2+} uptake, although some residual uptake was observed through either a MCUb complex, an unknown mitochondrial uptake pathway, or a compensatory protein expression. MCU-KO mice showed significant inhibition to epileptiform activity due to an increase in inhibitory tone at baseline.


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