ELEVATED INTRACELLULAR Ca$^{2+}$ ALTERS MITOCHONDRIAL PROTEIN IMPORT AND THE ACCUMULATION OF INTRAMITOCHONDRIAL PROTEINS IN NEURONS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Physiology
University of Toronto

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2010

ABSTRACT

Most (99%) mitochondrial proteins are nuclear-encoded and must be imported into mitochondria. Deficits in mitochondrial protein import (MPI) affect mitochondrial function and can cause neurodegenerative diseases. I hypothesized that MPI was regulated by \(\text{iCa}^{2+}\). In differentiated PC12 cells, treatment with the \(\text{Ca}^{2+}\) ionophore (A23187; 24h, 0.15uM) increased \(\text{iCa}^{2+}\), ROS generation and promoted neurite outgrowth. Western blot and flow cytometry in live cells showed that A23187 increased levels of mitochondrial proteins; mtHSP70 and mtGFP in mitochondria and autoradiography confirmed that A23187 increased the import of mtGFP. A23187 also slowed intramitochondrial mtGFP degradation. Increased MPI was not associated with mitochondrial biogenesis, but appeared partially dependent on cAMP. In rat cortical neurons, mtHSP70 also increased after A23187 treatment. These results show that, in neurons, increased \(\text{iCa}^{2+}\) can regulate MPI. Further, increased \(\text{iCa}^{2+}\) can slow intramitochondrial protein degradation. These results indicate that MPI is labile and may be altered in response to neuronal activity.
ACKNOWLEDGMENTS

First and foremost I would like to thank my supervisor, Dr. Linda Mills. Thank you for allowing me the opportunity to join your lab and to have such a wonderful graduate experience. Working with you, I learned a great deal not only about the basic sciences but I also learned a great deal about myself. Supervisors like you are few and far between and I appreciate your honesty, encouragement and guidance over the past 2 years.

I would like to show my gratitude to the rest of The Mills Lab, specifically Natasha, Jamie and Diana for their support as well. Know that I appreciate the time each of you took, especially in the early phases of my project, to help me around the lab. Thank you to the students and staff of The Eubanks Lab as well. Having friends for co-workers really made my graduate experience more enjoyable.

Special thanks go to Dr. Eubanks and Dr. Monnier for their time and effort as members of my committee and for their input regarding the direction of my project. I would also like to acknowledge all members of my defense committee for their contributions.

Thank you to my mother and two brothers. I know the stress of graduate school sometimes got to me and I appreciate your love and understanding. Thank you to my father, who played a huge role in motivating me to achieve my academic goals. I wish you were here to see this.

Finally, I would like to thank my fiancée, Christine for her love and support. Simply put, I do not think I would have succeeded without you by my side.
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<th>Description</th>
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<tbody>
<tr>
<td>A23187</td>
<td>Ca$^{2+}$ ionophore</td>
</tr>
<tr>
<td>AAA</td>
<td>ATPases Associated with diverse cellular activities</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchor protein</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-Bis(2-aminophenoxy)ethane-$N,N,N,N'$-tetraacetic acid</td>
</tr>
<tr>
<td>BHS</td>
<td>Blue excitation filter</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Ca$^{2+}$ ion</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CamKII</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase type II</td>
</tr>
<tr>
<td>CamKIV</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase type IV</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CN</td>
<td>Calcineurin</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>DCF</td>
<td>5-(and-6)chloromethyl-2',7' dichlorohydrofluoresceindiacetate, acetyl ester</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRP1</td>
<td>Dynamin-related protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FADH2</td>
<td>reduced form of flavin adenine dinucleotide (FAD)</td>
</tr>
<tr>
<td>FIS-1</td>
<td>Mitochondrial fission 1 protein</td>
</tr>
<tr>
<td>Fluo-3</td>
<td>N-[4-[(acetyloxy)methoxy]-2,7-dichloro-3-oxo-3H-anthen-9-yl]-2-[2-[(acetyloxy)methoxy]-2-oxyethyl]amino]-5-methyl[phenoxy][ethoxy][phenyl]-N-[2-[(acetyloxy)methoxy]-2-oxyethyl]-, (acetyloxy)methyl ester</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GHS</td>
<td>Green excitation filter</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HDDS</td>
<td>Human deafness dystonia syndrome</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks human cancer cell line</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Inter membrane space</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-Dalton</td>
</tr>
<tr>
<td>MEK1</td>
<td>Dual specificity mitogen-activated protein kinase kinase 1</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MnTBAP</td>
<td>Manganese (III) tetrakis (4-benzoic acid)porphyrin chloride</td>
</tr>
<tr>
<td>MPI</td>
<td>Mitochondrial protein import</td>
</tr>
<tr>
<td>Mt</td>
<td>Signifies mitochondrial association</td>
</tr>
<tr>
<td>MTG</td>
<td>Mitotracker green</td>
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<tr>
<td>mHSP70</td>
<td>Mitochondrial heat shock protein 70</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-demethylthiazol-2-yl)-2,5-dephenyltetrasolium bromide</td>
</tr>
<tr>
<td>mTFA</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide plus hydrogen</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NRF1/2</td>
<td>Nuclear respiratory factor 1 and 2</td>
</tr>
<tr>
<td>OD</td>
<td>Oxygen deprivation</td>
</tr>
<tr>
<td>OGD</td>
<td>Oxygen and glucose deprivation</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PAM</td>
<td>Presequence translocase-associated motor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC12</td>
<td>Rat pheochromocytoma cells</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLO</td>
<td>Poly-L-ornithine</td>
</tr>
<tr>
<td>Rh123</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma protein subfamily of GTPases</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>S35</td>
<td>Radioactive sulfur isotope</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SAM</td>
<td>Sorting and assembly machinery</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the means</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SQ</td>
<td>SQ22536 or 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the inner membrane</td>
</tr>
<tr>
<td>TOM20</td>
<td>Translocase of the outer membrane 20kDa</td>
</tr>
<tr>
<td>WCL</td>
<td>Whole cell lysate</td>
</tr>
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</table>
1.0 INTRODUCTION
1.1 Mitochondria

Mitochondria are critically important cellular organelles that serve as the driving force for all metabolic tasks in the eukaryotic cell. Mitochondria contribute to ATP production through oxidative phosphorylation, Ca\(^{2+}\) homeostasis, steroid synthesis, ROS production and apoptosis. Structurally, mitochondria are organized into 4 distinct compartments by an inner and an outer membrane. Mitochondria are about 1\(\mu\)m in length (Squitieri et al., 2004) and occupy about 10-20% of the cell volume in neurons (Ledda et al., 2000). Mitochondria contain their own DNA and the genome is organized into a single, circular chromosome. This chromosome shows many similarities with prokaryotes and it is currently believed that mitochondria originated from a symbiotic relationship between bacteria and eukaryotic cells that began millions of years ago (Andersson et al., 2002; Andersson et al., 1998). Most mitochondrial proteins however are encoded by the nucleus, synthesized on cytoplasmic ribosomes and must be targeted and subsequently imported into mitochondria.

1.2 Mitochondrial Functions

1.2.1 Oxidative Phosphorylation

All cells generate and store usable energy in the form of adenosine triphosphate (ATP). Neurons have a large energy requirement due to their constant activity and the need to maintain ion gradients. The brain in fact accounts for 20% of total oxygen consumption and receives 15% of the total cardiac output (Moreira et al., 2007). Mitochondria play a critical role in allowing cells to make ATP, in an oxygen dependent process called oxidative phosphorylation. Briefly, the initial oxidation of glucose to pyruvate in the cytosol and processing of acetyl CoA in the mitochondria by the citric acid cycle, create intermediate molecules NADH and FADH\(_2\) which enter the electron transport chain (ETC). The ETC is an assortment of proteins on the inner mitochondrial membrane that facilitate the transfer of electrons in exchange for pumping hydrogen atoms out of the matrix and into the inter-
membrane space. This process creates a concentration gradient of hydrogen ions and a pH gradient. The easiest pathway for hydrogen ions to regain equilibrium is through ATP synthase (complex V) thereby providing the energy necessary to create ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). Most of a cell’s energy is generated in the mitochondria through oxidative phosphorylation, but it is important to note that small amount of energy can be created via glycolysis or through the citric acid cycle through a process called substrate level phosphorylation. Oxidative phosphorylation is a dynamic process that can be modulated, in neurons for example or whenever energy demand is high (Grover et al., 2008). Additionally, mitochondria are often found clustered at locations where ATP is needed. For example, mitochondria traffic to active synapses (Verstreken et al., 2005) or to growth cones where they provide energy for cone motility, organelle transport and cytoskeleton assembly (Ruthel and Hollenbeck 2003).

1.2.3 Free radical generation and detoxification

Reactive oxygen species (ROS) is a term that describes chemically reactive molecules that contain oxygen. ROS are so highly reactive because of their unpaired valence electrons (Turrens, 2003). When present in large amounts, ROS has damaging effects on proteins, amino acids, lipids and DNA. Moderate levels can however participate in signaling pathways and are important in protein expression, gene expression, neurotransmitter release and apoptosis (Prabhakar et al., 2007; Avshalumov et al., 2007; Turrens, 2003).

Since most consumed oxygen within the cell is used in oxidative phosphorylation, mitochondria are the major source of cellular ROS; specifically the inner mitochondrial membrane and matrix (Reddy, 2006; Brookes et al., 2004). The inner mitochondrial membrane houses the electron transport chain proteins which leak electrons at complex I and complex III contributing to the production of superoxide (Reddy, 2006). Superoxide anions can be subsequently dismutated by manganese superoxide dismutase (MnSOD) into hydrogen peroxide and oxygen. Hydrogen peroxide can further break down to the highly
reactive hydroxyl radical via the fenton reaction or water if, fully reduced by catalase or glutathione peroxidase (Moreira et al., 2010; Yang et al., 2008; Lin and Beal 2006). The outer mitochondrial membrane is also a source of ROS via the oxidative deamination of primary aromatic amines (Reddy, 2006).

In the CNS, oxygen consumption and ATP production outpace all other cell types. Due to high levels of polyunsaturated fatty acids, poor oxidant defense and high iron content, the CNS is especially susceptible to free radical attack resulting in ROS damage (Rego and Oliveira 2003). Oxidative damage and mitochondrial dysfunction are specifically implicated in multiple neurodegenerative diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS) and even ageing (Yang et al., 2008; Mattson, 2007; Lin and Beal 2006; Rego and Oliveira 2003).

Oxidative damage only ensues when ROS levels overwhelm cellular antioxidant defenses and there are several signaling pathways that specifically depend upon low levels of ROS. ROS induced release of Ca^{2+} for example is responsible for the activation of various kinases like protein kinase C (PKC), which studies have linked to cancer proliferation via MAPK’s and oncogene activation (Gopalakrishna and Jaken, 2000). Several nuclear transcription factors are activated by ROS and include but are not limited to NF-kB, AP-1, and NFAT (Pande and Ramos, 2005; Rao and Hogan, 1997). Protein tyrosine phosphatases are also widely known to respond to ROS changes within the cell. Specifically, these phosphatases contain cysteine residues that are highly susceptible to oxidation, allowing them to be highly regulated by even mild oxidative stress (Salmeen and Barford, 2005). For a comprehensive review regarding ROS signaling, the reader is directed to Valko et al. (2007).

1.2.2 Ca^{2+} homeostasis

Ca^{2+} is a ubiquitous signaling molecule and second messenger that operates in virtually all cells. In nervous tissue, extracellular Ca^{2+} concentrations are usually about 1mM, but the
intracellular level of free Ca\textsuperscript{2+} is about 100nM due to its ability to bind cytosolic proteins or get sequestered in the mitochondria or ER (Pivovarova and Andrews, 2010). Under physiological conditions, Ca\textsuperscript{2+} is tightly regulated within the cell and within individual organelles because of its diverse signaling capabilities. Ca\textsuperscript{2+} originating from the extracellular environment can enter the cell either through voltage-gated channels, ligand-gated channels or through receptor operated channels. Calcium is also sequestered and released by intracellular stores (mitochondria or endoplasmic reticulum (ER)). This intracellular calcium release can also induce the influx of Ca\textsuperscript{2+} from the extracellular space via what has been termed store operated Ca\textsuperscript{2+} entry. Calcium extrusion mechanisms include the plasma membrane ATP/ Ca\textsuperscript{2+} pump and the Na\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger. The plasma membrane Na\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger can however operate in reverse mode, when the cell is depolarized for example, thereby driving transport of Na\textsuperscript{+} out of the cell and increased intracellular Ca\textsuperscript{2+}.

Both the ER and mitochondria are unique organelles because of their ability to control Ca\textsuperscript{2+} fluxes within their own volume, in their micro environment and also within the entire cell. Mitochondrial Ca\textsuperscript{2+} uptake occurs mainly through the mitochondrial Ca\textsuperscript{2+} uniporter and depends upon mitochondrial membrane potential and extra-mitochondrial Ca\textsuperscript{2+} concentrations (Sullivan et al., 2006). In excitable cells, mitochondrial Ca\textsuperscript{2+} extrusion occurs via the Na\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger, but transport rates are not as high as the uniporter and can result in the accumulation of mitochondrial Ca\textsuperscript{2+}. The Na\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger is coupled to the H\textsuperscript{+} gradient through the Na\textsuperscript{+}/ H\textsuperscript{+} exchanger, which can indirectly dictate Ca\textsuperscript{2+} extrusion. A second method of mitochondrial Ca\textsuperscript{2+} extrusion is through the H\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger located on the inner mitochondrial membrane. The H\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger however operates comparatively slower than the Na\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger and saturates at a lower concentration. The H\textsuperscript{+}/ Ca\textsuperscript{2+} exchanger is therefore not usually a major player in cellular Ca\textsuperscript{2+} clearance. (Chinopoulos and Adam-Vizi, 2010; Szabadkai and Duchen, 2008; Bernardi, 1999) Lastly, Ca\textsuperscript{2+} can be quickly released through transient opening of the mitochondrial permeability transition pore although this extrusion pathway it is often associated with a pathological condition (Kann and Kovas, 2007; Gunter et al., 2004).
The effects of transient increases in intracellular Ca\(^{2+}\) include the activation of gene expression, activating or inhibiting Ca\(^{2+}\) signaling pathways such as MAPK, PKA or PKC or Ca\(^{2+}\) enzymes such as calcineurin, calmodulin or calpain. The effects of intramitochondrial Ca\(^{2+}\) fluxes include the modulation of ATP production, modulation of neurotransmitter release and synaptic transmission, regulation of organelle trafficking, nuclear signaling, ROS production and cell death signaling (Starkov, 2010; Chinopoulos and Adam-Vizi, 2010; Szabadkai and Duchen 2008).

1.2.4 Apoptosis

Apoptosis or programmed cell death is a complex, multi-step process that can be initiated by a diverse range of signaling molecules/ stresses. These signals can be intracellular or extracellular in origin and include oxidative damage, Ca\(^{2+}\) imbalance, growth factors and cytokines to name a few (Kushnareva and Newmeyer, 2010). All of these effectors however converge at the level of the mitochondrion to induce programmed cell death.

The “point of no return” in the apoptotic pathway is permeabilization of the outer mitochondrial membrane and release of pro-apoptotic signaling factors into the cytosol (Borutaite, 2010). Permeabilization usually occurs either as a result of the opening of multiple mitochondrial permeability transition pores or through the Bcl-2 family of apoptotic proteins (Kushnareva and Newmeyer, 2010). Once the outer mitochondrial membrane is penetrated, mitochondria can release pro-apoptotic proteins such as Smac/DIABLO, HtrA2/Omi, AIF and cytochrome c (Green and Kroemer, 2004). These proteins initiate a caspase cascade that results in the digestion and degradation of all intracellular structures and nuclear material. For a more comprehensive review of apoptosis, see Borutaite (2010), Ott et al. (2009) and Pinton et al. (2008).
1.3 Mitochondrial protein import

As previously mentioned, present day mitochondria evolved from a symbiotic relationship between bacteria and eukaryotic cells that developed over millions of years (Andersson et al., 2003; Andersson et al., 1998). During the course of this co-evolution, a substantial portion of the mitochondrial genome is believed to have migrated to the host nucleus, leaving only a few genes behind. As a consequence, over 99% of all known mitochondrial proteins are nuclear encoded on cytoplasmic ribosomes and must be imported to the mitochondria (Bohnert et al., 2007). Maintenance of mitochondrial protein import is therefore critically important to cell function and survival.

Similar to gram-negative bacteria, mitochondria contain two membranes, which necessitate the import of proteins into 4 separate functional locations (Schmidt et al., 2010). Depending on the particular targeting sequences and guidance from chaperone molecules, nuclear encoded proteins can be inserted into the outer membrane, the intermembrane space, the inner membrane or the matrix. This highly specific task is carried out through the help of mitochondrial pre-protein receptor complexes called TOMs (translocases of the outer membrane) and inner mitochondrial membrane proteins called TIMs (translocases of the inner membrane) (see Figure 1). Additionally, mtDNA encoded proteins can also directly insert themselves in the inner mitochondrial membrane through the OXA complex (Bonnefoy et al., 2009).

To date, the majority of mitochondrial protein import studies have focused on yeast, fungus or isolated mitochondrial fractions (Hood and Joseph 2004). Little is known about MPI in mammalian cells and even less about MPI in neurons. There is mounting interest in mammalian mitochondrial protein import research, especially with respect to the multiple neurodegenerative diseases that have a mitochondrial dysfunction component (Sirk et al., 2007, Yang et al., 2008, Nicholls, 2009, Knott et al., 2008).
Figure 1: Mitochondrial protein import machinery.

(Adapted from Pfanner et al. 2008). Mitochondrial protein import (MPI) begins with pre-protein synthesis in the cytosol. Proteins that are targeted to the mitochondria are processed by chaperone molecules (mtHSP70, HSP100/Clp, HSP60) which help keep proteins in an unfolded/ import permissive conformation. Next, pre-proteins interact with the TOM receptors on the outer membrane including the surface receptors Tom’s, the channel-forming protein Tom40 and the smaller Tom proteins involved in stability and assembly of the TOM complex. Pre-proteins not destined for the outer membrane via the SAM complex, are carried to either the TIM23 complex or the TIM22 complex to be inserted into the mitochondrial matrix or the inner mitochondrial membrane respectively. Upon arrival in the matrix, pre-proteins are proteolytically cleaved by mitochondrial processing peptidases (MPPs) and with the cooperation of matrix chaperones, folded into mature, functional proteins.
1.3.1 Targeting and chaperones

The import of mitochondrial proteins begins when pre-proteins are synthesized in the cytosol and subsequently directed to the mitochondria. Mitochondrial targeting information exists on the pre-protein itself (Truscott et al., 2003) and can consist of several signaling mechanisms ranging from positively charged internal sequences and short C-terminal peptides which are not removed upon processing, to cleavable N-terminal amino acid sequences that are later cleaved off to allow correct folding and protein function (Laan 2010). Presequence containing proteins can further be classified into water soluble matrix proteins, membrane proteins or inter membrane space proteins. Presequences are usually about 15-55 amino acids in length and have a high content of basic, hydrophobic and hydroxylated amino acids (Vogtle et al., 2009; Heijne et al., 1989).

Mitochondrial chaperone molecules also assist with the targeting of pre-proteins to their corresponding destinations on the outer membrane, inter-membrane space, inner membrane or the mitochondrial matrix. Several protein chaperone families including the heat shock protein 70 family (HSP70) work to stabilize pre-proteins, protect them from damage and ensure proper folding. The ATP dependent chaperone mtHSP70 is of particular importance to mitochondrial function because of the significant role it plays in the import, folding and stabilization of matrix proteins (Voos and Rottgers, 2002). Deletion of the gene SSC1 encoding mtHSP70 in yeast is lethal under all conditions (Craig et al., 1987). For a complete review of mitochondrial chaperones, the reader is directed to (Laan et al., 2010; Voos and Rottgers, 2002).

1.3.2 Surface receptors and translocases of the outer membrane (TOMs)

Once a pre-protein is delivered to the outer mitochondrial membrane, the protein and associated chaperones interact with the TOM complex. The TOM complex is made up of
seven different subunits that can be divided into three functional categories: the surface receptors Tom20, Tom22 and Tom70; the channel-forming protein Tom40; and the smaller Tom proteins involved in stability and assembly of the TOM complex Tom5, Tom6, and Tom7 (Wiedemann et al., 2004). The TOM complex mediates the translocation and insertion of almost all nuclear encoded proteins through the outer mitochondrial membrane. The amino acid presequence of a mitochondrial protein usually takes the form of an amphipathic α-helix, which is specifically recognized by Tom20 and Tom22 (Paschen and Neupert, 2001). Tom20 is not like most other matrix proteins and is not imported in the classical sense. Tom20 is nuclear encoded and cytoplasmically transcribed/translated, however, it does not require the TOM complex itself to facilitate insertion into the outer membrane. Rather, Tom20 complexes with the outer mitochondrial membrane protein Mim1 to aid in the insertion of the α-helix of the translocase into the outer mitochondrial membrane (Becker et al., 2008).

From the edge of the outer mitochondrial membrane, a protein is then directed through the β-barrel transmembrane protein Tom40, which along with the smaller Tom proteins forms what is called the general import pore (GIP) (Hood and Joseph 2004). Proteins containing internal targeting sequences are usually recognized by Tom70, although they have been reported to interact with Tom20 and Tom22 (Pfanner and Geissler, 2001). These proteins require the guidance of cytosolic chaperones, which help prevent protein aggregation. HSP’s specifically are required to interact with several Tom70 molecules simultaneously before these proteins are able to move through the GIP and into the inter-membrane space. Proteins that are not destined for transfer to the inner mitochondrial membrane but rather to remain in the outer membrane must do so through the help of the sorting and assembly machinery (SAM). The SAM consists of the proteins Sam37, Sam35 and the essential β-barrel protein Sam50. These proteins function in concert with the TIM chaperone complex to insert larger proteins, with mostly with β-barrel configurations into the outer mitochondrial membrane (Bohnert et al., 2007; Wiedemann et al., 2004).
1.3.3 Translocases of the inner membrane (TIMs)

After release from the TOM complex, pre-proteins not destined for the outer membrane are carried to either the TIM23 complex or the TIM22 complex. Tim50 immediately binds pre-proteins with N-terminal pre-sequences as they exit the Tom40 channel to help stabilize and direct them to the inner mitochondrial translocases. Tim50 also functions to keep the Tim23 channel in the closed conformation in the absence of any pre-proteins. The TIM23 complex consists of Tim23, Tim17 and Tim44 which contain a membrane integrated domain and several transmembrane segments (Pfanner and Geissler, 2001). This complex continues to process precursors with cleavable N-terminal sequences destined for the inner mitochondrial membrane or matrix (Wiedemann et al., 2004). Inside the matrix, lies the import motor or presequence translocase-associated motor (PAM). The import motor is an ATP dependent multi-subunit molecule that consists of mtHSP70, Tim44 and the chaperone Mge1- all of which are essential for cell viability (Pfanner and Geissler, 2001). mtHSP70’s mechanism of action is still debated and evidence exists for at least two similar but distinct pathways. The competing theories are based on mtHSP70 functioning as a trap, to stop pre-proteins from uncontrolled backflow through Tim23 (Yamano et al., 2008) or active pulling of the pre-protein through successive ATP driven, conformational changes of mtHSP70 while anchored to Tim44 (Krayl et al., 2007). Recently, it has been proposed that a combination of both mechanisms may best explain the function of the PAM complex (Schmidt et al., 2010). Upon arrival in the matrix, pre-proteins are proteolytically cleaved by mitochondrial processing peptidases (MPPs) and with the co-operation of matrix chaperones, are folded into mature, functional proteins. Tim23, when associated with Tim21 is also able to direct protein traffic to the inner mitochondrial membrane and the matrix in a PAM independent process (Chacinska et al., 2005). Tim23 is able to switch between these two forms when directed to do so by the targeting information presented by the pre-protein.

Proteins containing internal signaling sequences are shuttled to the TIM22 complex after they are released by Tom40 for integration into the inner mitochondrial membrane. When released into the inter membrane space, these proteins are first bound by a Tim9-Tim10-Tim12
complex which then docks with the TIM22. This process activates the Tim22 channel, thereby initiating the process of lateral protein transfer into the lipid phase of the inner mitochondrial membrane. This process is ATP independent, and is powered through the differential mitochondrial membrane potential that exists between the matrix and the intermembrane space (Rehling et al., 2003).

Overall, the Tim complexes are highly regulated compared to Tom transport kinetics. This specificity and redundancy prevents the breakdown of electrochemical gradients through the free flow of ions consequently preserving mitochondrial ATP production capacity. The critical role of the Tim complexes has been supported by Hwang et al. (1989) who demonstrated that protein import can still occur despite rupture of the outer mitochondrial membrane and loss of TOM machinery.

1.3.4 Intramitochondrial protein turnover and cytoplasmic degradation

Mitochondrial proteins are degraded when they become damaged or unfolded. Mitochondrial proteins in the outer membrane have a half-life of about 4 days, while proteins of the inner membrane can have a half-life of up to 12 days (Ip et al., 1974; Brunner and Neupert, 1968). Through experiments carried out in our own laboratory, mitochondrial GFP half-life has been measured to be 50h. Proteins in the outer mitochondrial membrane may be tagged and degraded by the traditional ubiquitin-proteasome system (Escobar-Henriques et al., 2006), but proteins of the inner mitochondrial membrane or matrix must be degraded by a different system. In addition to the processing peptidases that cleave signal sequences inside the mitochondrial matrix, two other functionally distinct classes of intramitochondrial proteases exist, ATP-dependent proteases and oligo-peptidases. The ATP-dependent protease family within the mitochondria ensures protein quality/function and helps in the processing of intramitochondrial proteins. They function by forming multimeric protein complexes that have proteolytic microcompartments which degrade proteins into smaller peptides when they are damaged or are no longer useful. ATP hydrolysis is used to power the process of substrate unfolding and subsequent transport into a proteolytic cavity (Baker and Sauer
These proteases recognize targets through specific amino acid combinations termed ‘degradation tags’, which become exposed on the protein surface through endoproteolysis or protein unfolding. Additionally, the proteases themselves can be modified by adaptor proteins which alter substrate specificity and proteolytic activity (Erbse et al., 2006). In humans, the gene papaplegin encodes an ATP protease which when mutated or deleted, causes an autosomal recessive form of hereditary spastic paraplegia (HSP) (Casari et al., 1998). HSP is characterized by the progressive degeneration of motor axons resulting in weakness and spastic lower limb movement (Soderblom and Blackstone, 2006). The devastating nature of this loss of function underscores the critical nature of ATP proteases in mammals.

Oligopeptidases, are very poorly characterized at this time, but it is believed they function in conjunction with ATP-dependent proteases (Baker and Sauer, 2006) to further degrade proteins to smaller peptides and eventually amino acids (Krause et al., 1997). A recent study however showed that deletion of MOP112, a yeast oligopeptidase, had little effect on cell function or viability further complicating the function and requirement of this class of proteins.

1.3.5 Regulation of mitochondrial protein import

While little is currently known about how mitochondrial protein import is regulated especially in neurons, a few potential signaling pathways and effectors have been identified. In myocardial cells for example, treatment with thyroid hormone (TH) increased the import of malate dehydrogenase (MDH) and ornithine carbamoyl transferase, which the authors attributed to the increased expression of mHSP70 and Tom20 (craig et al., 1998). Other studies, in fact show that when Tom20 expression is artificially increased through transfection, the import of mitochondrial proteins is indeed increased as well. Tom20 inhibition resulted in decreased protein import, identifying Tom20 as a critical protein in determining the kinetics of import (Grey et al., 2000; Phan et al., in preparation). In a related
study using H9C2 cardiac mitochondria, mtHSP70 was similarly over expressed which increased mitochondrial protein import of MDH (Cikavecchia et al., 2002). Contractile activity achieved through electrical stimulation has also been identified as a unique regulator of mitochondrial protein import and mitochondrial biogenesis in muscle cells. Specifically, contractile activity has induced increases in mtHSP70, Tom20 and mitochondrial transcription factor A (mtTFA) which has resulted in increased import of proteins such as MDH, and mtTFA into the mitochondrial matrix (Gordon et al., 2001). mtTFA has been identified as playing an essential role in the replication of mitochondrial DNA and subsequently mitochondrial biogenesis.

Recent work from the Mills lab, has demonstrated that pulses of 50 mM KCl are able to modulate the import of mtGFP as well a physiological proteins including mtHSP70. Studies have shown that (Wright et al., 2001) paraquat pretreatment of isolated mitochondria inhibits or arrests the import of mitochondrial proteins. In support of this work, research from the Mills lab confirms that treatment with 200uM H$_2$O$_2$ for 24h inhibits the import of mtGFP in differentiated PC12 cells.

1.4 Mitochondrial dynamics: biogenesis, degradation, fission and fusion

Mitochondrial biogenesis is a highly complex process that involves the co-ordination of two distinct genomes and the action of several transcriptions factors and cofactors. Despite this complexity, mitochondrial biogenesis is a dynamic process where new mitochondria can be stimulated to be formed by a variety of environmental cues including ROS stress, increased ATP demand, depolarization, Ca$^{2+}$ and thyroid hormone binding (McLeod et al., 2005). Mitochondria, like their bacterial ancestors replicate using binary fission which must include replication of mitochondrial DNA as well as physical division of the organelle itself. Key factors that regulate the transcription of nuclear encoded, mitochondrial genes are the nuclear respiratory factors 1 and 2 (NRF1 and NRF2) (Virbasius CA et al., 1993; Virbasius JV et al., 1993), the peroxisome proliferator-activated receptor $\gamma$-coactivator-1 (PGC-1) family and
cAMP response element binding protein (CREB) (Diaz and Moraes, 2008). Up regulation of the gene encoding TFAM is essential for mitochondrial biogenesis because of its role mitochondrial genome replication. When mitochondria are found to be in excess within the cell or if they are compromised, they can be degraded to maintain cellular homeostasis. In the brain, mitochondrial half-life is estimated to be about 25 days (Menzies and Gold, 1971). The primary means of mitochondrial turnover is through autophagy and operates first through the collection of mitochondria into a phagosome. Next, the phagosome is combined with lysosomes to form an autolysosome (Kim et al., 2007). How mitochondria are targeted for degradation is currently unknown, but it is thought that outer membrane proteins play a role in identifying mitochondria in need of recycling (Kissova et al., 2004).

Mitochondria are dynamic organelles; they are continuously motile and frequently change their size and shape. Fission and fusion events, while they are related to mitochondrial biogenesis, can take place independently of mtDNA replication and organelle biogenesis. Mitochondria also can change their shape and size in response to external cues such as cellular stress, Ca\(^{2+}\) overload, cell division or apoptosis. In mammals, fusion requires the transdimerization of MFN1 or MFN2 molecules on the outer mitochondrial membrane, which bring two mitochondria close enough together to make contact. GTPase activity subsequently allows the membranes to fuse. The inner mitochondrial membrane fuses in a similar manner, but requires the action of OPA1 instead (Hoppins et al., 2007). Fission is accomplished through the self assembly of a protein the protein Drp1, which is recruited to the outer mitochondrial membrane by FIS-1. After recruitment, Drp1 self assembles into a spiral that wraps around the mitochondria, constricting the membrane and causing fission (Hoppins et al., 2007).

1.5 Mitochondrial neurodegenerative diseases

Mitochondrial dysfunction has been identified as a major consequence of and cause of many neurodegenerative diseases (see Table 1). Even without specific pathology, mitochondrial stress brought on by accumulated mtDNA mutations or ROS has heavily influence our
Table 1: Mitochondrial diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Classification</th>
<th>Protein mutation</th>
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<tr>
<td><strong>Diseases with mitochondrial involvement</strong></td>
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<tr>
<td>Alzheimer’s disease</td>
<td></td>
<td>APP, PSEN1, PSEN2</td>
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<tr>
<td>Parkinson’s disease</td>
<td></td>
<td>Parkin, DJ-1, α-synuclein, PINK1, LRRK2, HTRA2</td>
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<td>Amyotrophic lateral sclerosis</td>
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<td>SOD1</td>
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<td>Huntington’s disease</td>
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<td>Huntingtin</td>
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<td><strong>Mitochondrial targeting disorders</strong></td>
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<td>Primary hyperoxaluria type 1</td>
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<td>AGT</td>
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<td>Pyruvate dehydrogenase deficiency</td>
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<td>PDH E1α</td>
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<tr>
<td><strong>Protein translocation or import disorders</strong></td>
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<tr>
<td>Human deafness dystonia syndrome</td>
<td></td>
<td>TIMM8A/ DDP1</td>
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<tr>
<td>Dilated cardiomyopathy with ataxia</td>
<td></td>
<td>DNAJC19/ Pam18</td>
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<tr>
<td>Spastic paraplegia-13</td>
<td></td>
<td>Hsp60</td>
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<td>Friedreich’s ataxia</td>
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<td>Frataxin</td>
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theories on cellular lifespan and normal ageing in human beings (MacKenzie and Payne, 2007). Major neurodegenerative diseases that have a mitochondrial dysfunction component include Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis and Huntington’s disease (Moura et al., 2010; Lin and Beal, 2006; Damiano et al., 2010; MacKenzie and Payne, 2007; Devi and Anandatheerthavarada, 2010).

Diseases specific to mitochondrial targeting errors include primary hyperoxaluria type 1 (PH1) and pyruvate dehydrogenase (PDH) deficiency. In PH1, the continued mis-targeting of the vital liver protein alanine/glyoxylate aminotransferase 1 to the mitochondria instead of the peroxisome eventually causes kidney stone disease (Danpure et al., 2003). In PDH deficiency, a mutation of the N-terminal signaling sequences of PDH leads to less of this protein in the matrix. PDH is required to catalyze the oxidative decarboxylation of pyruvate to acetyl CoA and diminished functionality of this protein has been linked to widespread lactic acidosis (Takakubo et al., 1995).

There are also a number of diseases associated with defects in the translocation of proteins or protein import machinery itself, thus reducing or preventing the import of a wide variety of proteins. A ‘classical’ example of a progressive neurodegenerative disease that is caused by a sustained reduction of protein import is human deafness dystonia syndrome (HDDS). This disease is caused by a mutation to the intermembrane protein deafness dystonia peptide 1 (DDP1) which is homologous to the fungal Tim8. Dysfunction of this protein prevents formation of a DDP1/Tim13 complex, which in mammals, interferes with chaperone activity and the normal import of mitochondrial proteins such as Tim23 (Roesch et al., 2002). Clinically, HDDS is characterized by neurological deafness, dystonia, spasticity, cortical blindness and mental deterioration (Binder et al., 2003). Other mutations to proteins of the import pathway that cause disease include dilated cardiomyopathy with ataxia, which is caused by dysfunction in a protein similar to matrix importer Pam18 (Davey et al., 2006) and spastic paraplegia-13 which is caused by dysfunctional hsp60 (Bross et al., 2008; Hansen et al., 2010). Overall, these pathologies illustrate the critical importance of proper folding and assembly of proteins and maintenance of the mitochondrial import machinery.
1.6 A23187: A Ca^{2+} specific ionophore

Intracellular Ca^{2+} is highly regulated because of its broad signaling capabilities and severe consequences upon dysregulation. Ionophores have emerged as useful tools to experimentally manipulate intracellular/ intramitochondrial Ca^{2+} conditions and associated signaling pathways in a controlled manner (Reed and Lardy, 1972). The ionophore A23187 is a carboxylic acid antibiotic (Figure 2) produced by Streptomyces chartreusensis. A23187 functions as a mobile ion carrier that, when present in its anionic form, preferentially complexes with divalent cations at the outer surface of a biological membrane in a 2:1 ratio (2 ionophore: 1 cation) (Painter and Pressman 1983; Erdahl et al., 1996). This complex, with its charge internally compensated for and outward facing lipophilic alkyl groups then traverses the hydrophobic membrane with ease. Once through the membrane, the complex releases the cation and repeats the process in reverse (Painter and Pressman, 1983). A23187 is well characterized in biological systems and is widely used to move Ca^{2+} across membranes in exchange for 2H^{+} in what is termed as electroneutral transport (Erdahl et al., 1994; Reed and Lardy, 1972). Manipulating Ca^{2+} in intact cells is more complex than in artificial membrane systems or in individual organelles because of the various pumps, channels and other variables that control for Ca^{2+} imbalance. Experiments using A23187 however, show that in intact cells, the rise in [Ca^{2+}]_{m} closely mirrors the rise in [Ca^{2+}]_{c} (Abramov and Duchen, 2003).

A23187 has been used in the successful manipulation of intracellular Ca^{2+} to stimulate neuronal outgrowth in PC12 cells via Rap1 activation (Kano et al., 2001). In a similar study, A23187 increased protein tyrosine phosphorylation in PC12 cells, leading to Ras activation, MAP kinase activation and up regulation of the NGFI-A gene resulting in increased cell differentiation (Rusanescu et al., 1995; Changelian et al., 1989). In muscle cells, A23187 induced increases in cytoplasmic Ca^{2+}, which enhanced cytochrome c mRNA levels and cytochrome c expression. Additionally, the authors found that PKC\alpha activation stimulated this response while the MEK inhibitor, PD98059 inhibited the observed effect implicating the PKC pathway in transduction the Ca^{2+} signal (Freyssenet et al., 1999). These
Figure 2: Structure of the mobile ion carrier A23187.

The free acid form has a molecular weight of 523 and an elemental analysis of C_{29}H_{37}N_3O_6. A23187 preferentially binds divalent cations in a 2:1 (ionophore: cation) complex to transport them across biological membranes.
results suggest increased intracellular Ca\(^{2+}\) as a possible signal in the up regulation of nuclear encoded mitochondrial proteins.

1.6.1 Ca\(^{2+}\) modulation of ATP/ biogenesis

Mitochondria, have emerged as key players in Ca\(^{2+}\) regulation whose Ca\(^{2+}\) accumulation, buffering and release can drastically change the spatiotemporal distribution of intracellular Ca\(^{2+}\) signals (Szabadkai and Duchen, 2007). It is well known that regulation of ATP synthesis can be modulated by intracellular Ca\(^{2+}\) load. In fact, several mitochondrial enzymes including pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase are primarily regulated by matrix Ca\(^{2+}\) (Denton, 2009). In HeLA cells and primary cultures of myotubes, increased mitochondrial and cytosolic Ca\(^{2+}\) caused an increase in mitochondrial ATP and cytoplasmic ATP availability. The induced increase in matrix Ca\(^{2+}\) remained, but dissipated slowly even after the Ca\(^{2+}\) agonists were washed out; suggesting mitochondrial sequestration of Ca\(^{2+}\) prolonged the observed increase in metabolic activity (Jouaville et al., 1998). Since increases in work are often associated with Ca\(^{2+}\) increases, this represents a key mechanism which couples Ca\(^{2+}\) signaling with ATP supply and demand (Szabadkai and Duchen, 2007). Furthermore, in experiments that uncoupled ATP synthesis, increasing Ca\(^{2+}\) caused a significant increase NRF-1. Increased expression of this transcription factor as well as Ca\(^{2+}\) induced up regulation of PGC-1 (as discussed in section 1.4) results in the stimulation of mitochondrial biogenesis, demonstrating how a physiological stimulus can result in an increase in the energy generating capacity of the cell (Diaz and Moraes, 2007; Li et al., 1999).

1.6.2 Calmodulin: a Ca\(^{2+}\) sensing protein

The calmodulin family of proteins offers another pathway to transduce intracellular Ca\(^{2+}\) signals and affect nuclear gene expression. Calmodulin, expressed in almost all eukaryotic
cell types (Chin and Means, 2000), participates in a variety of processes including cell growth, proliferation, movement, metabolism, nerve growth and long term potentiation. Calmodulin controls the activity of various intracellular proteins through binding of Ca\(^{2+}\) ions. Upon binding Ca\(^{2+}\), calmodulin undergoes a conformational change. As a result, calmodulin becomes active and can bind proteins and transcription factors that otherwise cannot interact with Ca\(^{2+}\), thereby acting as another Ca\(^{2+}\) signal transducing agent (Burgoyne 2007). Key proteins that calmodulin modulates are the Ca\(^{2+}\) dependent protein kinases (CaMKs) involved in synaptic plasticity (Lisman et al., 2002) and calcineurin, the protein phosphatase involved in the activation of the transcription factor nuclear factors of activated T-cells (NFAT) (Hogan et al., 2003). Increased cytoplasmic Ca\(^{2+}\) load alone is able to increase calcineurin in muscle cells and neurons, which increased nuclear localized NFAT and NFkB function, leading to the transcription of particular genes involved in a cellular Ca\(^{2+}\) stress response and differentiation (Graef et al., 1999; Biswas et al., 1999; Stevenson et al., 2001). While several Ca\(^{2+}\) sensing pathway exist in the cell, many of which are coupled to mitochondrial function, it is not known how Ca\(^{2+}\) can specifically affect the import of nuclear encoded mitochondrial proteins, especially in neurons.

1.7 Studying mitochondrial protein import in neurons

Two neuronal models to assess the role of Ca\(^{2+}\) signaling in mitochondrial protein import were used. Differentiated PC12 cells were used extensively to establish the effects of A23187 on a neuronal cell line and primary rat cortical neurons were used to verify the findings in a more physiological model.

Designated PC12, the rat adrenal pheochromocytoma cell line originated from a tumor on the adrenal gland of an irradiated rat (Greene and Tischler, 1976). Adrenal cells are formed from multipotent neural crest cells, similar to the developmental pathway that exists in neurons or glia, shortly after embryo gastrulation in vertebrates. When PC12 cells are grown in the presence of nerve growth factor (NGF), they cease dividing, neurites are stimulated to extend
from the cell body, the cells become electrically excitable, they become increasingly sensitive to neurotransmitters such as acetylcholine (Ach) in addition to producing their own neurotransmitters, and the number of Ca$^{2+}$ channels increases (Greene and Tischler, 1976). Some disadvantages include the fact that PC12 cells simply are not a replacement for nerve cells and the possible accumulation of mutations over a large passage number. Overall, the ease of use, large availability, resiliency and ability to imitate sympathetic neurons make using cultured PC12 cells an invaluable tool in studying numerous problems in neurobiology.

To test experimental findings in a more physiologically relevant model, cortical neurons were harvested from rat brains and cultured. Rat cortical neurons provide an excellent platform to study neurological problems from a primary source (PC12 cells are considered a secondary cell line). Obtaining primary cultures is a more involved process and requires a high degree of cell culture and dissection experience. Careful dissection of only the cortex of E18 rat pups and incubation in neuronal permissive media yields cultures rich in neurons. For optimal results, cultures are allowed to mature for several days to a week to ensure normal neuronal maturation and expression of ion channels.

1.7.1 Green fluorescent protein

The green fluorescent protein (GFP), originally harvested from the Jellyfish *Aequorea Victoria*, is one of the most extensively used proteins in biology (Stepanenko *et al.*, 2008). One of the major advantages of GFP, is the protein’s ability to fold and fluoresce without additional cofactors or enzymes, making it a useful tool for use inside live, novel biological systems. Wild type GFP is a 238 amino acid protein and is excited at a wavelength of 395nm and emits light at 504nm (Chalfie, 1995; Tsien, 1998). The structure of GFP is typically a cylindrical β-barrel, with an α-helix running up the axis of the cylinder (Stepanenko *et al.*, 2008). In the center of this cylinder, attached to the α-helix but protected from damage or interference from solvent molecules, lies the chromophore. The GFP chromophore is made from Ser$_{65}$, Tyr$_{66}$, Gly$_{67}$, and becomes fluorescent only after nucleophilic attack of the amide
at Gly$_{67}$ on the carbonyl of Ser$_{65}$, a dehydration reaction, then dehydrogenation of the $\alpha$-$\beta$ bond at Tyr$_{66}$ (Tsien, 1998).

The Mills lab uses the mutant variant GFP-S65T, whose serine at position 65 is changed to a threonine, allowing for a faster, stronger, more stable fluorescence signal, that is excitable by the typical 488nm laser commonly used for fluorescein isothiocyanate (FITC) imaging (Tsien 1998). This mutant GFP was subsequently fused to the N-terminal mitochondrial targeting sequence of COX VIII, which was then ligated to a tet-responsive expression vector (pTRE, Clontech, #PT3215-5). A second cell line was developed which expressed the pTet-off vector (Clontech, #PT3073-5). The PC12 Tet-off cells were co-transfected with the pTRE/mtGFP vector, the result being a negative tet-inducible, mitochondrially targeted GFP protein (Sirk et al., 2003). mtGFP expression is controlled by the tet-responsive element (TRE) which is located just upstream of a tet operator containing mini-CMV promoter. Cells are induced to express mtGFP upon removal of tet, whereby a tet-controlled transactivator binds the tet operator allowing gene expression. When tet is present in the cell culture medium, it binds the tet transactivator, preventing access to the promoter site and subsequent transcription mtGFP (see Figure 3). Interestingly, because of the unfolded state pre-proteins are kept in during their import (Hood and Joseph 2004) and possible interference of the N-terminal amino sequence in normal mtGFP folding, mtGFP does not fluoresce until it is fully imported, processed and folded within the mitochondrial matrix. This has been verified by Sirk et al. (2003) and by my own experiments assessing lack of cytoplasmic fluorescence. Additionally, the lack of a cytoplasmic mtGFP signal is in part due to the rapid degradation of non-imported protein as evidenced by the low mtGFP protein levels isolated from cytoplasmic western blot fractions.

1.7.2 Techniques for studying mitochondrial protein import

The fact that mtGFP only fluoresces upon proper processing and folding in the matrix allows for accurate mitochondrial localization and imaging, and when coupled with the high-throughput of flow cytometry, makes this experimental model a powerful tool in studying the
Figure 3: Induction of mtGFP import in NGF differentiated PC12 cells.

(A) Schematic representation of a novel, negative tet-inducible, mitochondrially targeted GFP protein. The addition of tet to culture medium inhibits the synthesis of mtGFP, while tet-removal allows the expression and subsequent import of mitochondrially targeted GFP.

(B) Confocal image of a live, NGF differentiated PC12 cell expressing mtGFP. Individual mitochondria appear as elongated strands.
dynamics of mitochondrial protein import in living cells. Another feature of the negative tet-inducible construct is the ability to “turn off” mtGFP fluorescence and monitor intramitochondrial degradation. Flow cytometry allows the user to analyze thousands of cells per second using multiple fluorescent probes. In this way, information regarding the amount of imported mtGFP can be collected at the same time PI data is collected.

Western blots on whole cell fractions or mitochondrial fractions are also useful tools in monitoring changes in mitochondrial protein expression and intramitochondrial accumulation however one of the most accurate methods to measure a change in MPI remains the immunoprecipitation of radiolabeled mitochondrial proteins. This technique labels newly synthesized mitochondrial proteins which allows the user to track the localization of a particular protein during a set amount of time. Quantification of the resulting radioactive signal provides the user with information on the rate of import within a specified period of time.

1.8 Rationale

It is commonly recognized that neuronal survival depends upon mitochondrial function. Studies have shown that mitochondrial function depends upon maintenance of mitochondrial protein import and that 99% of all mitochondrial proteins are nuclear encoded and must be imported into the mitochondria. It can therefore be inferred that mitochondrial protein import is critical for neuronal function and survival- a statement supported by the vast number of diseases caused by a deficit in mitochondrial protein import or mitochondrial function. What are not known however, are the regulatory mechanisms which control mitochondrial protein import and if they can be modulated to ameliorate neurological diseases.

Previous studies using thyroid hormone showed increased import of mitochondrial proteins through an up-regulation of the protein import machinery. Studies on myocytes showed that electrical activity was also able to up-regulate parts of the protein import machinery which led to similar increases in mitochondrial protein import.
Studies from the Mills lab (Phan et al., in preparation) have confirmed that in neurons, up-regulation of import machinery, specifically transfection of a major translocase Tom20, was also able to increase the import of mitochondrial proteins. Similar to the electric induced depolarization of myocytes, recent data from the Mills lab (Fong et al., in preparation) demonstrated that depolarization (50uM KCl) increased the import of mitochondrial proteins in NGF differentiated PC12 cells. Ca\(^{2+}\) and cAMP pathways were involved in the KCl induced up-regulation of MPI providing a clear rationale for my current research on chronic increased Ca\(^{2+}\) regulation of mitochondrial protein import in neurons.

1.9 Hypothesis and specific aims

Hypotheses:

1. Intracellular Ca\(^{2+}\) regulates MPI in differentiated PC12 cells and primary neurons.

2. The effects of increased Ca\(^{2+}\) on MPI are independent of mitochondrial biogenesis.

3. The Ca\(^{2+}\) regulatory pathways involved in MPI include cAMP and calcineurin.

Specific Aims:

1. To determine if intracellular Ca\(^{2+}\) regulates MPI in differentiated PC12 cells and in primary neurons. Specifically, how does increasing Ca\(^{2+}\) alter the import, synthesis, or degradation of nuclear-encoded mitochondrial proteins?

2. To determine if increasing Ca\(^{2+}\) alters levels of Tom20 a ROS-sensitive translocase in mitochondria or mtHSP70 a key import chaperone. Tom20 itself is not imported into mitochondria, while mtHSP70 is imported across the outer mitochondrial membrane and is directed to the mitochondrial matrix.

3. To determine if increased Ca\(^{2+}\) alters mitochondrial biogenesis.

4. To identify the signaling pathways that regulate MPI, specifically the role of calcineurin and cAMP.
2.0 MATERIALS AND METHODS
2.1 The PC12 cell line

2.1.1 Creation of a tetracycline-off mtGFP expressing PC12 cell line

The Mills lab previously developed a tet-off mutant variant GFP-S65T PC12 cell line (Sirk et al., 2003). Briefly, GFP was fused to the N-terminal mitochondrial targeting sequence of COX VIII, which was then ligated to a tet-responsive expression vector (pTRE, Clontech, PT3215-5). A second cell line was developed which expressed the pTet-off vector (Clontech, PT3073-5). The PC12 Tet-off cells were co-transfected with the pTRE/mtGFP cells, the result being a negative tet-inducible, mitochondrially targeted GFP protein (Sirk et al., 2003). mtGFP synthesis is fully controlled by adding or removing tetracycline to or from the culture media (see Figure 5 for mtGFP turn-on and turn-off examples in PC12 cells). Tet responsiveness was confirmed by the lack of mtGFP in the mitochondria when incubated with tetracycline or doxycycline (will be referred to as mtGFP-off from now on).

2.1.2 Culturing PC12 cells

Undifferentiated mtGFP-off PC12 cells were grown in 100mm diameter polystyrene tissue culture dishes (Falcon, # 353003) and kept at 37° C in a humidified incubator consisting of 5% CO2 and 95% air. The cell culture medium used was RPMI 1640 (Gibco, #11875-093) supplemented with 10% horse serum (Gibco, #16050-122), 5% fetal bovine serum (Gibco, #16000-044) and 50U/ml (1ml/200ml media) of penicillin-streptomycin (Gibco, #15240-062). Cells were fed every other day by complete media exchange and passaged into new dishes weekly or until grown to 80% confluency. The optimal dose of tetracycline (Sigma, #T-7660) to inhibit mtGFP synthesis, while allowing for easy removal was previously determined to be 50ng/ml (Sirk et al., 2003).
Figure 4: FACS analysis of mtGFP signal in NGF differentiated PC12 cells +/- tet.

Flow cytometry. Green line represents mtGFP signal accumulation following tet removal. Signal increases for up to 3 days, at which point mtGFP fluorescence reaches a plateau (data not shown). All experiments assessing mtGFP import were carried out within 24h-48h of mtGFP induction. Grey line represents, cells previously maximally expressing mtGFP, with tet added at time zero to inhibit mtGFP synthesis. The curve shows the degradation of mtGFP signal over time. N=1 test experiment, 3 samples/ condition, 10 000 cells per sample.
2.1.3 Differentiating and plating PC12 cells- the experimental model

Differentiation was induced by the addition of 25ng/ml nerve growth factor 2.5S (NGF) (Harlan, #0005017) to the previously mentioned culture medium. Unless otherwise stated, all PC12 cells were differentiated using NGF and 15% serum for 5-6 days. Prior to the start of any experiments the cells were gently collected using Hank’s Balanced Salt Solution (HBSS) without Ca$^{2+}$ or Mg$^{2+}$ (Gibco, 14170-161), centrifuged for 5 minutes at 1500rpm, resuspended in 1% serum NGF RPMI media, counted using a hemocytometer and plate on 5% collagen coated dishes. The number of cells plated depended on the type of experiment and the available growth media of a particular type of dish used. Density of cells/growth area or cells/media was kept as close as possible between experiments but ultimately depended on the type of experiment (see experimental setup below for each experimental setup used).

2.2 Primary neurons

2.2.1 Dissection and plating of dissociated cells

Pregnant E18 Wistar rats (Harlan) were sacrificed by CO$_2$/O$_2$ inhalation followed by pure CO$_2$ inhalation according to University Health Network guidelines for animal care. The E18 rat pups were removed via dissection of the abdominal cavity then transferred to ice-cold phosphate buffered saline (PBS). Fetuses were separated from one another and their respective fetal membranes using fine forceps and scissors and transferred to a second dish containing ice-cold dissecting medium. To extract the cortices, fetal skulls were cut using curved fine scissors along the sagittal line from the snout to the back of the head. With the skull cut, the cortex could be exposed by applying pressure to the head. The top 1/3 of each hemisphere was excised using fine scissors and collected in 15ml centrifuge tubes (BD Falcon: #352097) filled with dissecting medium (see below). 3-4 cortices were collected per tube, and centrifuged at 1000 rpm for 3 min. The supernatant was removed and 5ml of papain solution (see below) was added to each tube. Care was taken not to over triturate the cell
pellet at this stage; rather the tissue was loosely mixed to allow uniform exposure to papain dissociation solution. Next, the cortices were incubated at 37°C on an orbital shaker for 30 min, before being centrifuged again at 1000 rpm for 5 minutes. The supernatant was removed and replaced with 5ml plating medium per tube (see below). Cortices were dissociated by trituration then collected into one larger volume which was further topped up to 50ml using plating medium. The entire 50ml volume was then poured through a debris strainer to remove any excess debris or undissociated tissue. Cortical neurons in suspension were counted using a hemocytometer and plated on PLO coated dishes. Generally, one litter of pups was plated onto 20, 100mm tissue culture dishes.

2.2.2 Culturing primary neurons

Cortical neurons were grown on 100mm diameter polystyrene tissue culture dishes (Falcon, # 353003) and maintained at 37°C in a humidified incubator consisting of 5% CO2 and 95% air. Cells were fed twice per week using maintenance medium (see below) via a 50% media exchange. Cortical neurons were allowed at least 4-5 days to mature in culture before being used in experiments. The following is a list of media and solutions used in the preparation and culturing of cortical neurons.

**Dissecting medium:** 250ml Ca\(^{2+}\), Mg\(^{2+}\) free HBSS (Invitrogen, #14170-161), 5.6ml 1M HEPES (Invitrogen, #15630-080), 1.25g sucrose (BDH, #B10274), 2.5g D-glucose (Sigma, #5767), and 100ml ddH2O to adjust osmolarity to 310-320mOsm, pH adjusted to 7.4.

**Plating medium:** Neurobasal medium (Invitrogen, #21103) with 2% B-27 with antioxidants (Invitrogen, #17504-044), 2mM Glutamax TM (Invitrogen, #35050-061), 1% FBS (Invitrogen, #16000-044), and 1x penicillin-streptomycin (Invitrogen, #15240-062).

**Papain solution:** 2mg/ml papain (Worthington, #3119) diluted in plating medium and filtered through a 20cc syringe with a 0.2um filter attached.
Maintenance medium: Neurobasal medium (Invitrogen, #21103) with 2% B-27 without antioxidants (Invitrogen, #10889-038) and 1x penicillin-streptomycin (Invitrogen, #15240-062).

2.3 Substrate preparation

2.3.1 Collagen

Preparation of a 100% collagen solution was previously completed in the Mills lab according to the Bornstein method. First, this required frozen rat tails to be thawed in 70% ethanol, and then rinsed in ddH₂O. Next, the collagen from the tendons in the tail was harvested and mixed with 0.1% acetic acid at a ratio of 1g/150ml and allowed to incubate at 4°C for 24-48h. The mixture was then centrifuged at 10 000 rpm for 30 minutes at 2°C to pellet insoluble fibers. The supernatant was collected, designated as a 100% collagen stock and frozen at -20°C. Desired concentrations (usually 5%) were achieved by simply diluting the 100% collagen stock in ddH₂O. Immediately before cell plating, dishes were coated for 20 minutes, rinsed with ddH₂O and left to dry at room temperature.

2.3.2 Poly-L-ornithine (PLO)

PLO (Sigma, #P-4638) was diluted in ddH₂O to a stock of 10mg/ml, aliquoted and frozen at -20°C. Working solutions were prepared by diluting one 75ul stock aliquot in 50ml ddH₂O, adding 0.19g of Borax (Sigma, #221732) and adjusting the pH to 8.4. PLO working solutions were sterilized by vacuum filtration prior to dish coating. To coat dishes, enough PLO working solution was added to fully cover the growth area of the dish and allowed to stand at room temperature overnight. The next day, remaining PLO was removed, dishes were rinsed in ddH₂O and air dried before use.
2.4 Reagent preparation

All compounds except for H$_2$O$_2$ (which was not frozen) were thawed immediately prior to use and diluted to a desired working concentration by adding RPMI cell culture media (1% serum + NGF). All experimental conditions were compared to cells treated with an equivalent volume of vehicle (DMSO, H$_2$O or NaOH) to serve as a control.

**Ca$^{2+}$ ionophore A23187:** (A23187- Sigma: #C7522), molecular weight 523.62, was prepared in DMSO (Sigma: #D8779) to a 2mM stock solution and frozen in 20ul aliquots at -20°C.

**Ionomycin from Streptomyces conglobatus:** (Ionomycin- Sigma: #I9657), molecular weight 709.00, was prepared in DMSO to a 5mM stock solution and frozen in 10ul aliquots at -20°C.

**Carbonyl cyanide m-chlorohenylhydrazone:** (CCCP- Sigma: #C2759), molecular weight 204.62, was prepared in DMSO to a 10mM stock solution and frozen in 50ul aliquots at -20°C.

**Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride:** (MnTBAP- Calbiochem: #475870), molecular weight 879.20, was prepared in 75mM sodium hydroxide (NaOH) to a 15mM stock solution and frozen in 20ul aliquots at -20°C.

**Hydrogen peroxide:** (H$_2$O$_2$- Sigma: #216763), 30% wt. solution in water (9.8M stock), was made fresh before the beginning of each experiment by diluting in RPMI to a desired concentration.

**Forskolin from Coleus forskohlii:** (Forskolin- Sigma: #F6886), molecular weight 410.50, was prepared in DMSO to a 10mM stock solution and frozen in 20ul aliquots at -20°C.

**SQ22536:** (SQ- Sigma: #S153), molecular weight 205.22, was prepared in H$_2$O to a 75mM stock solution and frozen in 20ul aliquots at -20°C.
2.5 Flow cytometry

Flow cytometry is a technique which allows the simultaneous quantification of 2 or more fluorescent signals, cell size and internal cell complexity. The Facscan Flow Cytometer (Becton Dickinson, San Jose, CA) is able to analyze thousands of cells per second by first aligning cells in a high-speed liquid stream, so that they can pass single file through a laser beam. This beam excites fluorescent molecules in the cell whose signal is quantified shortly after by a photomultiplier tube (PMT). It also collects data on the size of the cell based on the shadow it casts and the internal complexity of the cell based on the light scatter profile. An analog to digital conversion system converts the light signals into electrical signals which are then quantified by a computer. Quantified fluorescent values are given as the arithmetic mean of all cells measured. Gates can be setup using software so that only populations of interest are quantified. Debris or dead cells can be filtered out in this way.

All flow cytometry experiments were plated on either 12 well (BD Falcon: #353503) or 24 well (BD Falcon: #353047) plates coated in %5 collagen at a density of 100 000 to 150 000 cells/ml culture media. Immediately prior to collection, culture media was aspirated and cells resuspended in ice cold Ca$^{2+}$ and Mg$^{2+}$ free Phosphate Buffer Saline (PBS: 1mM KH$_2$PO$_4$, 10mM Na$_2$HPO$_4$, 137mM NaCl, and 2.7mM KCl, pH 7.4) and transferred to 5ml polystyrene round bottom tubes (BD Falcon: #352052) before being taken for measurement. All experimental conditions tested using flow cytometry were carried out in triplicate with at least 10 000 cells collected per sample. Unless otherwise stated, all flow cytometry experiments were performed on 5-6d NGF differentiated PC12 cells. Cells were differentiated in 15% serum at first, but experiments were carried out in 1% serum.

2.5.1 Analysis of mtGFP import

To measure the import of newly synthesized mtGFP into the mitochondria of PC12 cells, 5-6d differentiated cells were rinsed 3 times to remove tet and induce mtGFP expression. After
several hours, experimental conditions were applied and cells monitored for up to 5d. Upon experiment completion, the cells were collected in ice cold PBS and analyzed using the Facsca flow cytometer. mtGFP was excited using the 488nm air-cooled argon laser and the emitted light was collected in the FL1 channel of the PMT which collects light in the 510-545nm range. Unless otherwise stated, mtGFP-on and mtGFP-off cells were used in all experiments in addition to controls to ensure experimental values were in a detectable range. Additionally, gates were set to divide responding and non-responding cell populations so that only mtGFP expressing cells would be quantified. Non- mtGFP expressing cells were gated out based on low or close to zero signal; similar to that of mtGFP-off cells while debris was gated out based on relative particle size compared to the cell population being analyzed. Figure 4 shows that increased mtGFP fluorescence correlates with increased ug mtGFP protein levels within mitochondrially enriched western blots (Sirk et al., 2003).

2.5.2 Analysis of intramitochondrial mtGFP degradation

mtGFP degradation rate was measured in a similar manner to mtGFP import, except PC12 cells were maximally expressing mtGFP (at least 5 days in culture + NGF but without tet) before beginning the experiment. At the start of the experiment (t=0), A23187 and tet were added to all cells and samples were monitored up to 5 days using the Facsca flow cytometer to quantify mtGFP fluorescence. Media/ drug replacement occurred every 2 days. mtGFP values were expressed as a % of the t=0 mtGFP value and all values were graphed to give the mtGFP ½ life in control and A23187 treated cells.

2.5.3 Analysis of cell death

Cell death was measured using the fluorescent dye propidium iodide (PI) (Molecular Probes: #P1304) by adding 1ul of a 1mg/ml stock solution in H2O per 1ml of media. Cultures were incubated for 15 minutes prior to fluorescence reading using the Facsca flow cytometer. PI
Figure 5: Increased mtGFP protein corresponds to increased fluorescence.

To determine the relationship between mtGFP fluorescence and protein concentrations, equivalent volumes of whole cell lysates obtained from GFP expressing cells were loaded into a 96 well plate at concentrations ranging from 0 to 300ug and mtGFP fluorescence was measured using a fluorescent plate reader. A linear relationship ($f = y_0 + ax$) exists between the amount of soluble mtGFP protein and the fluorescence emitted within the range measured ($R^2 = 0.96$; N=4) (Sirk et al., 2003).
was excited using the 488nm air-cooled argon laser and the emitted light was collected in the FL3 channel of the PMT which only collects light with wavelengths greater than 650nm. Cell death was expressed as a % of PI positive cells versus total cells analyzed. 70% ethanol was used to generate a positive cell death control.

2.5.4 Analysis of reactive oxygen species

ROS was measured by incubating off-cells in CM-H2DCFDA (5-(and-6)chloromethyl-2',7'dichlorohydrofluoresceindiacetate, acetyl ester) (DCF) (Molecular Probes: #C6827) for 30 minutes. DCF was made up by dissolving 50ug of powdered DCF in DMSO to make a 4mM stock solution. The working solution was further diluted into RPMI to achieve the final working concentration of 7uM. After incubation cells were rinsed in PBS and read using the Facscan flow cytometer. DCF was excited using the 488nm air-cooled argon laser and the emitted light was collected in the FL1 channel of the PMT which collects light in the 510-545nm range. Due to the very broad emission spectrum of DCF, which overlapped into all detection channels, and the limitations of the FACS hardware, mtGFP nor PI could be assessed at the same time as DCF. 500uM H₂O₂ was added to cultures 1h prior to collection to create a positive DCF control.

2.5.5 Analysis of intracellular Ca²⁺

Intracellular Ca²⁺ was measured by incubating mtGFP-off cells in Fluo-3 AM Ca²⁺ dye (Invitrogen: #F1242) for 40 minutes. Fluo-3 AM was made up by dissolving 50ug of powdered Fluo-3 in DMSO to make a 2mM stock solution. This was further diluted in serum free RPMI and sonicated for 10 minutes to achieve a final working concentration of 4uM. After dye loading, cells were returned to their original media to allow for de-esterification of intracellular AM esters. Cells were then collected in Ca²⁺/ Mg²⁺supplemented HBSS (Invitrogen: # 14025-092). Fluo-3 was excited using the 488nm air-cooled argon laser and
the emitted light was collected in the FL1 channel of the PMT which collects light in the 510-545nm range. 10uM A23187 was added to cultures immediately prior to collection to create a positive Fluo-3 control.

2.5.6 Analysis of mitochondrial mass

Mitochondrial mass was measured by incubating mtGFP-off cells in mitotracker green FM (MTG) (Molecular Probes: # M7514) for 30 minutes. MTG was prepared in DMSO to a 1mM stock solution, before being diluted in media to a final working concentration of 20nM. After dye loading, cells were rinsed in PBS and read using the Facscan flow cytometer. MTG was excited using the 488nm air-cooled argon laser and the emitted light was collected in the FL1 channel of the PMT which collects light in the 510-545nm range. To establish a positive control, cells were simply incubated with a 50nM concentration of MTG for the previously mentioned time.

2.6 Confocal Imaging

For imaging experiments, all cells were plated on PLO or 5% collagen coated 35mm glass bottom dishes (Wilco Wells: #70671-62), at densities of 75 000-100 000 cells per dish. Images were captured using an inverted Nikon, scanning confocal microscope (Bio-rad MCR 600) equipped with an argon ion laser with 20x phase, 20x fluor or 60x PlanApo oil objective lenses. Confocal Assistant v4.02 was used for analysis of collected images. Pluronic (Invitrogen: #F127) was dissolved in DMSO to a working solution of 20% (w/v) and was used (as required) with any of the previously mentioned fluorescent dyes in a 1:1 ratio of dye: pluronic mixture. Pluronic facilitates solubilization of water-insoluble dyes and fluorescent indicators such as AM esters.
2.6.1 Imaging PC12 cells- mtGFP/phase

A23187 treated PC12 cells were prepared for confocal microscopy in much the same way as described in section 2.5.1. Cells were however imaged in cell culture media instead of being transferred to PBS and cells were plated on 35mm glass dishes at slightly lower cell densities to allow for greater image resolution of individual cells. The BHS filter block was used to obtain final images. The phase objective was used to obtain wide field images of groups of cells and cell morphology.

2.6.2 Imaging primary neurons- rhodamine 123

A23187 treated primary neurons were prepared for confocal microscopy in much the same way as described in section 2.2.1. Cells were however imaged on 35mm glass dishes at slightly lower cell densities to allow for greater image resolution of individual cells. Cells were incubated in Rhodamine 123 (Molecular Probes, #R302) for 30 minutes to allow resolution of individual mitochondria. Rh123 was prepared in ddH₂O to make a 0.5mM stock, which was further diluted in cell culture media to a final working concentration of 0.5uM. The GHS filter block was used to obtain final images.

2.7 Western blot

Total protein expression was assessed using 5-6 day differentiated PC12 cells and rat cortical neurons in the following way. PC12 cells were plated at a density of 3.5-4 x 10⁶ cells per 100mm polystyrene dish, while cortical neurons were prepared as described in 2.2.1 and treated at 4-5 days in vitro. After treatment with A23187, PC12 cells and rat cortical neurons were collected in ice-cold 1x PBS (10ml/ per dish) into 15ml centrifuge tubes then spun at 1000rpm for 4 min. The supernatant was removed and pellet was washed once in 1ml ice-
cold 1x PBS before being transferred to a 1.5ml Eppendorf tube (Sorenson: #16130) for a 30s spin at 9000rcf.

2.7.1 Preparation of whole cell lysates

Whole cell lysates were prepared by lysing the pellet obtained in section 2.7 in 200ul-300ul of cell lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Nonidet P-400, 0.5% sodium deoxycholate, 2 protease inhibitor cocktail tablets (Roche, #11836153001) adjusted to a final volume of 50ml with H₂O). Samples were tritutated and if required stored at -80°C.

2.7.2 Preparation of subcellular fraction

Mitochondrial fractions were obtained by resuspending the pellet obtained in section 2.7 in 150ul-200ul of digitonin release buffer (250mM sucrose, 17mM MOPS, 2.5mM EDTA, 0.8mg/mL digitonin, 2 protease inhibitor cocktail tablets adjusted to a final volume of 50ml with H₂O) for 1 minute at room temperature. Cell suspensions were then transferred to pre-cooled 2ml Dounce glass homogenizers where the cell membranes were disrupted with 50 low clearance strokes from a glass pestle (size B). The homogenates were then returned to 1.5ml Eppendorf tubes to pellet unbroken cells and nuclei by centrifuging at 1700rcf for 10 minutes at 4°C. The supernatants were then collected and centrifuged in an Eppendorf tube at 21 000rcf for 30 minutes at 4°C. Pellets contained the mitochondrial enriched fractions, while the supernatant contained the cytoplasmic fractions. Mitochondrial pellets were resuspended in 50ul-70ul of cell lysis buffer, vortexed and if required stored at -80°C.

2.7.3 Protein quantification

All protein quantification was carried out using the DC Protein Assay (BioRad: #500-0116) which is based on the Lowry protein quantification technique. The BioRad assay reaches
90% of maximum colour development within 15 minutes and remains stable for 1-2 hours. The assay is based on the reaction of protein with an alkaline copper tartrate solution and folin reagent. Colour change occurs in the presence of the amino acids tyrosine, tryptophan, cystine, cysteine and histidine and proportionally increases with increased amino acids. The resultant blue colour has a maximum absorbance at 750nm and a minimum absorbance at 405nm.

The assay was performed using a 96 well plate (BD Falcon: #353072) and absorbance was read at 630nm on an ELISA plate reader. Protein concentrations of interest were calculated by comparing them to a standard curve, generated by determining the absorbance of known concentrations of bovine serum albumin (BioRad: #500-0005). Protein quantifications were run in triplicates and the average concentration for each sample was recorded.

2.7.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared by adding a specific volume of cell homogenate to 2x loading buffer (62.5mM Tris-HCl (pH 6.8), 2% SDS, 0.00125% bromophenol blue, 10% glycerol, 715mM β-mercaptoethanol), before being adjusted to the volume of the largest sample with cell lysis buffer. Care was taken to calculate identical amounts of protein loading between samples. In general, 40ug of protein was loaded for whole cell lysates and 10-15ug of protein loaded for mitochondrial fractions. Prepared samples were then boiled for 5 min to denature proteins/mask charges and resolved on a 4% stacking gel at 40V, then through the 12% running gel at 80V submerged in a running buffer consisting of Tris-base, glycine, SDS, and H$_2$O. The PageRuler Protein Ladder (Fermentas: #SM1811) was run in all blots to ensure bands of interest resolved at the expected size. The proteins resolved in the gel were then transferred to an ethanol soaked PVDF membrane (PALL: #66543) overnight at 30V at 4 ºC in a transfer buffer consisting of Tris-base, glycine methanol and H$_2$O. The membranes were then washed in TBS-T (20mM Tris, 137mM NaCl, 3.8mM HCl, and 0.1% Tween-20, pH 7.6)
before being blocked for 1h at room temperature in a 5% non-fat milk/TBS-T solution to reduce non-specific antibody binding.

2.7.5 Detection of proteins

Membranes were incubated in the following primary antibodies overnight, washed in TBS-T, before being incubated in a HRP-linked secondary for 1h. Luminescence was triggered using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer: #NEL-105) and recorded on X-Ray film (Denville Scientific: #E3018). Antibodies were diluted in a 5% non-fat milk/TBS-T solution or a 5% BSA/TBS-T solution as required. To ensure binding specificity, all HRP-linked secondary antibodies were tested with and without the addition of a primary antibody (Shulyakova et al., in preparation). Different ug protein loads were run on the same blot to test for linearity. Results are shown in the appendix (see Appendix Figure 3).

mtGFP
1° Anti-mtGFP rabbit polyclonal antibody (Molecular Probes: #A6455). 1: 3000 dilution
2° HRP-linked anti-rabbit donkey antibody (Amersham: #NA934). 1: 15000 dilution

mtHSP70
1° Anti-mtHSP70 mouse monoclonal antibody (Thermo Scientific: #MA3-028). 1: 5000 dilution
2° HRP-linked anti-mouse sheep antibody (Amersham: #NA931). 1: 10000 dilution

mtTFA
1° Anti-mtTFA rabbit polyclonal antibody (Bio-vision: #3885-100). 1: 500 dilution
2° HRP-linked anti-rabbit donkey antibody (Amersham: #NA934). 1: 3000 dilution
Tom20
1° Anti-Tom20 rabbit polyclonal antibody (Santa Cruz: #SC11415). 1: 2000 dilution
2° HRP-linked anti-rabbit donkey antibody (Amersham: #NA934). 1: 5000 dilution
PGC1α
1° Anti-PGC1α rabbit monoclonal antibody (Cell Signaling: #2178). 1: 500 dilution
2° HRP-linked anti-rabbit donkey antibody (Amersham: #NA934). 1: 2000 dilution

CNα
1° Anti-CN α rabbit polyclonal antibody (Cell Signaling: #2614). 1: 1000 dilution
2° HRP-linked anti-rabbit donkey antibody (Amersham: #NA934). 1: 5000 dilution
FIS-1
1° Anti-FIS-1 rabbit polyclonal antibody (Santa Cruz: #SC98900). 1: 200 dilution
2° HRP-linked anti-rabbit donkey antibody (Amersham: #NA934). 1: 2000 dilution

GAPDH
1° Anti-GAPDH mouse monoclonal antibody (Calbiochem: #CB1001). 1: 15000 dilution
2° HRP-linked anti-mouse sheep antibody (Amersham: # NA931). 1: 5000 dilution

2.7.6 Quantification of bands

Bands of interest exposed on X-ray film were scanned and quantified using Quantity One software (BioRad). A box was drawn around the largest band of interest, which was copied and applied around all other bands of interest to obtain the optical density per equal area of analysis (OD/mm²). Directly above or below the band of interest, a second box was used to establish a background reading which would later be subtracted from the value of the band to give a net optical density of a particular band of interest. OD/mm² values for whole cell lysates were normalized to GAPDH bands and since no stable control for mitochondrial fractions was found, mt fractions were run in triplicates per sample.
2.8 Immunoprecipitation and autoradiography

2.8.1 Metabolic labeling

Differentiated PC12 cells were prepared for autoradiography in much the same way as western blots (see section 2.7). After treatment with DMSO control or A23187, instead of being collected in ice-cold PBS to prepare fractions, cells were incubated for 1h in cystine and methionine deficient RPMI (Sigma: #R7513) supplemented with 50uCi/ml of S\textsuperscript{35} (Perkin Elmer: #NEG772002MC), 1% dialyzed serum and NGF. After isotope incubation, cells were rinsed in cell culture medium and collected as previously described in section 2.7 All proteins containing cystine or methionine synthesized during this time would have incorporated the S\textsuperscript{35} tag allowing for the quantification of newly synthesized mtGFP in the mitochondria or PC12 cells.

2.8.2 Subcellular fractionation and immunoprecipitation

After cells were collected, they were fractionated to obtain mitochondrial pellets as described in section 2.7.2 and protein was quantified as described in section 2.7.3. 1ul of an anti-mtGFP antibody was added to 15ug of protein, which was then brought to a total volume of 400ul in a 1.5ml Eppendorf tube with the addition of cell lysis buffer. Samples were then rotated for 1h at 4\textdegree C to facilitate antibody binding. Next, 30ul of Protein G plus Protein A agarose beads (Calbiochem: #IP05) were added to each sample before being rotated again overnight at 4\textdegree C. To purify the immune complexes, samples were spun at 21 000rcf for 10 minutes at 4\textdegree C the following morning and then washed in cell lysis buffer. Samples were then spun at 21 000rcf for 10 minutes at 4\textdegree C, washed in a high salt buffer (500mM NaCl, 0.1% Nonidet P40, 0.05% sodium deoxycholate, 50nM Tris-HCl, pH 7.5), followed by a spin at 21 000rcf for 10 minutes at 4\textdegree C before being washed in a low salt buffer (0.1% Nonidet P40, 0.05% sodium deoxycholate, 50nM Tris-HCl, pH 7.5). Salt washes were repeated twice each.
2.8.3 SDS-PAGE, gel drying and detection of radiolabeled proteins

After the final wash, 50ul 1X SDS loading buffer (31.25mM Tris-HCl (pH 6.8), 1% SDS, 0.000625% bromophenol blue, 5% glycerol, 357.5mM β-mercaptoethanol) was added to each pellet before being boiled for 5 minutes and spun again at 21 000rcf for 10 minutes at 4°C. The supernatant (roughly 40ul) was then loaded into a 4% stacking gel/12% running gel and resolved at 80V in running buffer. After the protein ladder was fully resolved, gels were removed from their cassettes and dried overnight on a gel dryer (Fisher Scientific: #DBGD45). Dried gels were exposed against X-ray film for at least 24h to achieve appropriate signal from the S\textsuperscript{35} containing protein. Bands were quantified in the same manner as in section 2.7.6.

2.9 MTT reduction assay

Cellular MTT reduction capacity was measured by plating 5-6 day differentiated mtGFP-off PC12 cells in a 96 well plate (BD Falcon: #353072), using 8 wells per condition. Wells were coated in PLO and cells were plated at a density of 10 000 cells per well, with 100ul of culture medium per well. After the cells settled in the wells overnight, they were washed to remove tet from the culture medium and treated with 0.1uM, 0.15uM and 0.2uM A23187. After 24h, 10ul of 5mg/ml MTT stock solution was added to each well. MTT stock solution was prepared by diluting in cell culture medium then thoroughly vortexed immediately prior to use. Following a 2h MTT incubation, the cell culture medium was carefully aspirated as not to remove any of the recently formed formazan crystals. 100ul of 100% DMSO was used to dissolve the crystals and the plate was put on an orbital shaker for 10 minutes. Absorbance of the now coloured DMSO was measured on an ELISA plate reader at a wavelength of 570nm, with 630nm as a reference. Both the highest and the lowest absorbance values were excluded in the final calculation for mean absorbance and values were expressed as percent of control.
2.10 Statistical Analysis

Data were analyzed using the Microsoft Excel 2007 data analysis package or Graphpad Prism v5.03. The unpaired student’s t-test or one-way ANOVA with Tukey’s post hoc test were used where appropriate and statistical significance was achieved at a p-value less than 0.05. All data were expressed as mean ± SEM, graphed using Microsoft Excel 2007 and edited in Microsoft PowerPoint 2007.
3.0 RESULTS
3.1 Low dose A23187 did not cause cell death in differentiated PC12 cells.

The mobile ion carrier, ionophore A23187 has been widely used to study Ca\(^{2+}\) homeostasis in neurons and other cell types (Bernardi, 1999). In these studies, PI was used to establish a dose of A23187 that increased intracellular Ca\(^{2+}\) but did not cause cell death even after prolonged exposures. As shown in Figure 6, exposure to 0.15uM A23187 for 24h, 48h or 72h did not significantly increase cell death. At 24h, cell death was 9.17% ± 2.8% in DMSO control treated cells which was not significantly different from the 5.09% ± 1.6% observed in A23187 treated cells. At 48h, cell death was 11.9% ± 3.0% in control cells, which was not significantly different from the 6.5% ± 1.5% of dead cells in the A23187 treated culture. At 72h however, cell death in DMSO treated cells was 13.0% ± 1.7% which was significantly different than the 5.6% ± 1.7% observed in A23187 treated cells (p= 0.01). This result confirmed that 0.15uM A23187 was a safe and sub-lethal dose in the NGF differentiated PC12 cell model. In contrast positive controls (70% ethanol for 10 min) clearly caused massive cell death.

3.2 Sustained exposure to low dose A23187 decreased the capacity of differentiated PC12 cells to reduce MTT

Cellular capacity to reduce tetrazolium salts (MTT) to formazan is a generally accepted method to assess cell viability/metabolic activity, although there is some controversy regarding its specificity for mitochondrial enzymes (see discussion). Figure 7 shows that exposure to 0.1uM, 0.15uM, and 0.2uM A23187 for 24h significantly decreased MTT reduction capacity; values decreased by 77% ± 2% (p=0.001), 74% ± 2% (p=0.005) and 72% ± 3% (p=0.0003) respectively versus DMSO controls. These data show that there is a decrease in cell viability as evidenced by an approx 25% decrease in MTT reduction.
Figure 6: A23187 (0.15uM) did not induce cell death in differentiated PC12 cells.

Flow Cytometry. (A) Shows three representative histograms of control cells (DMSO), A23187 treated cells (0.15uM, for 72h) and positive cell death control cells (ethanol, 10 min, to generate maximal cell death). Red bars identify the cell populations that were labeled by PI (dead) cells (see text). The ‘gate’ for dead cells was established from the + controls (see text). (B) Cell death assessed at 24h, 48h and 72h post addition of A23187. Values are expressed as mean ± SEM, N= 4 experiments, 3 samples/experiment, 10 000 cells per sample. * denotes p<0.05 unpaired student’s t-test.
Figure 7: Sustained exposure to A23187 (24h) resulted in a moderate decrease in cell viability.

Shown are results from MTT reduction assay. 0.1uM, 0.15uM, and 0.2uM significantly decreased MTT reduction capacity/ metabolic rate at 24h. Values for MTT reduction are expressed as mean ± SEM. N= 3 experiments, 6 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
3.3 A23187 (24h) increased mtGFP levels in the mitochondria of PC12 cells

To assess the effect of increasing intracellular Ca\(^{2+}\) on MPI, differentiated PC12 cells stably transfected with a tet-regulated, mitochondrially targeted GFP (mtGFP) were exposed to A23187. Prior to each experiment, tet was washed out to induce mtGFP expression. In these experiments import was monitored by flow cytometry; values for mtGFP fluorescence were expressed as arbitrary fluorescence units (AU) unless otherwise indicated. Confirmed in PC12 cells +/- A23187, mtGFP fluorescence reflects only mtGFP that is imported into mitochondria and mtGFP fluorescence assessed by flow cytometry is proportional to mtGFP protein levels in mitochondria (Sirk et al., 2003- experiments carried out in PC12 cells, using the same mtGFP import model). Figure 8 shows that A23187 significantly increased mtGFP versus DMSO controls. mtGFP values for cells treated with 0.25uM and 0.5uM A23187 for 24h were 145.9AU ± 4.1AU (p=0.008) and 154.6AU ± 6.8AU (p=0.008) versus 125.2AU ± 3 in DMSO controls. A lower dose of A23187 (0.1uM) had no effect on the mtGFP signal changing from 125.2 ± 3AU to 122.0 ± 1.1AU. These results shows that sustained exposure to A23187 at concentrations above 0.1uM can increase mtGFP levels in the mitochondria of differentiated PC12 cells. Note: mtGFP values for DMSO control treated cells versus naive cells were unchanged, demonstrating the negligible effect of DMSO alone on mtGFP import (data not shown).

3.4 A23187 (0.15uM) increased mtGFP levels in mitochondria

To determine optimal A23187 treatment, the effects of 0.15uM A23187 on mtGFP (see Figure 8) were assessed at multiple time points. Figure 9 A shows representative raw data organized by four-quadrant statistics which allows for the simultaneous gating of mtGFP on/off expressing cells and PI positive/ negative cells into 4 distinct categories. This permits quantification of the mtGFP signal in live cells. Figure 9 B shows a representative trace of the log fluorescence intensity value (AU) in off cells (Pink line= mtGFP-off cells) versus the log fluorescence intensity value of GFP expressing cells (Blue line= Control cells, Green line= A23187 24h on/off). Shown in figure 9 C, A23187 (0.15uM) applied for 24h did not
Figure 8: Sustained A23187 (24h) increased mtGFP in mitochondria of PC12 cells.

Flow cytometry shows that exposure to 0.25uM and 0.5uM A23187 (24h) significantly increased mtGFP versus DMSO controls. Values expressed as mean ± SEM. N= 3 experiments, 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
Figure 9: A23187 (0.15uM) increased mtGFP in mitochondria.

Flow cytometry (A) Representative dot plots for control and A23187 showing mtGFP fluorescence in live cells (bottom right quadrant). (B) Representative histogram of mtGFP fluorescence in off cells, DMSO control and A23187 treated cells. (C) A23187 (0.15uM) increased mtGFP at 24h on/off, 48h and 72h. Values expressed as mean ± SEM. N= 8 experiments for 24h, N=10 experiments for 24h on/off, N=7 experiments for 48h and N=4 experiments for 72h. 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
significantly change mtGFP; the value changing to 174.9 ± 17.1 from 158.9 ± 17.7. A23187 (0.15uM) applied for 24h on/off (24h ionophore followed by 24h ionophore off = 48h total) significantly increased mtGFP from 168.5 ± 9.9 to 272.7 ± 17.3 (p=0.00002). A23187 (0.15uM) applied for 48h significantly increased mtGFP from 159.6 ± 13.9 to 266.2 ± 23.7 (p=0.001). A23187 (0.15uM) applied for 72h significantly increased mtGFP from 156.0 ± 24.2 to 229.4 ± 21.0 (p=0.03). This result shows that while sustained low dose (0.15uM) A23187 increases mtGFP, a 24hr treatment with ionophore followed by a 24h rinse results in an even larger increase in mtGFP in the mitochondria. A23187 24h on/off therefore became the preferred dose for future experiments. Note: 1% serum RPMI + NGF media osmolarity was measured for DMSO control and 0.15uM A23187 and were found to be 313mOsm and 312mOsm respectively (within normal range).

3.5 A23187 exposure slowed intramitochondrial mtGFP degradation

To determine if A23187 (0.15uM) slowed mtGFP turnover/degradation (which could contribute to the observed increase in mtGFP), PC12 cells expressing mtGFP were treated with 50ng/ml tet to arrest mtGFP protein synthesis. Subsequently the mtGFP signal was monitored for up to 5 days. This procedure allowed for the calculation of mtGFP half-life.

Figure 10 A shows that exposure to 0.15uM A23187 initially had no effect on mtGFP degradation. 24h after mtGFP synthesis was inhibited the mtGFP signal in both A23187 and DMSO control cells showed a significant decline as expected. The decline however was not different between A23187 and DMSO treated cells: decreasing to 76.6% ± 3.4% of max mtGFP (always on mtGFP controls) and 67.0% ± 4.0% respectively. Over time, the mtGFP degradation rate in A23187 treated cells slowed significantly compared to DMSO controls. At 48h, levels in A23187 treated cells were 60.2% ± 1.7% vs. 43.2% ± 2.9% in control cells (p=0.001). 3 days tet decreased mtGFP to 33.7% ± 3.8% in control cells and 54.3% ± 3.6% in A23187 treated cells (p=0.003) while 4 days tet decreased mtGFP to 27.6% ± 3.3% in control cells and 45.9% ± 4.6% in A23187 treated cells (p=0.008) and 5 days tet decreased
Flow cytometry (A) 50ng/ml tet was added to the media to suppress mtGFP synthesis and the decline in the mtGFP signal (mtGFP turnover) was monitored for 5 days. mtGFP ½ life was 49h in control cells versus 83h in A23187 treated cells. (B) Compares the effects of exposure to A23187 for 48h (blue histogram) to exposure to A23187 for 24h followed by a 24h washout of A23187 (orange histogram). Both treatments significantly slowed the decline in mtGFP. Values expressed as mean ± SEM. N=5 experiments for 24h on/off A23187. N=4 experiments for d1-d4. N=3 experiments for d5. 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
mtGFP to 18.7% ± 1.4% in control cells and 37.5% ± 5.7% in A23187 treated cells (p=0.01). Exponential decay ($y=Ae^{(-Bx)}$) was calculated with $R^2$ values of 0.9793 for control 0.9792 for A23187 treated cells. mtGFP half-life was calculated to be 49h in control cells versus 83h in A23187 treated cells. These data indicated that prolonged exposure to low dose A23187 (0.15uM) slowed intramitochondrial mtGFP degradation by 48h.

Figure 10 B shows the same data for 48h controls versus 0.15uM A23187 degradation (Blue histogram), compared to cells treated with 0.15uM A23187 for 24h on followed by 24h off (24on/24off cells) (Orange histogram). In A23187 treated cells, mtGFP declined to 66.3% ± 3.1% versus 48.3% ± 3.2% in DMSO controls (p=0.002). These data suggest that a transient (24h) exposure to A23187 was sufficient to impair mtGFP degradation to the same degree as a 48 hrs exposure.

3.6 A 24h pulse of A23187 (0.15uM) (24h on/off) increased mtGFP and mtHSP70 protein in the mitochondria PC12 cells

To assess the change in mtGFP protein content in the mitochondria and assess levels of other physiological mitochondrial proteins, western blots were performed on mitochondrial fractions. Western blot data is given as optical density units/mm² unless otherwise stated. Figure 11 A shows representative blots of mitochondrial fractions run in triplicates. The proteins selected for analysis were either imported or associated with the mitochondrial membrane; mtGFP, mtHSP70, mtTFA and Tom20. Figure 11 B shows that after A23187 (0.15uM) for 24h on/off mtGFP protein levels in mitochondria significantly increased from 44.62± 7.3 to 82.3± 9.2 (p=0.006). mtHSP70 protein levels increased significantly from 58.4± 3.0 to 77.6± 2.4 (p=0.001). Mitochondrial fractions probed for mtTFA were not significantly different after treatment with A23187, changing from 39.8± 10.4 to 40.9± 9.0. Control fractions probed for Tom20 were not significantly different from mitochondrial fractions from A23187 treated cells, changing from 99.4± 8.9 to 82.3± 7.9. Taken together these data show that A23187 was not only capable of increasing levels of mtGFP protein in
Figure 11: A23187 (0.15uM) for 24h on/off increased mtGFP and mtHSP70 protein levels in mitochondria.

Western blot of mitochondrial fraction (A) Representative western blots for mtGFP, mtHSP70, mtTFA and Tom20 after exposure to A23187 (0.15uM) for 24h on/off. (B) mtGFP and mtHSP70 protein levels in the mitochondria increased significantly after A23187 (0.15uM) for 24h on/off but not mtTFA or Tom20. Values expressed as mean ± SEM. N=5 experiments for mtGFP, mtTFA and Tom20. N=4 experiments for mtHSP70. Western blot fractions done in triplicate per sample, 10-15ug of protein per sample. * denotes p<0.05, unpaired student’s t-test.
mitochondria (a result that matches the increase in mtGFP fluorescence) but also increased levels of proteins that are components of the import machinery e.g. mtHSP70.

3.7 A23187 (0.15uM) for 48h increased mtGFP, mtHSP70 and mtTFA levels in mitochondria in PC12 cells

To assess if a longer dose of A23187 (0.15uM) would change protein content in the mitochondria, western blots were performed on mitochondrial fractions. Figure 12 A shows representative blots of mitochondrial fractions run in triplicates. As before, mtGFP, mtHSP70, mtTFA and Tom20 were analyzed. Figure 12 B shows that after A23187 (0.15uM) for 48h mtGFP protein levels in mitochondria significantly increased from 45.2± 5.1 to 111.2± 9.4 (p=0.0006), mtHSP70 protein levels increased significantly from 51.6± 7.5 to 89.6± 7.7 (p=0.001) and mtTFA protein levels increased significantly from 43.8± 7.1 to 70.6± 14.0 (p=0.04). Control fractions probed for Tom20 were not significantly different from A23187, changing from 74.9± 12.2 to 85.7± 14.8 after being treated with A23187 (0.15uM) for 48h. Taken together these data show that 48h A23187 was capable of increasing mtGFP, mtHSP70 (part of the protein import machinery) and mtTFA (involved in mitochondrial biogenesis) protein levels in mitochondria.

3.8 A23187 (0.15uM) for 24h on/off increased immunoprecipitated radiolabeled mtGFP in the mitochondria PC12 cells

To assess the affect of A23187 on the rate of newly synthesized mtGFP protein import, PC12 cells were radiolabeled for 1h with radioactive S35, followed by mtGFP immunoprecipitation of mitochondrial fractions. Figure 13 A shows triplicate samples of audioradiographic immunoprecipitated mtGFP blots from mitochondrial fractions. Figure 13 B shows that after A23187 (0.15uM) for 24h on/off the amount of radiolabeled mtGFP in the mitochondria increased from 10.7± 0.7 to 24.9± 4.8 (p=0.009). This result demonstrates that the rate of mtGFP import, over a 1h period was significantly increased in cells treated with A23187.
Figure 12: A23187 (0.15uM) for 48h increased mtGFP, mtHSP70 and mtTFA protein levels in mitochondria.

Western blot of mitochondrial fraction (A) Representative western blots for mtGFP, mtHSP70, mtTFA and Tom20 after exposure to A23187 (0.15uM) for 48h. (B) mtGFP, mtHSP70 and mtTFA protein levels in the mitochondria increased significantly after A23187 (0.15uM) for 48h but not Tom20. Values expressed as mean ± SEM. N=4 experiments for mtGFP. N=8 experiments for mtHSP70 and Tom20. N=6 experiments for mtTFA. Western blot fractions done in triplicate per sample 10-15ug of protein per sample. * denotes p<0.05, unpaired student’s t-test.
Figure 13: A23187 (0.15uM) for 24h on/off increased mtGFP import rate.

Autoradiography combined with immunoprecipitation of mitochondrial fraction (A) Representative autoradiograph for mtGFP after exposure to A23187 (0.15uM) for 24h on/off. (B) Radiolabeled mtGFP protein levels in the mitochondria increased significantly after A23187 (0.15uM) for 24h on/off. Values expressed as mean ± SEM. N=5 experiments. Autoradiograph fractions done in triplicate per sample. * denotes p<0.05, unpaired student’s t-test.
Data presented for autoradiography experiments are given as optical density units/mm² unless otherwise stated.

3.9 A23187 (0.15uM) for 24h did not slow intramitochondrial mtGFP protein degradation

To determine if A23187 (0.15uM) affects mtGFP protein degradation, PC12 cells maximally expressing mtGFP were treated with 50ng/ml tet at the beginning of the experiment to stop protein synthesis and mtGFP protein levels were measured after 24h. Slowed intramitochondrial degradation/turnover is manifested as higher levels of mtGFP protein in the mitochondria after the addition of tet. Figure 14 A shows representative blots of mitochondrial fractions run in triplicate, specifically the bands correspond to remaining control versus A23187 levels of mtGFP protein after expression was stopped. Figure 14 B shows that after A23187 (0.15uM) for 24h remaining mtGFP protein levels in mitochondria changed from 113.4± 11.2 to 165.9± 33.2 although this change was not statistically significant. A23187 for 24h does not have an effect on mtGFP degradation.

3.10 A23187 (0.15uM) for 24h on/off slowed intramitochondrial mtGFP degradation

To determine if A23187 (0.15uM) would slow mtGFP after 24h treatment, followed by a 24h wash, protein degradation was assessed at the 24h on/off time point. As before, PC12 cells maximally expressing mtGFP were treated with 50ng/ml tet at the beginning of the experiment to stop protein synthesis and mtGFP protein levels were measured after 24h on/off. Figure 15 A shows representative blots of mitochondrial fractions run in triplicate, specifically the bands correspond to remaining control versus A23187 levels of mtGFP after expression was stopped. Figure 15 B shows that after A23187 (0.15uM) for 24h on/off remaining mtGFP protein levels in mitochondria significantly increased, with A23187 (0.15uM) 24h on/off treatment changing protein levels from 68.8± 5.8 to 94.3± 9.7 (p=0.03).
Figure 14: A23187 (0.15μM) for 24h did not slow mtGFP protein degradation in mitochondria.

Western blot of mitochondrial fraction. 50ng/ml tet was added to the media to suppress mtGFP synthesis and the decline in the mtGFP protein (mtGFP turnover) was monitored (A) Representative western blot for mtGFP after exposure to 24h A23187 (0.15μM) +50ng/ml tet for 24h. (B) mtGFP protein levels were not significantly different in 24h A23187 (0.15μM) + 50ng/ml tet. Values expressed as mean ± SEM. N=3 experiments. Western blot fractions done in triplicate per sample. 10-15ug of protein per sample. unpaired student’s t-test.
Figure 15: A23187 (0.15uM) for 24h on/off slowed mtGFP protein degradation in mitochondria.

Western blot of mitochondrial fraction. 50ng/ml tet was added to the media to suppress mtGFP synthesis and the decline in the mtGFP protein (mtGFP turnover) was monitored (A) Representative western blot for mtGFP after exposure to A23187 (0.15uM) for 24h on/off and 50ng/ml tet for the full 48h. (B) mtGFP protein levels were higher in 24h on/off A23187 (0.15uM) treated cells +50ng/ml tet. Values expressed as mean ± SEM. N=4 experiments. Western blot fractions done in triplicate per sample. 10-15ug of protein per sample. * denotes p<0.05, unpaired student’s t-test.
3.11 A23187 (0.15uM) for 24h on/ off increased expression of mtGFP, mtHSP70 but decreased mtTFA protein expression

To determine if A23187 alters the expression level of mitochondrial proteins, thereby increasing the total protein available for future import, western blots were performed on whole cell lysates of NGF differentiated PC12 cells. Proteins assessed included the inducible mtGFP protein, physiological proteins including those required for MPI (mtHSP70, Tom20) and proteins involved in mitochondrial biogenesis (mtTFA, PGC1α). Figure 16 A shows representative blots of whole cell lysates, with corresponding Gapdh values used for normalization of protein load. Figure 16 B shows that after A23187 (0.15uM) for 24h on/off mtGFP expression increased from 71.1± 5.4 to 91.5± 8.0 (p=0.03), mtHSP70 expression increased from 69.1± 3.5 to 80.0± 4.2 (p=0.04), mtTFA protein expression however decreased significantly from 76.1± 5.0 to 59.0± 8.1 (p=0.04) while Tom20 expression levels also decreased from 110.4± 6.6 to 92.2± 7.1 (p=0.04). PGC1α protein expression however was not significantly different after A23187 exposure, changing moderately from 63.4± 5.0 to 60.4± 4.2. The following result demonstrates that A23187 treatment can alter the expression of selected proteins, while not affecting or reducing the expression of others.

3.12 A23187 (0.15uM) for 24h on/ off did not increase CN α expression, but did increase the CN α cleavage

To assess the involvement of CN α, a probable downstream target of the A23187 stimulated increase of intracellular Ca²⁺, western blots were performed on whole cell lysates. CN α expression was then assessed, as was the amount of CN α cleavage. Figure 17 A shows representative western blots of whole cell lysates of CN α and the CN α cleavage product respectively. Both are shown with matching Gapdh bands for normalization of protein load. Figure 17 B shows that after treatment with A23187, CN α expression was no different than in control cells, the values changing from 90.0± 8.8 in control cells to 81.7± 6.1 in treated cells. The cleavage product of CN α however was significantly changed, increasing from
Figure 16: A23187 (0.15uM) for 24h on/off increased mtGFP, mtHSP70 and decreased mtTFA and Tom20 protein expression in whole cell lysates of PC12 cells.

Western blot- whole cell lysates. (A) Representative western blot for mtGFP, mtHSP70, mtTFA, Tom20, PGC1α and the control protein Gapdh after exposure to A23187 (0.15uM) for 24h on/off. (B) mtGFP and mtHSP70 expression increased, while mtTFA and Tom20 expression decreased after exposure to A23187 (0.15uM) for 24h on/off. Values expressed as mean ± SEM. N=5 experiments for mtGFP, N=6 experiments for all other proteins. Proteins were normalized Gapdh to control for loading. 40ug of protein loaded per sample.* denotes p<0.05, unpaired student’s t-test.
Figure 17: A23187 (0.15uM) for 24h on/off increased cleaved calcineurin alpha (CN α) levels in whole cell lysates of PC12 cells.

Western blot- whole cell lysates. (A) Representative western blot for CN α and the control protein Gapdh after exposure to A23187 (0.15uM) for 24h on/off. (B) Cleaved CN α levels increased after exposure to A23187 (0.15uM) for 24h on/off. Values expressed as mean ± SEM. N=4 experiments. Proteins were normalized Gapdh to control for loading. 40μg of protein loaded per sample. * denotes p<0.05, unpaired student’s t-test.
42.2± 4.0 to 73.1± 7.2 (p=0.005) implicating CN α pathway modulation in the response to A223187 treatment.

3.13 A23187 (0.15uM) for 24h on/ off decreased FIS-1 expression

FIS-1 protein levels were assessed by western blot to determine the protein’s involvement in the mitochondrial fission and fusion events observed during and after A23187 removal. Figure 18 A shows a representative western blot of a whole cell lysates probed for FIS-1. Shown underneath are the Gapdh bands which serve as a control for protein loading. Figure 18 B shows that at 24h on/ off, A23187 (0.15uM) caused a significant decrease in FIS-1 expression as seen by a drop in protein levels from 55.3± 7.8 to 32.1± 4.5 (p=0.02).

3.14 A23187 altered cellular and mitochondrial morphology

To assess changes to mitochondrial morphology in response to A23187, mtGFP expressing PC12 cells were treated with the ionophore and morphology was assessed using confocal microscopy. Figures 19 A and B show control and A23187 (0.15uM) treated cells respectively. Mitochondrial beading is observed after 24h of A23187. This effect however is reversed upon ionophore removal. Figures 19 C and D show that at the 24h on/off time point the mitochondria in DMSO control treated cells are not morphologically different from the mitochondria in A23187 treated cells.

Phase contrast and confocal microscopy were used to assess the effect of Ca^{2+} induced differentiation via exposure to A23187. Experiments revealed A23187 (0.15uM) + NGF stimulated PC12 differentiation more than NGF treated cells alone. Figures 20 A and B show phase contrast images of DMSO control treated cells and 24h on/off A23187 (0.15uM) treated cells. Notice the increased number of neurites visible per cell and the increased length of each neurite.
Figure 18: A23187 (0.15uM) for 24h on/off decreased FIS 1 protein levels in whole cell lysates of PC12 cells.

Western blot- whole cell lysates. (A) Representative western blot for FIS 1 and the control protein Gapdh after exposure to A23187 (0.15uM) for 24h on/off. (B) FIS 1 protein levels decreased after exposure to A23187 (0.15uM) for 24h on/off. Values expressed as mean ± SEM. N=4 experiments. Proteins were normalized Gapdh to control for loading. 40ug of protein loaded per sample.* denotes p<0.05, unpaired student’s t-test.
24h mitochondrial morphology

A- Control

B- A23187

24h on/off mitochondrial morphology

C- Control

D- A23187

Figure 19: A23187 (0.15uM) altered mitochondrial morphology.

Confocal images. (A) Shows a DMSO control treated cell for 24h and (B) shows an A23187 (0.15uM) treated cell for 24h. Notice the beaded mitochondria after A23187 treatment. (C) shows DMSO control treated cell for 24h on/off and (D) shows an A23187 (0.15uM) treated cell for 24h on/off. Notice the mitochondria no longer appear beaded and are indistinguishable from control cells. Images were taken with a 60x oil objective lens with software set to zoom 3.0
Figure 20: A23187 (0.15uM) for 24h on/off enhanced PC12 cell differentiation.

(A) Shows a phase contrast image of DMSO control treated cells for 24h on/off. (B) Shows a phase contrast image of A23187 (0.15uM) treated cells for 24h on/off. Notice, cellular differentiation (longer neurites and more neurites) appears enhanced in A23187 treated cells. Images were taken with a 20x objective lens.
3.15 A23187 (0.15uM) for 24h on/off increased intracellular Ca\(^{2+}\) in PC12 cells

To provide direct evidence that the Ca\(^{2+}\) ionophore A23187 actually increased intracellular Ca\(^{2+}\), the Ca\(^{2+}\) indicator dye fluor-3 was used. As seen in Figure 21, no change was observed 1h, 3h, 6h and 12h after A23187 (0.15uM) was added with fluorescence intensity of fluor-3 changing from 59.2+ 10.9, 56.6+ 9.4, 64.5+ 11.1 and 58.2+ 9.8 in time matched controls to 61.1+ 11.7, 55.2+ 9.3, 61.9+ 12.0 and 74.8+ 13.3 respectively. At 24h however, Ca\(^{2+}\) levels in A23187 treated cells increased from 69.0+ 11.8 to 166.1+ 22.8 (p=0.04). Despite the removal of A23187, the 24h on/off time point still showed increased intracellular Ca\(^{2+}\) with a DMSO control value of 51.0+ 5.2 and an A23187 value of 100.0+ 11.7 (p=0.04). These results show that Ca\(^{2+}\) does not immediately increase after such a low dose of A23187, but does increase by 24h and 48h post addition of the compound.

3.16 A23187 (0.15uM) for 24h on/off increased ROS in PC12 cells

While it is widely known that Ca\(^{2+}\) dysregulation leads to increased ROS production by the mitochondria and cell as a whole, it was not know whether a low dose of A23187 shown to moderately increase intracellular Ca\(^{2+}\) would also drive ROS production. Figure 22 A shows a sample of raw flow cytometry data. The experiment demonstrates that at all given time points, ROS, as measured by DCF fluorescence was increased. The results showed consistent increases for each repetition at each time point, but the data were however pooled and expressed as % of control because of the high degree of variability in the DCF signal between experiments. Pooled data in Figure 22 B shows that 24h A23187 increased ROS by 140.5%+ 17.2% (p=0.03), 24h on/off A23187 increased ROS by 159.4%+ 17.2% (p=0.02) and 48h A23187 increased ROS by 194.4%+ 30.6% (p=0.02) when compared to respective DMSO controls.
Figure 21: A23187 (0.15μM) for 24h on/off increased intracellular calcium in PC12 cells.

Flow cytometry. Exposure to A23187 for 24h on/off significantly increased intracellular calcium at 24h and 48h post addition. Values expressed as mean ± SEM. N=3 experiments, 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
Figure 22: A23187 (0.15uM) increased intracellular ROS in PC12 cells.

Flow cytometry. (A) Sample of raw data. Exposure to A23187 for 24h, 24h on/off and 48h. (B) Pooled data shows A23187 significantly increased intracellular ROS at 24h, 24h on/off and 48h (Increased DCF fluorescence). Values expressed as mean ± SEM. N=3 experiments for 24h, and 48h. N=4 experiments for 24h on/off. 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
3.17 24h H$_2$O$_2$ reduced mtGFP in the mitochondria of PC12 cells with no increase in cell death

To assess the effect of a primary source of oxidative stress (not secondary to an increase in Ca$^{2+}$ or another cellular stress), PC12 cells were incubated in the presence of H$_2$O$_2$. Seen in Figure 23, H$_2$O$_2$ did not affect mtGFP at 25uM, 50uM or 100uM with mtGFP reading 150.1± 7.3 in control cells, 155.0± 10.0 for 25uM, 165.8± 9.2 for 50uM, and 156.0± 7.2 for 100uM. At 200uM H$_2$O$_2$ however, mtGFP was significantly reduced to 96.7± 11.0 (p=0.007). The data indicates that at a certain threshold above 100uM H$_2$O$_2$, ROS inhibits mtGFP import into PC12 cells.

To ensure that the previous reduction in mtGFP upon the addition of H$_2$O$_2$ was not caused by killing the cells, cell death was measured using PI at all of the previously used H$_2$O$_2$ concentrations. Figure 24 shows flow cytometry results for cell death after 24h of H$_2$O$_2$. Cell death in control cultures was found to be 15.9% ± 1.2% which was not significantly different from 13.9% ± 0.7% in 25uM H$_2$O$_2$ treated cells, 15.1% ± 1.8% in 50uM H$_2$O$_2$ treated cells, 14.1% ± 1.2% in 100uM H$_2$O$_2$ treated cells or even 16.1% ± 1.0% in 200uM H$_2$O$_2$ treated cells. This result demonstrates that at the concentrations used, H$_2$O$_2$ was not lethal.

3.18 24h on/ off A23187 (0.15uM) increased mitochondrial mass but did not up regulate biogenesis

An increase in mtGFP signal measured by flow cytometry could not only occur because of increased import but also if there was increased number of mitochondria. Using the dye mitotracker green, which accumulates in the mitochondria of actively respiring cells regardless of membrane potential, mitochondrial mass can be assessed. Figure 25 shows that control cells have a mitotracker green signal of 166.1± 15.6 and A23187 treated cells have a significantly higher signal of 246.6± 17.9 (p=0.002). This result suggests that mitochondrial mass was increased after A23187 treatment and provides support for increased biogenesis.
Figure 23: H$_2$O$_2$ for 24h decreased mtGFP fluorescence in the mitochondria of PC12 cells.

Flow cytometry. Exposure to 200uM H$_2$O$_2$ for 24h significantly decreased mtGFP in mitochondria. Values expressed as mean ± SEM. N=3 experiments, 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
Figure 24: H2O2 for 24h did not cause cell death in PC12 cells.

Flow cytometry. H2O2 did not increase cell death versus control treated cells at 25uM, 50uM, 100uM or 200uM. Values expressed as mean ± SEM. N=3 experiments, 3 samples/experiment, 10 000 cells per sample. unpaired student’s t-test.
Figure 25: A23187 for 24h on/off increased mitochondrial mass in PC12 cells.

Flow cytometry. A23187 (0.15uM) for 24h on/off significantly increased mitochondrial mass (measured via mitotracker green) vs DMSO control cells. Values expressed as mean ± SEM. N=4 experiments, 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
Figures 26 A and B show representative mitochondrial fractions of PC12 cells probed with anti-mtTFA and graphed raw data while Figures C and D show whole cell lysates probed with anti-mtTFA and anti-PGC1α. These data are a sample of a single N (sister culture to one of the previous flow cytometry mitotracker green experiments) of previously pooled and graphed data from Figure 11 and Figure 16. Overall, there was no increase in mtTFA import to the mitochondria and there was no increase in mtTFA or PGC1α expression and despite increases in mitotracker green.

3.19 24h forskolin did not increase mtGFP while 24h on/off forskolin significantly increased mtGFP

To determine if cAMP signalling pathways were involved in the Ca^{2+} induced upregulation of mtGFP import, the adenylate cyclase stimulating compound forskolin was used. Figure 27 A shows that a 24h treatment of various concentrations of forskolin did not significantly change mtGFP in the mitochondria of PC12 cells as measured by flow cytometry. The mtGFP signal in DMSO control treated cells was 135.7± 8, 135.7± 8.3 at 1uM forskolin, 149.4± 9.7 at 5uM forskolin, 160.2± 10.0 at 10uM forskolin, 112.5± 8.3 at 25uM forskolin (5uM and 25uM appear to be visually different, significance was not achieved using the student’s t-test, p=0.06 in each case). Figure 27 B however shows that a 24h on/off treatment of forskolin did increase mtGFP in the mitochondria of PC12 cells. The mtGFP signal in DMSO control treated cells was 219.6± 6, 258.6± 20.0 at 1uM forskolin, 311.8± 37.5 at 5uM forskolin (p=0.03), 311.3± 41.2 at 10uM forskolin (p=0.04), 293.2± 36.9 at 25uM forskolin. A ‘no DMSO’ control group was also run alongside these experiments to ensure the level of DMSO itself was not causing the observed change in mtGFP. The no DMSO control mtGFP signal was 213.4± 6.3.
Figure 26: A23187 (0.15uM) for 24h on/off did not up regulate biogenesis in PC12 cells.

Western blot- whole cell lysates and mitochondrial fractions. (A) Representative mitochondrial fraction western blot for mtTFA and the control protein Gapdh after exposure to A23187 (0.15uM) for 24h on/off. (B) mtTFA protein levels after exposure to A23187 (0.15uM) for 24h on/off. (C) Representative whole cell western blot for mtTFA, PGC1α and the control protein Gapdh after exposure to A23187 (0.15uM) for 24h on/off. (D) mtTFA and PGC1α protein levels after exposure to A23187 (0.15uM) for 24h on/off. Values expressed as mean ± SEM. Data shown are a representative set of sister cultures that match a single N from the previous mitochondrial mass flow cytometry experiment. Whole cell lysate proteins were normalized Gapdh to control for loading. 40ug of protein loaded per whole cell sample, 15ug of protein loaded per mitochondrial fraction sample.
Figure 27: 24h forskolin did not increase mtGFP while 24h on/off forskolin significantly increased mtGFP.

Flow cytometry. (A) Exposure to 1uM, 5uM, 10uM and 25uM forskolin for 24h did not alter mtGFP when compared to DMSO treated cells. (B) 5uM and 10uM forskolin exposure for 24h on/off significantly increased mtGFP when compared to DMSO treated cells. Values expressed as mean ± SEM. N=3 for 24h and for 24h on/off experiments, 3 samples/experiment, 10000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
3.20 24h on/off SQ22536 decreased mtGFP and attenuated the 24h on/off A23187 induced increase in mtGFP

To further establish the role of cAMP signaling in mitochondrial protein import, studies using the adenylate cyclase inhibitor SQ22536 were carried out. To determine if mtGFP levels could be down regulated through decreased intracellular cAMP just as mtGFP was up regulated through increased cAMP, adenylate cyclase was inhibited in PC12 cell cultures and the effects of mtGFP were quantified. Figure 28, flow cytometry revealed that at 24h on/off 100uM SQ22536 and 200uM SQ22536, mtGFP was moderately decreased. 100uM SQ22536 decreased mtGFP from 156.6± 3.3 to 142.9± 3.7 (p=0.009), while 200uM SQ22536 decreased mtGFP from 182.4± 4.3 to 158.6± 1.7 (p=0.008).

To establish a link between the Ca$^{2+}$ induced increases in mtGFP and the cAMP signaling pathway, the mtGFP signal in mitochondria of PC12 cells was assessed after incubation with A23187 (0.15uM) and A23187 (0.15uM) plus the adenylate cyclase inhibitor SQ22536 (100uM). Figure 29 B shows that after the addition of the ionophore A23187, mtGFP signal was 316.4± 13.0, while A23187 plus SQ22536 significantly decreased mtGFP to 270.9± 16.3 (p=0.03). Figure 29 A shows representative, raw data for all treatments compared to control, graphed together.

3.21 MnTBAP (100uM) nor A23187 (0.15uM) increased mtGFP at 24h alone, but MnTBAP + A23187 significantly increased mtGFP

As previously mentioned, increased intracellular Ca$^{2+}$ has the capacity to increase ROS production in the mitochondria and the cell as a whole. Figure 22 shows that while the Ca$^{2+}$ ionophore A23187 has the ability to increase mtGFP in PC12 cells ROS levels also increased during treatment. Figure 23 however shows that increased ROS, through the direct application of H$_2$O$_2$ decreased mtGFP. To further examine the role of ROS in the modulation of import, the antioxidant MnTBAP (a superoxide dismutase (SOD) mimetic) was used.
Figure 28: SQ22536 for 24h on/ off decreased mtGFP in mitochondria of PC12 cells.

Flow cytometry. 100uM and 200uM SQ22536 exposure for 24h on/off significantly decreased mtGFP when compared to H2O treated cells. Values expressed as mean ± SEM. N=7 experiments for control versus 100uM and N=3 experiments for control versus 200uM. 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, unpaired student’s t-test.
Figure 29: SQ22536 (100uM) decreased mtGFP when added to A23187 (0.15uM) for 24h on/off.

Flow cytometry. (A) SQ22526 (100uM) added together with A23187 (0.15uM) for 24h on./off significantly reduced mtGFP compared to A23187 (0.15uM) alone. (B) Shows representative, unanalyzed raw data for control, SQ22536, A23176 and A2+SQ treatments, graphed together. Values expressed as mean ± SEM. N=4 experiments, 3 samples/experiment, 10 000 cells per sample* denotes p<0.05, unpaired student’s t-test.
Figure 30 shows that at 24h, both 100uM MnTBP and 0.15uM A23187 did not have a significant effect on mtGFP changing from 217.6± 8.0 to 225.3± 10.6 and 243.9± 8.8 respectively. When MnTBAP and A23187 were added together however, mtGFP increased significantly to 294.4± 0.1 (p<0.05). MnTBAP + A23187 was also significantly different from every other treatment group (p<0.05).

3.22 MnTBAP and A23187 for 24h on/off both increased mtGFP, while MnTBAP + A23187 significantly increased mtGFP more than either treatment alone

Similar to the previous experiment, MnTBAP was assessed again but for a longer period (24h on/off instead of 24h as presented above) to examine if MnTBAP alone could modulate mtGFP. All combinations of MnTBAP with or without A23187 were assessed to determine if the antioxidant would further up-regulate import when added to A23187. The results of this experiment, shown in Figure 31 revealed an mtGFP signal of 380.9± 13.0 in NaOH control cells, 460.9± 15.7 in 100uM MnTBAP treated cells, 588.0± 12.2 in 0.15uM A23187 treated cells and 714.3± 24.9 in A23187+ MnTBAP treated cells. Each treatment was statically significant from every other treatment (p<0.05).

3.23 24h A23187 increased mtHSP70 and mtTFA but not Tom20 in the mitochondria of primary neurons

To validate data found in the PC12 cell model, western blots on mitochondrial fractions were repeated using primary neurons. Figure 32 A shows representative western blots of mitochondrial fractions run in triplicates. Proteins investigated include mtHSP70 and Tom20 (vital components of the import machinery) and mtTFA (a critical protein involved in mitochondrial genome replication). Figure 32 B shows that A23187 (0.15uM) for 24h significantly increased mtHSP70 protein levels in the mitochondria from 14.8± 2.5 to 36.8± 4.2 (p=0.001) and mtTFA protein levels in the mitochondria from 60.5± 3.4 to 70.9± 4.0 (p=0.04). Tom20 levels were not significantly different after A23187 treatment for 24h,
Figure 30: A23187 and MnTBAP (24h) increased mtGFP.

Flow cytometry. Both 24h MnTBAP (100uM) and 24h A23187 (0.15uM) did not increase mtGFP but A23187+MnTBAP significantly increased mtGFP in mitochondria. Values expressed as mean ± SEM. N=3 experiments, 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, one-way ANOVA, Tukey post hoc test.
**Figure 31:** 24h on/off MnTBAP and A23187 increased mtGFP more than either treatment alone.

Flow cytometry. Both 24h on/off MnTBAP (100uM) and 24h on/off A23187 (0.15uM) increased mtGFP and A23187+MnTBAP significantly increased mtGFP in mitochondria compared to MnTBAP or A23187 alone. Values expressed as mean ± SEM. N=3 experiments, 3 samples/ experiment, 10 000 cells per sample. * denotes p<0.05, one-way ANOVA, Tukey post hoc test.
Figure 32: A23187 (0.15uM) for 24h increased mtHSP70 and mtTFA protein levels in mitochondria of primary neurons.

Western blot of mitochondrial fraction (A) Representative western blots for mtHSP70, mtTFA and Tom20 after exposure to A23187 (0.15uM) for 24h. (B) mtHSP70 and mtTFA protein levels in the mitochondria increased significantly after A23187 (0.15uM) but not Tom20. Values expressed as mean ± SEM. N=5 experiments. Western blot fractions done in triplicate per sample, 10-15ug of protein per sample. * denotes p<0.05, unpaired student’s t-test.
changing from 94.4±7.3 to 86.2±9.3. Taken together, these data shows that 24h A23187 in primary neurons is capable of modulating mitochondrially imported proteins.

3.24 24h on/off A23187 increased mtHSP70 but not mtTFA or Tom20 in the mitochondria of primary neurons

To investigate whether, longer lapse time could further increase the levels of mitochondrial proteins (as seen with the PC12 data), western blots were performed on the mitochondrial fractions of primary neurons after 24h on/off A23187. Figure 33 A shows representative western blots of mitochondrial fractions run in triplicates of the same proteins analyzed earlier, mtHSP70, mtTFA and Tom20. Figure 33 B shows that A23187 (0.15uM) for 24h on/off significantly increased mtHSP70 protein levels in the mitochondria from 26.7±6.1 to 47.6±2.1 (p=0.008). mtTFA and Tom20 protein levels however were not significantly different changing from 47.6±2.1 to 38.1±2.2 and 66.0±7.5 to 69.4±11.4 respectively.

3.25 24h on/off A23187 did not increase expression of mtHSP70, mtTFA, PGC1α or Tom20 in the mitochondria of primary neurons

To determine if A23187 alters the expression level of mitochondrial proteins in cortical neurons, thereby increasing the total protein available for future import, western blots were performed on whole cell lysates. Proteins assessed included physiological proteins required for MPI (mtHSP70, Tom20) and proteins involved in mitochondrial biogenesis (mtTFA, PGC1α). Figure 34 A shows representative blots of whole cell lysates, with corresponding Gapdh values used for normalization of protein load. Figure 34 B shows that after A23187 (0.15uM) for 24h on/off total mtHSP70, mtTFA, PGC1α and Tom20 protein levels were not significantly different, changing from 42.5±1.3 to 46.8±2.1, 64.0±2.1 to 56.6±3.9, 74.7±3.5 to 73.7±0.1, and 25.0±1.7 to 24.5±2.0 respectively.
Figure 33: A23187 (0.15uM) for 24h on/off increased mtHSP70 protein levels in mitochondria of primary neurons.

Western blot of mitochondrial fraction (A) Representative western blots for mtHSP70, mtTFA and Tom20 after exposure to A23187 (0.15uM) for 24h. (B) mtHSP70 protein level in the mitochondria increased significantly after A23187 (0.15uM) for but not mtTFA or Tom20. Values expressed as mean ± SEM. N=4 experiments for mtHSP70 and mtTFA. N=3 experiments for Tom20. Western blot fractions done in triplicate per sample, 10-15ug of protein per sample. * denotes p<0.05, unpaired student’s t-test.
Figure 34: A23187 (0.15uM) for 24h on/off did not alter expression of mtHSP70, mtTFA, Tom20 or PGC1α in primary neurons.

Western blot - whole cell lysates. (A) Representative western blot for mtHSP70, mtTFA, Tom20, PGC1α and the control protein Gapdh after exposure to A23187 (0.15uM) for 24h on/off. (B) mtHSP70, mtTFA, Tom20 and PGC1α total protein expression levels were not changed after exposure to A23187 (0.15uM) for 24h on/off. Values expressed as mean ± SEM. N=3 experiments. Proteins were normalized Gapdh to control for loading. 40ug of protein loaded per sample.
3.26 A23187 altered mitochondrial morphology in primary neurons

To assess changes to mitochondrial morphology in rat cortical neurons after application of A23187, primary neurons were loaded with rhodamine 123 and imaged using confocal microscopy. Figure 35 A shows a DMSO control treated cell. Notice the tubular, elongated mitochondria throughout the cell body and processes. Figure 35 B shows an A23187 (0.15uM) treated cell after 48h. Notice the beading and aggregation of mitochondria in response to increased Ca$^{2+}$ load.
Figure 35: A23187 (0.15uM) altered mitochondrial morphology in primary cortical neurons.

Fluorescent confocal images, with rhodamine 123 labeled mitochondria. (A) shows a DMSO control treated cell for 48h and (B) shows an A23187 (0.15uM) treated cell for 48h. Notice the beaded/ aggregated mitochondria after A23187 treatment. Images were taken with a 60x oil objective lens with software set to zoom 3.0
4.0 DISCUSSION
Mitochondrial protein import is critical for neuronal function and survival. Little is known however, about how mitochondrial protein import is regulated, especially in mammalian neurons. Ca\(^{2+}\), one of the most common and widely available signaling molecules in the cell, has been implicated in previous work which showed that MPI could be modulated either through depolarization via electrical stimulation or through depolarization using KCl. The Ca\(^{2+}\) ionophore A23187 was used to elicit a global, chronic rise in intracellular calcium that occurred independent of ion channel function, which is in contrast to KCl treatment (Fong et al., in preparation). While A23187 can be classified as less physiological than KCl stimulation, A23187 allows for tight control over the intensity and length of the Ca\(^{2+}\) increase in addition to penetrating intracellular organelles such as the ER and mitochondria. For the first time, my work shows that increased intracellular Ca\(^{2+}\) alone is able to increase protein import to the mitochondria, slow intramitochondrial protein degradation and increase the expression of mitochondrial proteins independent of mitochondrial biogenesis. Furthermore, evidence suggests a Ca\(^{2+}\) mediated association to cAMP regulatory pathways and modulation of the calcineurin pathway. Uncovering what controls MPI could have wide-ranging implications for a variety of neurodegenerative diseases.

### 4.1 Ca\(^{2+}\) modulation of MPI

#### 4.1.1 Import evidence

The mtGFP/ flow cytometry assay has multiple benefits including high throughput and the ability to study MPI and intramitochondrial degradation in live cells. Flow cytometry also permits the testing of several parameters simultaneously such as cell death, mitochondrial mass, intracellular Ca\(^{2+}\) and ROS, with replications in a modest time frame. Measuring the mtGFP signal is a surrogate for measuring MPI; however, it is important to interpret changes in mtGFP signal with caution. Measuring cell fluorescence at 24h, 48h or 72h only provides evidence regarding the total mtGFP content of the mitochondrion at a particular time point. Variables that contribute to levels of proteins in mitochondria include, mitochondrial protein
import rate, mitochondrial protein expression, changes to the import machinery proteins, cytoplasmic and intra-mitochondrial protein degradation and mRNA stability.

In this study, import was studied in PC12 cells using the mtGFP protein import model three ways. mtGFP fluorescence in mitochondria was quantified using flow cytometry, mitochondrial protein levels were assessed in mitochondrial fractions by western blot and mtGFP protein import rate was measured by immunoprecipitation of radiolabeled protein.

A23187 significantly increased mtGFP in the mitochondria of PC12 cells. Flow cytometry experiments revealed that in live cells, mtGFP import was increased at 24h, but not at a lower dose. Higher doses of A23187, however caused cell swelling and were associated with neuronal cell death. Low dose A23187 (0.15uM) showed similar increases in mtGFP fluorescence in the 24h on/off model, 48h and 72h, with the largest increases observed at the 24h on/off time point. Given the potential for A23187 to cause Ca\(^{2+}\) dysregulation, the 0.15uM low dose A23187 was chosen for the majority of future experiments at the 24h on/off time point.

A23187 possesses intrinsic fluorescence (10uM A23187 demonstrated by Abramov and Duchen, 2003). To exclude the possibility that A23187 was causing auto fluorescence thus accounting for the increases in fluorescence detected by flow cytometry, 0.15uM A23187 was added to mtGFP off cells immediately prior to flow cytometry quantification. No increase in signal was detected, indicating A23187 at the doses used was not causing auto fluorescence. The mtGFP increase caused by increasing Ca\(^{2+}\) was validated using a second Ca\(^{2+}\) ionophore, ionomycin, which had the additional advantage of not being auto fluorescent (see Appendix Figure 1). Furthermore, to ensure that morphological changes were not affecting the mtGFP signal, an acute, high dose of the uncoupler CCCP was added to cells. After application of CCCP for 1h (previously determined to sufficiently alter mitochondrial morphology), no change in mtGFP signal was observed (see Appendix Figure 2).
A potential confound in using the mtGFP model to assess import in neurons, is the use of the cytomegalovirus (CMV) promoter. Identified by Wheeler and Cooper (2001), the hCMV promoter sequence reveals five CRE’s which, upon cell depolarization bind the newly, readily available CREB proteins to artificially up-regulate gene expression. Experiments from the Mills lab using KCl to depolarize PC12 cells noted increased intracellular Ca\(^{2+}\) during depolarization, which led to the hypothesis that Ca\(^{2+}\) increases, through A23187 could be driving mtGFP expression through CREB/ CRE-promoter stimulation. This however cannot be the case because mtGFP expression in our vector is controlled by a mini-CMV promoter which lacks all five CRE sites identified within the CMV promoter (clontech, 2010). When the CRE sites were experimentally mutated by Wheeler and Cooper (2001), promoter driven up-regulation of the gene of interest, no longer occurred. While CREB regulation may be involved further upstream of mtGFP or other mitochondrial protein transcription, direct artificially induced stimulation as a result of CRE binding sites on the promoter of mtGFP is not occurring.

To assess the effect of A23187 on protein levels in the mitochondria, western blots of mitochondrial fractions were assessed. This measured accumulated mitochondrial protein at a given time. Proteins examined included mtGFP, mtHSP70 and Tom20. mtGFP was probed to support the previous findings using flow cytometry and was found to be significantly higher at both 24h on/off A23187 and 48h A23187, providing strong support for Ca\(^{2+}\) modulation of this mitochondrial protein. mtGFP however, is an artificial protein and its response to Ca\(^{2+}\) may not represent native mitochondrial proteins’ responses to increased Ca\(^{2+}\).

mtHSP70 is an important, native protein chaperone involved in maintaining mitochondrial function. Specifically, This ATP-dependent chaperone binds unfolded proteins to stabilize them, prevent aggregation and promote efficient refolding upon delivery to the desired mitochondrial compartment (Goswami et al., 2010; Voos and Rottgers, 2002). It is imported to the mitochondrial matrix through a similar pathway as the N-terminal targeted mtGFP and inactivation of mtHSP70 leads to a complete breakdown of protein transport across the inner mitochondrial membrane. mtHSP70 was therefore studied to understand how native
mitochondrial proteins might behave in response to increased Ca$^{2+}$. As with mtGFP, mtHSP70 protein levels within the mitochondria were found to be significantly higher after A23187 treatment at both 24h on/off and at 48h. mtHSP70 levels in the mitochondria of primary cortical neurons were also examined and found to be significantly increased at 24h and 24h on/off.

Since mtHSP70 is part of the import machinery, increased levels of this protein provides special insight into a mechanism for the increase of other mitochondrial proteins. Indeed, evidence shows (Colavecchia et al., 2003; Craig et al., 1998; Ornatsky et al., 1995) that modification of import machinery proteins including mtHSP70 can modulate the import of other proteins destined for the mitochondria.

The second physiological protein assessed after A23187 treatment was the major translocase of the outer membrane and member of the TOM complex, Tom20. This protein was chosen due to its role as a major entry point through outer mitochondrial membrane for nuclear encoded mitochondrial proteins and because of its unique insertion method. Tom20 is not an intramitochondrial protein and is not imported in the classical sense but it is still associated with the mitochondrial membrane and can be assessed via western blot of mitochondrial fractions. Studies from the Mills lab have shown that a decrease in Tom20 resulted in reduced mtGFP import and that rescue of this deficit restores mtGFP import (Phan et al., 2006). After both 24h on/off and 48h of A23187 however, Tom20 levels within the mitochondria were not significantly changed. Additionally, A23187 after 24h and 24h on/off did not change Tom20 protein levels in the mitochondria of primary cortical neurons. While this result was unexpected given the increases observed in mtGFP and mtHSP70, it is entirely possibly for MPI to be up-regulated in the absence of changes to Tom20. mtHSP70 for example, increased protein import in C2C12 cells after mtDNA depletion with no change accompanying to Tom20 (Joseph et al., 2004). This result suggests that the import machinery is not operating at full capacity under normal conditions and that there is room to increase or decrease MPI without taking drastic changes such as modifying levels of a major translocase such as Tom20. How much import rate can be modified without modifying the TOM
complex proteins or up regulating gene expression is unknown, but is estimated to be low considering the low levels of mtGFP protein that normally accumulates in the cytoplasm (western blot of cytoplasmic fractions not shown) or the lack of fluorescence seen in the cytoplasm of mtGFP-on cells. Given the quick import or degradation of ectopic mitochondrial proteins, a large up-regulation in import rate would likely not result in an equally large increase in proteins within the mitochondria. Additionally, since Tom20 is not imported in the classical sense, it is possible that its anchoring to the mitochondria was hindered by increased Ca$^{2+}$ or associated increased ROS; thereby preventing any detectable increase in Tom20 protein levels in the western blots of mitochondrial fractions.

To address the impact of increased Ca$^{2+}$ on the rate of mitochondrial protein import, mtGFP expressing cultures were incubated in radioactive S35 for 1h after A23187 treatment. In support of the previously established evidence on the effect of A23187 on mtGFP import, more S35 tagged mtGFP was in the mitochondria of A23187 treated cells after 1h isotope incubation. Autoradiography remains the “gold standard” when measuring MPI and in this study, provides the most conclusive evidence thus far for increased protein import rate. It is possible that mRNA stability was increased as a result of increased intracellular Ca$^{2+}$. To address this, real time polymerase chain reaction (RT-PCR) could have been employed to check the relative levels of mRNA transcripts of mtGFP at various times before the 1h incubation of S35. Alternately, siRNA could have been introduced to control for excess mRNA and allowing only those proteins already translated to be imported.

To establish definitive evidence regarding the effect of increased intracellular Ca$^{2+}$ on MPI, experiments measuring import using A23187 need to be repeated in a low extracellular Ca$^{2+}$ environment. Alternatively, a Ca$^{2+}$ chelator such as BAPTA could be added to the cell culture to facilitate decreased extracellular calcium via Ca$^{2+}$ buffering, although sustained exposure to this drug should be monitored closely. If the increases to mtGFP are blocked in the presence of A23187 when coupled to low extracellular Ca$^{2+}$, it would confirm that A23187, specifically mediated through Ca$^{2+}$ was responsible for modulating MPI in PC12 cells. These experiments are currently underway.
4.1.2 Expression evidence

As a major signaling molecule and second messenger in neurons, much attention has been focused on identifying genes that are up regulated in response to depolarization, increased Ca\textsuperscript{2+} or other extracellular signals. Ca\textsuperscript{2+} dependent gene transcription is associated with the up regulation of over 300 genes in neurons, although each gene has a unique time course for being activated (Lin et al., 2008). Furthermore, most genes respond differently to Ca\textsuperscript{2+} increases depending on the route of entry, further complicating the matter (Lin et al., 2008). Ca\textsuperscript{2+} modulation of gene activity includes the activation of downstream Ca\textsuperscript{2+} binding proteins such as calmodulin, calcineurin and a host of other secondary kinases, phosphatases and transcriptions factors such as PKA, CamKII, CREB, NF-kb, NFAT and many others (Greer and Greenberg, 2008). Kim and Usachev (2009) recently showed that mitochondrial Ca\textsuperscript{2+} cycling facilitates the activation of the transcription factor NFAT, which is responsible for the control of synaptic plasticity, axonal growth and neuronal survival in neurons while Au et al., (2005) outlined Ca\textsuperscript{2+} dependent up regulation of mitochondrial electron transport chain gene expression in granulosa cells. Few studies however, have examined the role of Ca\textsuperscript{2+} fluxes on the expression of mitochondrial proteins or import machinery proteins. Studies from the Mills lab (Fong et al., in preparation) have showed that KCl was able to up regulate the expression of several mitochondrial proteins but it was not known at that time whether increased Ca\textsuperscript{2+} alone could cause similar changes.

Given the large increases in mtGFP in the mitochondria, it was expected that mtGFP expression levels would also be increased. Western blots from whole cell lysates showed that indeed, mtGFP expression was increased after an increase in intracellular Ca\textsuperscript{2+} (24h on/off A23187). Given that the mtGFP construct is driven by a mini-CMV promoter lacking all 5 CRE binding sites, CREB induced up regulation of this gene was highly unlikely. Alternatively, mtGFP could have undergone post-transcriptional changes or stabilization downstream of increased Ca\textsuperscript{2+} or cAMP, allowing for increased translation and subsequent import of a greater amount of mtGFP protein.
mtHSP70 protein levels isolated from whole cell lysates showed a similar increased, albeit modest expression after Ca\textsuperscript{2+} treatment. These results suggest that while MPI can be up regulated by Ca\textsuperscript{2+}, an increase in mitochondrial proteins does appear to not occur without an accompanying increase in gene expression. This idea is supported by the contention that mitochondrial proteins levels are relatively low in the cytoplasm and are either quickly imported following translation or rapidly degraded. Interestingly, mtHSP70 levels in whole cell lysates of primary cortical neurons were not significantly increased as they were in the mitochondrial fractions, pointing to the possible existence of a reserve pool of the transcript or protein in cortical neurons that can be imported before it is degraded in the cytoplasm.

Tom20 levels however, were significantly reduced after A23187 treatment, indicating either Tom20 expression was reduced by increased intracellular Ca\textsuperscript{2+} or that Tom20 is specifically sensitive to Ca\textsuperscript{2+} induced increases in ROS. Boengler et al., (2006) confirm the latter possibility, as they recoded decreased levels of Tom20 after oxidative stress (induced by ischemic injury in pig hearts). Interestingly, in primary cortical neurons, Tom20 levels in whole cell lysates were not significantly changed. While this was expected considering primary neurons also showed no change in mitochondrial Tom20 levels, it is surprising that Tom20 would be down regulated in PC12 (a secondary cell line) cells but not primary cortical neurons. Ca\textsuperscript{2+} modulation of the activity of Tom20 however cannot be excluded and could in part explain increases in MPI in the absence of changes to this major translocase.

4.1.3 Turnover evidence

Independent of increased gene expression or increased import rate, mitochondrial protein levels can also increase through reduced intramitochondrial degradation. Intramitochondrial degradation was monitored by using tet to inhibit mtGFP synthesis. mtGFP fluorescence and mtGFP protein levels were then followed over time to assess the effects of increased intracellular Ca\textsuperscript{2+} on mtGFP degradation.
Intramitochondrial degradation of mtGFP was unchanged after 24h A23187, but by 48h became significantly slowed and remained slowed for up to 5 consecutive days of A23187 treatment. Interestingly, cells treated with A23187 for 24h on/off showed impaired degradation to a similar degree as cells treated for 48h. Whether the Ca\(^{2+}\) increase is applied for 24h on/off or 48h, the reduction in degradation was similar indicating lasting effects of intracellular Ca\(^{2+}\) increases, lasting effects on the degradation or stability of intramitochondrial proteins or slowed intracellular Ca\(^{2+}\) clearance after A23187 removal.

Preliminary evidence gathered regarding mtGFP degradation in western blots shows that by 5d and 7d, the amount of remaining mtGFP in control cells is comparable to A23187 treated cells (data not shown), illustrating that while very little mtGFP remains after 5 days of degradation, the effect of increased intracellular Ca\(^{2+}\) appears transient. While mtGFP is an engineered protein, the targeting sequence is from the native mitochondrial protein cytochrome c oxidase (subunit VIII); allowing an extension of these findings to include native mitochondrially targeted proteins as well. My results show for the first time, that a small increase to intracellular Ca\(^{2+}\) reduces the degradation of intramitochondrial proteins. This serves to increase the total level of mitochondrial protein content; a task MPI struggles to complete during various neurological pathologies.

The mechanism through which mtGFP degradation was inhibited is not known but is likely to involve the major class of intramitochondrial proteases, AAA ATP-dependent proteases. As their name suggest, this family of proteases involved in intramitochondrial protein quality control and requires ATP to function (Langer 2000, Baker and Sauer 2006). ATP measurements were not directly performed on cell cultures after A23187 treatment but it is possible that plasma membrane Ca\(^{2+}\) ATPases used much of the available ATP supply to counteract the A23187 induced increase in intracellular Ca\(^{2+}\). Studies by Fong et al. (in preparation) in fact showed that after 48h KCl, intracellular Ca\(^{2+}\) was increased with a concomitant decrease in ATP. Interestingly, some classes of this protease possess specific metal cation dependent functionality. Yta10p protein expressed in yeast for example, showed reduced capacity to degrade proteins of the inner mitochondrial membrane in the presence of...
excess Ca\(^{2+}\) or magnesium (Pajic et al., 1994), offering an additional mechanism for the reduced degradation of mtGFP in PC12 cells.

The accumulation of mitochondrial protein can be interpreted as a negative consequence of Ca\(^{2+}\) stimulation. Studies show that a sustained inhibition of mitochondrial protein degradation is reminiscent of pathological conditions, as seen in the autosomal recessive form of hereditary spastic paraplegia, spinocerebral ataxia or sever phenotypes observe in yeast (Kaser and Langer, 2000; Atorino et al., 2003; Martinelli and Rugarli, 2009). These conditions cause chronic loss of function to mitochondrial maintenance and protein degradation, therefore a transient reduction in degradation caused by a pulse of Ca\(^{2+}\), while important with respect to quantification of total mitochondrial protein, is not likely to result in severe disturbances to mitochondrial function.

### 4.1.4 Toxicity

Since A23187 is often employed in studies of apoptosis or the pathology of neurodegeneration and ageing, toxicity was assessed. For these studies, the concentrations used range from 1uM to 50uM depending on the cell type and time course of Ca\(^{2+}\) dysregulation desired (Cho et al., 2010; Michel et al., 1994; Lukas and Jones, 1994).

After preliminary flow cytometry results were found demonstrating that A23187 was able to modify mtGFP in PC12 cells, it was necessary to determine if these concentrations of A23187 were in fact sub lethal. An extensive study on the effect of A23187 on NGF differentiated PC12 cell viability carried out by Lukas et al. (1994) revealed that A23187 for 24h had no impact on cell death until concentrations above 3uM and neurite loss did not occur until concentrations above 1uM. Functional state of the cell was assessed by cellular capacity to re-uptake dopamine and was not found to be impaired until concentrations of 1uM A23187 or higher. Furthermore, dopamine re-uptake was assessed at low dose (0.1uM) A23187 and was not different than controls for up to 48h (the latest time point tested).
Propidium iodide (PI), a fluorescent molecule which intercalates with exposed DNA, was used to assess cell death in my experiments. PI is membrane impermeable, so it does not intercalate with the DNA of viable cells, thereby providing a good measure of healthy versus compromised cells. It was found that 0.15uM A23187 for up to three days did not significantly increase cell death in NGF differentiated PC12 cells. In fact, after 3 days of A232187, cell death was significantly reduced indicating that the chosen concentration of A23187 was not only below toxic levels for PC12 cells but also neuroprotective. Cell viability was also assessed by MTT assay to compliment the previous findings. The MTT assay is used in biological systems to measure the metabolic activity of cells. Specifically, the tetrazolium salts used in this assay are primarily reduced by NADH in the mitochondria (Berridge et al., 2005) and a lower capacity to reduce MTT is thought to represent lower cellular and mitochondrial functional capacity (Mosmann, 1983). In my experiments, MTT reduction moderately albeit significantly decreased upon the addition of A23187 for 24h. This result, while important is very plausible after a mild \( Ca^{2+} \) stress. A small reduction in MTT, coupled with no increase in cell death however indicates a non-pathological cellular state after A23187 incubation. Additionally, recent evidence suggests that, MTT can and is often reduced in the cytosol, endoplasmic reticulum and plasma membrane (Bernas and Dobrucki, 2002) and therefore caution should be exercised when interpreting MTT results with respect to mitochondrial function alone.

4.2 Sub cellular mechanisms of action and \( Ca^{2+} \) pathways investigated

4.2.1 \( Ca^{2+} \) increases and calcineurin

The spatial and temporal pattern of \( Ca^{2+} \) elevation is significant in determining how the cell will respond to \( Ca^{2+} \) influx. Examples include how different concentrations of \( Ca^{2+} \) decide growth cone movement in neurons (Kater and Mills, 1991), or how large increases in \( Ca^{2+} \) instead of activating a signaling pathway can lead to neuronal cell death. Intracellular \( Ca^{2+} \) increases are facilitated by three mechanisms which, in the broadest sense consist of: increasing \( Ca^{2+} \) from intracellular stores, through channels/ pores on the plasma membrane,
or through a non-specific route facilitated through compounds like ionophores. While it is generally accepted that Ca\(^{2+}\) ionophores causes global, non-specific increase in intracellular Ca\(^{2+}\) they can and do however activate both intracellular Ca\(^{2+}\) stores and Ca\(^{2+}\) channels upon application. Dedkova et al. (2000) report that in fura-2 loaded Ehrlich ascites tumor cells, murine neutrophils and T-lymphocytes, the complex character of Ca\(^{2+}\) increase could not be explained by the ionophoric properties of ionomycin or A23187 alone. While ionophores create conditions for the increase of all methods of Ca\(^{2+}\) increase, depending on the concentration of ionophore used, the character of the increase in Ca\(^{2+}\) was different at different times. It was concluded that Ca\(^{2+}\) concentrations in the range of 1x10\(^{-9}\)M to 1x10\(^{-6}\)M activated native plasma membrane or IP3 dependent-ER Ca\(^{2+}\) transport mechanisms within seconds-minutes, while only at higher concentrations of ionophore did the non-specific increases of Ca\(^{2+}\) from the extracellular space become apparent (Dedkova et al., 2000). While the concentration of A23187 used in my experiments (1.5x10\(^{-7}\)M) was similar to those used by Dedkova et al. (2000), the time course of Ca\(^{2+}\) increases in my experiments were very different. Ca\(^{2+}\) increases were not detected at 1h (earliest time point tested using flow cytometry) and were not significantly increased until 24h and 48h. This suggests that while an early Ca\(^{2+}\) spike probably did occur, the cell quickly adapted and compensated for this change.

It is important to note that no precedent using ionophores at such low concentrations in excitable cells, has been set. While Dedkova et al. (2000) show interesting results, many of their experiments used ionomycin, which transports Ca\(^{2+}\) in a 1:1 complex and can therefore transport Ca\(^{2+}\) faster and more efficiently. Perhaps only after enough A23187 had accumulated in the membranes of the cell or when A23187 was able to overcome the cell’s native capacity to deal with increased Ca\(^{2+}\) did intracellular Ca\(^{2+}\) levels increase.

Although the concentration of A23187 used in the current study is identified with activating native signaling mechanisms, the time course of the observed Ca\(^{2+}\) increase suggests Ca\(^{2+}\) influx through the ionophoric mechanism. Interestingly, the Ca\(^{2+}\) increases observed at 24h and 24h on/off coincided with mtGFP and other physiological protein increases. No Ca\(^{2+}\)
increases were detected at any time before 24h (through Fluor3 use in flow cytometry or confocal microscopy), nor were any mtGFP changes detected at any time before 24h on/off unless a slightly higher dose of A23187 was used. This time course is consistent with the time it takes to alter protein expression in a biological system. Higher (sub lethal) doses of A23187 were used for 2h to 4h pulses but very little change to mtGFP levels occurred, perhaps more pulses or a higher concentration would have yielded a different result.

When Ca\(^{2+}\) increases within the cell, it binds the Ca\(^{2+}\) binding protein calmodulin (CaM). The Ca\(^{2+}\)/CaM complex then binds to Calcineurin (CN). CN is a serine/threonine protein phosphatase consisting of a catalytic \(\alpha\) subunit and a regulatory \(\beta\) subunit. CN is ubiquitously expressed in mammalian cells and selectively enriched in neurons where it acts as an effector of Ca\(^{2+}\) signaling by regulating the phosphorylation of proteins known to participate in neuronal development, cAMP signaling, long term potentiation (LTP), gene regulation and apoptosis (for review, see Hogan et al., 2003; Rusnak and Mertz, 2000). CN expression was studied due to its role in mitochondrial to nucleus communication and the involvement of this type of coordination during the events of mitochondrial protein import. Additionally, CN \(\alpha\) contains the catalytic subunit of calcineurin; therefore, measuring the expression of this subunit could provide information not only on protein expression levels, but activity levels too.

Despite the high probability of increased CN epression or activity after Ca\(^{2+}\) stimulation, CN \(\alpha\) was not found to increase after A23187 treatment. This seemingly perplexing result can be explained by the simple fact that ROS both cleaves and inactivates CN. Indeed, Lee et al. (2007) confirmed CN inactivation via H\(_2\)O\(_2\) dependent cleavage in mouse cortical neurons. In response to A23187 however, both intracellular Ca\(^{2+}\) and ROS increases. The CN \(\alpha\) cleavage product was therefore examined in western blots and a strong increase was observed after A23187 treatment. This result demonstrates that while the Ca\(^{2+}\) ionophore A23187 is able to increase intracellular Ca\(^{2+}\) and initiate CN signaling pathways, it does not because of the concomitant increase in intracellular free radicals. Similarly, after addition of cyclosporin A
(a known CN inhibitor) to KCl treated cells, mtGFP import levels were unaffected indicating no involvement in the import of mitochondrial proteins (Fong et al., in preparation).

The transient decrease in CN activity and predicted decrease in the downstream transcription factor NFAT may help explain why cell death (apoptosis mediated through the fas ligand/receptor pathway and/or the bcl-2 antagonist of cell death pathway (Bad)) was suppressed after A23187. In neurons the cytoplasmic NFATc4 isoform is dephosphorylated by activated CN, uncovering its nuclear localization sequence, leading to nuclear translocation and Fas gene transcription. Once the Fas ligand binds the Fas receptor, the death inducing signaling complex (DISC) is activated, leading to caspase dependent apoptosis. CN activated NFATc4 has been implicated in neuronal death following brain ischemia (Shioda et al., 2007). FK506 a potent CN inhibitor was successfully used to block Fas-ligand binding, which stopped apoptotic cell death. Furthermore, the mitochondrially dependent pro-apoptotic protein Bad is translocated to the mitochondria upon dephosphorylation by CN. Bad then binds to and inhibits the function of the pro-survival protein basal cell lymphoma extra large (Bcl-XL) offering an additional pathway by which inactivation of CN following A23187 treatment could lead to neuroprotection. Overall, inactivation of CN by A23187 indicates that this pathway is not involved in MPI, but a transient decrease in CN can and does limit the induction of apoptosis in neurons.

### 4.1.2 ROS signaling

Reactive oxygen species (ROS) are products of normal cellular metabolism but, if left unchecked or suddenly increased through ischemic injury for example, can have deleterious consequences for the cell. Low to moderate levels of ROS however have beneficial effects and normally participate in a wide variety of signaling pathways. ROS signaling is involved in growth factor signaling, the modulation of protein tyrosine phosphatases, serine/threonine kinases (evidenced by the ROS inactivation of CN) and nuclear transcription factors such as AP-1, NFKB, HIF-1 and NFAT (Valko et al., 2007). ROS signaling is also responsible for the regulation of various physiological functions including cell adhesion, growth, differentiation,
the immune response and apoptosis underscoring their importance in the day to day functionality in biological systems (Valko et al., 2007).

After moderate increases in intracellular Ca\(^{2+}\), ROS levels were increased in PC12 cells. The increase in Ca\(^{2+}\) was sub lethal; therefore it is possible that ROS was acting as a second messenger not an inducer of cellular damage/ death. These increases in ROS coincide with the temporal increase in, mtGFP, physiological proteins and decreased degradation. Increases in ROS may also help explain the increases in the expression and import of mtHSP70. This member of the heat shock protein 70 family is up regulated in response to cellular stress, including increased free radicals, to help maintain mitochondrial function (Lowenstein et al., 1991; Lee et al., 2001; Giffard et al., 2004). Over expression of mtHSP70 has been specifically implicated in the reduction of astrocyte cell death, maintenance of mitochondrial membrane potential and preservation of ATP levels following oxygen glucose deprivation (OGD) (Voloboueva et al., 2008). Up regulation of mtHSP70 plays a vital role in maintaining import and is also likely responsive to moderate ROS signaling.

Deleterious effects of ROS were demonstrated in response to a direct H\(_2\)O\(_2\) insult. As predicted by the down regulation/ damage to mitochondrial protein import machinery in response to oxidative stress (Boengler et al., 2006), PC12 cells imported less mtGFP when incubated with H\(_2\)O\(_2\). Therefore it is believed that the Ca\(^{2+}\) induced changes in MPI, and accompanying increase in ROS are in opposition to the effects of direct oxidative damage induced by H\(_2\)O\(_2\). To further examine the relationship between Ca\(^{2+}\), ROS and MPI, PC12 cells were treated with the antioxidant MnTBAP. MnTBAP acts as a superoxide dismutase (SOD) mimetic, catalyzing the breakdown of the highly reactive O\(_2^-\) anion to H\(_2\)O\(_2\). Simultaneous incubation with A23187 and MnTBAP resulted in a significant increase in mtGFP in the mitochondria of PC12 cells when compared to control, MnTBAP alone and A23187 alone. MnTBAP however, while detoxifying dangerous free radicals, does so at the expense of a temporarily increased H\(_2\)O\(_2\) load. Preliminary evidence using the H\(_2\)O\(_2\) sensitive dye DCF suggests that in PC12 cells, MnTBAP maintains and even increases H\(_2\)O\(_2\) levels when compared to A23187 treated cells (data not shown). Perhaps different concentrations of
MnTBAP may have been tried, in an effort to benefit from the antioxidant properties of MnTBAP without over stimulating the catalysis of $O_2^-$ to $H_2O_2$. Taken together, these results show that oxidative stress induced by the direct application of $H_2O_2$ does reduce import in live PC12 cells, but that moderate increases in ROS and intracellular $Ca^{2+}$ synergistically increase the import of mitochondrially targeted proteins.

### 4.2.3 Biogenesis

A critical pathway that could have been activated by increased intracellular $Ca^{2+}$ was the mitochondrial biogenesis pathway. Given the increases observed in artificial and native mitochondrial proteins in response to $Ca^{2+}$, it remains a possibility that these increases were simply due to increased physical number of mitochondria per cell and therefore more mitochondrial protein per cell. The biogenesis pathway has been well characterized and requires the participation of $Ca^{2+}$ signaling to activate the protein kinases CamK IV or PKC, which proceed to activate nuclear transcription factors such as PGC1α, CREB, NRF1/2 and NFAT (Pejznochova et al., 2010; Scarpulla, 2008; Mercy et al., 2005). PGC1α in fact plays a critical role in regulating the expression of several nuclear encoded mitochondrial proteins necessary for mtDNA replication, electron transport chain function, MPI and mitochondrial biogenesis (Diaz and Moraes, 2008).

The transcription factor mtTFA is a critical protein that is up regulated by this pathway and is instrumental in replication of the mitochondrial genome. In the current study, mtTFA protein levels were assessed in mitochondria and found to be either increased or unaffected depending on the time course of the $Ca^{2+}$ increase. Results in PC12 cells and primary cortical neurons show that only directly after $Ca^{2+}$ stimulation, were mtTFA protein levels increased in the mitochondria. Other time points such as 24h on/off, when A23187 was initially applied for 24h then removed showed no increase in mtTFA levels in the mitochondria of PC12 cells or primary neurons. This result suggests that while increased $Ca^{2+}$ is able to change the levels
of mtTFA within the mitochondria, the effect is only transient and disappears once Ca\textsuperscript{2+} levels begin to return to baseline. This increase however, may be due to an up regulation of the protein import machinery and not activation of the biogenesis pathway per se. To further address the issue, expression levels of mtTFA were assessed. Expression was not increased in primary cortical neurons and was in fact decreased in PC12 cells, which supports the idea that mitochondrial biogenesis was not initiated. PGC1α protein levels in whole cell lysates of PC12 cells and primary neurons were also assessed. An increase in this protein would most certainly reflect induction of the mitochondrial biogenesis pathway, regardless of the occasional increases in mtTFA. PGC1α expression however, remained unchanged after intracellular Ca\textsuperscript{2+} increases in both PC12 cells and primary cortical neuron.

Despite recent evidence (Diaz and Moraes, 2008; Liang et al., 2010; Mercy et al., 2005) showing that both cell depolarization and Ca\textsuperscript{2+} increases alone could induce mitochondrial biogenesis, the inconsistent changes in mtTFA levels and the lack of change to PGC1α observed in my studies does not support this contention. One of the major differences however between those studies and my work, was the method employed to increase intracellular Ca\textsuperscript{2+}. A23187, at low concentrations induces a small, generally channel independent progressive change in intracellular Ca\textsuperscript{2+}, and therefore may activate different intracellular signaling mechanisms than those required for biogenesis. In fact, Liang et al. (2010) demonstrated the specific involvement of voltage gated L-type Ca\textsuperscript{2+} channels by abolishing KCl induced Ca\textsuperscript{2+} up regulation of biogenesis through application of the Ca\textsuperscript{2+} channel blocker nifedipine. Ca\textsuperscript{2+} can affect the import or expression of several mitochondrial proteins and stimulate mitochondrial biogenesis (Mercy et al., 2005; Liang et al., 2010), but Ca\textsuperscript{2+} increases through voltage sensitive channels, represent a unique retrograde signaling pathway for increased mitochondrial biogenesis not activated by global, intracellular Ca\textsuperscript{2+} increases. In support of this idea Hood et al. (2001), who previously demonstrated up regulation of mitochondrial biogenesis through contractile activity, used A23187 alone to replicate the increase but had only limited success. A23187 was able to increase the import of nuclear encoded, mitochondrial proteins; however, other factors critical for biogenesis in muscle cells remained unchanged. It was concluded that Ca\textsuperscript{2+}, while a critical component of
mitochondrial biogenesis activation, was only a single player in a complex signaling pathway.

Protein concentration measured via the modified Lowry method was also used to compare total protein concentration isolated from mitochondrial fractions to check for large increases, which could signify increased mitochondrial number. Given equal cell plating and equal re-suspension in cell lysis buffer, protein concentrations were not significantly changed after increased intracellular Ca\(^{2+}\), again supporting the hypothesis that biogenesis was not induced (data now shown).

Lastly, mitochondrial mass was assessed using the membrane permeable, mitochondrially localized dye, mitotracker green. After Ca\(^{2+}\) ionophore treatment, mass was recorded to be 150\% of control cells. When cells incubated with mitotracker were viewed under confocal microscopy however, mitochondrial number was not noticeably increased after A23187 treatment. The large increase in mitotracker green fluorescence was unexpected considering the key regulator of mitochondrial biogenesis, PGC1\(\alpha\) was not increased. The fact that mitotracker fluorescence increased without an increase in mitochondria number exposes a significant weakness when using this dye to assess biogenesis.

It has however been recently reported that during its selective accumulation in the mitochondria, mitotracker green covalently binds to mitochondrial proteins by reacting with their thiols on cysteine residues (Presley et al., 2003). It is possible that the increased availability of proteins inside the mitochondria after A23187 treatment allows for increased mitotracker green retention rates, and therefore increased fluorescent signal. Alternatively the morphological changes observed after A23187 treatment could have been responsible for the changes to mitotracker green signal. To test this hypothesis, an acute, high dose of the uncoupler CCCP was added to cells to alter mitochondrial structure. After 1h CCCP, no change in mitotracker green signal was observed. Slight mitochondrial swelling resulting from increased intracellular Ca\(^{2+}\) could have also caused an increase in mitotracker signal, simply allowing for more dye accumulation per mitochondria, although again no such
mitochondrial volume increases were obvious when examined using confocal microscopy. Taken together, future use of mitotracker green should be closely monitored for any non-specific interactions with cellular parameters such as increased Ca\(^{2+}\) load and results should be interpreted with caution.

To summarize, mtTFA import and expression, PGC1\(\alpha\) expression and mitochondrial mass were assessed in PC12 cells and primary cortical neurons. Increases in all of these parameters have been strongly implicated mitochondrial biogenesis and are the minimum standard used to determine if biogenesis is occurring. In response to increased intracellular Ca\(^{2+}\), mtTFA was up regulated in the mitochondria of neurons, while mtTFA and PGC1\(\alpha\) expression was unchanged. Despite the puzzling increases in mitotracker green signal, it was concluded that without increases in all three of these three parameters biogenesis was not a factor following elevated intracellular Ca\(^{2+}\).

### 4.2.4 cAMP and Ca\(^{2+}\) signaling pathway convergence

A major intracellular signaling pathway that is activated by increased Ca\(^{2+}\) is the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway. cAMP is a second messenger used in intracellular signal transduction that is synthesized in the cytosol from ATP by the enzyme adenylate cyclase (AC). Increased cAMP within the cell binds to and activates PKA which can increase or decrease the activity of a variety of proteins through phosphorylation or alter gene transcription through the activation of CREB (Sands and Palmer, 2008; Lonze and Ginty, 2002). Of the 10 different AC isoforms in mammals, the two that exist in neurons (AC1 and AC8) are both directly stimulated by increases in Ca\(^{2+}\) (Cooper, 2003; Ferguson and Storm, 2004).

To assess the convergence of A23187 induced increases in intracellular Ca\(^{2+}\) and cAMP signaling pathways forskolin, an AC activator, was investigated in mtGFP expressing PC12 cells. Forskolin operates by directly stimulating AC, catalyzing the ATP to cAMP reaction,
which provides an effective method of increasing intracellular levels of cAMP. Forskolin added for 24h at various concentrations did not have an effect on mtGFP in PC12 cells; however, it did significantly increase mtGFP after 24h on/off suggesting cAMP involvement in the import of mitochondrial proteins.

Next, the AC inhibitor SQ22536 (Haslam et al., 1978) was used to test whether mtGFP import could be down regulated in response to decreased intracellular availability of cAMP. My results show that while SQ22536 induced decreases in mtGFP were moderate, the decreases occurred consistently and were therefore statistically significant. These results indicate that while cAMP is readily able to increase mtGFP levels, reductions in cAMP while not as effective in modulating import, are still able to reduce mtGFP in PC12 cells. Interestingly, when A23187 and SQ22536 were both added to cultures, mtGFP levels were also found to be decreased when compared to A23187 treated cells, indicating convergence of cAMP signaling and Ca\(^{2+}\) signaling on MPI. Future experiments regarding cAMP however, should assess the response of physiological proteins and the possible involvement of decreased intramitochondrial degradation to be sure of pathway convergence.

Beyond increases in cytoplasmic cAMP caused by either increased Ca\(^{2+}\) or forskolin, very little is known regarding the mechanism of cAMP regulation of MPI. CREB, which is activated by cAMP through PKA and CamK IV, is widely known to activate the expression of many nuclear and mitochondrial genes via CRE binding sites in their promoters (Johannessen et al., 2004). CREB signaling has been implicated in apoptosis, oxidative stress, plasticity, mitochondrial biogenesis and neuronal growth. In the last decade, co-localization studies have demonstrated CREB accumulation in the mitochondria (Cammarota et al., 1999; Lee et al., 2005) while more recently, CREB was shown to be imported into the mitochondria through the TOM complex (De Rasmo et al., 2009). Once imported to the mitochondria, CREB directly affects the transcription of mitochondrially encoded proteins of the respiratory chain demonstrating that cellular energetic demands can cause an increase in the import of transcription factors and increase in selective mitochondrial proteins. Any other
mitochondrial proteins whose promoters contain CRE binding sites would presumably be subject to similar up regulation by Ca\(^{2+}\) induced CREB increases.

The recent discovery of PKA anchor proteins (AKAP’s) on the outer mitochondrial membrane also couples cAMP/ Ca\(^{2+}\) signaling, through activated PKA, to mitochondrial function and survival. This type of protein complex allows for the local regulation of cAMP/ PKA signaling events through the modification of the stimulus intensity and temporal resolution. The protein products of the gene AKAP1 are expressed in PC12 cells and actively bind PKA in a complex at the mitochondrial/ cytosol interface Feliciello et al. (2005). AKAP’s demonstrate a direct link between cAMP and the mitochondria, independent of classical mitochondria to nuclear signaling associated with events like mitochondrial biogenesis. Examples include Feliciello et al. (2005) and Gisinberg et al. (2003) who showed that AKAP complexes actually recruited mRNA coding for the F0-f subunit of ATP synthase and MnSOD in HeLa cells to the outer mitochondrial membrane. Feliciello et al. (2005) also discovered that AKAP complexes were associated with ribosomes and mitochondrial protein import machinery. AKAP’s have also been reported to be necessary for cAMP/PKA dependent regulation of L-type Ca\(^{2+}\) channels, providing a mechanism not only for signal transduction directly to the mitochondria but also for spatiotemporal modulation or enhancement of native intracellular Ca\(^{2+}\) signaling (Bunemann et al., 1999; Johnson et al., 1994).

Taken together, AKAP’s link Ca\(^{2+}\)/cAMP signaling events to activation of native Ca\(^{2+}\) channels, the increased translation and subsequent import of mitochondrial proteins. It is currently not known how AKAP’s are regulated (Carlucci et al., 2008), and further work is needed to address the role of second messengers and signaling networks on the number or stability of AKAP’s. Discovering how AKAP’s are regulated could have tremendous implications regarding our current understanding of MPI and mitochondrial dynamics.
4.2.5 Cellular and mitochondrial morphological changes

Traditionally, PC12 cell differentiation has been stimulated by a NGF driven extracellular signal regulated kinase (Ras/ERK) pathway (Cowley et al., 1994; Klesse et al., 1999) but it can also synergize with the cAMP/PKA signaling pathway (Hansen et al., 2003). Previous work from the Mills lab demonstrated KCl enhanced differentiation of PC12 cells. Concomitant with KCl treatment was increased intracellular Ca\(^{2+}\); therefore, differentiation was monitored in the presence of a Ca\(^{2+}\) ionophore. A23187 induced increases in intracellular Ca\(^{2+}\) enhanced PC12 cell differentiation by increasing the number and length of neurites as early as 24h. This suggested a shared pathway between KCl and Ca\(^{2+}\) induced increases in differentiation, most likely converging downstream in the ERK pathway via PKA or MEK1 (Hansen et al., 2003; Hilborn et al., 1997). Treatment with the AC activator forskolin enhanced the differentiation of PC12 cells in a similar way implicating cAMP signaling pathways in PC12 differentiation (data not shown). While it is unknown if cell differentiation and protein import events are linked, Ca\(^{2+}\) increases through the use of A23187 do appear to interact with and activate native signaling and gene transcription pathways in ways that agree with existing literature.

Other changes in response to increase intracellular Ca\(^{2+}\) include the transient beading of mitochondria in PC12 cells followed by a return to long tubule formations. The mitochondria of cortical neurons appeared to be more sensitive to increased Ca\(^{2+}\), although cells were incubated for a full 48h instead of 24h on/off before being imaged (as done in PC12 cells). Mitochondria, through the tight regulation of specialized fission and fusion proteins, are able to change their morphology in order to communicate, migrate, adapt to energy demands or adapt to otherwise altered cellular conditions (Knott et al., 2008). One such protein examined was FIS-1, a mitochondrial fission protein that regulates fission events. Anchored to the outer mitochondrial membrane, this protein recruits another fission protein, Drp1 to the mitochondria (Stojanovski et al., 2004). Drp1 then oligomerizes into chains that wrap around the mitochondria, constricting it, causing organelle fission (Hoppins et al., 2007). FIS-1 protein levels were measured in whole cell lysates of PC12 cells and were found to be
significantly reduced. Fission/ fusion events depend on the relative levels of each type of protein and decreased FIS-1 would provide a permissive environment for fusion to occur. Less FIS-1 protein at the 24h on/off time point suggests that the once beaded mitochondria were fusing to become elongated and strand-like which supports the morphological data collected in mtGFP expressing PC12 cells.

Reduced levels of FIS-1 in HeLa cells and Drp1 in PC12 cells have been associated with significant protection from apoptotic stimuli and support the decrease in cell death I observed after A23187 (Lee et al., 2004; Cribbs and Strack 2007). Recent evidence shows that Drp1 can be phosphorylated at a single serine residue by PKA, causing the inactivation of this protein while the phosphatase CN causes its activation (Cribbs and Strack, 2007). Whether or not Ca^{2+} induced increases in cAMP/PKA and inactivation of CN by ROS could modify Drp1 levels was not tested but future experiments on this protein could help clarify the link between apoptosis and fission proteins.

It is however plausible that A23187 caused mild, acute stress causing the wide distribution of mitochondria throughout the cell possibly to help buffer the increased Ca^{2+} load before fusing again post A23187 removal. With respect to MPI, mitochondrial shape change drastically alters the surface area and organization of inner and outer mitochondrial membranes. Therefore, morphological changes can potentially affect import machinery, although further studies manipulating mitochondrial dynamics and MPI are required.

4.3 Summary

4.3.1 Summary and working model

Most mitochondrial proteins are nuclear-encoded and must be imported into mitochondria by a process known as mitochondrial protein import (MPI). The goal of this study was to identify the signaling pathways that regulate MPI in neurons. Using a neuronal cell line, differentiated PC12 cells that expressed a mitochondrially targeted GFP (mtGFP) and rat
cortical neurons, my results indicate that intracellular Ca\textsuperscript{2+} is involved in the regulation of MPI and the regulation of intramitochondrial protein turnover. MPI was quantified by autoradiography, western blot and in live cells by flow cytometry. Treatment with a sub lethal dose of the Ca\textsuperscript{2+} ionophore A23187 (0.15\textmu M for 24h on/off) increased the import of mtGFP. Levels of mtTFA and mtHSP70- two other mitochondrially targeted proteins also increased but levels of Tom20 were unaffected. mtGFP turnover in mitochondria assessed by flow cytometry and western blot and were initially unaffected, but by 48h slowed significantly in PC12 cells exposed to A23187. Expression of mtGFP and mtHSP70 also increased in the absence of changes to Tom20, a major translocase of the outer mitochondrial membrane that shows particular susceptibility to ROS. These results suggest that the up regulation of mtHSP70 may be a key event in the regulation of the import of matrix targeted mitochondrial proteins.

The addition of A23187 increased PC12 differentiation and altered mitochondrial morphology but did not increase expression of mtTFA or PGC1α; the absolute minimum evidence for mitochondrial biogenesis. The addition of A23187 increased Ca\textsuperscript{2+} as expected, increased levels of ROS and led to CN inactivation via proteolytic cleavage, ruling out this signaling pathway in the modulation of MPI. Using an activator and an inhibitor of adenylate cyclase, cAMP/PKA signaling was shown to modulate the Ca\textsuperscript{2+} induced changes in MPI, although AC inhibitors were not able to completely block A23187 induced increases in mtGFP.

Figure 36 shows a working model of the role of intracellular Ca\textsuperscript{2+} in the regulation of MPI. My results suggest that MPI is regulated by multiple mechanisms that involve interplay between Ca\textsuperscript{2+}, ROS, the second messenger cAMP, the nuclear genome and the mitochondrial genome. Interestingly, levels of Tom20 a major translocase of the outer membrane was unaffected, and appeared to be decreased, in whole cell lysates.
4.3.2 Limitations and future directions

The limitations of the current study include the non-physiological method used to produce small increases in intracellular Ca\(^{2+}\). While ionophores have been widely used and are known to increase intracellular Ca\(^{2+}\), through channels and release from intracellular stores, pathways that rely heavily on spatiotemporal Ca\(^{2+}\) signaling may not be affected, particularly at the low doses used. Future experiments should focus on activating native mechanisms to induce increases in intracellular Ca\(^{2+}\), e.g. using the second messenger IP3 to help release Ca\(^{2+}\) from intracellular stores (inositol triphosphate pathway), or using plasma membrane Ca\(^{2+}\) channel agonists such as FPL 64176 or Bay K 8644. A review of the literature suggests that transient increases in Ca\(^{2+}\) could have different effects. For example, although sustained activation of Ca\(^{2+}\) channels seen in glutamate neurotoxicity is associated with increased ROS and cell death, the transient activation of Ca\(^{2+}\) channels are not associated with the increased levels of ROS. Additionally, since PKC/MAP kinase pathways were activated in muscle cells in response to increased Ca\(^{2+}\), it would be imperative to test whether neurons showed a similar pattern of kinase activity that could modulate MPI.

Another caveat using ionophores is their capacity to transport cations other than Ca\(^{2+}\). This is generally not found to be a major confound in biological systems, but non-Ca\(^{2+}\) transport by ionophores can occur. This was addressed by validating preliminary results with a second ionophore, ionomycin, although it too suffers from similar specificity weaknesses. Increased intracellular Ca\(^{2+}\) not only affected import rate but also the expression and degradation of at least one mitochondrial protein, mtGFP. Finally, increased intracellular Ca\(^{2+}\) could plausibly have affected multiple other pathways including those that regulate mitochondrial fission/fusion.

To fully understand the consequences of changes in MPI a deeper understanding of how and why mitochondrial proteins are degraded is required. Considering the novel nature of my observation that mtGFP turnover decreased in response to increased intracellular Ca\(^{2+}\), this pathway demands our attention.
Figure 36: Proposed working model for the effects of increased intracellular calcium on MPI in neurons.

The effects of A23187 modulation of cAMP are summarized in the following figure. Solid lines represent pathways supported in this study, while dashed lines represent pathways inferred from existing literature. Each line-drawn pathway terminates at a unique result supported by evidence from the current study.
A good place to start this investigation would be to assess cellular ATP levels to determine if they are sufficient after A23187 administration to sustain the ATP-dependent mitochondrial AAA peptidases. Further understanding of mitochondrial dynamics as they affect mitochondrial function would also be critical in our current understanding of MPI. It is plausible that the dynamic changes observed in mitochondrial morphology induced by A23187 reflect a mitochondrial membrane re-organization that modulates the MPI machinery and/or the mechanisms that regulate intramitochondrial protein degradation.

4.3.3 Conclusion

Taken together, the current study demonstrates that, for the first time that changes in intracellular Ca\(^{2+}\) can alter the import of nuclear encoded mitochondrial proteins in the mitochondria of neurons. Increased Ca\(^{2+}\) has multiple effects on mitochondrial signals and mitochondrial proteins. It builds upon previous evidence established regarding the KCl induced up regulation of MPI, while adding novel information regarding the effects of increased Ca\(^{2+}\) on mitochondrial morphology, intramitochondrial protein degradation and extending our work on MPI to primary cortical neurons. These results indicate that MPI is labile and may be altered in response to neuronal activity. While we are only scratching the surface regarding our understanding of MPI in neurons, research such as this carries important implications regarding the day-to-day functionality of the mitochondria and multiple neurodegenerative diseases associated with mitochondrial dysfunction.
5.0 APPENDIX
Appendix Figure 1: A23187 (24h on/ off) and Ionomycin (24h on/ off) increased mtGFP in mitochondria of PC12 cells.

Flow cytometry shows that exposure to varying concentration of ionomycin (green bars) showed similar increases in mtGFP as previous A23187 treatments (blue bars) versus respective DMSO controls. Values expressed as mean ± SEM. N= 1 trial experiment, 3 samples/ experiment, 10 000 cells per sample.
Appendix Figure 2: Acute mitochondrial shape change did not modify mitochondrial mass.

Flow cytometry. (A) Mitochondrial shape change induced by high-dose 10uM or 20uM CCCP (1h) did not drastically modify mitotracker green fluorescence in PC12 cells. (B) Mitochondrial shape change induced by high-dose 10uM or 20uM CCCP (1h) did not drastically modify mtGFP fluorescence in PC12 cells. Values expressed as mean ± SEM. N=1 trial experiment for A and B, 3 samples/ experiment, 10 000 cells per sample.
Appendix Figure 3: Protein ug loading control. Sample immunoblot and quantification of Gapdh.

Western blot. (A) Representative immunoblot of cell lysates probed with anti-gapdh antibody, then an HRP-linked secondary antibody. (B) Histogram of quantified protein to demonstrates linear relationship between ug amount of protein loading and luminescent signal captured by x-ray film. Values expressed as optical density units / mm².
6.0 REFERENCES


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