MOLECULAR AND CELLULAR INSIGHTS INTO MITOCHONDRIAL CONTRIBUTIONS TO NEURONAL AUTOPHAGY: LINKS TO ENERGETICS AND MITOPHAGY

By

Yea Seul Shin

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Florey Institute of Neuroscience and Mental Health
Department of Anatomy and Neuroscience
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne

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ABSTRACT

Neurones are essential for brain homeostasis and as highly metabolic cells rely on mitochondrial oxidative phosphorylation (OXPHOS) for energy. The integrity and functionality of mitochondria are critical for neuronal survival, and the involvement of dysfunctional mitochondria is recognized as a common theme amongst various neuropathologies. New evidence has suggested that the inappropriate clearance of dysfunctional mitochondria via autophagy (termed mitophagy) determines the pathogenesis of neurodegenerative diseases such as Parkinson’s disease. Mechanistic studies of mitophagy have been undertaken using mammalian cell lines, but this research lacks relevance to neuropathologies. This thesis investigates triggers of autophagy/mitophagy in primary neurones, and specifically if disruption of mitochondrial bioenergetics triggers neuronal autophagy, and mitophagy in particular.

Cultures of primary cerebellar granule cells (CGCs) were utilized and inhibitors of the OXPHOS complexes (rotenone, 3-Nitropropionic acid, antimycin A, potassium cyanide and oligomycin targeting complex I-V, respectively), were employed to induce bioenergetic dysfunction of mitochondria. Initial investigations using MTT cell viability assay, phase contrast microscopy and cellular membrane permeabilization detected by propidium iodide staining, determined appropriate concentrations of OXPHOS inhibitors which induced effective mitochondrial damage producing slow neuronal degeneration. From this baseline adverse effects of OXPHOS inhibitors on mitochondrial bioenergetics were documented by monitoring reductions in cellular ATP level, mitochondrial membrane potential ($\Delta \Psi_m$) and oxygen consumption rate (OCR) of CGCs. $\Delta \Psi_m$ was rapidly dissipated in CGCs exposed to the inhibitors of complexes I, III and IV (rotenone, antimycin A and potassium cyanide, respectively), whilst the inhibitor of complex II, 3-Nitropropionic acid, produced a much slower reduction of $\Delta \Psi_m$. Employing Seahorse XF24 technology allowed an incisive readout of mitochondrial functional changes where significant bioenergetic impairment was observed subsequent to inhibition of complexes I and II, which are core components of energy metabolism regulating the redox balance (NAD$^+$/NADH levels) and TCA cycle.
Existent evidence indicates depolarization of $ΔΨ_m$ triggers mitophagy in mammalian cell lines, however CGCs display contrasting results where $ΔΨ_m$ depolarization via complex III and IV inhibition was insufficient to elicit mitophagy despite the inducer of mitophagy, PINK1, being mobilized to mitochondria. In contrast, inhibition of complexes I and II induced mitophagy, as indicated by PINK1 mobilization and disappearance of the pH-sensitive fluorescence mitophagy reporter, mt-Rosella. Western immunoblotting of the general autophagy marker, LC3, and monitoring of acidic vesicles with monodansylcadaverine revealed activation of autophagic flux in CGCs exposed to inhibitors of complexes I-IV, indicating general autophagy in response to bioenergetic impairment irrespective of mitophagy induction.

Results presented herein reveal the complexity of neuronal mitophagy and that $ΔΨ_m$ may not be a necessary trigger of neuronal mitophagy. Thus inhibition of individual respiratory complexes, and notably complexes I and II, may underlie the triggering of mitophagy in primary neurones, where different mechanisms induce mitophagy in neurones compared to immortalized cell lines. This difference may be due to the unique bioenergetic dependence of neurones. Understanding the mechanisms of mitophagy and autophagy in primary neurones provides valuable insights into therapeutic approaches for neurodegenerative diseases.
Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the PhD except where indicated,

(ii) due acknowledgement has been made in the text to all other material used,

(iii) the thesis is less than 100,000 words in length, exclusive of tables, figure legends, bibliography and appendices.

______________________________

Yea Seul Shin
Publications

During the course of my studies the following manuscripts have been published. Publication which is directly related to the work described in this thesis is denoted by *.


Abstracts


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Yea Seul Shin
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<tbody>
<tr>
<td>3-NP</td>
<td>3-Nitropropionic acid</td>
</tr>
<tr>
<td>AA</td>
<td>Antimycin A</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>ACD</td>
<td>Autophagic cell death</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionnic acid</td>
</tr>
<tr>
<td>AMPK</td>
<td>5'-Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy-related genes</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CGC</td>
<td>Cerebellar granule cells</td>
</tr>
<tr>
<td>CN</td>
<td>Cyanide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>EndoG</td>
<td>Endonucleus G</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FIP200</td>
<td>FAK-family interacting protein of 200 kDa</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>Glu</td>
<td>L-glutamate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H⁺</td>
<td>Protons</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>Htt</td>
<td>Huntingtin protein</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMPase</td>
<td>Inositol monophosphate</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule associated protein 1 light chain 3</td>
</tr>
<tr>
<td>LY294002</td>
<td>2-(4-morpholiny)-8-phenylchromone</td>
</tr>
<tr>
<td>MDC</td>
<td>Monodansylcadaverine</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>Mfn1</td>
<td>Mitofusin 1</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Rapamycin-sensitive mTOR complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Rapamycin-insensitive mTOR complex 2</td>
</tr>
<tr>
<td>NADH/NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NBM</td>
<td>Neurobasal medium</td>
</tr>
<tr>
<td>NDP52</td>
<td>Nuclear dot protein 52 kDa</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OMMP</td>
<td>Outer mitochondrial membrane permeabilization</td>
</tr>
<tr>
<td>Omp25</td>
<td>Outer membrane protein 25</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>OPTN</td>
<td>Optineurin</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>Oli</td>
<td>Oligomycin</td>
</tr>
<tr>
<td>OSCP</td>
<td>Oligomycin-sensitive conferring protein</td>
</tr>
<tr>
<td>PARL</td>
<td>Presenilin-associated rhomboid-like protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor-γ coactivator 1α</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PI3K-III</td>
<td>Class III phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PINK1</td>
<td>Phosphatase and tensin homolog (PTEN) induced putative kinase 1</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RAP</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>Rag GTPases</td>
<td>Ras-related GTPase</td>
</tr>
<tr>
<td>RHEB</td>
<td>Ras homolog enriched in brain</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Rot</td>
<td>Rotenone</td>
</tr>
<tr>
<td>secs</td>
<td>Seconds</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Sequestosome-1</td>
</tr>
<tr>
<td>STS</td>
<td>Stauroporine</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR-DNA binding protein</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
</tr>
<tr>
<td>TIM23</td>
<td>Translocase of the inner membrane 23</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51-like kinase 1</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-protease system</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage-dependent anion channel 1</td>
</tr>
</tbody>
</table>
CHAPTER ONE

General Introduction
1.1. Overview: Reflections on Mitochondria, Neurones, Quality Control and Responses to Cellular Stress

Purpose of Overview

This thesis is concerned with the relationship between mitochondrial bioenergetic insufficiency in mammalian neurones and the intracellular disposal of dysfunctional mitochondria. This topic of research brings together several strands of individual research themes in biochemistry, cell biology and neuroscience. Therefore, it is appropriate to provide a structured literature review and critical analysis of relevant research, on which the present project is based. In this preliminary overview section, I present a brief summary of the main themes pertinent to the development of my research project, without extensive literature referencing, aside from some books and major review articles that cover these broad topics extensively. The overview thus serves to introduce and to contextualise main themes, as a prelude to the more detailed considerations of individual topics that lead more directly to the research issues of my thesis.

1.1.1. Mitochondria: Major Players in Bioenergetics and Cell Biology

For many years, mitochondria were best known as the organelles of cellular ATP production by the process of oxidative phosphorylation. The double-membrane organization at the periphery of each mitochondrion, together with the invaginations of the inner mitochondrial membrane (IMM) to form the cristae, represents an almost iconic
image of these organelles (Alberts et al., 2002) (Fig. 1.1A, B). The enzymes of the respiratory chain and ATP synthase are embedded on the inner membrane. These, together with a host of soluble enzymes of the tricarboxylic acid cycle (TCA cycle) in the mitochondrial matrix space and as well as their substrates and products, provide the biochemical machinery that enables the energy derived from catabolism of sugars and fatty acids to be harnessed for the production of ATP from ADP and inorganic phosphate. Concurrent with the latter process, molecular oxygen is used to re-oxidise reduced adenine nucleotides (NADH) and flavin nucleotides (FADH$_2$) via the electron transport chain. These striking energy transformations, which utilise chemiosmotic principles, have at their core the energised IMM, which carries a voltage potential across it ($\Delta V_m$), as well as generating a proton motive force that drives ATP synthesis (Alberts et al., 2002) (Fig. 1.2).

Mitochondria also contain a host of metabolic pathways, some of which are general to mitochondria in all tissues (e.g. TCA cycle and fatty acid oxidation), while others are found more prominently in specialised tissues (e.g. some aspects of sterol biosynthesis). Further, there are various types of transport processes that enable metabolites to be transported into and out of mitochondria. Some of these involve active transporters in the inner membrane; others require elaborate metabolic shuttle pathways. Ion transport is another important aspect of mitochondrial function in cells, specifically across the inner membrane. The IMM has very strict inherent permeability barriers (including, of course, to protons (H$^+$)), unlike the much more porous outer mitochondrial membrane (OMM). These ion transport functions of the IMM are not only intimately involved in the process of oxidative phosphorylation (OXPHOS) (e.g. translocation of H$^+$ and phosphate ions), but also embrace transport of ions, such as those of iron and calcium. Such ions are involved in various metabolic processes and cellular regulation and signal transduction, more broadly. Mitochondria, as represented in the electron micrograph of Fig. 1.1A, were initially thought to be a population of discrete particles inhabiting the cytosol of the cell. More recent studies have revealed a complex process of dynamic changes of mitochondrial morphology in living cells. Thus, mitochondria are recognised to undergo fusion or fission processes that proceed continuously in cells. Fission leads to the formation of elongated mitochondrial filaments traversing relatively long distances in the cytosol; fission is the breakdown of such structures, generating more discrete organellar particles (Westermann, 2008). Such mitochondrial dynamics also include the gross
Figure 1.1. Ultrastructure and schematic representation of mitochondria

(A) Ultrastructure of mitochondrion visualized by transmission electron microscopy. (B) Mitochondrion consists of double membranes, inner and outer membrane, which separate the mitochondrial matrix and intermembrane space (adapted from Alberts et al., 2002).
Figure 1.2. Summary of biochemical and metabolic machinery involved in generation of energy

Food molecules from cytosol are processed via enzymes in the mitochondrial matrix and citric acid cycle (or TCA cycle), from which redox cofactors such as NADH are generated. Electrons from NADH are passed through the electron transport chain, eventually leading to reduction of oxygen and pumping of protons (H+) into the inter mitochondrial space (space between inner and outer mitochondrial membrane). Due to the accumulation of H+ in the intermembrane space, pH and voltage potential (or mitochondrial membrane potential, $\Delta \psi_m$) are formed across the inner membrane. $\Delta \psi_m$ drives translocation of H+ into the matrix while ADP is phosphorylated to form ATP via ATP synthase (adapted from Alberts et al., 2002).
intracellular displacement (or transport) of individual mitochondrial entities, which is an important feature of mitochondria in neurones (as discussed below). Indeed, the interaction of mitochondria with cytoskeletal elements, particularly but not exclusively microtubules, is an important aspect of mitochondrial localization and mobilization in cells. In this context, it is important to note the frequent association of mitochondria with membranes of the endoplasmic reticulum, which may have functional significance, for example Ca$^{2+}$ handling in cells.

Given the abovementioned contributions that mitochondria make to the function and homeostasis of cells, a completely unexpected aspect of these organelles was the recognition in the mid 1990’s that mitochondria play a decisive role in the apoptotic pathway leading to cell death. This role came about with the discovery that cytochrome c, a key protein of the respiratory chain, was able to trigger the activation of caspases in the cytosol. Further, other proteins of the intermembrane space (IMS) between the IMM and OMM were found to participate in other aspects of the induction or maintenance of caspase activation leading to apoptosis, or also known as programmed cell death (PCD)-Type I. Indeed, studies of the mitochondrial role in cell death revealed that many members of the Bcl-2 family are involved in the outer mitochondrial membrane permeabilization (OMMP) that leads to the migration of IMS proteins into the cytosol (and even in the reverse direction), some of which are involved in cell death signalling (Smith et al., 2008). Indeed mitochondria contribute to both caspase-dependent and -independent PCD (Galluzzi et al., 2012; Higgins et al., 2010; Nagley et al., 2010). The injury-dependent signalling pathways downstream of mitochondria may crosstalk and determine the adoption of one or more death pathways, namely PCD-Type I apoptosis, -Type II autophagic cell death or -Type III programmed necrosis (Nagley et al., 2010).

An overview of mitochondria would not be complete without brief mention of the evolutionary origin and present-day biogenesis of these organelles. Mitochondria are derived in an evolutionary sense from endosymbiotic bacteria that inhabited the cytosol of the earliest primitive eukaryotic cells (Dyall et al., 2004). Over billions of years, these endosymbionts gave up substantial portions of their prokaryotic genomes to the nuclear chromosomes of the host cells by some sort of intracellular gene transfer. Thus, the mitochondria of the higher eukaryotes of today, including mammals, have relatively
small genomes, with the bulk of the proteins of mitochondria being encoded in nuclear DNA. The residual genes in mitochondrial DNA (mtDNA) of humans and other mammals encode 13 proteins of the respiratory chain and ATP synthase; all such proteins embedded are in the IMM. The vast majority (approx. 99%) of the one thousand or so proteins of mitochondria are imported from the cytosol, following their biosynthesis on ribosomes that translate mRNAs specified by nuclear genes. The import process has many distinctive features of protein trafficking and sorting to the various compartments of the organelle (e.g. matrix, IMM, IMS and OMM). For its part, mtDNA also encodes two rRNA species that are part of mitochondrial ribosomes plus 22 mitochondrial tRNA species that are part of the intrinsic mitochondrial protein synthesising system. This mitochondrial protein biosynthetic system translates the 13 mRNAs encoded by mtDNA for production of the abovementioned proteins of the respiratory chain and ATP synthase. Strikingly it still retains some features (e.g. sensitivity to antibiotics in particular) of the primitive bacterial systems from which it evolved.

These considerations led to the recognition in the 1980’s that some diseases in humans arose from mutations in mtDNA, paralleled the much longer known respiratory mutants in lower eukaryotes such as yeast discovered more than 30 years before. These human mitochondrial diseases predominantly affected tissues of high energy demand such as nerves or muscles, but could also affect endocrine or sensory tissues leading to diabetes, blindness or deafness (Wallace, 1992). However, not all diseases with explicit mitochondrial structural or functional defects arise from mtDNA mutations: many are caused by changes to nuclear genes affecting mitochondrial proteins directly or proteins involved in the assembly of mitochondrial respiratory chain complexes (Shoubridge, 2001). Further, many neurodegenerative diseases not strictly classified as mitochondrial diseases, as such, are known to have their aetiology and progression strongly influenced by mitochondrial processes in the affected cells. These include Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease (HD) and amyotrophic lateral sclerosis (ALS) (Beal, 2005), expanded on in a later section of this overview.

Finally it is pertinent to comment on how mitochondria may be subjected to quality control both under physiological and pathological conditions. These processes of mitochondrial turnover during natural cell development and function, as well as during
the response of cells to trauma or insult, involve controlled proteolysis on one hand, and sequestration by the machinery of autophagy, on the other. These aspects are dealt with a little more fully in a subsequent section of the Overview below (section 1.1.3.1). Before overviewing those aspects, it is useful to introduce the special features of mitochondria in neuronal cells.

1.1.2. Special Features of Mitochondria in Neurones

Brain function in humans and animals is well known to need a continuous supply of oxygen. Neurones are the building blocks of the nervous system and possess unique phenotypic and bioenergetic features. These metabolically active cells have high energy demands and consume 20% of all oxygen and 25% of glucose in resting human body where the brain only accounts for 2% of the body weight (Silver & Erecinska, 1998). Unlike other types of cells, most neurones lack the ability to store glycogen (Obel et al., 2012) and those in the vertebrate brain almost exclusively rely on complete oxidation of glucose via mitochondrial OXPHOS for energy supply.

This absolute reliance of neurones on mitochondrial OXPHOS is reflected by the relative distribution of mitochondria in neurones (Van Laar & Berman, 2013). The morphologies of neurones vary in size and shape according to their specific roles and functions in the brain. Their specialized structures can be broadly divided into three parts, namely dendrites, cell body and axon. The specialized morphology of neurones reflects the functional heterogeneity in each segment of neurones, and these specialized structures are designed to communicate with the surrounding neurones by transmitting and receiving information via electrochemical signalling mechanisms. The excitability of the neuronal membrane allows neurones to transmit action potentials along the axons to the presynaptic areas of axon endings where the electrical signal is translated into chemical information in the form of neurotransmitters. The release of neurotransmitters at synapses leads to the subsequent active interaction of neurotransmitters at the postsynaptic area of adjoining neurone, where the signal is received and passed on to the downstream neurone.
in electrical pulses. This electrochemical communication process accounts for the high metabolic demand of neurones, as there is high energy requirement for constant maintenance and restoration of the ionic gradient across the neuronal membrane, as well as for uptake and recycling of neurotransmitters (Alle et al., 2009; Attwell & Laughlin, 2001). Furthermore, mitochondrial uptake of $\text{Ca}^{2+}$ into the mitochondrial matrix as part of regulating cytosolic $\text{Ca}^{2+}$ level also contributes to brain energy demand. The highly polarized structure of a neurone shows functional heterogeneity between neuronal sub-compartments, which exhibit different energy requirements according to their specific functions. The correlation between the differential distributions of mitochondria in sub-compartments of neurones in adaptation to the local energy requirement can be observed (Amadoro et al., 2014), emphasizing the critical role of mitochondria as the primary source of ATP for neurones. The local mitochondrial density is especially high at structures of neurones such as growth cones, presynaptic and postsynaptic endings, as well as nodes of Ranvier. In all these locations mitochondrial function is essentially important in maintaining important neuronal activities.

In order to meet differential energy demands at specific sites of neurones, mitochondria are found to be transported to the parts of neurones with high energy demand (Reynolds & Rintoul, 2004). Mitochondria are synthesized in the cell body and many are distributed via translocation to the other parts of the neurone. For example, translocation of mitochondria takes place along axonal microtubules in anterograde and retrograde directions, via kinesin motor proteins and cytoplasmic dynein, respectively. For this process, assembly and association of motor proteins initially occurs in the cell body, enabling mitochondria to be transported along the axon to meet cellular energy demand (Reynolds & Rintoul, 2004). Interestingly, metabolically inactive and degenerating mitochondria with lower membrane potential have been encountered in the distal axons of the neural tips of primary neurones (Barrientos et al., 2011). Such “disabled” mitochondria could have one of two fates. First, they show a tendency to be transported back to the soma in retrograde direction. Second, they could be engulfed by autophagosomes, in a process termed “mitophagy” (to be discussed below). The mitochondrial membrane potential was found to be an important factor in mitochondrial transportation along the axon, where mitochondria with higher potential are transported towards the growth cones, whereas those with lower membrane potential are transported back to the soma in retrograde manner (Miller & Sheetz, 2004).
Thus a high correlation between mitochondrial defects and neurodegenerative diseases would not be surprising because of the high dependency of neurones on mitochondrial oxygen supply and function. In particular, dysfunctional mitochondrial respiratory chain complexes were highlighted in previous studies involving post-mortem brain tissues of patients and animal models of PD and HD which showed severe deficiency in the mitochondrial respiratory chain complexes I, II and III (Gu et al., 1996; Schapira et al., 1990). Additionally, impaired mitochondrial function was linked to other neurological diseases such as in AD and ALS, in which the dysfunction of complex IV of the respiratory chain and loss of mitochondrial membrane potential, as well as an elevation of level of cytosolic Ca\(^{2+}\), have been reported, respectively (Beal, 2000). As the efficiency of mitochondrial electron transport through respiratory complexes may decrease with ageing, neurones in ageing brain face an increased susceptibility towards oxidative damage by an increased production of reactive oxygen species (ROS). ROS produced by dysfunctional mitochondria can induce further damage to mitochondrial protein homeostasis, which engenders a vicious cycle of further damage.

Given the important roles of mitochondria combined with the post-mitotic and non-proliferating nature of neurones, damaged mitochondria need to be cleared efficiently in order to maintain a healthy pool of mitochondria. This consideration emphasises the importance of mitochondrial quality control.

1.1.3. Mitochondrial Surveillance System: Quality Control of Dysfunctional Mitochondria

Thus far, this overview has outlined the crucial functions of mitochondria in neurones and how neurones have an absolute reliance on the correct function of mitochondria, which is especially true at synaptic termini where there is a high demand
for a constant supply of energy and buffering of Ca$^{2+}$ due to ongoing regulation of neurotransmitters. Therefore, it is not surprising to find impaired mitochondrial function in neurones threatened by advancing neurodegenerative disease; furthermore, failure to clear damaged mitochondria has been linked to aetiology of neurodegenerative diseases (Chen & Chan, 2009). In order to survey and maintain mitochondrial homeostasis, cells are known to employ several surveillance mechanisms at the molecular, organellar and cellular levels.

Mitochondrial damage arises from proteotoxic stresses such as aggregation of unfolded proteins, presence of misfolded proteins or bioenergetic stresses such as loss of $\Delta \Psi_m$. As mentioned previously, the mitochondrial genome only holds a few genes coding for respiratory complexes while the majority of mitochondrial protein content is encoded by nuclei. The nuclei-encoded mitochondrial protein precursors are imported into the mitochondria in a relatively unfolded state through narrow pores of tightly regulated translocase of the outer membrane, the TOM complexes (Kovermann et al., 2002; Rehling et al., 2003). The intra-organellar mitochondrial quality control machineries such as chaperone families, Hsp60, Hsp70 and Hsp78, and proteases assist maturation of precursors to ensure correct folding, assembling into multimeric complexes, and positioning within mitochondria. These proteins also participate in refolding or degradation of misfolded and unfolded proteins at a molecular level of quality control. The proteases also take part in protein quality control by degrading proteins with destabilizing mutations and oxidative modifications that are beyond repair. In addition, a substrate of AAA+ proteases, ClpXP, was suggested to assist in inducing mitochondrial specific unfolded protein response and promote activation of transcription of nuclear encoded mitochondrial chaperone genes to strengthen mitochondrial protein homeostasis and to attenuate accumulation of misfolded proteins in mitochondria (Haynes et al., 2007; Haynes & Ron, 2010; Livnat-Levanon & Glickman, 2011). The ubiquitin-protease system (UPS) also plays a part in turning over of short lived proteins, which are retro-translocated to the OMM to be ubiquitinated and degraded by proteasome, which is a complex protein digestion machinery (Ciechanover et al., 2000; Margineantu et al., 2007). In addition, production of ROS as a by-product during respiration of mitochondria or due to damage in respiratory chain complexes also adds to the proteomic damage. Such deleterious effects of ROS are countered by the antioxidants present within mitochondria, such as glutathione.
The dynamic nature of mitochondria offers another aspect of mitochondrial quality control, where mitochondria constantly undergo fusion in a way to mix and dilute damaged mitochondrial content, such as mutated mitochondrial DNA or misfolded proteins, to the neighbouring healthy mitochondria to reduce the damage. Mitochondrial fusion provides a significant mitochondrial quality control for post-mitotic neurones as neurones are unable to divide to dilute out affected mitochondria between cells. Furthermore, neurones can become susceptible by accumulating damaged mitochondria, whereas other mitotic tissues can divide and dilute affected mitochondria to decrease the magnitude of damage on cells. Once the mitochondrial damage becomes too extreme and causes dissipation of $\Delta \Psi_m$, damaged parts of mitochondria are inhibited to fuse with the adjacent healthy mitochondria. The segregation of dysfunctional mitochondria from the rest of the mitochondrial network limits the damage and allows selective turnover by a lysosome-dependent selective degradation process called autophagy (Mizushima, 2009; Wang & Klionsky, 2011; Youle & Narendra, 2011). Abovementioned functions of mitochondrial quality control are reviewed more extensively elsewhere (Baker et al., 2014), and this thesis will more focus on the autophagic removal of damaged mitochondria as a means of maintaining the integrity and functionality of mitochondria.

1.1.3.1. Selective Autophagy of Mitochondria

Autophagy is now recognized as the cellular housekeeping process that allows lysosomal dependent degradation of cytoplasmic contents. The primary function of autophagy is a non-selective degradation of cytoplasmic components to remove damaged organelles and dysfunctional proteins from the cell, thus maintaining functional homeostasis of the cell. Autophagy can additionally be recruited in response to starvation and degrade proteins to provide amino acids as substrates for production of energy. Interestingly, autophagy can also be associated with cell death, as well as with cell maintenance or rejuvenation. Thus, in type II programmed cell death dying cells exhibit typical morphologies of autophagy; further, autophagy is required for such death (Nagley et al., 2010). Whether autophagy is protective or a cellular death mechanism depends on the cell type, the nature and extent of injury and other parallel cellular responses (Yue et
al., 2009). In a number of neurodegenerative diseases, decreased autophagic function is evident, which may be associated with accumulation of misfolded proteins. In this context, autophagy has a protective mechanism degrading misfolded proteins within the cell (Rubinsztein, 2006; Rubinsztein et al., 2007).

Lastly, depending on the types of molecular involvement previous to fusion into lysosomes, processes of autophagy are categorized into three major subtypes, microautophagy, chaperone-mediated autophagy and macroautophagy. Among the three subtypes of autophagy, chaperone-mediated autophagy is well recognized as a highly specialized form of autophagy which degrades certain misfolded proteins (e.g. α-synuclein) by chaperone-mediated guidance to the lysosome. On the other hand, macroautophagy deals with both selective and non-selective bulk degradation of damaged organelles such as mitochondria and ubiquitinated proteins with a long half-life (Levine & Klionsky, 2004). This selective form of autophagy allows degradation of selective proteins or organelles such as peroxisomes, endoplasmic reticulum, ribosomes, the nucleus, intracellular pathogens, protein aggregates, lipid droplets, and mitochondria. The majority of the consideration in this thesis deals with specific degradation of mitochondria via macroautophagy, or also known as mitophagy, as will be discussed in more detail in Literature Review section 1.5.4.
1.2. Outline of Thesis Project and Specific Focal Points of Chapter 1

The overall goal of this project is to elucidate the relationship between mitochondrial bioenergetic disruption and the activation of mitophagy, as well more general autophagy. The specific aims are presented at the end of this chapter.

The topics to be covered by more detailed review and where appropriate, critical analysis, are those most relevant to the thesis topic and goals of the research. These will include:

2. Involvement of dysfunctional mitochondria in neurodegenerative diseases
3. General review on autophagy.
4. Regulation of mitophagy in mammalian cells, particularly in neurones.

Other topics, not directly relevant to the thesis are dealt with in less detail and not reviewed exhaustively, and they include, cell death, mitochondrial diseases, and other forms of autophagy.
1.3. Neuronal Mitochondria: Physiological Function

The central nervous system (CNS) has the highest energy demand of body organs as it consumes about 20% of oxygen inspired at rest, although it consists of only 2% of the body weight (Silver & Erecinska, 1998). Neurones are largely responsible for this extraordinary metabolic rate of the CNS, as the brain uses approximately 80% of energy to support events associated with neuronal firing, including action potential propagation, maintenance of membrane potentials, and release and uptake cycling processes of the major neurotransmitters GABA and L-glutamate (Glu) (Astrup et al., 1981; Shulman et al., 2004). Neurones meet most of their energy demands through mitochondrial OXPHOS, as they have limited glycolytic capacity (Ames, 2000; Erecinska et al., 2004; Mayevsky & Chance, 1975; Obel et al., 2012). Mitochondria are synthesized in neuronal cell bodies and the highest density of mitochondria is known to be distributed in perinuclear regions (Collins et al., 2002). However, neurones face a difficult challenge in providing sufficient energy, as they are highly compartmentalized cells stretching over long distances. Neuronal segments distal from the cell body, such as dendrites and axons, also have high energy demand linked to their specific functional roles in neurotransmission and especially the generation of action potentials. In order to meet their energy requirement, mitochondria display a heterogeneous distribution in different parts of neurones, presumably reflecting differences in metabolic demands (Collins et al., 2002). Distribution of mitochondria is also affected by the activity-dependent regulation of neuronal mitochondrial trafficking, where mitochondrial motility is greatly reduced at sites of high electrical activities (Ohno et al., 2011; Zhang et al., 2010). Highly energized mitochondria were observed to be present at greater density at the periphery of neurones where events involving Ca$^{2+}$ and neurotransmission are substantially localized (Collins et
al., 2002). The functional status and heterogeneous distribution of mitochondria in neurons show that mitochondria meet the diverse energy demands of neurons, and that it is critical to maintain homeostasis of mitochondria for neuronal survival. Thus, it is important to consider the important functional roles of mitochondria.

Mitochondria are cellular organelles which are present in the vast majority of eukaryotic cells (Scheffler, 2001). Although mitochondria vary in shape and sizes across different types of cells, they have the same principal organization in their structures. The two membranes of mitochondria, OMM and IMM, compartmentalize mitochondria such that they possess an IMS between the two membranes, with the mitochondrial matrix located in the innermost area of mitochondria (Polyakov et al., 2003; Scheffler, 2001). Protein constituents in the matrix differ from those in the IMS. OMM and IMM have contrasting permeability characteristics, the IMM only allows oxygen, carbon dioxide and water molecules to freely permeate across the membrane, but permeability of small molecules and ions, such as H\(^+\), is limited compared to the OMM (Lemasters, 2007). The IMM is, therefore, equipped with specific transporters and exchangers such as H\(^+\) pumps and adenine nucleotide transporter, which exchanges ATP for ADP, to allow specific transport of molecules across that membrane. Such permeability characteristics of IMM are important for establishing the electrochemical gradient across the membrane for ATP synthesis via OXPHOS. The highly invaginated structures of the IMM are also known as cristae and they serve to significantly increase the total surface area for metabolic involvement, providing greater capacity for ATP generation via electron transport chain (ETC) (Polyakov et al., 2003). Many of the metabolic pathways occur within the mitochondrial matrix, such as the TCA cycle, the urea cycle, fatty acid oxidation, biosynthesis of haem and others (Beal, 2005; Howell et al., 2003; Scheffler, 1999). Mitochondria also play an important role in buffering and tightly modulating cytosolic Ca\(^{2+}\), which is an important regulator of key enzymes in the cell and also involved in neurotransmitter release (Babcock et al., 1997). Furthermore, disruption of intracellular Ca\(^{2+}\) regulation leads to cell death, as in L-Glu-mediated excitotoxicity (Scheffler, 2001).

As much as mitochondria are involved in neuronal homeostasis, they also play an equally important role in mediating PCD, as the IMS of mitochondria contains pro-apoptotic factors such as cytochrome c and Smac/DIABLO. Injury to mitochondria can
cause cytochrome c release from the mitochondrial IMS into cytosol, which then aids in forming the apoptosome by binding to Apaf1 and pro-caspase 9. The apoptosome activates downstream caspases, which cleave cellular components and eventually induce cell death (Danial & Korsmeyer, 2004; Nagley et al., 2010). Other apoptogenic proteins involved in programmed necrosis (recently defined as caspase-independent apoptosis (Galluzzi et al., 2012), such as endonuclease G (EndoG) and apoptosis-inducing factor (AIF) are embedded at the IMM and are released to induce caspase-independent chromosomal DNA cleavage termed generically PCD (Nagley et al., 2010; Smith et al., 2008). Moreover, dysfunction of mitochondrial energy metabolism can lead to neuronal death due to reduced production of ATP and the generation of ROS as the by-products of mitochondrial respiration. ROS can cause oxidative damage to mitochondrial components such as crucial enzymes and mitochondrial DNA (Beal, 2005). Given the importance of the physiological roles of mitochondria, it is hardly surprising to see numerous neurological diseases caused by a disruption of mitochondrial function or mutations of mitochondrial DNA (Beal, 2005; Chan, 2006). In summary, the brain function is highly dependent on mitochondria as the main source of energy via OXPHOS, and dysfunction of energy supply as well as other metabolic functions of mitochondria could play a number of pathological roles in neurones (Beal, 2005).

1.3.1. Energy Production via Mitochondrial OXPHOS

Mitochondrial OXPHOS is a major cellular source of ATP. ATP is produced via OXPHOS as a result of serial redox reactions across the respiratory chain complexes I-IV, also known as the ETC, where electron transfer is coupled to pumping of H⁺ to allow a formation of electrochemical gradient across the IMM to drive phosphorylation of ADP into ATP at F₁F₀ ATP synthase, complex V (Larsson, 2010) (Fig. 1.3). The ETC consists of four multi-subunit redox-enzymes, complex I (NADH ubiquinone oxidoreductase), complex II (succinate ubiquinone oxidoreductase), complex III (ubiquinone-cytochrome c reductase), and complex IV (cytochrome oxidase). Additionally there is the complex V, F₁F₀ ATP synthase. The ETC is embedded in the IMM at which the charge and chemical.
Figure 1.3. Five enzyme complexes of mammalian oxidative phosphorylation system

Complexes I–IV constitute the electron transport chain, and the addition of complex V (ATP synthase) completes the oxidative phosphorylation system (adapted from Larsson, 2010).
potential are established due to the separation of charged ions between mitochondrial matrix and IMS. OXPHOS takes advantage of this property in producing ATP.

The four ETC enzyme complexes are complemented with prosthetic groups and each enzyme catalyses independent redox reactions to allow transfer of electrons to oxygen via electron carriers, ubiquinone and cytochrome c. The OXPHOS begins with a transfer of electrons from oxidation of NADH and succinate at complex I and II, respectively. Ubiquinone, which resides within the membrane lipid bilayer, shuttles electrons from complex I or II to complex III. Complex III is reduced and electrons are passed on to complex IV via cytochrome c, where oxygen molecules become the final acceptor of electrons and H$_2$O is produced as a result (Scheffler, 1999). Transfer of electrons through the ETC is coupled to pumping of positively charged H$^+$ at complex I, III and IV from mitochondrial matrix into the IMS, giving rise to charge and concentration differences across the impermeable IMM (Nicholls & Ferguson, 2004). Consequently, a potential called the proton motive force is established across the IMM, which is comprised of two components, the electrical gradient also known as the mitochondrial membrane potential ($\Delta \Psi_m$), and the pH gradient. The majority (up to 80 %) of the proton motive force is comprised of the $\Delta \Psi_m$ component (Murphy et al., 1999; Nicholls, 2002), hence $\Delta \Psi_m$ is considered the key parameter of mitochondrial function and bioenergetics, and also as one of the main critical determinants of neuronal survival. The electrochemical potential drives H$^+$ translocation down the gradient from the IMS into the matrix through complex V, the ATP synthase, thereby allowing phosphorylation of ADP into ATP (Kwong et al., 2006; Nicholls, 2002).
1.3.2. Mitochondrial Electron Transport Chain Components

1.3.2.1. Complex I NADH:Ubiquinone Oxidoreductase/NADH dehydrogenase

Complex I serves as the first entry step feeding electrons into the electron transport chain of mitochondria. It is by far the largest enzyme of the respiratory chain and its structure was determined in the late 1990s (Friedrich & Bottcher, 2004; Grigorieff, 1999; Guenebaut et al., 1998). This 44 subunit enzyme can be functionally divided into three different domains; a dehydrogenase unit, a hydrogenase-like unit and a transporter unit (Lenaz & Genova, 2010). The low resolution structure of the complex from electron microscopy revealed an L-shaped structure where the dehydrogenase and hydrogenase-like domains form a short arm protruding into the matrix, while the transporter unit is deeply integrated into the IMM (Lenaz & Genova, 2010), forming a long arm of the complex. The prosthetic groups such as flavin mononucleotide (FMN) and iron-sulfur (Fe-S) clusters aid the transfer of electrons within the complex (Lenaz & Genova, 2010).

Complex I is a $\text{H}^+$ pump which is capable of producing $\text{H}^+$ gradient across the IMM by converting the chemical energy of NADH (Santo-Domingo & Demaurex, 2012). NADH, which is derived from multiple reactions of oxidation of nutrients in mitochondria, such as the TCA cycle, is bound and oxidised at the NADH oxidizing site of the dehydrogenase unit where 2 electrons are donated to the primary electron entry point of the complex, FMN. The electrons are transferred along a series of Fe-S clusters and reach the N2 Fe-S cluster, which is located at the interface with the hydrophilic domain and membrane domain (Lenaz & Genova, 2010) (Fig. 1.4). N2 is considered to be the site of ubiquinone binding where electron is directly donated to the mobile electron carrier, ubiquinone (Hinchliffe & Sazanov, 2005). As the electrons pass through the complex to the final destination ubiquinone, the transporter domain allows translocation of $\text{H}^+$ across the membrane, providing almost 40 % of the $\text{H}^+$ flux used for the production of ATP (Brandt, 2006; Sazanov, 2007; Walker, 1992; Yagi & Matsuno-Yagi, 2003). How the transfer of electrons and $\text{H}^+$ pumping are coupled in this complex still remains unclear,
Figure 1.4. Structure of complex I, NADH dehydrogenase

The L-shaped enzyme human complex I has a basic arrangement of the 45 subunits. A schematic representation of the electron pathway from NADH through FMN and the Fe-S clusters (N1 to N6) to ubiquinone (or coenzyme Q (CoQ)) is represented with lines and arrows (adapted from Lenaz & Genova, 2010).
although, the conformational changes of complex I induced by the reduction of ubiquinone is considered to be closely linked to $H^+$ translocation across the IMM via the transporter domain (Baradaran et al., 2013; Brandt, 2006).

Complex I is inhibited by a variety of naturally occurring and synthetic inhibitors which are grouped into three categories; Type A quinone antagonists, Type B semiquinone antagonists and Type C quinol antagonists (Degli Esposti, 1998; Lenaz & Genova, 2010). Such a variety of inhibitors suggests different modes of action or different binding sites of the complex I inhibitors. The pathway of the electron from NADH in complex I involves transfer of two electrons to the final destination ubiquinone via a series of iron-sulfur clusters, where the last iron-sulfur cluster, the N2 centre contains two binding sites for ubiquinone. The sites of ubiquinone binding are the active sites for complex I inhibition, and rotenone (Rot) is the best known inhibitor of complex I (Degli Esposti, 1998) (Table 1.1).

1.3.2.2. Complex II Succinate:Ubiquinone Oxidoreductase/Succinate dehydrogenase

Complex II, also known as succinate dehydrogenase, is part of the mitochondrial respiratory chain but it differs from the rest of the complexes because of its direct linkage via the metabolic TCA cycle to the ETC. Without creating transmembrane $H^+$ gradient, complex II feeds electrons to the ETC by catalysing oxidation of succinate to fumarate as part of the TCA cycle and supplies electrons directly to ubiquinone in the lipid bilayer of the inner membrane to deliver electrons to complex III (Scheffler, 2011).

Complex II has a relatively simple structure and consists of four main units, the succinate dehydrogenase unit and two anchor units, which reside in the IMM. Succinate dehydrogenase consists of a large flavoprotein subunit (Fp), which contains covalently linked FAD, and three non-haem iron-sulfur protein subunits (Ip) on the matrix side of the membrane. The two hydrophobic anchor subunits (chains C and D) in the IMM anchor the whole complex to the IMM (Van Vranken et al., 2015). They also contain b-
type cytochrome and ubiquinone binding site (Lenaz & Genova, 2010; Scheffler, 2011) (Fig. 1.5).

Upon binding of succinate to the Fp subunit, it catalyses oxidation of succinate to fumarate and the resulting two electrons are accepted by covalently bound FAD and transferred through three non-haem Fe-S clusters. Successful conductance of electrons through the Fe-S centres leads to reduction of ubiquinone, which is bound to the two anchor proteins in the IMM. Reduced ubiquinone transfers electrons to complex III for further ETC activity (Lenaz & Genova, 2010). Unlike other complexes, complex II does not couple electron transfer to H⁺ translocation across the membrane, so thus it does not contribute to H⁺ gradient. The absence of H⁺ translocation indicates that although complex II is important for driving OXPHOS by providing electrons to the chain and ultimately reduce oxygen, it does not play a direct role in producing energy via Complex V ATP synthase because it fails to contribute to the electrochemical gradient (Lenaz & Genova, 2010). However, inhibition of complex II by pharmacological agents, such as 3-Nitropropionic acid (3-NP), not only prevents blocks electron transport by inhibiting the feeding of electrons to the ETC but also inhibits a part of TCA cycle of oxidation of succinate to fumarate and causes an overall metabolic dysfunction of cells (Huang et al., 2006) (Table 1.1).

1.3.2.3. Complex III Ubiquinol:Cytochrome Oxidoreductase/Cytochrome bc₁ complex

Complex III goes by two names, cytochrome bc₁ complex according to its structural components, and ubiquinone-cytochrome c oxidoreductase/cytochrome c from its functional role involved in catalysing oxidation of ubiquinol and reduction of cytochrome c. The architecture and topology of the complex was revealed by the groups of Yu, Deisenhofer and colleagues (Xia et al., 1997), who pictured complex III as having two bc₁ monomers forming a homodimeric structure perpendicular to the IMM. Each monomer is constituted from di-haem cytochrome b₅ and b₆, which form the majority of the transmembrane helices, cytochrome c₁ and Rieske Fe-S protein, spanning the complex peripherally (Papa et al., 2012; Raha & Robinson, 2000) (Fig. 1.6A, B).
Table 1.1

Direct inhibition of mitochondrial respiratory chain complexes by site-specific inhibitors and their function. The table shows the name of each agent used, the target binding site and the function primarily affected. The term “site-specific” applies to all agents used here except for CCCP, which has a more generalized action to dissipate membrane potential, as indicated.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Binding Site</th>
<th>Function affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone (Rot)</td>
<td>Complex I&lt;br&gt;NADH ubiquinone oxidoreductase&lt;br&gt;N₂ Fe-S cluster site</td>
<td>Non-competitive inhibitor&lt;br&gt;Blocks transfer of electrons from Fe-S centres to the ubiquinone-binding site, inhibiting electron transfer to Complex III</td>
</tr>
<tr>
<td>3-Nitropropionic acid (3-NP)</td>
<td>Complex II&lt;br&gt;Succinate ubiquinone oxidoreductase&lt;br&gt;Binds to Arg297 residues at the active site (substrate binding site)</td>
<td>Irreversible inhibitor&lt;br&gt;Blocks electron transfer to Complex III&lt;br&gt;Metabolic poison: prevents oxidation of succinate to fumarate in Krebs cycle</td>
</tr>
<tr>
<td>Antimycin A (AA)</td>
<td>Complex III&lt;br&gt;Ubiquinone-cytochrome c reductase&lt;br&gt;Binds to the Q₉ site within complex III</td>
<td>Specific inhibitor of Complex III, inhibiting electron transfer from semiquinone to the Q₉ site, blocking the flow of electron from cytochrome b to cytochrome c₁</td>
</tr>
<tr>
<td>Potassium cyanide (KCN)</td>
<td>Complex IV&lt;br&gt;Cytochrome oxidase&lt;br&gt;Binds to the cytochrome c oxidase haem α₃-CuB binuclear centre</td>
<td>Non-competitive inhibitor&lt;br&gt;Blocks electron transfer to oxygen&lt;br&gt;Inhibits both cellular oxygen utilization and ATP production</td>
</tr>
<tr>
<td>Oligomycin (Oli)</td>
<td>Complex V&lt;br&gt;FₒF₁ ATP synthase&lt;br&gt;Binds to the proton conducting Fₒ sector</td>
<td>Specific for mitochondrial ATP synthase&lt;br&gt;Blocks proton translocation across the IMM, inhibiting phosphorylation of ADP to ATP</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)</td>
<td>Phospholipid bilayers</td>
<td>Dissipates the mitochondrial inner membrane potential by acting as a proton ionophore to generate a widespread nonspecific proton leak</td>
</tr>
</tbody>
</table>
Figure 1.5. Structure of complex II, succinate dehydrogenase monomer of Escherichia coli

Complex II consists of a large flavoprotein subunit (Fp) (SdhA, orange) and three non-haem Fe-S protein subunits (Ip) (SdhB, green) on the matrix side of the membrane. The two hydrophobic anchor subunits (SdhC, red, and SdhD, blue) in the IMM anchor the whole complex to the IMM. FAD, oxaloacetate (OAA), haem b, and ubiquinone (UQ) are shown in light green, light blue, yellow, and pink, respectively. The Fe-S clusters are represented as red and grey, respectively (adapted from Lenaz & Genova, 2010).
Figure 1.6. Structure of bovine mitochondrial complex III, cytochrome bc₁ complex

(A) Represents a complete structure of the 11-subunit bovine mitochondrial cytochrome bc₁ complex in the dimeric form. The redox active metal centres are colour coded: cytochrome b (green), cytochrome c₁ (yellow) and the redox cofactors (cyt b, cyt c₁ and 2Fe.2S cluster) are represented in red (adapted from Papa et al., 2012). (B) illustrates the route of electron transfer through complex III via a series of redox reactions via Q cycle and the specific inhibitors of complex III (adapted from Raha & Robinson, 2000).
The overall reaction of this complex includes oxidation of two ubiquinols from complex I and II and subsequent transfer of electrons to cytochrome c in the IMS via Rieske Fe-S clusters and cytochrome c. Complex III also operates similarly to complex I, which couples electron transfer to generation of the H\(^+\) gradient driving synthesis of ATP at complex V by releasing four H\(^+\) into the IMS (Scheffler, 2011; Trumpower & Gennis, 1994).

The redox reaction of ubiquinone coupled to pumping of H\(^+\) at complex III is known as the Q-cycle (Mitchell, 1976; Mitchell, 1975; Trumpower, 1990). The Q-cycle is a series of redox reactions that involves sequential oxidation and reduction of ubiquinol (fully reduced form of ubiquinone, also written as QH\(_2\)) and ubiquinone (fully oxidized form of ubiquinone, Q), and the reduction of cytochrome c concomitantly with pumping of H\(^+\) into the IMS. Complex III has two quinone binding sites that are responsible for the Q-cycle, named as Q\(_N\) near the matrix side, and Q\(_P\) near the outer surface of IMM. The Q-cycle is initiated by an entry of ubiquinol, or QH\(_2\), from complex I and II into the Q\(_P\) site of complex III. QH\(_2\) is fully oxidized at the site and donates 2 electrons to complex III. One electron goes to the Rieske Fe-S cluster which subsequently passes the electron to cytochrome c. Ultimately, the electron is transferred to soluble cytochrome c in the IMS and which will eventually transfer electrons to complex IV of the ETC. The second electron is transferred to the Rieske Fe-S cluster which subsequently passes the electron to cytochrome c. Ultimately, the electron is transferred to soluble cytochrome c in the IMS and which will eventually transfer electrons to complex IV of the ETC. The second electron is transferred to the Rieske Fe-S cluster which subsequently passes the electron to cytochrome c. Ultimately, the electron is transferred to soluble cytochrome c in the IMS and which will eventually transfer electrons to complex IV of the ETC. The second electron is transferred to the Rieske Fe-S cluster which subsequently passes the electron to cytochrome c. Ultimately, the electron is transferred to soluble cytochrome c in the IMS and which will eventually transfer electrons to complex IV of the ETC. 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1.3.2.4. Complex IV Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase)

Mammalian complex IV, also known as cytochrome c oxidase, is the fourth member of the mitochondrial electron transport chain. It is the final site of electron transport where cytochrome c from complex III is re-oxidised and passes on electrons to oxygen molecules to reduce them to water. At the same time, electron transfer is coupled to H$^+$ pumping across the membrane, giving a net transfer of four H$^+$ into the IMS.

Complex IV is a member of the super-family of haem-copper proteins containing up to 13 subunits. The three largest subunits, I, II and III form the functional core of the enzyme and are encoded by the mitochondrial genome and synthesized in the mitochondrial matrix. Other subunits perform regulatory functions and support in insulation, stabilization or assembly of the complex (Scheffler, 2011). The subunit II is positioned near the surface of the complex facing the IMS, allowing a close contact with the dinuclear Cu$_A$ domain and electron carrier cytochrome c. Electrons are donated from cytochrome c to the Cu$_A$ domain from which electrons are transferred to haem a and to the bimetallic haem a$_3$-Cu$_B$ redox site at subunit I (Lenaz & Genova, 2010) (Fig. 1.7). Oxygen molecules approach the haem a$_3$-Cu$_B$ reaction centre for conversion to water by accepting electrons from haem a$_3$-Cu$_B$ (Lucas et al., 2011). The reduction process of oxygen creates a high chemical potential, which pumps H$^+$ against the electrochemical gradient across the IMM during the electron transfer steps (Branden et al., 2006). Consequently, the redox reaction catalysed by complex IV results in a charge separation across the membrane by movement of positive charges into the IMS as electrons are transferred to oxygen, giving rise to the potential energy utilized for ATP production via complex V. Due to the conversion of oxygen molecules into water at complex IV during electron transfer, oxygen consumption of a cell is considered to directly reflect OXPHOS in mitochondria. Many procedures determining bioenergetics of mitochondria, such as the Seahorse XF analyser, measure the oxygen consumption rate to evaluate mitochondrial dysfunction in disease settings. Cyanide (CN) binds to the haem a$_3$-Cu$_B$ binuclear centre where it blocks the electron to oxygen, thereby inhibiting both cellular oxygen utilization and ATP production (Way, 1984) (Table 1.1).
Figure 1.7. **Structure of complex IV, cytochrome c oxidase**

Complex IV is a member of super-family of haem-Cu proteins containing up to 13 subunits. Electron transfer (indicated in light grey) from cytochrome c through complex IV to the catalytic center of the enzyme (haem a$_3$/Cu$_{II}$), and the translocation of H$^+$ across the inner mitochondrial membrane (IMM) are illustrated (adapted from Lenaz & Genova, 2010).
1.3.2.5. Complex V/F\textsubscript{1}F\textsubscript{0} ATP Synthase

While not strictly part of the ETC, ATP synthase is discussed here as a key component of OXPHOS. Mitochondrial F\textsubscript{1}F\textsubscript{0}-ATP synthase, or the complex V of the electron transport chain is the final complex of OXPHOS from which ATP is synthesized by energy provided by the electrochemical gradient generated by complex I, III and IV of the respiratory chain (Capaldi, 1994; Nijtmans et al., 1995). The F\textsubscript{1}F\textsubscript{0}-ATP synthase is reversible and can work in both synthesis and hydrolysis of ATP. Synthesis of ATP involves the core functional subunits of complex V, F\textsubscript{1} and F\textsubscript{0}, which work in coordination to synthesize ATP from ADP and inorganic phosphate (Pi). F\textsubscript{1} domain is composed of three copies of subunits α, and β, and one subunits of each γ, δ and ε and is situated facing the mitochondrial matrix. F\textsubscript{0} contains subunits c (a ring structured oligomer that is embedded in the IM), a, b, d, F6, OSCP (oligomycin-sensitive conferring protein) and other accessory subunits e, f, g and A6L. F\textsubscript{0} spans the IMM and is connected to F\textsubscript{1} domain by the central stalk of complex V constituted by the F\textsubscript{1} γ, δ and ε subunits, and a peripheral stalk formed by F\textsubscript{0} subunits b, d, F6 and OSCP (Devenish et al., 2000; Jonckheere et al., 2012) (Fig. 1.8).

The first step to the synthesis of ATP occurs with the transfer of H\textsuperscript{+} through the F\textsubscript{0} domain of the enzyme at the expense of transmembrane proton motive force. Transfer of H\textsuperscript{+} down the potential gradient causes rotation of the c-subunit oligomer ring of F\textsubscript{0} (Cox et al., 1984), and δ and ε subunits of F\textsubscript{1} domain which are bound to the c-subunit subunits (Weber & Senior, 2003), a reaction also known as “rotary catalysis” (Devenish et al., 2008). The rotation of asymmetric δ subunit causes conformational changes in α and β subunit hexamer, at which each 120° of the δ subunit rotation opens catalytic sites located at the interface of α and β subunits. Freshly synthesised ATP molecules are liberated from the opening catalytic sites, followed by binding of inorganic phosphate and ADP molecule. As the δ subunit rotates again, bound ADP and phosphate are occluded and ATP synthesis takes place. The newly formed ATP molecule is released when the δ subunit makes a full 360° turn and once again opens the catalytic sites of α and β subunits (Weber & Senior, 2003). Oligomycin (Oli) is a well-known inhibitor of F\textsubscript{0}F\textsubscript{1} ATP synthase where it binds to the proton conducting F\textsubscript{0} sector, and blocks H\textsuperscript{+} translocation.
across the IMM, thus inhibiting phosphorylation of ADP to ATP (Devenish et al., 2000) (Table 1.1).

1.4. Broad Aspects of Mitochondrial Involvement in Neurodegenerative Disease

Neurodegenerative diseases are a group of brain pathologies characterized by progressive degeneration of specific subtypes of neurones and synapses of the nervous system, often causing impairment of motor and cognitive functions. Neurodegeneration is caused by multifactorial mechanisms involving genetic, environmental and endogenous factors which are interrelated to cause cellular dysfunction, eventually leading to PCD (Jellinger, 2010). In addition to these risks, ageing seems to be the primary risk factor for the majority of neurodegenerative diseases such as PD, HD, AD and ALS. In all of these diseases, selective vulnerability of neurones at specific anatomical areas of the brain gives rise to a characteristic clinical phenotype. With an increase in the ageing population due to improvement of life expectancy and quality of medical care, the occurrence of neurodegenerative diseases has also increased, thus there are urgent needs to develop new therapeutic strategies. Although the aetiology and mechanisms of neuronal degeneration have been the subjects of extensive research for the last few decades, current treatments can only provide limited benefit in alleviating clinical symptoms and only have limited effects on prevention or progression of the disease. As the search for therapeutic strategies has gained momentum, biochemical and genetic research have revealed converging lines of evidence which have emphasized dysfunctional mitochondria as a key common theme of neurodegeneration. As discussed elsewhere in Chapter 1, mitochondria are the gatekeepers for cellular survival in regulating the intrinsic pathway of PCD and are the major source of both cellular ATP and ROS. Further, combined with the high energy demand in neurones, primarily met by mitochondrial OXPHOS, and the limited regenerative capacity of post-mitotic neurones, it is not surprising to find dysfunctional mitochondria as a convergence point for neurodegeneration. Thus it is important to
Figure 1.8. Schematic representation of yeast mitochondrial ATP synthase, complex V (F₁Fₒ ATP Synthase)

F₁ domain is composed of three copies of subunits α, and β, and one subunit of each γ, δ and ε and is situated facing the mitochondrial matrix. Fₒ contains subunits c (a ring structured oligomer that is embedded in the IMM), a, b, d, F6, OSCP (oligomycin-sensitive conferring protein) and other accessory subunits e, f, g and A6L. Fₒ spans the IMM and is connected to F₁ domain by the central stalk of complex V constituted by the F₁ γ, δ and ε subunits, and a peripheral stalk formed by Fₒ subunits b, d, F6 and OSCP (adapted from Devenish et al., 2000).
investigate the physiological function of mitochondria specifically in neurones and how they participate in neuronal degeneration.

1.4.1. Overview of Mitochondrial Involvement and Neuronal Degeneration

Since mitochondria play an important role as key coordinators of neuronal survival and death, mitochondrial pathobiology takes a centre stage in age related-neurodegenerative diseases such as PD, AD, HD and ALS. The following section will review the involvement of mitochondria in pathophysiology of neurodegenerative diseases, although a detailed coverage of all aspects of autophagy in these neuropathologies is beyond the scope of this thesis. However, selected examples relevant to its involvement in neurodegenerative conditions can be found in Section 1.5.3.

1.4.1.1. Parkinson’s Disease

PD is an age-related disease, which affects approximately 2% of all ageing population, and whose incidence is increasing in Western societies. PD is characterized by a progressive degeneration of dopaminergic neurones in the upper brainstem, and the subsequent deficiency of the neurotransmitter dopamine contributes to the clinical symptoms of PD, such as resting tremor, muscular rigidity, bradykinesia and postural instability (Polymeropoulos et al., 1997). Another major pathological identifier of PD is the presence of intracytoplasmic inclusions of α-synuclein and accumulation of its insoluble aggregates called Lewy bodies in many of the brain regions, such as substantia nigra, locus coeruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei and the central and peripheral divisions of the autonomic nervous system (Polymeropoulos et al., 1997; Spillantini et al., 1998). While there has been an intense research on the molecular pathways for progression of PD, the current treatment for PD
only provides symptomatic relief by replacing depleted dopamine with its precursor, L-DOPA.

The involvement of mitochondrial dysfunction in PD has been well recognized as the core theme underlying the pathogenesis of PD. Particularly, the inhibition of complex I has been considered as a major contributor to the development and progression of PD. Studies employing post-mortem brain tissue from PD patients revealed specific defects of respiratory complex I activity in the substantia nigra (Schapira et al., 1990), which was further supported by subsequent animal studies using complex I inhibitors, such as Rot, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat and maneb. Animals treated with these inhibitors represent experimental models of PD, showing preferential degeneration of dopaminergic neurones and pathological and behavioural features analogous to those found in PD (Betarbet et al., 2000; Duty & Jenner, 2011; Jenner, 2001; Meco et al., 1994; Thiruchelvam et al., 2003). Strikingly, α-synuclein appeared to contain a mitochondrial targeting sequence and was shown to predominantly accumulate in the IMM causing inhibition of respiratory complex I in a dose-dependent manner, especially in the PD-vulnerable regions of the brain such as substantia nigra and striatum (Liu et al., 2009a; Zhang et al., 2008). The accumulation of α-synuclein in these areas was accompanied by decreased complex I activity and subsequent production of ROS, providing evidence that mitochondrial accumulated α-synuclein may directly interact and interfere with the function of complex I (Devi et al., 2008). Furthermore, α-synuclein was found to interact with the mitochondrial dynamic machinery such that mitochondrial morphology and mitochondrial DNA were defective in transgenic mice expressing mutant α-synuclein (Martin et al., 2006).

The importance of general mitochondrial homeostasis in PD recently gained much attention, as studies in post-mortem brains of PD patients showed down-regulation of PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α) which is known to play a central role in the regulation of cellular energy metabolism and mitochondrial biogenesis (Liang & Ward, 2006; Zheng et al., 2010). Down-regulation of PGC-1α in PD patients suggests that general dysfunction in mitochondrial bioenergetics may be a risk factor in exacerbating PD pathophysiology. Along the same line, recent genetic studies of early-onset PD patients showed mutations in PD related genes such as PINK1
(Phosphatase and tensin homolog (PTEN) induced putative kinase 1; Park6), Parkin (Park2) and DJ-1 (Park7) (Andres-Mateos et al., 2007; Bonifati et al., 2003; Kitada et al., 1998; Valente et al., 2001). These are the most common mutations found that affect mitochondrial function in familial cases of PD, where the products of these genes are associated with regulating mitochondrial integrity and function via interaction between mitochondrial dynamics and autaphagic machinery (Trempe & Fon, 2013). In particular, PINK1 and Parkin are involved in the specific elimination of dysfunctional mitochondria via the lysosome-dependent autophagic pathway, a process termed mitophagy (Jin & Youle, 2012) (See Section 1.5.3).

1.4.1.2. Alzheimer’s Disease

AD is the most common form of dementia affecting more than 35 million people worldwide, and being the most prevalent in the aged population of 65 years and older. Symptoms involve deterioration of memory and other cognitive domains with eventual death within 3-9 years after diagnosis (Kukull et al., 2002; Querfurth & LaFerla, 2010).

The key neuropathological identifiers of AD are an accumulation of amyloid beta (Aβ) peptides, which are the main component of the amyloid plaques found in the AD brain, and the intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau proteins (Querfurth & LaFerla, 2010). The neurotoxicity induced by these components is still actively under investigation, but it is generally accepted that the presence of misfolded Aβ proteins in the ageing brain can lead to oxidative and inflammatory damage, and consequently energy failure and synaptic dysfunction causing neuronal death (Carrillo-Mora et al., 2014). In particular, accumulation of Aβ in mitochondria was observed in AD patients and transgenic AD mice, in which the chronic exposure to soluble Aβ was associated with an increased level of hydrogen peroxide (H₂O₂) and diminished activity of complex III and IV, reflecting impaired mitochondrial energy production (Caspersen et al., 2005; Hansson Petersen et al., 2008; Manczak et al., 2006; Rhein et al., 2009). Indeed, the pathological increase of Aβ in AD brains targets mitochondrial function, promoting progressive synaptic impairment due to enhanced...
vulnerability of neurones to increased oxidative stress and dysfunctional cellular energy metabolism (Mao & Reddy, 2011; Mattson & Liu, 2002).

Interestingly, accumulating data from human studies and *in vitro* and *in vivo* experiments provide evidence for Aβ associated mitochondrial dysfunction and subsequent bioenergetic failure as early events that occur in AD pathogenesis. Studies involving positron emission tomography imaging and various biochemical assays also revealed the dysfunction of general mitochondrial metabolism involving reduced glucose metabolism, decreased mitochondrial respiration and altered expression and decreased activity of mitochondrial energy producing enzymes, and importantly these changes preceded clinical diagnosis of AD (Fukuyama *et al.*, 1994; Minoshima *et al.*, 1997; Yao *et al.*, 2009).

NFTs are another pathological hallmark of AD and are composed of aggregates of hyperphosphorylated tau (Bancher *et al.*, 1989). Tau is a microtubule-associated protein which is abundantly expressed in neuronal axons, and it is involved in regulating the dynamics of microtubules in the cytoskeleton to facilitate axonal transport of organelles to nerve terminals (Ittner & Gotz, 2011). In the pathological setting of AD, tau is hyperphosphorylated and forms aggregates of paired helical filaments, which ultimately contribute to the neurodegeneration (Garcia & Cleveland, 2001). Neurones are well known for their highly elongated morphology, and they require efficient delivery of organelles, such as mitochondria, from soma to axons, dendrites and synapses. Cell culture studies indicate hyperphosphorylation of tau results in detachment of tau from microtubules, causing impairment of axonal transport of mitochondria and energy deprivation at the synapses, eventually causing synapse degradation and neuronal damage (Mandelkow *et al.*, 2003; Stamer *et al.*, 2002; Thies & Mandelkow, 2007; Trinczek *et al.*, 1999). Furthermore, N-terminal-truncated tau was found to localize to mitochondrial membranes where tau fragments exert toxic effects by dramatically impairing OXPHOS of mitochondria and causing pathological synaptic changes (Amadoro *et al.*, 2012; Atlante *et al.*, 2008). Transgenic animal models overexpressing mutant tau protein exhibit pathological features resembling those AD patients, such as dysregulation of mitochondrial respiratory complex chain components, impaired mitochondrial respiration and ATP synthesis with consequent higher levels of ROS in aged transgenic mice (David
et al., 2005). For all the reasons above, a great emphasis has been placed on mitochondrial dysfunction as a hallmark of AD.

1.4.1.3. Huntington’s Disease

HD is a dominantly inherited neurological disorder caused by expression of abnormal polyglutamine expansion in the gene for the huntingtin protein (Htt). HD is characterized by preferential and progressive degeneration of striatal (GABAergic) medium spiny projection neurones and cortical neurones, followed by movement disorder and cognitive deficits (Bates, 2005; Ferrante et al., 1991). The length and dosage of the pathologic polyglutamine expansions of Htt directly correlates with the clinical severity of the disease, and aggregates of mutant Htt protein have been found in pathological sites in the post-mortem brains of patients with HD and of HD mouse models (DiFiglia et al., 1997; Mangiarini et al., 1996; Reddy et al., 1998). Although many cellular pathways have been extensively investigated to find the cause of the selective premature death of striatal neurones, there are no disease-modifying therapies currently available to treat or delay or to prevent HD progression (Costa & Scorrano, 2012). Several lines of evidence demonstrated abnormal energy metabolism and dysfunctional bioenergetics of mitochondria in post-mortem HD brain. In addition, an adverse effect of mutant Htt on mitochondria was observed, suggesting an involvement of dysfunctional mitochondria in the pathogenesis of HD (Reddy & Shirendeb, 2012).

Mitochondrial involvement in the pathogenesis of HD gained much credence when biochemical studies of the HD brains showed impaired activity of respiratory chain complexes, TCA cycle enzymes and abnormal bioenergetics. Post-mortem HD brain tissue displayed compromised mitochondrial respiration with significantly reduced activities of complexes II, III and IV, and showed localized hypometabolism in HD caudate nucleus, implying selective susceptibility of the caudate nucleus during the pathogenesis of HD (Brennan et al., 1985; Browne et al., 1997; Gu et al., 1996). Furthermore, ATP production and respiration of mitochondria were significantly impaired in striatal cells of mutant Htt mice (Orr et al., 2008). The mitochondrial defect identified in post-mortem HD caudate nucleus paralleled that found in HD neurotoxin
animal models. Animals exposed to the chronic administration of an irreversible inhibitor of complex II, 3-NP, developed selective striatal lesions that replicate pathological features of HD, and possessed abnormal energy metabolism as displayed by HD brain (Beal et al., 1993; Brouillet & Hantraye, 1995). Indeed, our laboratory showed that 3-NP induced PCD of striatal GABAergic neurones with redistribution of “pro-apoptotic” mitochondrial proteins (Diwakarla et al., 2009a). Interestingly, complex II serves as one of the core targets associated with the TCA cycle, where blockade of any of its enzymes may cause mitochondrial dysfunction. Thus, effective functioning of TCA cycle is critical as neurones exclusively rely on glucose as their primary energy source. Other work demonstrated impaired functions of TCA cycle enzyme, aconitase, in HD brain (Tabrizi et al., 1999), decreased metabolism of glucose and a significant increase in lactate level in basal ganglia in striatum (Martin et al., 1992), further supporting the involvement of dysfunctional mitochondrial bioenergetics in HD pathogenesis. The striatum is clearly more susceptible and sensitive to defects in mitochondrial bioenergetics than other brain regions (Pickrell et al., 2011), consistent with the neuropathological features associated with HD.

The aggregates of mutant Htt represent a major pathological hallmark of HD, and are known to interfere a number of cellular functions, including transcriptional regulation (Steffan et al., 2000; Yu et al., 2002), axonal transport (Borrell-Pages et al., 2006) and recognition of autophagic cargo for degradation (Martinez-Vicente et al., 2010). In particular, mutant Htt has been shown to have an adverse role on mitochondrial dynamics and morphology by interacting with the mitochondrial protein dynamin-related protein 1 (Drp1). Mutant Htt enhances Drp1 GTPase enzymatic activity and causes excessive mitochondrial fragmentation, blockage of mitochondrial movement and abnormal distribution, leading to defective axonal transport of mitochondria and selective synaptic degeneration (Chang et al., 2006; Trushina et al., 2004). Mitochondria of abnormal structure were observed in post-mortem brain of both juvenile and adult-onset HD, further supporting the adverse role of Htt on mitochondria (Johri & Beal, 2012). In addition, mutant Htt was also found to interfere with the expression of PGC-1α, an upstream gene involved in mitochondrial biogenesis and function (Cui et al., 2006). Inhibition of PGC-1α expression could limit the ability of affected neurones to respond to energy demand in HD, and consistent with this idea, reduced expression of PGC-1α target genes was observed in striatum of post-mortem brain of HD patient (Weydt et al., 2006).
All these abovementioned results indicate that mitochondrial impairment is clearly associated in HD pathogenesis.

**1.4.1.4. Amyotrophic Lateral Sclerosis**

ALS is a neurodegenerative disease involving progressive degeneration of upper and lower motor neurones in the brain, brainstem and spinal cord, eventually resulting in muscle weakness, muscle atrophy, spasticity, and leading to paralysis and death (Rowland & Shneider, 2001). ALS occurs mainly in sporadic forms, and only a small proportion of ALS is inherited in familial forms as a result of mutations in genes, such as copper-zinc superoxide dismutase (SOD1/ALS1), Alsin (ALS2), senataxin (ALS4), FUS/TLS (fused in sarcoma, ALS6), and DNA/RNA binding proteins called TDP-43 (TAR-DNA binding protein) (Da Cruz & Cleveland, 2011) and valosin-containing protein gene (VCP) (Abramzon *et al.*, 2012; Ayaki *et al.*, 2014; Ishikawa *et al.*, 2012). Approximately 20% of familial ALS cases are associated with mutations in the SOD1 gene, and animal models of ALS with SOD1, TDP-43 or FUS mutations have become valuable predictors of the human disease as they progressively develop pathologic features of motor neurone degeneration (Kato, 2008). The common signature within motor neurones that degenerate in ALS is the accumulation of TDP-43 (Neumann *et al.*, 2006), which is considered a key substrate for disease development (Ling *et al.*, 2013). Although there has been extensive research investigating the molecular pathogenesis of the disease, the aetiology is still largely unknown. However, increasing evidence points toward involvement of mitochondrial damage and dysfunction in the pathogenesis of ALS.

Motor neurones are the largest type of neurones in human brain being of great length (up to a metre or longer in length) and possessing large diameter axons. Due to these morphological features, they have high energy demand and require efficient axonal transport of organelles, such as mitochondria, to the metabolically active sites. Particularly, axon terminals of motor neurones rely on mitochondrial OXPHOS for energy for maintenance and restoration of ionic gradients across the membrane during neuromuscular activity (Mattson *et al.*, 2008). Considerable evidence suggests that
dysfunctional Ca\textsuperscript{2+} homeostasis and Glu excitotoxicity (Kruman et al., 1999; Rothstein & Kuncl, 1995), increased levels of oxidative stress (Beal et al., 1997; Cai et al., 2005; Cutler et al., 2002; Pedersen et al., 1998), and triggering of apoptotic cascades (Sathasivam et al., 2001), which are all related to dysfunction of mitochondria, could be the potential pathogenic mechanisms in ALS. Structural abnormalities of mitochondria were clearly observed in neuromuscular junctions, skeletal muscle, liver, and motor cortex of ALS patients (Atsumi, 1981; Menzies et al., 2002a; Menzies et al., 2002b; Sasaki & Iwata, 1999). Transgenic animal models of ALS with SOD1, TDP-43 or FUS mutations also show swollen mitochondria and deformed cristae (Johri & Beal, 2012). A massive degeneration and accumulation of abnormal mitochondria was also observed in dendrites of motor neurones at onset of the disease long before the neurones start to degenerate in SOD1 mutant mice (Kong & Xu, 1998), suggesting mitochondrial defects may represent a critical role in pathogenesis of ALS. Furthermore, disrupted axonal transport was documented in both ALS patients and mutant SOD1 transgenic mice, which may hinder mitochondrial energy supply to the metabolically active axonal terminals (De Vos et al., 2007; Magrane & Manfredi, 2009).

In addition, direct involvement of mutant SOD1 in mitochondrial function was suggested by a study which showed misfolded mutant SOD1 primarily targeted the axonal mitochondria of motor neurones in vivo, and similarly, a proportion of misfolded mutant SOD1 proteins were also found to bind to the cytoplasmic surface of mitochondria (Vande Velde et al., 2011). Although SOD1 is mostly cytosolic (Weisiger & Fridovich, 1973), mutant SOD1 have been found to localize to the mitochondrial IMS and matrix (Higgins et al., 2002; Mattiazzi et al., 2002; Okado-Matsumoto & Fridovich, 2001; Sturtz et al., 2001), and may potentiate mitochondrial damage through abnormal interactions with chaperone proteins (Okado-Matsumoto & Fridovich, 2002; Shinder et al., 2001), disrupt mitochondrial protein import (Liu et al., 2004), and cause loss of mitochondrial membrane potential via impairment of the mitochondrial respiratory chain (Carri et al., 1997). In fact, early changes in bioenergetics of mitochondria were observed before any clinical symptoms arose in SOD1 mutant mice, wherein decreased mitochondrial respiration, especially at complex IV of the respiratory chain, was observed in brain and spinal cord (Kirkinezos et al., 2005). Mutant SOD1 was also demonstrated to alter morphology of mitochondria from an elongated form to more spherical shape (Vande Velde et al., 2011), and affect the dynamics of mitochondria by causing...
impairment of mitochondrial fusion in both axons and cell bodies (Magrane et al., 2012). Moreover, the interaction between mutant SOD1 and mitochondria may cause changes in the redox potential of mitochondria, resulting in an increased production of ROS, and motor neurones are well documented to be particularly vulnerable to oxidative stressors (Cassina et al., 2008).

The importance of mitochondrial homeostasis in ALS pathogenesis gained more weight when treatments and genetic manipulations targeting prevention of mitochondrial impairment showed suppressive effects on disease progression in SOD1 mutant mice. For instance, inhibitors of mitochondrial permeability transition pore formation such as cyclosporine A and nortriptyline delayed disease onset and increased survival of ALS mice (Keep et al., 2001; Wang et al., 2007). Further, dietary supplementation with creatine enhanced neuronal energy availability reducing oxidative stress, protected motor neurones and extended survival of SOD1 mutant mice (Klivenyi et al., 1999).

The rapid advances in ALS research have allowed a considerable understanding of the pathogenesis of the disease involving mitochondrial dysfunction in TDP-43 proteinopathies. TDP-43 is a DNA- and RNA-binding protein primarily located in the nucleus, and it is believed to be involved in regulation of transcription and splicing (Bose et al., 2008). The abnormal processing and aggregation of TDP-43 has been reported in a number of neurodegenerative diseases including in the brain tissue of ALS patients (Liscic et al., 2008; Mackenzie et al., 2007). Although it is still illusive how TDP-43 mutations affect mitochondrial function, some findings point in the direction of a direct toxic effect of TDP-43 on mitochondria through impaired autoregulation of TDP-43 protein expression (Hong et al., 2012). Furthermore, transgenic animals expressing mutant TDP-43 display mitochondrial changes which resemble those observed in ALS patients. These changes include abnormal mitochondrial trafficking and morphology, mitochondrial clustering and aggregation of mutated TDP-43 around mitochondria causing adverse effect on OXPHOS (Braun et al., 2011; Magrane et al., 2014; Shan et al., 2010; Stribl et al., 2014; Wang et al., 2013; Xu et al., 2010). For these reasons, mitochondrial dysfunction is recognized as one of the key elements that contribute to the ALS pathology.
1.4.2. Overview

Given the important roles of mitochondria combined with the post-mitotic and non-proliferating nature of neurones, damaged mitochondria need to be cleared efficiently in order to maintain a healthy pool of mitochondria. Quality of mitochondria is maintained largely by a number of mechanisms such as chaperones and anti-oxidant molecules within mitochondria, proteins which control dynamics of mitochondria by aiding fusion or division in response to the magnitude of mitochondrial damage, and lastly via selective autophagy of mitochondria, which will be the main focus of the last part of this literature review.
1.5. Mitochondrial Quality Control through Autophagy

1.5.1. Autophagy: General Definition and Physiological Function

Autophagy is a cellular housekeeping process that sequesters and delivers cytoplasmic components to the lysosomes for degradation. The term “Autophagy” was coined by de Duve from his electron microscope observations of single or double membrane vesicles containing cellular parts of the cytoplasm and organelles which eventually fused with lysosomes (De Duve & Wattiaux, 1966). Based on these morphological observations, several major functions of autophagy have been described. Initially, the main function of autophagy was believed to be an inducible process in response to nutrient or growth factor deprivation and to maintain energetic balance by non-selectively sequestering and degrading parts of the cytosol to obtain amino acids and fatty acids, which were then utilized in energy production or protein synthesis (He & Klionsky, 2009). Autophagy was also found to play a role in cellular defence by removing pathogens or toxic compounds that enter the cell (Ravikumar et al., 2010). Furthermore, selective autophagy has been shown to occur constitutively at a basal level to ensure homeostasis of the intracellular proteome or organelles by selectively targeting misfolded/aggregated proteins and damaged organelles for lysosomal-mediated degradation (Komatsu et al., 2005). On the contrary, autophagy can also participate in cell death mechanisms despite the above mentioned protective roles of autophagy. Autophagy has been viewed as a part of PCD-Type II autophagic cell death, which is influenced by regulatory genes of autophagy and exhibits morphological features of autophagy during cellular death (Higgins et al., 2011; Marino et al., 2014).

Autophagy can occur in 3 subdivided categories depending on the mode of delivery of cargo to the lysosome; microautophagy, chaperone-mediated autophagy and
Macroautophagy (Mizushima, 2007; Mizushima et al., 2008) (Fig. 1.9). Microautophagy involves invagination and pinching off of lysosomal membranes, introducing small quantities of cytoplasm into the lysosomal lumen for degradation. Chaperone-mediated autophagy selectively targets proteins with a peptide motif, KFERQ, and transports such proteins to the lysosome for degradation (Ravikumar et al., 2010). Macroautophagy on the other hand, mediates degradation of organelles at the largest scale compared to the other two pathways, by sequestering a portion of cytoplasm that contains proteins, sugars, lipids, RNA, and organelles such as mitochondria and endoplasmic reticulum into double-membrane vesicles called autophagosomes (Blomgren et al., 2007; Nixon, 2006; Shintani & Klionsky, 2004). Of these three known autophagic routes, macroautophagy will be the focus of this thesis and will be referred to as autophagy herein.

1.5.2. Understanding Molecular Machineries of Mammalian Autophagy

Electron microscope studies of autophagy enabled the autophagy process to be tracked and characterized into several distinctive steps, involving formation of the cup-shaped phagophore at the phagophore assembly site, which matures into a double-membrane structure known as autophagosome. Autophagosomes containing long-lived proteins and organelles fuse with lysosomes to breakdown the cargo, a process followed by a release of degraded molecules into the cytosol for recycling (He & Klionsky, 2009; Mizushima, 2007) (Fig. 1.10). These processes are termed as an autophagic flux, which involves a complicated interaction of different complexes of proteins (Mizushima et al., 2010). Genetic screens using yeast Saccharomyces cerevisiae as a simple experimental model (Takeshige et al., 1992) allowed a breakthrough that proved incisive to our understanding of mammalian machinery involved in autophagic processes, and identified 30 or more autophagy-related (Atg) genes as essential for autophagy (Xie & Klionsky, 2007). Many of these genes were found to be involved in the formation and assembly of autophagosomes in the cytoplasm for the delivery to the lysosome during the process of autophagic flux (Klionsky, 2007). About half of yeast Atg genes were found to be
Figure 1.9. Three different forms of selective autophagy which target specific subgroups of substrates: microautophagy, chaperone-mediated autophagy and macroautophagy

There are three types of autophagy, macroautophagy, microautophagy and chaperone-mediated autophagy, and the term “autophagy” employed in this thesis indicates macroautophagy (adapted from Mizushima et al., 2007).
Figure 1.10. Schematic diagram of the steps of autophagy

The class III PI3K-III complex made up of Vps15, Vps30/Atg6, Atg14, and Vps34 (PtdIns3K) mediates nucleation of the phagophore membrane, enwrapping cytosolic proteins, protein aggregates and organelles (such as mitochondria). Atg12-Atg5-Atg16 and Atg8-PE conjugates are recruited to the phagophore, and phagophore is expanded together with the transmembrane protein Atg9 to form autophagosome. Upon vesicle completion, autophagosome is fused with lysosome and cargo is degraded by lysosomal proteases (adapted from He & Klionsky, 2009).
conserved in mammalian cells, allowing understanding of molecular machineries and signalling cascades of mammalian autophagy regulation.

1.5.2.1. Induction of autophagy

One of the main regulators of autophagy is known as mammalian target of rapamycin (mTOR). mTOR is a master regulator of nutrient and energy availability within the cell and regulates cell growth, protein synthesis, and nutrient transport, while negatively regulating catabolic processes such as autophagy (Wullschleger et al., 2006). mTOR is found in two distinct multiprotein complexes, rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2), where the inhibition of the former lifts its inhibitory effect on autophagy in response to starvation, hence inducing autophagy (Hall, 2008). The mTORC1 pathway can be inactivated by several factors such as low energy (low AMP/ATP ratio), deprivation of growth factors (such as insulin) or nutrients (such as amino acids) and pharmacological inhibition by rapamycin (RAP).

mTOR is predominantly modulated by phosphatidylinositol-3-kinase (PI3K)-protein kinase B (also known as Akt)-dependent pathway (Hahn-Windgassen et al., 2005). The PI3K pathway is activated by the binding of insulin (or growth factors) to insulin receptor (IR) on the cell surface (Ravikumar et al., 2010) (Fig. 1.11). Activated PI3K induces conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), recruiting phosphoinositide-dependent kinase 1 (PDK1) and Akt to the cell membrane. Akt, an upstream positive regulator of mTOR, is then phosphorylated, resulting in the phosphorylation of tuberous sclerosis complex 2 (TSC2) and thereby the inactivation of tuberous sclerosis complex 1 (TSC1)-TSC2 (Fig. 1.11). TSC1-TSC2 inactivation allows GTP-bound state of Ras homolog enriched in brain (RHEB), which is required for the activation of mTORC1 (Yu et al., 2015). Inhibitors of PI3K activity, such as 3-methyladenine, wortmannin and 2-(4-morpholino)-8-phenylchromone (LY294002) can downregulate autophagy (Blommaart et al., 1997; Seglen & Gordon, 1982). 5’-Adenosine monophosphate-activated protein kinase (AMPK) also acts as a regulator of mTORC1 by sensing changes in the cellular
energy state as in the intracellular AMP/ATP ratio, and inhibits mTOR signalling by directly phosphorylating TSC2 (Fig. 1.11) (Meijer & Codogno, 2006). Recent evidence shows that an inhibition of AMPK activity by compound C or a dominant negative construct strongly suppressed autophagy, indicating AMPK is required for mammalian autophagy (Meijer & Codogno, 2007; Meley et al., 2006). Apart from the ratio of intracellular AMP/ATP, mTORC1 can also be activated by the level of amino acids via Rag GTPases (Ras-related GTPase), hence suppressing autophagy in nutrient-rich environment (Demetriades et al., 2014) (Fig. 1.11).

However, not all autophagy inducers converge on mTOR; several other mTOR-independent pathways also exist, such as an activation of series of proteins via intracellular cAMP levels and intracytosolic Ca\(^{2+}\) levels from the endoplasmic reticulum (ER) stores (Williams et al., 2008). Small molecule autophagy enhancers such as trehalose, small-molecule enhancers of rapamycin (SMERs), inositol monophosphatase (IMPase) inhibitor L-690,330, and the calpain inhibitor calpastatin were also found to facilitate autophagy in mTOR-independent regulation of autophagy (Mizushima, 2007; Ravikumar et al., 2010), however, their mechanisms of action are not well understood.

1.5.2.2. Biogenesis of autophagic vacuoles

Inhibition of mTOR initiates the formation of autophagosome formation via steps involving dephosphorylation and phosphorylation of Unc-51-like kinase 1 (ULK1)-Atg13-FIP200 (FAK-family interacting protein of 200 kDa) complex signals downstream of mTORC1 to regulate autophagosome synthesis (Fig. 1.11, inset). The formation of the autophagosome is likely the most complex step of autophagy, and involves recruitment of multiple Atg genes. Autophagosome formation starts from isolation of a membrane called phagophore from pre-existing organelles such as ER and Golgi network. The assembly of phagophore and elongation of the double membrane is mediated by an activation of class III phosphatidylinositol 3-kinase (PI3K-III), Vps34/Beclin-1/Atg14L complex and a combined action of the Atg12-Atg5 and Atg8 (also known as microtubule associated protein 1 light chain 3, LC3) bound to phosphatidylethanolamine (PE) conjugation complexes (Fig. 1.10). An experiment in the yeast *Saccharomyces cerevisiae* model...
Autophagy is regulated by PI3K-Akt-mTOR pathway. The most typical trigger of autophagy is nutrient starvation, which is translated by binding of insulin or growth factors to insulin receptor, thereby activating PI3K. Activation of PIK3 leads to phosphorylation of Atk which directly phosphorylates and inhibits TSC2, which is a negative regulator of mTOR. AMPK also acts as a regulator of mTORC1 which senses the changes in the intracellular AMP/ATP ratio. Rag GTPases also activates mTORC1 in the presence of amino acid. Inhibition of mTOR by pharmacological agent or starvation ultimately results in initiation of autophagy (dotted inset) (adapted from Ravikumar *et al.* 2010).
identified the Apg12p-Apg5p (Atg12-Atg5 homolog) conjugation system as an essential step for autophagy (Mizushima et al., 1998a) and which was well conserved in human cells (Mizushima et al., 1998b). Apg7p (Atg7) was required for the conjugation reaction as an Apg12p-activating enzyme, hence playing a critical role in autophagosome formation (Tanida et al., 1999). As the membrane of phagophore elongates, it sequesters a portion of cytosol containing the proteins and organelles to be degraded, and the two ends of the phagophore close to form a double-membrane autophagosome. During the process of autophagosome formation, the cytosolic form of LC3 (LC3-I) is conjugated to PE to form the LC3-PE conjugate (LC3-II), which is recruited to autophagosomal membranes and covers the inner and outer membrane of an autophagosome.

1.5.2.3. Lysosome-mediated degradation of autophagic vacuoles

The completion of the autophagosome maturation process leads to a delivery and fusion of autophagosomes to lysosomes to form an entity known as autolysosome (Fig. 1.10). The inner membrane of autophagosome is degraded within the lysosome exposing the contents to enzymatic degradation by hydrolases in an acidic environment. The resultant molecules are recycled as building blocks of other newly synthesized macromolecules or used as substrates for energy production (Klionsky et al., 2008). LC3-II is the only Atg protein known to remain associated with autophagosomes even after fusion with lysosomes, and due to its specific association with autophagosomes, but not with other vesicular structures, its cellular level has been widely employed as a useful marker for autophagic activity (Klionsky et al., 2008).

Autophagy is the only pathway known in mammalian cells that is involved in turning over organelles, and compromised function of autophagy have been documented in a number of different pathological settings including diabetes, cancer and neurodegenerative diseases (Ravikumar et al., 2010). Especially in the case of neurodegenerative diseases, changes in autophagic flux have been suggested to participate in the development of symptoms of neurodegeneration (Puyal et al., 2012).
1.5.3. Dysfunctional Autophagic Flux in Neurodegeneration

Neurones in the brain are unique in their morphological and functional properties compared to other peripheral cells of the body. In particular, neurones are at least partially protected from nutrient deprivation by glial cells, which support neurones by releasing neurotrophins and nutrient. Peripheral nutrient supplies through the blood-brain barrier via hypothalamic regulation ensure supply of glucose to neurones is not compromised during times of starvation (Boland & Nixon, 2006). Therefore, neurones have less dependence on starvation-induced autophagy and it is hard to detect markers of autophagic flux at basal level in healthy neurones as they are protected from induction of large-scale autophagy. However, recent studies in animal models indicated neuronal autophagy was more sensitive to a variety of stresses and injuries than starvation, which rarely induced autophagy (Yue et al., 2009). Importantly, the role of selective autophagy at basal level is highlighted by a study in Atg7 gene knockout mice which showed impairment of constitutive turnover of cytoplasmic components, resulting in accumulation of abnormal organelles and ubiquitinated proteins (Komatsu et al., 2005). Given that the accumulation and aggregation of disease-associated proteins is a common pathological feature in many age-related neurodegenerative diseases such as PD, AD, HD and ALS, dysfunction in autophagic flux may play an important role in disposal of aggregates of ubiquitinated proteins in these diseases. Indeed, an immuno-electron microscope study has revealed accumulation of immature autophagic vacuoles in cortical biopsies from patients of AD, particularly within neuritic processes and synaptic terminals (Nixon et al., 2005). Remarkable accumulation of immature autophagic vacuoles in dystrophic neuritic processes suggested brains with AD pathology may have impaired autophagic flux whereby transportation of autophagosomes to the lysosomes and maturation of autophagosomes into autolysosomes were impaired, thereby preventing neuroprotective mechanisms of selective autophagy (Nixon et al., 2005). Accumulation of autophagic vacuoles was also observed in neurones from frontal cortex brain biopsies of patients with HD (Tellez-Nagel et al., 1974). Moreover, an in vitro study showed degradation of aggregate-prone proteins associated with neurodegenerative diseases was mediated by autophagy and inhibition of distinctive steps of autophagic flux caused intracytoplasmic accumulation of these proteins, while pharmacological enhancement of autophagy by RAP assisted their clearance (Ravikumar et al., 2002). Furthermore,
treatment of transgenic TDP-43 mice, which exhibit pathological aggregates of TDP-43 as in ALS, with autophagic activators showed reduction in neuronal loss, learning and memory deficits and improved locomotor function (Wang et al., 2012). A recent large scale exome sequencing study of ALS patients confirms that defects in proteostasis, or protein homeostasis, play a pivotal role in ALS (Johnson et al., 2010). Key evidence for this involvement is the cytoplasmic accumulation and aggregation of toxic misfolded proteins (SOD1, TDP-43, FUS and C9ORF72 translation products) which are implicated in motor neurone death in ALS (Ling et al., 2013). The role of autophagy in pathogenesis of PD was also demonstrated when autophagy and chaperone-mediated autophagy were able to degrade intracellular inclusions of a-synuclein in degenerating dopamine neurones and inhibition of autophagy led to the accumulation of toxic proteins (Webb et al., 2003). Neurones are particularly prone to delayed autophagic degradation of toxic proteins due to their post-mitotic nature and highly polarized morphology, where dysfunctional autophagic clearance of ubiquitinated proteins can lead to accumulation of aggregated forms of toxic proteins and cause neuronal degeneration (Hara et al., 2006; Komatsu et al., 2006). Although whether abundant accumulation of autophagic vacuoles in these neurological conditions reflects enhancement of autophagy or an impairment of degrading process in the lysosome is still unclear. Moreover, there is a growing literature from Genome Wide Association Studies demonstrating that compromised autophagic and/or lysosomal mechanisms underlie and thus contribute to the pathology of neurodegenerative disorders. Thus for example in frontotemporal degeneration, a clinically heterogeneous syndrome resulting commonly in dementia, proteins involved in lysosomal and autophagic pathways were implicated recently in frontotemporal dementia (Ferrari et al., 2014). All these observations emphasize protein degradation via the autophagy-lysosome pathway is likely to play an important role in modulating pathogenesis of toxic protein aggregation in these neurodegenerative diseases. Strategies that improve proteostasis and clear these misfolded proteins (Friedman et al., 2015; Renna et al., 2010) in neurones are therefore an attractive therapeutic approaches for neuropathologies.

As the autophagic flux gained attention for its role of clearing mutant protein aggregates, a failure to clear damaged organelles such as mitochondria by autophagy (also known as mitophagy) began to emerge with genetic studies of PD. Failure to clear damaged mitochondria in the cell can result in neuronal death as mitochondria act as the
core regulator of PCD by releasing pro-apoptotic molecules which cause neuronal death. In fact, genetic screens of patients suffering from early-onset PD showed mutations of the gene PARK6 (Valente et al., 2001) and PARK2 (Kitada et al., 1998) whose products (PINK1 and Parkin, respectively) are involved in quality control of mitochondria through mitophagy, and which will be discussed in more detail in the next section.

1.5.4. Quality Control of Mitochondria: Mitophagy

Mitochondrial defects have been considered as the epicentre of neurodegenerative diseases. Since mitochondria are so critically involved in cellular metabolism and cell death mechanisms, timely removal of damaged or dysfunctional mitochondria is essential for neuronal survival. Mammalian cells employ a number of surveillance systems to maintain the quality of mitochondria, such as intra-organelar proteases and the ubiquitin-proteasome system (UPS), which control homeostasis of mitochondria at the molecular level by degrading short-lived proteins that are misfolded or modified beyond repair (Baker et al., 2014). The dynamic nature of mitochondria also assists the maintenance of mitochondrial function by fusing damaged mitochondria with other neighbouring mitochondria to dilute any damages that may have occurred inside the organelle. However, if the damage is too great, the damaged fraction of mitochondria is segregated from the healthy remainder by fission machineries in order to isolate the damage and to protect the remaining healthy population of mitochondria (Baker et al., 2014). The segregated mitochondria are selected by the autophagic machinery and enter lysosomes for permanent degradation. Based on the previous electron microscopic observations of mitochondria being enclosed in autophagosomes, a term mitophagy was first given by Lemasters to describe a targeted degradation of damaged mitochondria (Lemasters, 2005).
1.5.4.1. Selective autophagy of mitochondria: PINK1-mediated mitophagy

The importance of timely removal of dysfunctional mitochondria via mitophagy was emphasized when genetic screens of early-onset PD patients revealed mutations of genes involved in mitochondrial quality control, namely, PARK2 (Kitada et al., 1998), and PARK6 (Valente et al., 2004). The loss-of-function mutations of the products of these genes have been recognized as the major cause of familial Parkinson’s disease known so far (Lansbury & Brice, 2002), suggesting the mitochondrial dysfunction observed in PD patients could be the result of failure of mitochondrial quality control.

PINK1 is a gene product of PARK6. The sequence analyses of PINK1 revealed the N-terminal sequence of PINK1 contained a mitochondrial targeting sequence, indicating a close association of PINK1 with mitochondria (Valente et al., 2004). Further studies on the subcellular distribution and the metabolism of PINK1 protein revealed cytoplasmic and mitochondrial distribution of PINK1 in cultured cells (Lin & Kang, 2008; Petit et al., 2005; Takatori et al., 2008) where the full length protein was constitutively localized, imported and cleaved by presenilin-associated rhomboid-like protein (PARL) in the IMM of mitochondria (Jin et al., 2010; Takatori et al., 2008), giving two N-terminally cleaved fragments as well as a full length protein. The majority of mutations of PINK1 associated with PD were positioned within the kinase domain including an ATP binding pocket, and the C-terminus, which assists in optimal kinase activity (Deas et al., 2009; Silvestri et al., 2005; Woodroof et al., 2011). These mutations affected the kinase activity of PINK1, but failed to alter its mitochondrial localization, further confirming the action of PINK1 on mitochondria (Silvestri et al., 2005; Valente et al., 2004). A detailed study of the physiological function of the putative kinase activity of PINK1 revealed its neuroprotective properties against stress-induced mitochondrial damage (Valente et al., 2004). Pathological features caused by the mutation of PINK1 at the putative kinase domain overlapped with some of the main pathological features of PD, such as decreased activity of respiratory chain complex I, increased susceptibility of mitochondria to oxidative stress, impaired proteasome function and an increased α-synuclein aggregation (Liu et al., 2009b; Valente et al., 2004). Loss of PINK1 function induced abnormal mitochondrial morphology in mammalian neurones (Hoepken et al., 2007; Wood-Kaczmar et al., 2008), and bioenergetic deficits in mitochondria in terms of reduced
respiration and ATP synthesis, which were similar to those induced by pharmacological inhibition of complex I by Rot in rats displaying PD-like phenotypes (Betarbet et al., 2006; Liu et al., 2009b). Thus, PINK1 has been suggested to play a neuroprotective role against intrinsic or environmental stresses such as oxidative stress, and in maintenance of mitochondrial homeostasis. The loss of kinase function of PINK1 is suggested to be associated with the aetiology of PD.

In addition to the above-mentioned roles of PINK1, an increasing literature has revealed the involvement of PINK1 as an early determinant of mitophagy and as a regulator of Parkin, the gene product of PARK2 (Clark et al., 2006; Yang et al., 2006). Parkin is an E3 ubiquitin ligase protein whose loss-of-function mutations in the ubiquitin-like domain has also been characterized as one of the most common causes for early-onset PD (Kitada et al., 1998). Parkin is also considered to be involved with maintenance of mitochondrial function, morphology and integrity, and its recruitment to mitochondria is regulated by the kinase activities of the upstream effector, PINK1 (Greene et al., 2003; Whitworth et al., 2005). Genetic rescue studies further confirmed that PINK1 and Parkin acted in a common pathway by regulating mitochondrial physiology to ensure mitochondrial integrity and function, and that mitophagy was initiated by PINK1-mediated recruitment of Parkin (Clark et al., 2006; Yang et al., 2006). Cell culture and genetic studies using PINK1-null Drosophila suggested a protective role of PINK1 on cellular function which was abrogated by the mutations, and similar pathologic phenotypes were observed in Parkin-null Drosophila models, including defects in mitochondrial morphology and increased sensitivity to oxidative stress (Clark et al., 2006; Valente et al., 2004). Together, the discovery of PINK1/PARK mutations in early-onset PD (Valente et al., 2004) and the gene rescue studies supported a direct molecular link between mitochondrial dysfunction and the pathogenesis of PD.

1.5.4.2. Molecular mechanisms of PINK1-mediated mitophagy

The key insights into the molecular pathways regulating PINK1-Parkin biology were revealed by Youle’s group, who reported selective recruitment of PINK1 and Parkin to depolarized mitochondria, promoting mitophagy (Jin et al., 2010; Matsuda et al., 2010;
Narendra et al., 2010). In healthy mitochondria, PINK1 was constitutively imported into mitochondria via general mitochondrial import machinery, TOM (translocase of the outer membrane) and TIM23 (translocase of the inner membrane 23), in a ΔΨm-dependent manner (Jin et al., 2010; Okatsu et al., 2012) (Fig. 1.12). The entry of PINK1 into the IMM was followed by constitutive cleavage and degradation by mitochondrial protease, PARL (Jin et al., 2010). In the event of a mitochondrial insult that led to depolarization of IMM, import of PINK1 to the IMM was inhibited, which eventually prevented the proteolytic cleavage of PINK1 by PARL, thereby allowing the full length PINK1 to accumulate on the OMM of depolarized mitochondria (Fig. 1.12). PINK1 was experimentally shown to selectively stabilize on the OMM at low membrane potential among a bioenergetically diverse population of mitochondria (Narendra et al., 2010). Upon stabilization, PINK1 directly induced mitophagy by recruiting Parkin and autophagy receptors through phosho-ubiquitin signals (Geisler et al., 2010; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014; Lazarou et al., 2015). The phospho-ubiquitin signals acted as the “digest me” signals on damaged mitochondria and directly recruited autophagy receptors such as optineurin (OPTN) and nuclear dot protein 52 kDa (NDP52) to induce mitophagy (Lazarou et al., 2015). Parkin amplified these PINK1-generated mitophagy signals by generating poly-ubiquitin chains on mitochondrial proteins such as VDAC1 (voltage-dependent anion channel 1) (Geisler et al., 2010; Sun et al., 2012), mitochondrial fusion-promoting factors, Mitofusin 1 (Mfn1) and 2 (Mfn2) (Chan et al., 2011; Gegg et al., 2010; Glauser et al., 2011; Poole et al., 2010), and other OM proteins such as TOM40, TOM70 and outer membrane protein 25 (Omp25) (Yoshii et al., 2011), which were further phosphorylated by PINK1. The poly-ubiquitin chains on the Parkin substrates were recognized and bound to the autophagic adaptor protein p62/SQSTM1 (Geisler et al., 2010), which caused recruitment of the Atg12-Atg5 complex and Atg8-LC3 phagophore, and its proximity to damaged mitochondria was suggested to execute an elimination of damaged mitochondria via autophagic pathway (Narendra et al., 2008). Furthermore, ubiquitination of Mfn proteins by Parkin caused an inhibition of fusion of damaged mitochondria to the other healthy mitochondrial network, thus preventing the damage from spreading into the remaining healthy population and priming the damaged mitochondria for general autophagic degradation (Ziviani & Whitworth, 2010). Together, the molecular pathways indicate loss of function mutations in PINK1 and Parkin can lead to an inhibition of the selective removal of damaged mitochondria, and that the subsequent accumulation of defective mitochondria may contribute to PD pathogenesis.
1.5.4.3. Mitophagy in Neuronal Systems

Most of the research that established the importance of PINK1 and Parkin recruitment to mitochondria in response to mitochondrial damage has relied on non-neuronal or immortalized cell cultures (Narendra et al., 2010; Vives-Bauza et al., 2010). Studies that have used Drosophila and mammalian cell lines such as SH-SY5Y, mouse embryonic fibroblast and HeLa cells as the cellular system, showed a robust PINK1-dependent recruitment of cytosolic Parkin to depolarized mitochondria (Geisler et al., 2010; Jin et al., 2010; Kim et al., 2008; Matsuda et al., 2010; Michiorri et al., 2010). However, the direct evidence showing PINK1-Parkin mediated mitophagy in mature neurones is somewhat more controversial, and the relevance and existence of PINK1- and Parkin-dependent mitophagy in neuronal systems still remain to be fully delineated.

Studies that employed primary cortical neurones or pluripotent stem cells (IPS)-derived dopaminergic neurones showed contradictory results with respect to the recruitment of Parkin to mitochondria (Cai et al., 2012; Joselin et al., 2012; Rakovic et al., 2013; Seibler et al., 2011; Van Laar et al., 2011). As the culture conditions of primary neurones and the composition of the media in which the neurones were grown vary between different neuronal types, the comparison between studies has proven to be difficult. One example here is a study by Joselin and colleagues, who showed the presence of antioxidants in the media in the form of the B-27 supplement, which is a conventional supplement for neuronal culture, affected the recruitment Parkin to mitochondria in primary cortical neurones. By comparison, the treatment with CCCP (carbonyl cyanide m-chlorophenyl hydrazone) in the absence of B-27 supplement induced a robust Parkin translocation to mitochondria in the same neuronal system (Joselin et al., 2012). A study that also employed primary cortical neurones demonstrated a slow but significant redistribution of Parkin to mitochondria subsequent to CCCP treatment. However the modifications of the culture methods and the maintenance of the neurones by their growth on glial beds with an apoptosis inhibitor, z-VAD-fmk, in order to mitigate any stressful environmental conditions following CCCP treatment, may have masked the real physiological reaction of neurones to mitochondrial damage (Cai et al., 2012). These results indicate that the conditions of neuronal culture may play an important factor in examining PINK- and Parkin-mediated mitophagy in primary
PINK1 is constitutively imported into mitochondria in healthy mitochondria in a ΔΨ\textsubscript{m} dependent manner. The entry of PINK1 into the IMM is followed by constitutive cleavage and degradation by PARL (not shown). At damaged mitochondria with low ΔΨ\textsubscript{m}, PINK1 is stabilized on the OMM and autophosphorylated at Ser228/Ser402, which lead to the recruitment of Parkin on mitochondria and subsequent poly-ubiquitination of OMM mitochondrial proteins to be recognized by the autophagic machineries (adapted from Okatsu et al., 2012).
neurones. Another interesting factor to be taken into consideration would be the unique metabolic properties of neurones. Unlike immortalized cell lines, which employ the glycolytic pathway and depend less on mitochondria for ATP synthesis, primary neurones have a rather different energetic dependence which predominantly relies on mitochondrial OXPHOS for the generation of ATP. This differential bioenergetic dependence is also thought to underlie the conflicting outcomes of PINK1-Parkin-mediated mitophagy between cell lines and primary neuronal cultures. Van Laar and colleagues (2011) have reported that a global application of CCCP to primary cortical neurones failed to show Parkin translocation to mitochondria for initiation of mitophagy. On the contrary, HeLa cells in the same study showed a rapid and robust localization of Parkin to mitochondria (Van Laar et al., 2011). Interestingly, when HeLa cells were forced to rely on mitochondrial respiration for energy by blocking glycolysis, translocation of Parkin to depolarized mitochondria was not observed (Van Laar et al., 2011), emphasizing how the unique metabolic requirements of neurones play an important role in recruiting mitophagy. Furthermore, studies using IPS cells from skin fibroblasts taken from PD patients with PINK1 gene mutation showed the endogenous level of Parkin was insufficient to induce mitophagy in dopaminergic neurones (Rakovic et al., 2013). Taken together, direct evidence supporting PINK1-Parkin-mediated mitophagy in primary neurones still remains a topic of controversy.
1.6. Significance of Study

1.6.1. Previous Beart & Nagley Laboratories’ work and its relation to this Thesis

Collaborative research in the Beart and Nagley laboratories has involved characterizing the diversity of PCD pathways in neurones. The general approach was to insult primary cultured neurones with stressors that mimic the neuropathological settings such as age-related chronic neurodegeneration and acute neuronal death caused by stroke. The initial focus was primarily on apoptosis (PCD-Type I), in which mitochondria play key roles as a core regulator via the intrinsic pathway, although there was early recognition of both caspase-dependent and caspase-independent mechanisms (Beart et al., 2007; Lim et al., 2006). Progress in the field of neuronal injury typically lagged behind that in other areas; thus, it was not until 2005-2010 that there was wide awareness that programmed necrosis (PCD-Type III), which often displayed many features resembling apoptosis, made important contributions to brain injury. Ongoing work further recognized components of programmed necrosis particularly in injuries caused by excitotoxicity and oxidative stress (Beart et al., 2007; Diwakarla et al., 2009a; Diwakarla et al., 2009b; Higgins et al., 2009). The different types of PCD, whilst they are not the focus of this thesis, have been the subject of numerous reviews (Galluzzi et al., 2009; Galluzzi et al., 2015), including from our own laboratory (Higgins et al., 2010; Nagley et al., 2010).

The interest in the involvement of autophagy in neurodegeneration arose from extensive earlier observations where multiple PCD pathways including apoptosis, autophagy (PCD-Type II; autophagic cell death) and programmed necrosis could potentially occur in parallel, with cross-talk between pathways, to induce diverse phenotypes of neuronal death in response to various insults (Nagley et al., 2010) (Fig. 1.13). In particular, this work on autophagy and autophagic cell death (ACD) started with the observation of insult-dependent recruitment of autophagic mechanisms in response to
Figure 1.13. Diverse pathways leading to cell death

The schematic indicates key stages that occur after a cell experiences a particular stressor from either outside or within the cell (the death trigger). The primary response is elicited by the immediate perturbation of cellular structures and functions. Subsequent intracellular signalling pathways are activated (secondary response), which, after processing (e.g. through interaction of competing members of the Bcl-2 family of proteins), lead to a “decision”. This determines the adoption of one or more death pathways that result in the possible outcomes listed at the foot of the schematic. Mitochondria play a key role in many of these death pathways, which may operate through redistribution to the cytosol of apoptogenic proteins. Other aspects of mitochondrial involvement (not shown here) include disrupted energisation of these organelles (and consequent imbalances in ion fluxes and loss of ATP production) and generation of secondary fluxes of ROS. Endoplasmic reticulum (ER) stress is sometimes invoked, for example in protein aggregation which becomes determinant when putative toxic protein intermediates are not destroyed by the UPS. Cross-talk between pathways (double-headed horizontal arrows) signifies the upstream and downstream interactions between pathways. Upstream cross-talk may influence the signal processing phase, and includes the interactions between ER and mitochondria mediated by Ca\(^{2+}\); downstream cross-talk affects the final death mechanism and morphological indications that typify the various death modes. Caspase involvement is obligatory for “pure” apoptosis (PCD-Type I), but is less evident in other apoptotic-like outcomes. The currently applied various death classification systems are summarized below the schematic, embracing the PCD-Type nomenclature and the more recently defined mechanistic terms for cell death modes. The concept of the apoptosis-necrosis continuum integrates the differential involvement of these various death pathways and outcomes, to help explain the often complex patterns of neuronal death (mix of PCD-Types I, II and/or III). In unregulated (accidental) necrosis, there is a rapid general breakdown of many aspects of cellular integrity. This characteristically includes dissipated $\Delta\psi_m$ with consequential losses of both ion gradients across the IMM and ATP production (adapted from Nagley et al., 2010).
neuronal injury (Higgins et al., 2011). Little attention was paid to autophagy in brain until the mid-2000s, because healthy neurones contained few autophagic vacuoles (Nixon et al., 2005), and displayed low levels of autophagosome-bound LC3-II relative to LC3-I in response to perturbation (Yu et al., 2005). With identification of the role of autophagic genes and the consequences of their knockout on brain function (Boland & Nixon, 2006), more details of the significance of autophagy in the brain began to emerge.

The evidence for complexity of cell death pathways in neurones was first found in the study of neuronal death mechanisms manifested by two different types of insults inducing different patterns of caspase involvement in neuronal PCD (Beart et al., 2007). This study was initiated to gain a better understanding of the underlying death mechanisms and the involvement of the intrinsic mitochondrial pathway in an excitotoxic injury mediated via the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of L-Glu receptor. Although both AMPA and staurosporine (STS) are known to cause PCD and manifest morphological features of apoptosis (Cheung et al., 2000), AMPA-mediated PCD showed different mechanistic profiles of engagement of the mitochondrial apoptogenic machinery in death signalling compared to STS-mediated apoptosis (Beart et al., 2007). Some of the distinguishing features of apoptotic process under AMPA treatment involved activation of calpain in response to a rapid increase in Ca^{2+}, recruitment of Bax to mitochondria and depolarization of ΔΨm. Furthermore, the release of AIF subsequent to calpain activation and delayed caspase-3 activation suggested extensive cross-talk between caspase-dependent and caspase-independent pathways during AMPA-mediated excitotoxicity. These features differ from the “classical apoptosis” produced by STS in which Bax recruitment to mitochondria is followed by subsequent collateral release of cytochrome c and SMAC into the cytosol without depolarization of ΔΨm, and release of Omi and AIF with relatively early activation of caspase-3 (Beart et al., 2007).

Neurones display PCD with cross-talk to produce insult-dependent cell death across the apoptosis-necrosis continuum, PCD-Type I alone was also found to cause differential recruitment of intrinsic mitochondrial pathway and cross-talk between caspase-dependent/-independent mechanisms to produce PCD in primary neurones. This complexity of the neuronal death pathways was emphasized in a detailed study by
Diwakarla and colleagues (2009b), who examined neuronal death under various stresses implicated in neurodegenerative diseases such as kainic acid, STS, H$_2$O$_2$ and trophic-factor withdrawal. Exposure of primary cerebellar granule cells (CGCs) to various insults produced characteristic progression of injury, wherein all insults recruited mitochondrial mechanisms. The dissection of the patterns of engagement of death pathways displayed time- and insult-dependent redistribution of mitochondrial IMS proteins, and differential interplay across a continuum from caspase-dependent to -independent signalling and Bax- and calpain-dependent/independent pathways. Sometimes here the differences noted were quite subtle in terms of their linkage to mitochondrial mechanisms and presumably reflected their complex regulation in neurones (Higgins et al., 2010; Nagley et al., 2010).

In addition to the differential insult-dependency of neuronal PCD, the type of neurone was also found to be another key factor that determines the profile of PCD. The engagement of death machinery was further examined in GABAergic neurones, which are involved in Huntington’s disease and stroke injury. Compared to CGCs, which are exclusively glutamatergic, primary cultures of GABAergic neurones showed slow-onset kinetics in PCD with insult-dependent recruitment of the intrinsic mitochondrial pathway. The results were somewhat different from what had been observed in primary cultures of CGCs and this study has further supported the notion of plasticity of cell death activation dependent upon injury paradigm and cell type. Indeed the complexities found here in the patterns of PCD in primary neurones are suggestive of diversity beyond the recommendations outlined in the updated cell death nomenclature (Galluzzi et al., 2012).

The multifaceted phenomenon of neuronal PCD, which encompasses extensive cross-talk between classical apoptosis, autophagy and programmed necrosis was elucidated in primary culture of murine cortical neurones. Cell death induced by oxidative stress was characterized as a mixture of autophagic cell death, regulated necrosis and involvement of differential patterns of IMS protein release into the cytosol by a caspase-independent mechanism (Higgins et al., 2011). Cortical neurones under oxidative stress produced biochemical and morphological profiles of injury which differed from the classical apoptosis, and showed a parallel recruitment of ACD and PCD-Type III highlighting the complexity of cell death mechanisms manifested in neurones.
Furthermore, the nature of the involvement of autophagy and ACD in cell death mechanism was injury-dependent. Through collaborations with Professor Rod Devenish, whose laboratory at Monash University had established various techniques for evaluating autophagy in cell lines, further insights into neuronal autophagy were obtained. Initially STS-mediated apoptosis was found to induce “good” autophagy as a protective mechanism against neuronal death, as autophagy was up-regulated under STS treatment and did not participate in the execution of apoptotic cell death. However, primary cortical neurones exposed to an acute oxidative stress generated by $\text{H}_2\text{O}_2$ underwent neuronal death that was dependent on, and regulated by, autophagy in absence of caspase activation. In contrast to STS-mediated cell death, that $\text{H}_2\text{O}_2$-activated ACD was suggested as an alternative cell death pathway when apoptotic pathway was disrupted by blockage of caspase activation by $\text{H}_2\text{O}_2$. Therefore, cross-talk between cell death pathways was proposed as an essential part of cellular termination if one pathway was impeded (Higgins et al., 2011). This work was extended to a model of chronic oxidative stress with transient manifestation in parallel of ACD and programmed necrosis, with ACD occurring before being overwhelmed by necrosis (Higgins et al., 2012).

Building on the above observations on the involvement of autophagy in neurones as PCD-Type II ACD, my interest turned to the role of autophagy in neurones in pathological conditions. Although autophagy can be induced by starvation, the pathway can also take place even at nutrient-rich conditions at basal level. Studies done in genetically modified mice have highlighted autophagy as a “constitutive” process and as an important mechanism for neuronal homeostasis for its key role in global turnover of cellular components such as misfolded aggregated dysfunctional proteins and organelles such as expired mitochondria (Komatsu et al., 2005), which are often found in neurodegenerative diseases. Constitutive autophagy is an energy requiring process especially during the process of sequestration of cellular components (Plomp et al., 1989; Plomp et al., 1987). As energy crises occur in acute brain injuries such as stroke, and in chronic neuropathologies in which dysfunctional bioenergetics of mitochondria are implicated, a logical extension of the previous work in autophagy is to explore how energy deficits influence the regulation of autophagic function in primary neurones.
1.6.2. Mitochondrial Quality Control in Neurones: What are the Triggers for Mitophagy?

Neurones are metabolically active cells that have high energy demand for their functions and they are well known for their predominant reliance on mitochondrial OXPHOS to meet their energy demand. Keeping mitochondria functional is, therefore, vital for cellular survival. Several mechanisms of cellular mitochondrial quality control exist; these include: chaperones and proteases to continuously monitor the integrity of mitochondrial proteome; antioxidant enzymes to reduce oxidative damages by ROS; fusion and fission of damaged part of mitochondria in response to mitochondrial damage and dissipation of $\Delta \Psi_m$; and macroautophagy of the whole organelle whose damage is beyond repair (Baker et al., 2014; Kotiadis et al., 2014). Of those mechanisms, macroautophagy of mitochondria, also known as mitophagy, has been extensively investigated over the last 5 years. This phenomenon was stimulated by genetic studies of early-onset PD that revealed loss-of-function mutations in genes regulating mitochondrial quality control in patients. The mutation of products of these genes, PARK6 and PARK2, or more generally known as PINK1 and Parkin, respectively, have been recognized as the most common cause of recessive PD known so far (Kitada et al., 1998; Valente et al., 2001). They are suggested to protect cells from stress-induced mitochondrial dysfunction by activating clearance of damaged mitochondria via mitophagy. The molecular mechanisms of PINK1 and Parkin pathways have been intensively investigated mainly in yeast cells, Drosophila and immortalized cancer cell lines, where the loss of $\Delta \Psi_m$ was found to activate processes involving PINK1 and Parkin, which orchestrate entry of mitochondria into the lysosome-mediated cascade of autophagic degradation. Mitophagy, however, has not been widely investigated in primary neurones to date, with the small existent literature suggesting that the activation and the extent of mitophagy were less pronounced in neurones than in non-neuronal cells (Van Laar et al., 2011).

Hence, this study focuses on investigating the mechanisms of mitochondrial quality control in primary cultured neurones and thus has potential therapeutic relevance. In order to investigate this process, a series of pharmacological insults will be applied to cultured neurones, aimed at disruption or inhibition of particular steps or processes of OXPHOS.
Fortunately a plentiful array of specific pharmacological insults is readily available to achieve these broad goals (See Table 1.1). The consequences of such disruption will be assessed bioenergetically to study the effects of bioenergetic dysfunction of mitochondria in recruiting mitophagy, in particular, and autophagy more broadly.

In order to address the above issues, murine cerebellar granule cells (CGCs) cultured in vitro were chosen for this study. CGCs are the largest homogenous neuronal population in the mammalian brain and closely resemble the nature and behaviour of neurones in vivo (Kramer & Minichiello, 2010). Not only are CGCs relatively easy to manipulate, but the high purity achieved in primary cultures of granule cells that comprise over 95% of whole population make them an ideal model system for elucidating the molecular basis of neuronal function. As a reference for the autophagic and mitophagic activities recruited by pharmacological inhibitors of the respiratory chain, rapamycin (RAP) (Chapter 6) and CCCP (Chapter 5 & 6) were used in this study. The former is commonly used as an inducer of general autophagy (macroautophagy) by the inhibition of mTOR pathway. The latter acts as a protonophore which rapidly depolarizes the $\Delta \Psi_m$, and it has been reported to induce mitophagy in cell lines (Narendra et al., 2010). As the pathways to triggering mitophagy in primary neurones have not yet been characterized under bioenergetic deficit, the overall goal of the research reported in this thesis is to elucidate the nature of the triggers that can lead to destruction of mitochondria by mitophagy in primary cultured neurones.
1.6.3. Specific Aims of the Research

The considerations above lead to several questions being raised relating to triggering of mitophagy in neurones by dysfunctional mitochondria subsequent to bioenergetic crisis. The specific aims of my thesis are as follows.

1. To characterize the cellular and mitochondrial bioenergetic responses of CGCs to an array of pharmacological inhibitors of mitochondrial function.
2. To determine the extent of activation of mitophagy induced by each pharmacological insult to CGCs.
3. To evaluate the activation of macroautophagy, in general, induced by treatment of CGCs with each pharmacological insult.

In broad terms, achievement of these aims will establish the relationship between particular types of mitochondrial bioenergetic disruption and the activation of mitophagy, as well more general autophagy.

1.6.4. Organization of the Thesis

In dealing with the above specific aims at an experimental level, the thesis is organized into a series of Chapters that contain the methodology of the techniques applied and the results of the research undertaken.

Chapter 2 contains a description of the general methods employed, basically organized as a Materials and Methods section, which is applicable to most of the studies reported in the subsequent results Chapters (3-6). The major part of this chapter describes
establishment of homogenous population of CGCs for subsequent experiments to be performed. Specific methods used in particular phases of the work are described in the relevant results Chapter, where necessary.

Chapter 3 reports how the cell culture conditions have been set up for the subsequent experimental studies, and addresses Aim 1 by describing the general responses of the CGCs to the set of pharmacological insults developed for testing the central propositions of the thesis. Biochemical and cytochemical methods such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay and propidium iodide (PI) staining of membrane permeabilized cells showed time-dependent and concentration-dependent decreases in cellular viability after exposure to pharmacological inhibitors of respiratory chain complexes. Appropriate doses of inhibitors of the complexes were chosen based on IC$_{50}$ values and surveys of phase contrast microscopic images. This series of studies is the essential baseline for future work and established the effective concentrations of inhibition of the mitochondrial respiratory complexes that produced neuronal injury and gave insights into the time-course of neuronal dieback.

The research Aim 1 continues to be addressed in Chapter 4, where the specific deficits in mitochondrial bioenergetic function affected by each of the pharmacological agents were examined by luciferase-based ATP measurement, live cell imaging of ΔΨ$_m$, assessed by the fluorescent dye tetramethylrhodamine methyl ester (TMRM), and the Seahorse Extracellular Flux Analyzer (XF24) to measure biochemical indices of bioenergetic status of the mitochondria in live cells. Significant decreases in cellular ATP were observed in time-dependent and concentration-dependent manners and simultaneous real-time measurement of oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of CGCs showed changes in mitochondrial OXPHOS and glycolysis during inhibition of respiratory complexes. A rapid drop in ΔΨ$_m$ in CGGs was observed as early as half an hour after some drug treatments. The outcome of these results provided a confirmation of the efficacy of the inhibitory actions of the chosen doses of each pharmacological insult, which involved multifaceted characterization of bioenergetic failure of CGCs after the inhibition of respiratory chain complexes.
The research reported in Chapter 5 addresses Aim 2 which deals with the issue of mitophagy induced by the various insults applied by inhibiting respiratory chain complexes. Techniques used to study mitophagy involve observation of mobilization of PINK1 proteins to mitochondria by immunofluorescence imaging. The results demonstrate that PINK1, the upstream initiator of mitophagy, was recruited to mitochondria in primary neurones with bioenergetic defects caused by inhibition of respiratory chain complexes I-IV. The recruitment of mitophagy in primary neuronal system was further investigated by live imaging of cells transfected with vector expressing a biosensor named Rosella. Rosella biosensor is comprised of a mitochondrial targeting sequence and a fast-maturing pH-stable red fluorescent protein fused to green fluorescent protein that is sensitive to changes in pH of the surroundings of organelle. CGCs treated with complex I and II inhibitors showed significant reduction in green fluorescence (fluorescence lost in acidic pH) as compared to red fluorescence (pH stable), indicating that pH of mitochondrial location switched from cytoplasmic to acidic environment under complex I and II inhibition, indicative of translocation of mitochondria to lysosomes during mitophagy. In contrast, CGCs treated with CCCP, a conventional mitophagy inducer in Drosophila and cell lines, was revealed as an unsuitable tool in the study of mitophagy in primary neurones.

In Chapter 6, which is relevant to Aim 3, the question of more general autophagy in the treated cells is addressed. For this purpose, an immunoblotting technique was utilized to detect a marker of autophagy (LC3-I to LC3-II transition), and additionally, fluorescence imaging of an auto-fluorescent marker of acidic vacuoles, monodansylecadaverine (MDC), was employed. Elevated autophagy flux and increased intensity of the general autophagy marker were detected in response to the inhibition of all ETC complexes. Thus, although the bioenergetic dysfunction caused by complex III and IV inhibition failed to show significant induction of mitophagy in CGCs, it was able to recruit general autophagy.

Each of the result Chapters 3-6 contains a discussion section in which the particular sets of data obtained are appraised and placed into the context of the relevant Aim of the study as whole.
The thesis continues with Chapter 7, which presents a more general discussion of the results obtained and covers broader issues than those dealt with in particular results chapters. This General Discussion also integrates some of the findings and considers them in terms of previous and emerging research findings of others in this and related fields.

Following the detailed listing of References cited in the thesis, there are three appendices in which specific sets of data or other information are collated and presented. These items are summarised or cited in the relevant place in the main text.
CHAPTER TWO

Materials and Methods
2.1. Materials

Neurobasal™ medium (NBM), Ca\(^{2+}\), Mg\(^{2+}\)-free Hank’s Balanced Salt Solution (HBSS), B-27 supplement, L-glutamine, qualified fetal bovine serum (FBS), penicillin-streptomycin and HEPES buffered salt solution, Lipofectamine2000 and Opti-MEM I (serum reduced media), minimal essential medium (MEM) and MEM without phenol red were purchased from Gibco™ Invitrogen Corporation (Melbourne, Australia).

Trypsin from porcine pancreas, trypsin inhibitor from glycine max (soybean), potassium chloride (KCl), poly-D-lysine, MgSO\(_4\).7H\(_2\)O, deoxyribonuclease I from bovine pancreas (DNase I), D-glucose, sodium pyruvate, bovine serum albumin lyophilized powder (BSA) and aphidicolin were purchased from Sigma-Aldrich (Sydney, Australia).

Sterile NUNC cell culture plates (Copenhagen, Denmark) and Falcon cell culture 6-well plates (Corning, USA) were used for cell culture. Glass coverslips (13 mm round) and glass microscope slides for confocal microscopy were purchased from Menzel-Glaser (Braunschweig, Germany). Fluorescent mounting medium was purchased from Dako (Dako Ltd). Syringes (25 ml) and 15 gauge needles were from Terumo® (Melbourne, Australia) and filter units were from Sarstedt (Germany).

Dimethyl sulfoxide (DMSO; 100 % (v/v)), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton-X 100 (TX-100), ethylenediaminetetraacetic acid (EDTA), RPMI 1640 growth medium, paraformaldehyde, propidium iodide (PI), ammonium persulfate, TWEEN® 20, TEMED, rapamycin (RAP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), monodansylcadaverine, sodium hydroxide (NaOH), rotenone (Rot), 3-nitropropionic acid (3-NP), antimycin A (AA) and potassium cyanide (KCN) were purchased from Sigma-Aldrich (Sydney, Australia). H\(_2\)O\(_2\) (30 % (w/v)) was purchased from Merck (Melbourne, VIC, Australia) and STS was purchased from Tocris Cookson (Bristol, U.K.).
Lumi-Light Substrate Kit and complete protease inhibitor cocktail tables were purchased from Roche Diagnostics (Castle Hill, Australia). SeeBlue® Plus2 Pre-Stained protein standard marker was purchased from Invitrogen (Melbourne, Australia). Bio-Rad Dc Assay kit, reagents A, B and S were all purchased from Bio-Rad Laboratories (Hercules, USA). Bio-Rad protein assay standard, Immuno-Blot® PVDF membrane, Acrylamide/Bis. Hyperfilm™ ECL film were purchased from Amersham Biosciences Pty Ltd (Castle Hill, Australia). Skim milk powder was purchased from Diploma (Victoria, Australia). Secondary antibodies horse radish peroxidise conjugated goat anti-mouse was purchased from Chemicon International Inc (Melbourne, Australia). IRDye® 680RD Secondary Antibodies and IRDye® 800CW secondary antibodies were purchased from Li-Cor (Lincoln, NE, USA). Primary antibody Anti-SDHA mouse monoclonal antibody was purchased from Abcam and primary antibody monoclonal anti-β-actin (mouse IgG2a isotype) was purchased from Sigma-Aldrich (Melbourne, Australia) and primary anti-glial fibrillary acidic protein (GFAP) was from Promega (WI, USA). Primary antibody against the N terminus of LC3 (microtubule-associated protein 1 light chain 3B) mouse was purchased from Nanotools (Antikorpertechnik GmbH). Secondary antibodies, Alexa Fluor® 488 (goat anti-mouse and rabbit), Alexa Fluor® 568 (goat anti-rabbit), tetramethylrhodamine methyl ester (TMRM) and Hoechst 33342 were purchased from Molecular Probes®, Invitrogen (Melbourne, Australia).

Mitophagy biosensor, Rosella construct was a kind gift from Dr Carlos Rosado from Monash University (Rosado et al., 2008). PINK1 primary antibody (anti-rabbit) was a kind gift from Dr Janetta Culvenor from University of Melbourne (George et al., 2010).

Adenosine 5'-triphosphate (ATP) Bioluminescent Assay (FLAA) and ATP powder (A1852) were purchased from Sigma-Aldrich (Sydney, Australia). Phosphosafe™ Extraction Reagent was purchased from Novagen® (EMD Millipore, Billerica, MA) and clear bottomed opaque sterile 96-well plates which were designed for luminescence reading (CLS3788) were purchased from Sigma Aldrich (Sydney, Australia).
All reagents and solutions used for Seahorse XF24 analyses were purchased from Seahorse Bioscience (North Billerica, MA). Measurement of mitochondrial respiration with Seahorse XF24 was performed by using XF Cell Mito Stress Test Kit (Part number: 103015-100) and the Kit contains oligomycin, rotenone/antimycin A and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

All other chemicals used to prepare buffers, solutions and media were of cell culture or molecular biology grade, and were purchased from BDH laboratories (Melbourne, Australia) unless otherwise stated. Milli-Q water (mQH$_2$O) (Millipore) was used to prepare all reagents, and in those instances where sterile solutions or equipment were required, filter sterilization using 0.2 µm filter units (Sarstedt, Germany) or autoclaving at 121°C for 20 min was performed, respectively.
2.2. Methods

2.2.1. Animals

All experimentation was approved by the Ethics Committee of the Florey Institute for Neuroscience and Mental Health and was undertaken according to the guidelines of the National Health and Medical Research Council (NHMRC, Australia). Animals were obtained from The Florey Institute of Neuroscience and Mental Health Core Animal Services. The cerebella of Swiss White mice (postnatal day (PND) 6-8) were used throughout all experiments to establish primary neuronal cultures.

2.2.2. Primary Cerebellar Granule Cell Culture

2.2.2.1. Primary cerebellar granule cell culture

All equipment was sterilized by spraying with 80% ethanol prior to dissection and the whole procedure was performed under sterile conditions. Swiss White mice at PND 6-8 were decapitated using a pair of scissors, making sure the decapitation line was low enough to prevent the cerebellum from being damaged. The removed heads were transferred to ice-cold HBSS pH 7.4 containing 3 mg/ml BSA, 1.16 mM MgSO₄, 1 mM sodium pyruvate, 10 mM HEPES, 7.6 mM D-Glucose. The skin and skull were cut in the midline and carefully removed around the brain to reveal the cerebellum. Under a
dissecting microscope (Industrial and Scientific Supply Company, Australia), the cerebellum was gently pulled away from the rest of the brain using a pair of fine curved forceps and placed in fresh ice-cold HBSS/BSA where any visible blood vessels and meninges surrounding cerebellum were removed on an ice-cold Petri dish. Dissected cerebella were transferred to a sterile 50 ml centrifuge tube and centrifuged at 1000 x g for 1 min. The supernatant was removed using a sterile Pasteur pipette and cerebella were chemically digested in 20 ml of pre-warmed HBSS/BSA (37°C) containing 0.2 % trypsin, 80 µg/ml DNase in a shaking water bath (37°C) for 30 min. The digestion was stopped by adding 20 ml of HBSS/BSA containing 83.2 µg/ml trypsin inhibitor, 12.8 µg/ml DNase, 1.4 mM MgSO₄ and the tube was centrifuged at 1200 x g for 3 min. The supernatant was removed by sterile Pasteur pipette and the pellet was mechanically digested in HBSS/BSA containing 0.52 mg/ml trypsin inhibitor, 80 µg/ml DNase, 2.66 mM MgSO₄ by 15 strokes of trituration with a 14 gauge needle mounted on a 20 ml sterile syringe (Terumo, Melbourne, Australia) to obtain a single cell suspension. The suspension of cells was centrifuged at 1200 x g for 3 min, and the supernatant was carefully removed by aspiration via sterile Pasteur pipette, taking care not to disturb the pellet. The resultant pellet was resuspended in NBM containing B-27 components (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum.

2.2.2.2. Cell counting

Prior to seeding cells into multi-well plates, the cellular density of the suspension was determined by using a Neubauer haemocytometer (Weber Scientific, Sheffield, UK). The cell suspension was thoroughly mixed to establish an even distribution of cells in the solution, and 10 µl of cell suspension was placed in each edge of haemocytometer as a representative of the cell suspension. The number of cells within the gridded area of the counting chamber was directly counted using a fluorescence microscope (Olympus U-TB190) and the average of four cell counts were used to determine the density of cells in original mixture. The cellular density was determined by the following formula:

Cell density (cells/ml) = Average of cell count x 10,000 cells/ml

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The seeding volume was determined from the above formula and cells were seeded in multi-well plates using an Eppendorf multi-pipette at a density of $0.12 \times 10^6$ cells/well for 96-well plates, $0.2 \times 10^6$ cells/well for 48-well plates, $0.35 \times 10^6$ cells/well for 24-well plates and $2 \times 10^6$ cells/well for 6-well plates (c.f. Diwakarla et al., 2009b).

2.2.2.3. Growth and maintenance of cultures

Experiments were performed on neurones plated on NUNC\textsuperscript{TM} plates (Copenhagen, Denmark). The plates were pre-coated with poly-D-lysine ($50 \mu g/ml$ per well) for 2 h or overnight in 37°C to promote cell adhesion to the plates. The poly-D-lysine solution was aspirated and the plates rinsed once with sterile mQH\textsubscript{2}O and left to dry in the sterile hood at room temperature. Cells were seeded at appropriate cell densities and left to grow in NBM in the presence of 10 % fetal bovine serum for 24 h on 0 day \textit{in vitro} (DIV), in a humidified incubator (5 % CO\textsubscript{2}, 8.5 % O\textsubscript{2} and N\textsubscript{2} at 37°C). At 1 DIV, the medium was fully replaced by NBM containing aphidicolin (1 µg/ml) to restrain non-neuronal cell proliferation (Diwakarla et al., 2009b; Giardina et al., 1998), and a final concentration of 3 % (v/v) B-27 supplement to support viability of neuronal culture. Half medium changes were conducted at 4 DIV and included aphidicolin (1 µg/ml) and 3 % (v/v) B-27 supplement and all experiments were performed at 7 DIV.

2.2.3. Drug Treatment

Cerebellar granule cells (CGCs) grown in multi-well plates were exposed to specific concentrations of insults at 7 DIV. Stock solutions of drugs were prepared by dissolving drugs in DMSO (STS, Rot, AA, RAP), NaOH (3-NP) or mQH\textsubscript{2}O (KCN). Serial dilutions of stock solutions of drugs were made in MEM containing 25 mM K\textsuperscript{+},
and drugs were equilibrated for 1 h at 37°C in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C). The final concentrations of the solvent were kept to 0.03-0.2% for DMSO and 0.04% for ethanol (Mercer et al., 2005). All drugs were prepared fresh for each experiment except STS and RAP, which were kept as stock solutions at -20°C until required for use. Complete media changes were performed to expose CGCs to drugs or vehicle that contained solvents only. Detailed descriptions of the concentrations of drugs and duration of treatment are described at each appropriate chapter of the thesis.

2.2.4. General Immunocytochemistry Procedure

CGCs grown in 24-well plate or 48-well plate or on coverslips were fixed for 10 min at room temperature with pre-warmed phosphate-buffered saline (PBS: 137 mM NaCl, 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, pH 7.4) containing 4 % (w/v) paraformaldehyde. Cells were washed three times with PBS and permeabilized for 5 min with 0.3 % (v/v) TX-100 in PBS at room temperature. Cells were washed three times with PBS and non-specific binding was blocked using PBS containing 1 % BSA (w/v) for 1 h at room temperature. The blocking buffer was made by dissolving 1 % BSA into PBS and then filtering the solution with a 0.45 µm syringe filter. CGCs were incubated overnight at 4°C overnight with primary antibodies diluted in blocking solution. Any unbound primary antibody was removed with three times wash with PBS prior to incubation with the secondary antibody conjugated with Alexa Fluor® diluted in blocking solution for 3 h at room temperature in dark. Appropriate dilutions of primary and secondary antibodies are described at each appropriate chapter of the thesis. Cells were gently washed with PBS three times at room temperature. Immunolabelled CGCs that were grown in multi-well plates for fluorescence microscopy were stored in PBS and kept at 4°C in dark, while those grown on coverslips for confocal microscopy imaging were carefully removed from the plates by gently lifting the coverslips from the well, and mounted on glass microscope slides using the Dako fluorescent mounting medium and left to dry in the dark at room temperature. Slides were stored at 4°C until required for imaging by microscopy.
2.2.5. Western Immunoblotting

2.2.5.1. Protein extraction

CGCs grown in 6-well plates were exposed to insults according to Section 2.2.2 and 2.2.3. Cells were washed once with ice-cold PBS (pH 7.4) and 100 µl of cold (4°C) radioimmune precipitation assay (RIPA) buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1 % (w/v) SDS, 1 % (v/v) Triton X-100, protease inhibitor tablet dissolved according to the manufacturer’s instructions) was added into each well. The cells from each experiment condition were gently removed by scraping and transferred into Eppendorf tubes and shaken on ice (150 rpm for 1 h with a Ratek Orbital Mixer Incubator). The collected samples were centrifuged at 14,000 x g for 5 min at 4°C and supernatant containing whole cell extracts were stored at -20°C until ready for use.

2.2.5.2. Protein assay

Protein concentration of the lysates was determined using the commercially available DC Bio-Rad Protein Assay Kit (Sydney, Australia) according to the manufacturer’s instructions. This assay kit is based on Lowry assay (Lowry et al., 1951) which involves a reaction of protein with copper in an alkaline medium and the subsequent reduction of Folin reagent to produce species with a characteristic blue colour. Standards comprised of BSA (0-2 mg/ml) were simultaneously assayed with experimental samples and the absorbance of the both the standards and the samples were measured at a wavelength of 750 nm using a Ceres UV900C microplate reader (Bio-Tek Instruments, Inc., Winooski, USA). A standard curve was generated using GraphPad Prism v.4.0. (GraphPad, San Diego, USA) to determine the protein content of the samples.
2.2.5.3. General procedures of detection of a protein by immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize the proteins of interest following drug treatment. Equal amounts of protein (~10 µg) were prepared in loading buffer (4 x loading dye, 10 % v/v β-mercaptomethanol) and denatured at 95°C for 5 min. Protein and protein standard marker (SeeBlue Plus2 pre-stained protein marker) were loaded onto SDS-polyacrylamide separating gel (375 mM Tris-HCl, pH 8.8, Acrylamide/Bis, 3.5 mM SDS, 2.2 mM ammonium persulfate, 0.0005 % (v/v) TEMED) with a 4% stacking gel (125 mM Tris-HCl, pH 6.8, 4 % Acrylamide/Bis, 3.5 mM SDS, 2.2 mM ammonium persulfate, 0.0005 % (v/v) TEMED). Detailed description of the appropriate percentage of Acrylamide/Bis in the separating gel is given in the appropriate chapter. Protein was electrophoresed at 100 V for 1.5 h in running buffer (25mM Tris, 192 mM glycine, 3.5 mM SDS) using the mini-protean 3 gel system (Bio-Rad Laboratories, Sydney, Australia). Bio-Rad filter paper and Polyvinylidene difluoride (PVDF) membrane (0.2 µm) were soaked in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) with 20 % (v/v) methanol for 15 min while the gels were soaked in transfer buffer for 10 min. The PVDF membrane and gels were layered in the order of filter paper, PVDF membrane, gel and filter paper in a Bio-Rad Mini Trans-Blot® Cell (Sydney, Australia) and proteins were transferred to membrane at 100 V for 1 h in transfer buffer with 20 % (v/v) methanol which was kept cool with an icepack during the transfer. After the transfer, PVDF membrane was washed with Tris-buffered saline (TBS: 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) to remove any methanol remaining from the transfer buffer, and blocked with 5 % (w/v) skim milk in TBS for 1 h at room temperature. The membrane was subsequently incubated with 1 % (w/v) skim milk in TBS containing primary antibody overnight at 4°C on a rocking platform (Ratek Instruments, Melbourne, Australia). Membranes were washed three times with TBS containing 0.1 % (v/v) Tween-20 (TBST), and subsequently incubated for 3 h at room temperature on a rocking platform with 1 % (w/v) skim milk in TBST containing HRP-conjugated secondary antibodies (1:1,000), or 1 h at room temperature with IRDye 680 conjugated secondary antibodies diluted in TBST (1:10,000) for Odyssey Classic infrared imaging system (Li-Cor, Lincoln, NE, USA). Membranes were washed three times with TBST, and protein was detected using Lumi-Light Western blotting substrate kit and exposure of membranes to ECL x-ray film and developed in an Agfa CP1000 automatic film processor (Agfa-Geveaert Limited, Melbourne, Australia). Alternatively, membranes
were washed three times with TBST to remove excess secondary antibodies and infrared fluorescence was detected by Odyssey Classic infrared imaging system. To verify that approximately equal amounts of protein were loaded blots were re-probed using primary antibodies specific for β-actin (anti-mouse 1:5000) in 1% (w/v) skim milk in TBS and above procedures were repeated for visualization of β-actin on the membranes.

2.2.5.4. Data analysis for Western immunoblotting

Densitometric analysis of Western immunoblots was performed using ImageJ software (obtained from the NIH web site: http://rsb.info.nih.gov.ij) or Image Studio™ Software to measure the area and density of protein bands after subtracting the background of the autoradiographic film. A rectangle was drawn around individual band and its optical density was normalized to the amount of β-actin of the same sample on the same gel. The values were expressed as a percentage of the amount of immunoreactivity observed in the control sample within the same gel and values were averaged across three or four independent cultures. Data are given as a mean ± SEM and were subjected to two-way ANOVA followed by Bonferroni’s post hoc test or Kruskal-Wallis analysis with the Dunn’s test for post hoc comparisons, where P < 0.05 was considered significant.
CHAPTER THREE

Characterization of Primary Cerebellar Granule Cell Cultures and Responses to Inhibitors of Mitochondrial Oxidative Phosphorylation
3.1. Introduction

Tissue culture techniques have been extensively employed in recent years for studying neurobiology. Cells dissociated from regions of the central nervous system (CNS), such as spinal cord and cerebellum, can be maintained in primary culture and provide specific properties of the CNS. Consequently, *in vitro* culture allows investigations of specific neurobiological aspects of growth, differentiation, effects of drugs and metabolic activities (Fedoroff & Hertz, 1977). In particular, primary neuronal cultures from postnatal rodent cerebellum have been widely used for such studies because of the advanced knowledge of cerebellar structure, which features well characterized layers of neurones whose postnatal development and neuronal synaptic connection profiles are precisely documented (Burry & Lasher, 1975; Contestabile *et al.*, 1973; Lasher & Zagon, 1972).

Cerebellar granule cells (CGCs) are excitatory glutamatergic neurones in the granular layer of the cerebellar cortex which give rise to parallel fibres to relay information to Purkinje cells of the molecular layer of the cerebellar cortex (Hirano *et al.*, 1986; Purves D, 2001; Purves *et al.*, 2001). CGCs are the most abundant population of neurones in the mammalian brain (Contestabile, 2002; Shepherd, 2004) allowing a high yield of viable CGCs from tissue culture of cerebellum. Furthermore, the cerebellum is a prominent structure of postnatal mammalian brain and its straightforward isolation process makes CGC culture most attractive for research. CGCs are isolated from PND 7 mice, the age at which the cerebellum starts to generate the main subtypes of neurones and their major synaptic connections (Messer, 1977). However, as found in all neuronal cultures, dissociating CGCs disturbs synaptic connections or circuitry between surrounding neurones, such as mossy fibres and Purkinje cells *in vivo*. In order to overcome the synaptic differences found between cellular networks *in vitro* and *in vivo*, a depolarizing agent KCl is added to the medium to mimic the synaptic connections between cells (See *et al.*, 2001). Mild depolarization of CGCs also prolongs neuronal viability in *in vitro* culture (Didier *et al.*, 1989; Gallo *et al.*, 1987; Lasher & Zagon, 1972; Thangnipon *et al.*, 1983) by activating voltage-dependent calcium channels (VDCCs) (Ichikawa *et al.*, 1998; Zurgil & Zisapel, 1984). The Ca\(^{2+}\)-mediated signalling pathways
promote synthesis of brain-derived neurotrophic factor (BDNF) (West et al., 2001) which mediates CGC development (Kokubo et al., 2009).

Primary CGC culture can provide a high purity homogenous population of CGCs, indeed up to 95 % by using antimitotic agents such as aphidicolin or cytosine arabinofuranoside, which inhibit proliferation of non-neuronal cells without interference of the differentiation of CGCs in vitro (Diwakarla et al., 2009b; Giardina et al., 1998; Messer, 1977; Thangnipon et al., 1983). Homogeneity of the culture is further maintained during the process of isolation of cells due to a low survival rate of large neurones such as Purkinje and Golgi cells which display different developmental profiles in postnatal cerebellum. These neurons do not adhere when plated to be grown in monolayer on tissue culture plates and are washed away during media changes (Kramer & Minichiello, 2010), thus allowing high purity of the CGC population in the culture. Due to the high abundance and homogeneity, primary culture of CGCs has been used extensively to study molecular pathways of fundamental neuronal functions such as PCD, neuronal migration and differentiation (Bilimoria & Bonni, 2008; Contestabile, 2002; Diwakarla et al., 2009b). This homogeneity is a real positive in cellular work since it allows direct interpretation of biochemical and molecular interactions, which otherwise are disadvantaged in mixed cell cultures. In addition, monolayer culture of CGCs can provide accessibility of cellular surfaces to chemical probes and labels to allow non-destructive observation of cellular growth and morphological features that change following delivery of neurotoxic agents. The homogeneity of CGCs in primary culture means they are especially amenable to studies of neuronal injury and they have been extensively utilized to this end. Indeed, our laboratory has worked with this primary culture model for some 20 years (Carroll et al., 1998; Cheung et al., 1998a; Diwakarla et al., 2009b; Giardina & Beart, 2001; Giardina et al., 1998) and most recently has documented the profiles of neuronal injury (apoptosis and programmed necrosis) induced by various stressors (Diwakarla et al., 2009b).

There is a wide awareness in the current literature of the existence of a spectrum of cell death types which crosstalk to produce diverse phenotypes of neuronal death depending on injury paradigm and cell types (Higgins et al., 2010; Nagley et al., 2010). This complexity of cell death mechanisms manifested in neurones has already been well
documented in studies where various stressors were used to mimic pathological settings of neurodegenerative diseases. Agents evaluated by our laboratory included: staurosporine (STS) as an inducer of apoptosis, hydrogen peroxide (H$_2$O$_2$) as an oxidative stressor, low K$^+$ to mimic trophic factor withdrawal, and kainate for inducing excitotoxicity (Beart et al., 2007; Diwakarla et al., 2009a; Diwakarla et al., 2009b; Higgins et al., 2009; Higgins et al., 2011; Higgins et al., 2012). These studies revealed the insult-dependent nature of neuronal injury and the multifaceted nature of PCD that underlies neurodegeneration. However, the current literature on PCD possesses many unknowns in terms of how bioenergetic failure ensuing from inhibition of OXPHOS complexes translates into neuronal injury. Although the application of pharmacological inhibitors of OXPHOS is well documented in animal models of human neuropathologies such as PD, HD, AD and ALS (Borlongan et al., 1997; Cannon et al., 2009; Ferrante, 2009; Jung et al., 2002; Moran et al., 2012; Parker et al., 1994; Ramaswamy et al., 2007; Sherer et al., 2003; Vis et al., 1999), there remain here many unknowns in terms of cause and effect on cellular and molecular changes related to consequent injury to OXPHOS complexes. Thus, this thesis required prior characterization of cellular changes related to injuries caused by OXPHOS inhibition by pharmacological inhibitors in order to observe their subsequent effect on neuronal mitochondrial biology. The determinant role of intracellular ATP levels in the apoptosis-necrosis continuum (Eguchi et al., 1997; Los et al., 2002; Nicotera & Melino, 2004; Proskuryakov & Gabai, 2010) was also taken into consideration, as a rapid energy depletion due to mitochondrial injuries could lead to necrotic-type of cell death (Skulachev, 2006).

Although the aim of this chapter of the thesis was not to dissect the complex cell death mechanisms manifested in neurones under bioenergetic stress, it was considered important to characterize the type of cell death in broad terms so as to avoid rapid accidental cellular demise that makes biochemical and morphological assessment difficult due to loss of cellular structural integrity and release of cellular cytoplasm into the extracellular space (Galluzzi et al., 2015). Different types of cell death are often characterized by involvement of specific enzymes involved during the cell death process (e.g. caspases, calpains and cathepsins), functional aspects and immunological characteristics, and cell death can also be classified according to the morphological features of dying cells. Morphological hallmarks of apoptotic-like cell death include rounding-up of the cell, reduction in cellular volume (pyknosis) and fragmentation of
nuclei (karyorrhexis), which can be easily detected under light microscopy and by using fluorescent DNA-binding stains, such as Hoechst (Kroemer et al., 2009). Furthermore, the use of a membrane-impermeable nuclear and chromosome counterstain, such as propidium iodide, can distinguish cells undergoing necrotic cell death which usually begins at the cell surface and exhibits early lysis of the plasma membrane before any significant alterations in nuclear morphology occurs (Collins et al., 1997; McCarthy & Evan, 1998). Conversely, the plasma membrane remains intact till the later stage in apoptotic-like cell death, providing a clear distinction from necrotic cell death (Elmore, 2007).

The focus of this particular segment of thesis investigation is therefore to fully document the inhibitory profiles (concentration- and time-dependence) of selected pharmacological agents acting upon mitochondrial OXPHOS in primary culture of CGCs via a careful “titration” of drug-induced injury. The overall desired outcome of the characterization of neurotoxicity profiles of all drugs was to determine the appropriate concentration of drugs to prevent cells from unregulated necrosis and therefore expedite subsequent cellular morphological and biochemical analyses secondary to bioenergetic dysfunction. This strategy will thus provide a baseline for the subsequent analyses of mitochondrial biology in the subsequent chapters of the thesis.
3.2. Methods

3.2.1. Primary Cerebellar Granule Cell Cultures

Primary cultures of CGCs were prepared from 6-8 day-old SWISS White mice according to Section 2.2.2. Briefly, cerebella were dissected in ice-cold isolating solution (HBSS pH 7.4 containing 3 mg/ml BSA, 1.16 mM MgSO4, 1 mM sodium pyruvate, 10 mM HEPES, 7.6 mM D-Glucose) and the meninges were carefully removed. The isolated cerebella were placed in fresh ice-cold isolating solution and they were chemically and mechanically dissociated and centrifuged. The cells were resuspended in NBM containing B-27 supplement (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum. Cells for fluorescence studies of MAP2 and GFAP immunolabelling and PI labelling were seeded in 48-well plates (0.2 x 10^6 cells/well), and cells for MTT cell viability assays were seeded and grown in 96-well plates (0.12 x 10^6 cells/well). All multi-well plates were pre-coated with poly-D-lysine (50 µg/ml). Neurones were left to grow in NBM containing B-27 components (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum for 24 h in a humidified incubator (5 % CO2, 8.5 % O2 and N2 at 37°C). Medium was fully replaced by NBM containing aphidicolin (1 µg/ml) to restrain non-neuronal cell proliferation (Diwakarla et al., 2009b; Giardina et al., 1998), and final concentration of 3 % (v/v) B-27 supplement. Half medium changes were conducted at 4 DIV and included aphidicolin (1 µg/ml) and 3 % of B-27 supplement and all experiments were performed at 7 DIV.
3.2.2. Immunofluorescence Staining of Cultured Cells by MAP2 and GFAP

CGCs are the largest homogenous neuronal population in the mammalian brain (Kramer & Minichiello, 2010). Cultures of CGCs in vitro previously have been shown to contain over 95% of neurons (Giardina et al., 1998) making them an ideal model system for elucidating the molecular basis of neuronal function. Homogeneity of CGC culture used in this study was examined by staining CGCs with markers of neurones (microtubule-associated protein 2; MAP2) and glial cells (glial fibrillary acidic protein; GFAP). MAP2 is a neuron-specific marker which is only found in post-mitotic neuronal cells in a wide variety of mammals (Izant & McIntosh, 1980), whereas GFAP is used to mark astrocytes in the brain (Raff et al., 1979).

Cultures were grown in 48 well plates and fixed with 300 µl of pre-warmed 4% paraformaldehyde solution pH 7.4 (4 g of paraformaldehyde dissolved in 100 ml PBS; 137 mM NaCl, 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, pH 7.4) for 10 min at room temperature. Cells were washed three times with pre-warmed PBS, and cells were permeabilized for 10 min with 0.3% (v/v) TX-100 in PBS. Cells were briefly washed with PBS and blocking with 1% BSA dissolved in PBS for 1 h at room temperature to prevent non-specific binding of antibodies. After blocking, cells were incubated with primary MAP2 antibody (anti-mouse, 1:200) and GFAP (anti-rat 1:100) diluted in PBS containing 1% BSA overnight at 4°C. Primary antibodies were aspirated and cells were washed with PBS three times and incubated with anti-mouse Alexa 488 (1:200) and anti-rabbit Alexa 568 secondary antibodies (1:200) diluted in PBS containing 1% of BSA for 3 h at room temperature in dark. After the incubation, secondary antibodies were removed and cells were washed three times with PBS. Images from random fields were taken with an Olympus 1x71 inverted fluorescence microscope, which was connected to the Olympus C-5050 digital camera, at an excitation/emission wavelength of 495/519 nm (green channel) and 578/603 nm (red channel).
3.2.3. Determination of Cell Viability

3.2.3.1. Preparation and exposure of cell cultures to drugs

All pharmacological inhibitors of mitochondrial respiratory chain complexes, Rot, 3-NP, AA and KCN were weighed and prepared fresh on the day of experiment. Stock solutions of Rot and AA were made by dissolving drugs in DMSO to give a concentration of 10 mM and 2 mM, respectively. 3-NP was dissolved in 100 mM of NaOH to give a concentration of 100 mM and KCN was dissolved in mQH2O to make a 1 M stock solution. Oli was dissolved in ethanol to make a 100 mM stock. STS was dissolved in DMSO to make a stock solution of 100 µM and stored in the freezer at -20°C for future experiments. H2O2 was made fresh on the day of experiment by diluting 30 % (w/v) H2O2 stock solution with MEM to give a final stock concentration of 10 mM. The final concentrations of the solvent were kept to 0.03-0.2% for DMSO and 0.04% for ethanol (Mercer et al., 2005) and vehicle controls were made by adding the appropriate % of the solvent to MEM. Drugs were further diluted in MEM to give the final concentration for the experiment and kept in a humidified incubator (5 % CO2, 8.5 % O2 and N2 at 37°C) for 1 h to allow the drug solutions to be warmed and equilibrated. For drug treatment, growth medium was removed and replaced by MEM containing drugs or vehicle control with a full medium change. Neuronal injury with drugs was induced for various time points during incubation in a humidified incubator (5 % CO2, 8.5 % O2 and N2 at 37°C).

3.2.3.2. MTT Assay: Function of mitochondria

CGCs were grown in 96-well plates at a density of 0.12 x 10^6 cells/well. Drugs were diluted in MEM and 100 µl was added to each well and incubated for 24 h for the concentration responses and 1-24 h for time-dependent changes in cell viability (Table 3.1). Cellular viability was assessed by colorimetric assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; an index of mitochondrial function)
Table 3.1. Drug concentrations and duration of treatment

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosporine (STS)</td>
<td>100 - 300 nM</td>
<td>2, 4, 6, 8, 12, 24</td>
</tr>
<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>20 - 50 µM</td>
<td>2, 4, 6, 8, 12, 24</td>
</tr>
<tr>
<td>Rotenone (Rot)</td>
<td>10 - 1000 nM</td>
<td>1, 4, 8, 16, 24</td>
</tr>
<tr>
<td>3-Nitropropionic acid (3-NP)</td>
<td>20 - 500 µM</td>
<td>1, 4, 8, 16, 24</td>
</tr>
<tr>
<td>Antimycin A (AA)</td>
<td>10 - 1000 nM</td>
<td>1, 4, 8, 16, 24</td>
</tr>
<tr>
<td>Potassium cyanide (KCN)</td>
<td>30 - 300 µM</td>
<td>1, 4, 8, 16, 24</td>
</tr>
<tr>
<td>Oligomycin (Oli)</td>
<td>0.1 - 1 µM</td>
<td>1, 4, 8, 16, 24</td>
</tr>
</tbody>
</table>
reduction assay as previously described (Beart et al., 2007). MTT was dissolved in RPMI 1640 growth medium to a final concentration of 5 mg/ml. MTT solution was filter-sterilized through a 0.2 µm filter and stored at -20°C in aliquots. MTT solution (10 µl) was directly added to the original culture medium (final concentration 0.5 mg/ml) at the time of cessation of drug treatment. Subsequent to MTT addition, cells were incubated in the humidified 37°C incubator for 30 min. The culture medium containing MTT was completely aspirated and 100 µl of DMSO was added to each well to dissolve formazan crystals and the plate was briefly shaken to further enhance dissolution. The quantity of formazan formed was determined by colorimetric analysis (absorbance at 570 nm) on a Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA). Raw values from drug-treated cells was standardized against cells incubated in 0.1 % TX-100 (100 % cell death) and expressed as percentage of vehicle control values (0 % cell death). Assays were performed with n = 3-4 replicates. Phase contrast images were taken to examine morphological features of neurones after drug treatment by Olympus 1 x71 inverted microscope and Olympus C-5050 digital camera.

3.2.3.3. Propidium iodide: Marker of cellular membrane disintegration

Propidium iodide (PI) is a fluorescent molecule which binds to nucleic acid with an excitation/emission wavelength of 535 nm/617 nm. Its membrane impermeable property enables it to be used to evaluate cellular viability since it only stains cells with compromised cell membranes. On the other hand, Hoechst 33342 is a cell membrane permeable nuclei dye, which gives blue fluorescence under UV light when bound to double strand DNA. Due to its cell permeable property, Hoechst can be used to stain all live and dead cells in the culture to observe nuclei morphology, or to count the whole number of cells available in the culture. PI stock was made by solution in mQH$_2$O to give a stock concentration of 1 mg/ml. The stock was kept at 4°C and fresh stock was prepared after 6 months.

Cultures were grown in 48 well plates and exposed to drugs as described in Section 2.2.2 and Section 3.2.3.1 for 1, 4, 8 and 24 h. After drug treatment, cells were incubated with PI (10 µg/ml) and Hoechst 33342 (5 µg/ml) diluted in PBS (pH 7.4) for 10 min in a
humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C). Cells were washed four times with pre-warmed PBS (pH 7.4) to remove unbound dye and immediately fixed with 300 µl of PBS containing 4 % paraformaldehyde (pH 7.4) for 10 min at room temperature. Cells were washed three times with PBS at room temperature and images from random fields (10-15 images, total number of cells 250-500) were taken by Olympus 1x71 inverted fluorescence microscope at an excitation/emission wavelength of 535 nm/617 nm for cells stained positive for PI and UV channel (350 nm/461 nm) for Hoechst stained cells. Cells stained with PI and Hoechst were counted and expressed as a percentage of PI stained cells over the total number of cells stained by Hoechst.

3.2.4. Data Analysis

The detection of neuronal and glial populations in CGC culture was assessed in a single immunocytochemical staining experiment where random images were captured from duplicate wells (13 pictures per well) was performed. The MAP2 and GFAP labelled cells were manually counted and expressed as a percentage of the total number of cells per image (300-500 cells were counted per picture). Cell viability data are given as a mean ± SEM of experiments performed across three to six independent experiments measured in quadruplicate wells. Concentration-response curves were generated by non-linear regression analysis using a computer-assisted curve fitting program (GraphPad Prism v.4.0. San Diego, CA, USA). Similarly, the time-dependent graphs were generated by GraphPad where data was presented as mean ± SEM from quadruplicate determinations in two independent experiments and are expressed as a percentage of the control. Statistical significance (* P < 0.05; ** P < 0.01) was examined by one-way ANOVA, followed by Dunnett’s post hoc test for intra-group comparison. Plasma membrane integrity was monitored by PI labelling, where data are presented as mean ± SEM from quadruplicate determinations in two independent experiments and are expressed as a percentage of the total number of cells. Statistical significance (** P < 0.01, ### P < 0.0001) was examined by two-way ANOVA, followed by Bonferroni post hoc test.
3.3. Results

3.3.1. Establishment of CGC Culture

Primary CGCs were cultured from postnatal Swiss mice and the development of the CGC culture was followed from 0 DIV to 7 DIV by phase contrast microscopy. Newly plated cells were dispersed evenly over the whole surface of the plate in the form of spheres, and after 24 h in vitro, cells already had started formation of neuritic processes (Fig. 3.1). A fine network of axon fibres presumably connecting between neurones became more apparent by 3 DIV and complex neuritic networks continued to develop over the course of culture period. By 7 DIV, neurones had formed extensive connections and round small cell bodies homogenous in shape and size, displaying the basic morphology characteristic of mature CGC described in vivo (Palay & Chan-Palay, 1974). Previous studies have shown that CGC cultures grown for 7-8 DIV exhibit functional properties of mature neurones (Carroll et al., 1998; Hack et al., 1995; Wong et al., 2001), therefore all subsequent experiments were performed at 7 DIV.

3.3.2. Composition of the Established CGC Culture

One of the advantages of CGC culture for studying molecular and biochemical events is its highly homogenous population of cells. Previous studies have shown that primary cultures grown in serum-free NBM and B-27 supplement permit optimal viability of neurones (Brewer & Cotman, 1989), and that antimitotic agents, such as aphidicolin, allow the establishment of high purity of CGC culture by inhibiting the growth of glial population (Giardina et al., 1998). The purity of the primary CGC culture established for this work was assessed by double immunofluorescence cytochemistry of
the neuronal marker, MAP2, and the astrocytic marker, GFAP, of CGCs at 7 DIV. Nucleic acid stain, Hoechst 33342, was also added to visualize nuclei of the cells. Fluorescence photomicrographs were taken of random fields of the culture and the total number of glial cells from representative images was counted and expressed as the percentage of the total number of neurones present in the field. Results showed more than 98 % of the cell population was stained with MAP2 (99 ± 0.4 %), while a small proportion of cells was stained with GFAP (0.6 ± 0.4 %), demonstrating the majority of the cell culture consisted of neurones (Fig. 3.2). As previous study has already proven, the use of mitosis inhibitor, aphidicolin (1-2 µg/ml) was able to inhibit proliferation of astrocytes in the culture, while allowing neuronal differentiation and growth (Diwakarla et al., 2009b; Giardina et al., 1998).

3.3.3. Characterization of Neurotoxicity of Stressors

The overall aim of this chapter was to determine appropriate concentrations of the inhibitors of mitochondrial OXPHOS complexes inducing a slow form of cellular injury, while avoiding rapid necrotic cell death, to allow subsequent monitoring of mitochondrial function. Previous study has revealed that severe neurotoxicity on neurones can induce necrosis, while weaker insults lead to slower degeneration of neurones featuring PCD (Bonfoco et al., 1995; Cheung et al., 1998b; Diwakarla et al., 2009b). Therefore, profiles of neurotoxicity of the inhibitors were first investigated in order to determine the appropriate concentrations of drugs that would induce slow cell death and allow observation of the morphological or biochemical changes of CGCs in response to bioenergetic inhibition. Cells at 7 DIV were exposed to various concentrations of drugs for 24 h and the consequent cytotoxicity was determined by the MTT cell viability assay. Morphological changes of neurones in response to drugs were monitored in parallel by phase contrast microscopy to correlate cellular changes with MTT viability data.
Figure 3.1. Temporal development of cerebellar granule cell (CGC) in culture

CGCs were maintained on poly-D-lysine coated multiwell plates, and images from representative fields were captured at (A) DIV 0, (B) DIV 1, (C) DIV 2, (D) DIV 3, (E) DIV 4, (F) DIV 5, (G) DIV 6 and (H) DIV 7. After 24 h of plating, neurones started to grow and project neurites to form complex connections with surrounding neurones over the remaining culture period. By DIV 7, neurones have formed extensive connections and homogenous in shape and size. Scale bar = 50 µm.
Figure 3.2. Immunocytochemical staining of CGCs with MAP2, GFAP and Hoechst 33342

Images from representative fields were captured at DIV 7 after performing double immunofluorescence staining with (A) neuronal marker, MAP2, (B) marker of astrocytes, GFAP and (C) nuclear staining, Hoechst 33342. Quantitative data showing the proportion of neurones and astrocytes in the culture revealed >98 % of cells of cultures were neurones. Data represent the mean ± SEM of a single experiment. Scale bar = 50 µm.
3.3.3.1. Neurotoxic profiles of STS and H\textsubscript{2}O\textsubscript{2}

Apoptosis (PCD-type I cell death) is characterized by cellular shrinkage with condensation of the cytoplasm, nuclear fragmentation and the subsequent formation of apoptotic bodies containing fragments of cytoplasmic organelles and nucleus (Kerr et al., 1972). On the other hand, necrosis or unregulated accidental necrosis is characterized by rapid swelling of the organelles and the cytoplasm, followed by loss of the plasma membrane integrity and subsequent lysis of the cells (Schweichel & Merker, 1973). STS is documented as the “gold standard” apoptosis-inducer in cells \textit{in vitro} and has been used widely to study mechanisms of apoptotic cell death pathway (Beart \textit{et al.}, 2007; Bertrand \textit{et al.}, 1994; Diwakarla \textit{et al.}, 2009b). On the contrary, H\textsubscript{2}O\textsubscript{2} is known as an oxidative stress inducer that triggers apoptotic machinery at low concentration, but at high concentration H\textsubscript{2}O\textsubscript{2} causes cell death by necrosis (McKeague \textit{et al.}, 2003; Palomba \textit{et al.}, 1999).

The neurotoxic profiles of the apoptotic inducer, STS, and the oxidative stressor, H\textsubscript{2}O\textsubscript{2}, have been previously examined in CGC culture, and they were shown to cause a slow form of cell death at low concentrations (Diwakarla \textit{et al.}, 2009b). Therefore, STS and H\textsubscript{2}O\textsubscript{2} were applied to CGCs as control treatments to document the slow form of cell death in CGC culture and to advance analyses of the actions of OXPHOS inhibitors. CGCs at 7 DIV were exposed to MEM containing various concentrations of STS (1 nM - 300 µM) (Fig. 3.3) and H\textsubscript{2}O\textsubscript{2} (1 µM - 1 mM) (Fig. 3.4) for 24 h. The concentration-response curves for each stressor were generated from the MTT cell viability assay from which the concentrations of the inhibitors required to reduce the viability of cells to 50 % of vehicle control (IC\textsubscript{50}) were determined. The corresponding morphological changes of CGCs were monitored by phase contrast microscopy.

Cell viability results showed concentration-dependent reductions of cell viability for both drugs (P < 0.0001). STS induced significant cell death at 100 nM (40 ± 6 % of control; P < 0.001), with an IC\textsubscript{50} at 34 ± 4.4 nM, causing maximal cell death at 300 nM (20 ± 4 % of control) (Fig. 3.3A). As expected, typical morphological features of apoptotic cell death were apparent, where thinning and breakdown of neurites, shrinkage
of the cell body and overall loss of neuronal networks were clearly evident at the concentration ranges of 200 - 300 nM (Fig. 3.3D-F). H$_2$O$_2$ produced rapid cell death at 30 µM (12 ± 5 % of control; P < 0.001), with an IC$_{50}$ value of 16 ± 0.1 µM and maximal cell death at 1000 µM (1.4 ± 0.6 % of control) (Fig. 3.4A). The morphological changes of CGCs exposed to oxidative stress were similar to that of STS-treated neurones, and included cell shrinkage and neuritic disintegration at concentrations higher than IC$_{50}$, at 40 µM - 50 µM (Fig. 3.4). The morphological features of necrotic-like swelling were not observed in STS- and H$_2$O$_2$-treated neurones at the employed concentrations and the majority of neurones manifested a slow-form of cell death morphologies. Interestingly, phase contrast microscopy revealed that CGCs exposed to STS and H$_2$O$_2$ at their IC$_{50}$ showed much less damage of their overall neuronal morphology, indicating that STS- and H$_2$O$_2$-induced metabolic damage preceded significant morphological damages.

### 3.3.3.2. Time-dependent neurotoxicity of STS and H$_2$O$_2$

As stated above, severe toxicity has previously been shown to lead to necrosis, while milder concentration can result in a slow degeneration by apoptosis (Bonfoco et al., 1995). Therefore, the neurotoxicity profiles of the drugs during the early phase of treatment was further examined by time-course analyses using the MTT assay to document time-dependent responses of cellular function. Guided by a previous publication, where concentrations at or higher than IC$_{50}$ values were used for STS and H$_2$O$_2$ in the same culture system (Diwakarla et al., 2009b), the time-course evaluation (2, 4, 6, 8, 12, 24 h) of cellular injury caused by STS and H$_2$O$_2$ was performed at two concentrations (STS 100 nM & 200 nM; H$_2$O$_2$ 20 µM & 30 µM) above or at IC$_{50}$ values previously found by the MTT assay (Fig. 3.5). As expected, higher concentrations of STS (200 nM) and H$_2$O$_2$ (30 µM) produced time-dependent losses of cell viability (P < 0.05; P < 0.001, respectively). STS at 200 nM was able to exert a significant cytotoxic effect at 24 h (P < 0.05; 53 ± 17 % of control) and H$_2$O$_2$ at 30 µM produced rapid loss of cell viability as early as 2 h (P < 0.05; 38 ± 10 % of control). Both lower concentrations of STS (100 nM) and H$_2$O$_2$ (20 µM) did not cause significant cytotoxic effect on CGCs over this time-course, however, data at 24 h of incubation with STS at 100 nM were suggestive of a decrease in cell viability.
Figure 3.3. CGCs treated with staurosporine (STS) at various concentrations

Cellular viability after exposure to STS at various concentration was determined at 24 h using the MTT assay (A). Data represent mean ± SEM from quadruplicate determinations in 6 independent experiments. Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC_{50} of STS for 24 h, (B) Vehicle control, (C) 100 nM, (D) 200 nM, (E) 250 nM and (F) 300 nM. Scale bar = 50 µm.
(A)

Cell viability (% control) vs. log [STS] M

(B)

(C)

(D)

(E)

(F)
Figure 3.4. CGCs treated with H\textsubscript{2}O\textsubscript{2} at various concentrations

Cellular viability after exposure to H\textsubscript{2}O\textsubscript{2} at various concentration was determined at 24 h using the MTT assay (A). Data represent mean ± SEM from quadruplicate determinations in 3 independent experiments. Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC\textsubscript{50} of H\textsubscript{2}O\textsubscript{2} for 24 h, (B) Vehicle control, (C) 20 µM, (D) 30 µM, (E) 40 µM and (F) 50 µM. Scale bar = 50 µm.
Figure 3.5. Time-dependent changes in cell viability of CGCs treated with STS and H$_2$O$_2$

CGCs were treated with (A) apoptosis inducer, STS 100 nM (B) STS 200 nM (C) oxidative stressor, H$_2$O$_2$ 20 µM and (D) H$_2$O$_2$ 30 µM. Cells showed time-dependent decrease in cell viability for higher concentrations of both insults. Data represent mean ± SEM from quadruplicate determinations in 4 independent experiments. Asterisks indicate significant reductions in cellular viability (* P < 0.05; ** P < 0.01) compared to control by one-way ANOVA, followed by Dunnett’s post hoc test.
3.3.4. Characterization of Neurotoxicity of Inhibitors of the Mitochondrial OXPHOS

3.3.4.1. Neurotoxicity induced by exposure to mitochondrial OXPHOS inhibitors

Guided by the insights from the cell viability studies for STS and H₂O₂, cytotoxic profiles of the inhibitors of mitochondrial respiratory complexes and F₁F₀ATP synthase were also investigated using the MTT cell viability assay (Fig. 3.6). CGCs were exposed to various concentrations of inhibitors: Rot (10 nM - 10 µM), 3-NP (10 µM - 10 mM), AA (1 nM - 3 µM), KCN (10 µM - 10 mM) and Oli (30 nM - 30 µM) for 24 h, and cell viability data were appraised relative to the cell viability of control cells treated with appropriate vehicle. Cell viability data showed concentration-dependent reductions in viability for all drugs (P < 0.0001 Rot, 3-NP, AA, Oli; P < 0.05 KCN) (Fig. 3.6). The rank order of potency of the drugs to inhibit each OXPHOS complex was evaluated according to the IC₅₀ values from concentration-response curves: AA > Rot > Oli > 3-NP > KCN (Table 3.2). AA was the most toxic inhibitor with an IC₅₀ value of 42 ± 1.3 nM, Rot was the second most toxic drug with an IC₅₀ of 100 ± 17 nM, while Oli, 3-NP and KCN induced milder cytotoxicity with IC₅₀ values of 4.7 ± 3.1 µM, 78 ± 33 µM and 1.2 ± 0.5 mM, respectively (Table 3.2).

3.3.4.2. Apoptotic-like injuries observed during exposure to inhibitors of mitochondrial respiratory chain complexes

The neurotoxicity of OXPHOS inhibitors was examined carefully by establishing concentration-dependent curves generated from MTT cell viability data. In order to compare the concentration-dependent morphological changes that occurred in response to drug treatments, phase contrast microscopy was performed in parallel with MTT assays. Exposure to Rot induced the first sign of cell death at 300 nM (P < 0.05; 64 ± 6.6 % of control) and maximal cell death at 10 µM (44 ± 5.8 % of control) as shown by cell
viability data (Fig. 3.6). Phase contrast images revealed that changes in morphological features of Rot-treated neurones resembled apoptotic-like injury induced by STS and H$_2$O$_2$. Treatment with Rot at concentrations < IC$_{50}$ value, such as 10 nM for 24 h treatment, was sufficient to induce thinning of neuritic networks and shrinkage of the cell bodies (Fig. 3.7), thus indicating that morphological damage preceded metabolic damage. Cell death was more apparent at 24 h incubation of 30 nM and higher concentrations, with definite loss of neuritic networks and shrinkage of cell bodies (100 - 1000 nM) (Fig. 3.7).

3-NP produced concentration-dependent reductions in cell viability where the first significant damage was observed at 100 µM (P < 0.05; 70 ± 5.0 % of control) and maximal death at 10 mM (36 ± 4.9 % of control) (Fig. 3.6). The neuritic network started to thin from 100 µM and more structural damage indicated by shrinkage of the cell bodies and thinning of neurites was noted at 300 µM. Discontinuity of neurites was more clearly evident with further shrinkage of the cell bodies at the concentration of 500 µM, indicating extensive damage at this concentration (Fig. 3.8). Contrary to findings for Rot-exposed CGCs, treatment of CGCs with 3-NP at a concentration < IC$_{50}$ value did not produce morphological damage. Structural damage was obvious when neurones were exposed to higher concentrations than the IC$_{50}$ value.

AA induced significant cell death at a concentration of 100 nM (P < 0.05; 78 ± 4.1 % of control) and the maximal toxicity was found at 3 µM (46 ± 3.3 % of control) (Fig. 3.6). Similar to the cytotoxic profile shown by Rot, treatment with AA at concentrations < IC$_{50}$ value was sufficient to cause morphological changes that represented a slow form of neuronal degeneration. Shrinkage of cell bodies was clearly evident at 24 h treatment of AA at 10 nM, and progressive degeneration of neurites and extensive apoptotic-like cellular damage was observed at the range of 30 nM - 1000 nM (Fig. 3.9).

In contrast to other respiratory complex inhibitors, KCN produced more variance in its concentration-response curves for cell viability (Fig. 3.6). Both significant metabolic dysfunction and maximal cell death were observed at 10 mM of KCN (P < 0.05; 10 ±
Figure 3.6. Concentration-response curves for cell viability of CGCs treated with respiratory chain complexes I-IV and F$_{1}$F$_{0}$ ATP synthase (complex V) inhibitors

CGCs were treated with various concentrations of OXPHOS complex I-V inhibitors (A) Rot, (B) 3-NP, (C) AA, (D) KCN and (E) Oli at 24 h. Cells showed concentration-dependent decreases in cell viability for all inhibitors. Data represent mean ± SEM from quadruplicate determinations in 3-5 independent experiments.
Actual and relative potencies of OXPHOS inhibitors were determined from concentration-dependent cell viability using the MTT assay. IC$_{50}$ values (mean ± SEM) were derived from three independent experiments employing quadruplicate determinations. The potency of drugs were compared using a non-parametric Kruskal-Wallis Test, which revealed significant differences in IC$_{50}$ values across all drugs (P < 0.0001).

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ (M)</th>
<th>Mean ± SEM</th>
<th>Rank of Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rot</td>
<td>1.4 x 10$^{-7}$</td>
<td>9.8 x 10$^{-8}$</td>
<td>7.7 x 10$^{-8}$</td>
</tr>
<tr>
<td>3-NP</td>
<td>1.4 x 10$^{-4}$</td>
<td>2.8 x 10$^{-5}$</td>
<td>6.6 x 10$^{-5}$</td>
</tr>
<tr>
<td>AA</td>
<td>4.2 x 10$^{-8}$</td>
<td>4.3 x 10$^{-8}$</td>
<td>3.9 x 10$^{-8}$</td>
</tr>
<tr>
<td>KCN</td>
<td>6.4 x 10$^{-4}$</td>
<td>8.9 x 10$^{-4}$</td>
<td>2.2 x 10$^{-3}$</td>
</tr>
<tr>
<td>Oli</td>
<td>3.2 x 10$^{-6}$</td>
<td>2.6 x 10$^{-7}$</td>
<td>1.1 x 10$^{-5}$</td>
</tr>
</tbody>
</table>
2.6 % of control) after 24 h drug treatment (Fig. 3.10). Despite having a high IC₅₀ of 1.2 ± 0.5 mM, neurones exhibited morphological features of cell death after 24 h incubation with concentrations < IC₅₀ (100 µM - 300 µM). A low level of disintegration of neuritic networks was observed at 30 µM of KCN, which worsened at 100 µM, where shrinkage of cell bodies and breakage of neurites were more visible. Neurones treated with KCN over the range of 200 µM - 300 µM showed prominent cellular damage with non-necrotic type of cell death (Fig. 3.10).

Overall, concentration-dependent reductions in cell viability were observed for all inhibitors of mitochondrial respiratory chain complexes, and light microscopic analyses revealed characteristics of apoptotic-like injury (breakage of neurites and shrinkage of cell bodies) with the complete absence of early necrotic-like swelling.

### 3.3.5. Time-dependent Neurotoxicity Profile of Inhibitors of the Mitochondrial OXPHOS

The aim here was to evaluate the time-course of neurotoxicity of the inhibitors to examine the extent of metabolic dysfunction caused during exposure to drugs over the period 1-24 h. Based upon the morphological observations by phase contrast microscopy when neurones were treated with a range of inhibitor concentrations, two concentrations of each inhibitor were chosen as follows: Rot 10 nM and 30 nM, 3-NP 100 µM and 300 µM, AA 10 nM and 30 nM, KCN 100 µM and 300 µM and Oli 0.1 µM and 1 µM (Table 3.3). Cell viability was monitored over time using the MTT assay.

Fig. 3.11 shows cell viability data for Rot and 3-NP at the two concentrations. Both lower and higher concentrations chosen for Rot (10 nM and 30 nM) and 3-NP (100 µM and 300 µM) exerted significant time-dependent toxicity on CGCs (P < 0.05 for Rot; P < 0.001 for 3-NP). Slow reductions in cell viability were observed over the time-course
Figure 3.7. CGCs treated with rotenone (Rot) at various concentrations

Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC₅₀ of Rot for 24 h: (A) vehicle control, (B) 10 nM, (C) 30 nM, (D) 100 nM, (E) 300 nM and (F) 1000 nM. Scale bar = 50 µm.
Figure 3.8. CGCs treated with 3-Nitropropionic acid (3-NP) at various concentrations

Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC$_{50}$ of 3-NP for 24 h: (A) vehicle control, (B) 20 µM, (C) 100 µM, (D) 200 µM, (E) 300 µM and (F) 500 µM. Scale bar = 50 µm.
Figure 3.9. CGCs treated with antimycin A (AA) at various concentrations

Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC$_{50}$ of AA for 24 h: (A) vehicle control, (B) 10 nM, (C) 30 nM, (D) 100 nM, (E) 300 nM and (F) 1000 nM. Scale bar = 50 µm.
Figure 3.10. CGCs treated with potassium cyanide (KCN) at various concentrations

Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC$_{50}$ of KCN for 24 h: (A) vehicle control, (B) 30 µM, (C) 100 µM, (D) 200 µM, (E) 230 µM and (F) 300 µM. Scale bar = 50 µm.
Table 3.3. Concentrations of OXPHOS inhibitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentrations</th>
<th>Site of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rot</td>
<td>10 nM, 30 nM</td>
<td>Complex I</td>
</tr>
<tr>
<td>3-NP</td>
<td>100 µM, 300 µM</td>
<td>Complex II</td>
</tr>
<tr>
<td>AA</td>
<td>10 nM, 30 nM</td>
<td>Complex III</td>
</tr>
<tr>
<td>KCN</td>
<td>100 µM, 300 µM</td>
<td>Complex IV</td>
</tr>
<tr>
<td>Oli</td>
<td>0.1 µM, 1 µM</td>
<td>Complex V</td>
</tr>
</tbody>
</table>
studied and significant reductions in cell viability were observed with longer incubation times for all treatments, indicating these concentrations were able to cause slow metabolic dysfunction. Time-dependency studies for AA showed negligible cellular injury of AA 10 nM over the time-course; although there was apparent decreased cell viability at 30 nM, it failed to reach statistical significance. KCN at 100 µM and 300 µM and Oli 1 µM were able to cause injury at the longer time intervals of treatment (P < 0.05). However, Oli 0.1 µM failed to produce significant reductions in cell viability (Fig. 3.12).

3.3.5.1. Time course labelling with propidium iodide after exposure to inhibitors of the mitochondrial OXPHOS

Accidental necrotic cell death is accompanied by rapid swelling of the cell body which eventually leads to rupture of the plasma membrane (Boujrad et al., 2007; Edinger & Thompson, 2004). Cells that undergo necrotic-like damage or death can be detected by membrane-impermeable DNA binding dye, PI, during the early hours of drug exposure (Diwakarla et al., 2009a; Diwakarla et al., 2009b; Higgins et al., 2009; Higgins et al., 2012). CGCs treated with OXPHOS inhibitors were monitored over time to examine if the previously chosen two concentrations of inhibitors (Rot 10 nM and 30 nM, 3-NP 100 µM and 300 µM, AA 10 nM and 30 nM, KCN 100 µM and 300 µM and Oli 0.1 µM and 1 µM) would lead to membrane permeability changes indicative of early necrotic cell death. CGCs were double stained with PI and cell-permeant nuclear counterstain, Hoechst 33342, and random fields were captured over time by fluorescence microscopy (535/617 and 350/461 nm for PI and Hoechst 33342, respectively) at 1, 4, 8 and 24 h (Fig. 3.13). PI-labelled cells were counted and presented as percentage of total number of cells.

Time-course monitoring of PI staining upon addition of OXPHOS inhibitors to CGCs revealed significant increases in percentage of PI labelled cells with longer incubation times for all treatments at higher concentration, indicating these concentrations were able to cause cellular demise (Fig. 3.13). As expected, vehicle treated control cells displayed negligible staining with PI with the percentage of PI-positive cells not exceeding 4 % of total number of cells for all time-course. Inhibition produced by Rot 30 nM affected a slow increase in percentage of PI-positive cells. The first significant
Figure 3.11. Time-dependent changes in cell viability of CGCs treated with inhibitors of respiratory chain complexes I-II

CGCs were treated with inhibitors of respiratory chain complexes I and II, (A) Rot 10 nM, (B) Rot 30 nM, (C) 3-NP 100 µM and (D) 3-NP 300 µM. Cell viability was determined using the MTT assay. Cells showed time-dependent decrease, in cell viability for all inhibitors. Data represent mean ± SEM from quadruplicate determinations in 2 independent experiments and are expressed as a percentage of the control. Asterisk indicates a significant reduction to cell viability (* P < 0.05; ** P < 0.01) compared to control by one-way ANOVA, followed by Dunnett’s post hoc test.
Figure 3.12. Time-dependent changes in cell viability of CGCs treated with inhibitors of respiratory chain complexes III, IV and F$_{0}$F$_{1}$ATP synthase

Cells were treated with inhibitors of respiratory chain complexes III-V, (A) AA 10 nM, (B) AA 30 nM, (C) KCN 100 µM, (D) KCN 300 µM, (E) Oli 0.1 µM and (F) Oli 1 µM. Cell viability was determined using the MTT assay. Cells showed time-dependent decrease in cell viability for KCN and Oli. Data represent mean ± SEM from quadruplicate determinations in 2 independent experiments and are expressed as a percentage of the control. Asterisk indicates a significant reduction to cell viability (* P < 0.05) compared to control by one-way ANOVA, followed by Dunnett’s post hoc test.
Cell death (PI uptake) of CGCs treated with mitochondrial OXPHOS inhibitors was monitored over time (1, 4, 8 and 24 h). CGCs were treated with mitochondrial OXPHOS inhibitors at two concentrations (A) Rot 10 nM and 30 nM, 3-NP 100 µM and 300 µM, (B) AA 10 nM and 30 nM, KCN 100 µM and 300 µM and Oli 0.1 µM and 1 µM. Data are expressed as a percentage of total number of cells and represent mean ± SEM from quadruplicate determinations in single representative experiment, similar data were found in a further experiment. Two-way ANOVA revealed a significant effect of treatment (P < 0.0001). (** P < 0.01, ## P < 0.0001; Bonferroni post hoc test).
increase was observed at 8 h (12 ± 5.5 % of total number of cells; P < 0.01) and maximum staining occurred at 24 h (21 ± 6.1 % of total number of cells; P < 0.001). Similarly, incubation with 3-NP 300 µM caused a slow rise in cell death, reaching maximal cell death after 24 h incubation (12 ± 2.6 %; P < 0.05) (Fig. 3.13A). Relatively few cells were stained positive for PI at early time points (1 h) for Rot and 3-NP, indicating the majority of cells were undergoing a slow-form of non-necrotic cell death. On the contrary, inhibition of complex III and IV by AA 30 nM and KCN 300 µM, respectively, showed more rapid cell death (4 h) (Fig. 3.13B). In agreement with cell viability data from the MTT assay, treatment with KCN 300 µM revealed rapid cellular demise, indicated by 73 ± 2.8 % of total number of cells incorporating PI as early as 4 h incubation (P < 0.001). The proportion of PI-positive cells remained steady for the remaining time course of KCN 300 µM treatment (8 h, 65 ± 4.1 %; 24 h, 76 ± 4.9 % of total number of cells), suggesting 4 h of incubation was enough to cause rapid injury and membrane permeability. AA 30 nM exposure also showed a similar profile with PI labelling as early as 4 h (27 ± 4.4 % of total number of cells; P < 0.001), which increased to 57 ± 3.6 % and 51 ± 4.8 % of cell death after exposure to AA for 8 & 24 h incubation, respectively. However, both high and low concentrations of Oli failed to induce significant permeabilization of cell membranes over the time-course studied.
3.4. Discussion

The experiments described in this chapter examined the neurotoxic profiles (concentration- and time-dependence) of selected inhibitors known to act upon mitochondrial OXPHOS complexes in rodent primary CGCs, with the specific aim being to establish relevant experimental concentrations of the targeted inhibitors to be employed in subsequent investigations.

CGCs have been strategically used as a unique model system for the study of neuronal function. They offer the unique advantage of a highly homogeneous population allowing interpretation of direct actions of pharmacological agents on neurones without interference from other subtypes of neurones or non-neuronal cells (Kroemer et al., 1995). Furthermore, CGC cultures develop morphological and biochemical features of mature CGCs seen in vivo, such as extensive neuritic networks, production and release of L-Glu (Schousboe et al., 1989), allowing insights into in vivo aspects of neuronal function. Immunocytochemical analysis and morphological observation revealed that CGCs cultured here were comprised almost exclusively of neurones and demonstrated less than 2 % glial contamination. As shown by the previous literature, maintenance of CGC cultures under serum-free conditions (Barnes & Sato, 1980) with 1 µg/ml mitosis inhibitor, aphidicolin, prevented the proliferation of non-neuronal cells such as astrocytes. The morphological development of CGCs in vivo was in agreement with earlier observations (Cheung et al., 1998a; Diwakarla et al., 2009b), showing complex network of neurites and small and round cell bodies.

Determination of the neurotoxic profiles of OXPHOS inhibitors on CGCs was performed using the MTT cell viability assay. The MTT assay has been widely used to determine cell viability due to its suitability for high throughput screening and ease of use (Cheung et al., 1998b; Giardina & Beart, 2002; Sheean et al., 2013). The reduction of yellow tetrazolium MTT into a purple formazan product directly reflects metabolic activity of cellular dehydrogenases with concomitant oxidation of NADH and NADPH.
(Altman, 1976) and the resultant absorbance is considered as directly proportional to the number of viable cells (Mosmann, 1983). MTT reduction by viable neurones is often translated into a general index of mitochondrial function (Beart et al., 2007; Cheung et al., 2000; Diwakarla et al., 2009a; Diwakarla et al., 2009b; Mosmann, 1983). When CGCs were exposed to various concentrations of STS, H$_2$O$_2$ and OXPHOS complex I-V inhibitors for 24 h, each inhibitor displayed a different profile of neurotoxicity: all concentration-response curves indicated decreases in cell viability with increasing concentration. IC$_{50}$ values were determined from concentration-response curves and generation of the rank order of potency revealed the inhibitor of complex III, AA, had the highest potency to cause cellular demise, followed by the inhibitor of complex I, Rot (Table 3.2). These two drugs were able to cause 50 % of cell death over 24 h in nanomolar range (AA 42 ± 1.3 nM; Rot 100 ± 17 nM). Complex V F$_{1}$F$_{0}$ ATPase inhibitor Oli (4.7 ± 3.1 µM), complex II inhibitor 3-NP (78 ± 33 µM) and complex IV inhibitor KCN (1.2 ± 0.5 mM) were found to be less potent.

Based on the IC$_{50}$ values determined, features of cellular degeneration in response to OXPHOS inhibition were concurrently observed. Careful dissection of the mode of cell death involved here was not a specific aim of this thesis, therefore, cells were broadly classified as undergoing apoptotic-like cell death or fast necrotic cell death. The first and most readily observed outcome following exposure of cells to drugs was altered morphology of cells in the monolayer cell culture. Hence morphological alterations were used as an index of toxicity or type of cell death (Kerr et al., 1972; Rello et al., 2005). As expected, STS- and H$_2$O$_2$-induced cellular damage led to apoptotic-like morphology, such as shrinkage of cell bodies and disintegration of neurites. H$_2$O$_2$ in particular, is known as an oxidative stressor that induces apoptotic-like PCD at low concentrations but unregulated necrosis at higher concentrations (Lennon et al., 1991; Saito et al., 2006). There were no signs of necrosis with the range of concentrations of H$_2$O$_2$ employed, indicating H$_2$O$_2$ at the concentrations used for this thesis was causing apoptotic-like PCD. Based on the observations found for STS- and H$_2$O$_2$-treated neurones, the end point observation of morphological changes following exposure to inhibitors of OXPHOS also resembled features of apoptotic-like cell death, such as shrinkage of cell bodies and disintegration of neurites. Morphological features of necrosis such as rapid swelling of cell bodies were not observed in neurones treated with OXPHOS inhibitors at the range
of concentrations employed, suggesting neurones were undergoing a slow-form of cell death.

Interestingly, in contrast to STS- and H₂O₂-induced cellular damage, the morphological damage of CGCs from Rot, AA and KCN-induced OXPHOS inhibition was observed at concentrations < IC₅₀ values, whereas any morphological changes manifested in neurones by STS and H₂O₂ treatment were only visible at concentrations > IC₅₀. The differences observed between STS- and H₂O₂-induced damage and those found for the OXPHOS inhibitors might be explained by their varying modes of inhibition of neuronal viability. STS- and H₂O₂-induced cell death does not directly involve injury per se to the mitochondrial ETC, whereas the OXPHOS inhibitors mentioned above are likely to directly inhibit the core enzymes responsible for ATP synthesis and ultimately cause ROS synthesis via inhibition of complex I, III and IV, respectively. Such direct inhibition of mitochondrial enzymes may affect important cellular functions involving neuronal metabolism such as the TCA cycle and loss of ATP, causing failures in neurotransmitter release and recycling which are important features of neuronal function. Such actions may lead to early cellular degeneration involving disintegration of neurites. In fact, a study on PD revealed Rot-induced inhibition of complex I in primary midbrain neuronal cultures resulted in microtubule depolymerisation, causing selective degeneration of dopaminergic neurones by disrupting vesicular transport along microtubules and synaptic dysfunction (Hongo et al., 2012; Ren et al., 2005), further supporting the present evidence for morphological alterations with neuritic disintegration during Rot treatment. Cellular dehydrogenases may still be functional during extensive morphological alterations because the integrity of organelles still remains functional and maintained in apoptotic bodies until a late stage in the process of slow apoptotic cell death (Elmore, 2007; Ziegler & Groscurth, 2004). Relative to other respiratory complex inhibitors, 3-NP was able to cause significant morphological damage as well as metabolic dysfunction at the IC₅₀ value. The majority of MTT metabolism is suggested to be conducted by succinate dehydrogenase (Lobner, 2000; Slater et al., 1963) whose inhibition is induced by 3-NP. Therefore the reduction of MTT observed in 3-NP-treated CGCs may directly be indicating the function of complex II.
Based on the IC\textsubscript{50} values and the morphological changes induced by OXPHOS inhibitors, two concentrations of each inhibitor at which the neurones still retained their overall morphology were chosen (Table 3.3), and the time-courses of their effects on cell viability were monitored. STS at 200 nM showed a slow decrease in cell viability, reaching significant reduction in cell viability after 24 h of incubation. Significant cell death was not observed during the early hours of exposure (2-8 h), observations consistent with the characteristics of apoptotic-like cell death, which exhibited a slow progression to complete cell death. A similar trend of slow degeneration was observed with all OXPHOS inhibitors at both high and low concentrations chosen, except KCN 300 µM. Cytochemical studies using PI showed consistent results where few PI-labelled were observed at early time points (1-4 h) for neurones treated with Rot 10 nM, 30 nM, 3-NP 100 µM, 300 µM, AA 10 nM, KCN 100 µM and Oli 0.1 µM, 1 µM. Although AA 30 nM and KCN 300 µM caused more rapid injury as indicated by the decrease in cell survival and increase in PI-positive cells observed at 4 h of incubation, a negligible number of cells was stained with PI at earlier timepoint (i.e. at 1 h of incubation), strongly suggesting that necrosis was not involved. Indeed Diwakarla \textit{et al.} (2009b) have suggested that early PI labelling of CGCs in the first 2 h is likely to represent by necrosis. Additionally, the morphological changes of KCN-treated neurones failed to show necrotic features (i.e. swelling), suggesting different mechanisms leading to cellular demise may underlie KCN-induced injury. The cell death induced by Oli was not detailed as for OXPHOS inhibitors of complex I-IV, but it showed time- and concentration-dependent injury that was PI-independent. Importantly, the patterns of PI labelling, especially those found for AA and KCN, need to be taken in the context of the slow loss of neuronal networks and absence of neuronal swelling. Clearly none of OXPHOS inhibitors evaluated cause neuronal death by unregulated necrosis.

Involvement of mitochondrial dysfunction is clearly shown in studies in which OXPHOS inhibitors were utilized to induce pathophysiological symptoms of neurodegenerative diseases, such as PD, HD, AD and ALS (Moran \textit{et al.}, 2012). In addition, OXPHOS inhibitors have been used as tools for investigations of acute injuries such as ischemic injury (Isaev \textit{et al.}, 2004), or pharmacological studies investigating neuroprotective properties of drugs in primary CGC cultures (Cho & Seong, 2002; Olsen \textit{et al.}, 1999). Such studies have employed drugs at much higher concentrations, up to 10 fold greater, compared to used here (Table 3.4) (Budd & Nicholls, 1996; Cho & Seong,
Table 3.4. Concentrations of OXPHOS inhibitors used in other literatures

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentrations</th>
<th>Duration</th>
<th>Cell type</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rot</td>
<td>10 nM, 30 nM</td>
<td>4 h, 24 h</td>
<td>Mouse CGCs</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>1 h</td>
<td>Rat CGCs</td>
<td>Isaev <em>et al.</em>, 2004</td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>0.5 h</td>
<td>Rat CGCs</td>
<td>Budd &amp; Nicholls, 1996</td>
</tr>
<tr>
<td></td>
<td>0.14 mM</td>
<td>48 h</td>
<td>mesencephalic cultures</td>
<td>Mercer <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>3-NP</td>
<td>100 µM, 300 µM</td>
<td>4 h, 24 h</td>
<td>Mouse CGCs</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>20 h</td>
<td>Rat CGCs</td>
<td>Isaev <em>et al.</em>, 2004</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>3 h</td>
<td>Rat CGCs</td>
<td>Olsen <em>et al.</em>, 1999</td>
</tr>
<tr>
<td></td>
<td>2.8 mM</td>
<td>48 h</td>
<td>Striatal cells</td>
<td>Diwakarla <em>et al.</em>, 2009a</td>
</tr>
<tr>
<td>AA</td>
<td>10 nM, 30 nM</td>
<td>4 h, 24 h</td>
<td>Mouse CGCs</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>0.1 µM</td>
<td>1.5 h</td>
<td>Rat CGCs</td>
<td>Isaev <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>CN-</td>
<td>100 µM, 300 µM</td>
<td>4 h, 24 h</td>
<td>Mouse CGCs</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>1 h</td>
<td>Rat CGCs</td>
<td>Cho &amp; Seong, 2002</td>
</tr>
<tr>
<td>Oli</td>
<td>0.1 µM, 1 µM</td>
<td>4 h, 24 h</td>
<td>Mouse CGCs</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>30 min</td>
<td>Rat CGCs</td>
<td>Budd &amp; Nicholls, 1996</td>
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</tbody>
</table>
2002; Diwakarla et al., 2009a; Isaev et al., 2004; Mercer et al., 2005; Olsen, 1999). The differences here can be explained by the different durations of drug-treatment inducing acute injuries in models of ischemia or neurotoxicity (i.e. 0.5 h to 1 h). However, chronic use of inhibitors of OXPHOS complexes at lower concentrations allows observation of changes to mitochondrial bioenergetics and subsequent cellular changes that occur in CGCs. Other literature investigating cellular and molecular pathways of cell death also employed chronic incubation of neurones with Rot (30 nM, 24 h) and 3-NP (5 mM, 48 h) in cultured rat mesencephalic dopaminergic neurons and striatal GABAergic neurones, respectively (Diwakarla et al., 2009a; Lim et al., 2007), validating the use of such low concentrations of OXPHOS inhibitors for this thesis.

In conclusion, as guided by morphological observations, cell viability assays and cytochemical assessment, the concentrations of OXPHOS inhibitors chosen in this study revealed a slow-form of cell death with apoptotic-like features. These investigations were not *per se* mechanistic seeking only to establish a baseline for more incisive work assessing mitochondrial bioenergetic dysfunction caused by OXPHOS inhibition. Thus the next thesis chapter describes a detailed investigation of bioenergetic dysfunction of CGCs, specifically of mitochondria, caused by these inhibitors.
CHAPTER FOUR

Bioenergetic Dysfunction and Mitochondrial Oxidative Phosphorylation: Strategic Analysis of the Inhibitors of Oxidative Phosphorylation
4.1. Introduction

In Chapter 3, the cytotoxicity profiles of the specific inhibitors of the complexes involved in OXPHOS were investigated. To this baseline, strategic analyses of the functional aspects of mitochondria were further investigated in order to confirm the adverse effects of OXPHOS inhibitors on bioenergetics and to seek insights into their precise actions on key mitochondrial mechanisms in the cultured neurones used here.

4.1.1. Mitochondrial Bioenergetics

A major functional role of mitochondria is the generation of ATP via OXPHOS. OXPHOS involves a series of redox reactions across the ETC through which electrons from oxidation of NADH at complex I and succinate at complex II are transferred to complex III via ubiquinol. Ultimately, electrons are accepted by oxygen molecules that are reduced to water at complex IV. Thus, measurement of consumption of oxygen by complex IV represents the overall activity of functional ETC. Transfer of electrons across the ETC is coupled to translocation of $H^+$ across the IMM via complexes I, III and IV. The resultant net accumulation of $H^+$ in the IMS gives rise to an electrical ($\Delta \Psi_m$) and chemical or concentration gradient ($\Delta p_{H_m}$) between the IMS and the matrix due to the continuous pumping of $H^+$ by ETC complexes. These two components are collectively termed the proton motive force (Brand & Nicholls, 2011) which is responsible of driving $H^+$ down the gradient into the mitochondrial matrix through $F_1F_0$ ATP-synthase (Complex V), thereby phosphorylating ADP into ATP and completing the OXPHOS (Nicholls, 2002). The mitochondrial membrane potential ($\Delta \Psi_m$), generates the majority of the proton motive force (Murphy et al., 1999; Nicholls, 2002; Perry et al., 2011) and plays a critical role for maintaining the physiological function of the respiratory chain to generate ATP. Additionally, $\Delta \Psi_m$ also plays a prime role in the storage of $Ca^{2+}$ in the
mitochondrial matrix and the generation of ROS (Nicholls. 2004). Collapse of $\Delta \Psi_m$ leads to abolishment of mitochondrial Ca$^{2+}$ uptake and may lead to pathological consequences such as excitotoxic cell death (Bianchi et al., 2004; Verkhratsky & Toescu, 2003). Therefore, alterations in $\Delta \Psi_m$ can be used as an index of dysfunctional mitochondria as it is considered the key parameter of mitochondrial health and functional bioenergetics, and also as one of the main critical determinants of neuronal survival. Overall, the oxygen consumption, generation of ATP and maintenance of $\Delta \Psi_m$ are tightly coupled and regulate the mitochondrial bioenergetics.

Before proceeding to the investigations of the adverse impact of OXPHOS inhibitors on mitochondrial biology, it is pertinent to overview the specific actions of chosen inhibitors on OXPHOS complexes.

4.1.2. Specific Inhibitors of OXPHOS Complexes

ETC consists of four multi-subunit redox-enzymes, complex I (NADH ubiquinone oxidoreductase), complex II (succinate ubiquinone oxidoreductase), complex III (ubiquinone-cytochrome c reductase) and complex IV (cytochrome oxidase). Together with the complex V, $F_1F_0$ ATP synthase, these complexes complete the components of OXPHOS. The inhibitors of OXPHOS are well known, and this chapter will employ specific inhibitors to directly induce mitochondrial bioenergetic dysfunction.

Complex I is inhibited by a variety of naturally occurring and synthetic inhibitors which are grouped into three categories; Type A quinone antagonists, Type B semiquinone antagonists and Type C quinol antagonists (Degli Esposti, 1998; Lenaz & Genova, 2010). Such a wide variety of inhibitors suggests different modes of action or different binding sites of the complex I inhibitors. The pathway of the electrons from NADH within complex I involves transfer of two electrons to the final destination ubiquinone via a series of iron-sulfur clusters, where the last iron-sulfur cluster, N2, acts
as a direct electron donor to ubiquinone (Fato et al., 2009). The sites of ubiquinone binding are the active sites for complex I inhibition, and Rot is the best known specific inhibitor of complex I. Rot is a plant-derived naturally occurring poison which has been widely used as a pesticide, insecticide or fish poison. It acts as a non-competitive, high-affinity inhibitor of complex I by binding near the site of ubiquinone reduction. More specifically, Rot inhibits the transfer of electron from N2 iron-sulfur cluster to ubiquinone while allowing partial reduction of ubiquinone to ubisemiquinone and eventually generates ROS (Earley & Ragan, 1984; Fato et al., 2009; Lenaz & Genova, 2010; Magnitsky et al., 2002; Okun et al., 1999) (Fig. 4.1). The blocked transfer of electrons to ubiquinone means electrons are not passed to complex III, thereby restricting the respiration and reduce ATP production. Furthermore, as electrons are restrained from being transferred to ubiquinone, oxygen becomes the new acceptor of electrons at complex I, producing ROS as a by-product and initiating a self-amplifying cycle of events that further damages mitochondrial function (Schapira, 2010). In fact, Rot has been categorised as a strong inducer of increase in ROS production among other types of Complex I inhibitors (Fato et al., 2009). The dose-dependent effect of Rot is well documented in the literature, and also it has been widely used to mimic the pathological features of PD (Cannon et al., 2009).

Complex II is a unique enzyme which is one of the essential enzymes involved in the TCA cycle as succinate dehydrogenase. Unlike other ETC complexes, pumping of H+ across the IMM is not coupled to the transfer of electrons in complex II. 3-NP is a well-known specific, irreversible suicide inhibitor of complex II, and has been widely used to produce a model of HD both in vivo and in vitro (Borlongan et al., 1998; Borlongan et al., 1997; Garcia et al., 2002). 3-NP is a cell permeable plant- and fungi-derived toxin and its specificity for complex II comes from its structure which is similar to the substrate of complex II, succinate. Upon binding at the enzyme’s active site, 3-NP slowly oxidizes to 3-nitroacrylate and covalently binds to the Arg297 residue at the active site of the enzyme, causing an irreversible inactivation of complex II (Huang et al., 2006) (Fig. 4.2). Due to the unique role of complex II in mitochondrial metabolism where a coordinated catalysis of two key biological pathways, namely TCA cycle and ETC, occur, inhibition of complex II by 3-NP not only results in the inhibition of quinone reduction leading to impairment of the electron flow through ETC (where succinate is an electron donor), but
also in metabolic inhibition of TCA cycle by inhibiting oxidation of succinate to fumarate (Fig. 4.2) (Iverson et al., 2012; Wojtovich et al., 2013).

Complex III is a site where oxidation of ubiquinols and subsequent transfer of electrons to complex IV occur via cytochrome c. Due to its critical role in cellular respiration, complex III has been considered as a promising target for several antibiotics and agricultural fungicides (Li et al., 2014). All complex III inhibitors target either of the quinone binding sites Q_P or Q_N, and there are two different subclasses of inhibitors based on their site of action (Xiao et al., 2014). AA is a highly site-specific inhibitor of complex III, which binds to the Q_N site of complex III and blocks electron transfer between haem b_H to ubiquinone (Q) to semiquinone (QH^-) during Q cycle, thereby inhibiting oxidation of b_H and only allowing a half turnover of the enzyme (Gao et al., 2003; Kim et al., 1998; Li et al., 2014; Raha & Robinson, 2000; Rieske et al., 1967; Xia et al., 1997) (Fig. 4.3). Inhibitory kinetic studies of porcine complex III showed that AA is a slow tight-binding specific inhibitor of complex III, which undergoes a conformational change by cleaving and forming hydrogen bonds at the Q_N site (Li et al., 2014). Similar to complex I, electron transfer at complex III is also accompanied by the production of ROS, presumably through electron leakage to molecular oxygen. With binding of AA at the Q_N site, blockage of electron transfer not only inhibits transfer of electrons to complex IV, but also induces a significant increase in production of superoxide anions, giving rise to oxidative stress (Turrens et al., 1985) and inhibition of H^+ translocation into the IMS (Scheffler, 2011).

Cyanide is a non-competitive inhibitor of complex IV, also known as cytochrome c oxidase, the terminal enzyme in the mitochondrial respiratory chain. Cyanide has a chemical structure similar to that of oxygen and its inhibitory role is exerted by binding to the haem a3-Cu_B binuclear centre and blocking electron transfer to oxygen to cause an inhibition of cellular oxygen consumption (Faxen et al., 2005; Isom & Way, 1984; Nuskova et al., 2010; Petersen, 1977) (Fig. 4.4). Consequently, its direct inhibition of respiration leads to inhibition of ATP production via Complex V (Nelson, 2006; Way, 1984). Cyanide has a high affinity for the haem a3-Cu_B binuclear centre (Leavesley et al., 2008) due to its ability to form a complex with metal components in complex IV, such as iron, zinc or copper metals. Although cyanide also targets other enzymes such as nitrate
In uninhibited complex I, electrons derived from oxidation of NADH are transferred to FMN and ultimately to ubiquinone (Q) via the N2 Fe-S centre. Rot blocks the transfer of electrons to ubiquinone (Q; blockage of electron flow from N2 to Q by Rot is indicated in gray shade), resulting in ubisemiquinone (CoQ$_1^\cdot$) and superoxide production (ROS) (adapted from Fato et al., 2009).
Figure 4.2. Mechanism of the inhibitory action of 3-NP on complex II

Functional complex II accepts electrons derived from the oxidation of succinate into fumarate. 3-NP is an analogue of the substrate of complex II, succinate. Upon binding to the active site of complex II, 3-NP establishes a covalent bond with complex II, thereby permanently inhibiting the electron transfer from succinate to FAD, and ultimately to ubiquinone (Q) (adapted from Wojtovich et al., 2013).
3-nitropropionic acid

TCA Cycle
Figure 4.3. Mechanism of the inhibitory action of AA on complex III

The route of electron transfer through Complex III involves a series of redox reactions allowing transfer of electrons to Fe-S centre and cyto c1 to cytochrome c and recycling of ubiquinone via Q cycle. AA binds to the Q_N site and inhibits reduction of ubiquinone to ubisemiquinone. As a result, semiquinone is generated at the Rieske protein site (FeS) which reduces oxygen to form superoxide (ROS). H+ uptake from the matrix is also inhibited, thereby inhibiting the H+ pumping mechanism. Other inhibitors of complex III, such as myxathiazol and stigmatellin, are also indicated as the inhibitors of the Q_P site (adapted from Raha & Robinson, 2000).
Figure 4.4. Mechanism of the inhibitory action of KCN on complex IV

Electrons from cytochrome c are passed to the electron acceptor, Cu₄, from which electrons are further transferred to haem a and the catalytic site, haem a₃-Cu₇, where oxygen is reduced. Cyanide (KCN) binds to haem a₃-Cu₇ binuclear center to inhibit binding and reduction of oxygen to H₂O. The H⁺ transfer pathway (from Asp 132 to Glu 286) is subsequently inhibited, causing prevention of H⁺ translocation to IMS (adapted from Faxen et al., 2005).
reductase (Lorimer et al., 1974) and myoglobin (Ver Ploeg et al., 1971), brain is one of the major target organs for cyanide as brain has a higher sensitivity to cyanide (Way, 1984). As a consequence of cyanide inhibition of complex IV, electron transfer from haem a₃ to molecular oxygen is blocked, inhibiting the whole respiratory chain activity and causing cytotoxic anoxia (Egekeze & Oehme, 1980). Additionally, H⁺ translocation across the IMM from matrix to IMS is also inhibited, generating excess ROS at complex I and III, which further contributes to cellular dysfunction (Gunasekar et al., 1998; Jones et al., 2000).

Complex V, the F₄Fₒ ATP synthase, has a number of inhibitors which inhibit either of the Fₒ or Fᵢ domains. The best known inhibitor of Fₒ domain is Oli, which binds directly to Fₒ subunits a and c, and blocks the flow of H⁺ through the channel (Fig. 4.5) (Devenish et al., 2000; Hong & Pedersen, 2008; Symersky et al., 2012). As a result, ATP synthesis is inhibited and the flow of H⁺ back to the matrix and electron transfer through the ETC are greatly reduced, under conditions in which the IMM is otherwise impermeable to H⁺. However, under conditions in which the IMM is rendered permeable to H⁺, either by mitochondrial damage or by the action of chemical protonophores such as CCCP, Oli blocks ATP synthase but there is no effect on electron transport through ETC.

The above mentioned inhibitors inhibit generation of ATP via OXPHOS and their inhibitory actions can be evaluated by several molecular techniques which assess various aspects of mitochondrial biology. This chapter addresses several aspects of mitochondrial function in order to assess the consequences of bioenergetic dysfunction imposed by each inhibitor by measuring cellular levels of ATP, ΔΨₘ and oxygen consumption rate (OCR). The technical basis of each method is described in the next section.
4.1.3. Techniques used to Assess Bioenergetic Dysfunction caused by the Inhibitors of OXPHOS Complexes

4.1.3.1. Measurement of cellular ATP using luciferase-luciferin based bioluminescence assay

Hydrolysis of ATP releases a large amount of free energy required for cellular metabolism and it is one of the key end-products of mitochondrial OXPHOS. Cellular injury results in a rapid decrease in cytoplasmic ATP level; therefore, ATP has been widely used as a marker of functional integrity of living cells (Crouch et al., 1993). Since ATP in neurons is synthesized mostly by mitochondrial OXPHOS (Surin et al., 2012), the level of ATP serves as a reliable index of mitochondrial function. Bioluminescence assay based on luciferase-luciferin enzymes is the most widely used method for cellular ATP determination due to the extreme specificity of the enzyme for ATP, high reproducibility and cost effectiveness (Crouch et al., 1993; Karamohamed & Guidotti, 2001; Lemasters & Hackenbrock, 1979; Rhodes & McElroy, 1958). This assay is based on the detection of light generated during the oxidation of D-luciferin in the presence of magnesium, oxygen and ATP from sample. Firefly luciferase catalyses the oxidation process of luciferin and the amount of light produced from the reaction of luciferase-luciferin assay is directly proportional to the level of cellular ATP levels in the sample.

\[
\text{ATP} + \text{luciferin} + \text{O}_2 \xrightarrow{\text{luciferase, Mg}^{2+}} \text{Oxluciferin} + \text{AMP} + \text{PPi} + \text{Light}
\]

Thus, the decrease in the level of cellular ATP due to the inhibitory actions of OXPHOS inhibitors was examined by quantifying the intensity of light released from samples that were treated with OXPHOS inhibitors using a luminometer.
Figure 4.5. Mechanism of the inhibitory action of Oli on complex V

Schematic representation of yeast mitochondrial ATP synthase including $F_1$ sector where generation of ATP occurs, and central stalks connecting the $F_O$ subunit to $F_1$ subunit. $H^+$ are depicted as passing through the proton channel across the IMM to reach the $F_1$ sector (adapted from Devenish et al., 2000).
4.1.3.2. Monitoring mitochondrial membrane potential using TMRM

The potential energy stored in the $\Delta \Psi_m$ plays a fundamental role in mitochondrial functions, including the production of ATP, buffering of Ca$^{2+}$, importation of precursor enzymes into mitochondria and synthesis of mitochondrial proteins (Chen, 1988). Thus $\Delta \Psi_m$ is a key indicator of cell health whose measurement directly allows an assessment of mitochondrial function. TMRM is a cell permeable cationic fluorescence dye that is widely used to monitor $\Delta \Psi_m$ by fluorescence imaging (Scaduto & Grotyohann, 1999). TMRM is a relatively non-toxic, cell-permeant, cationic dye (Scaduto & Grotyohann, 1999) which can be used to estimate $\Delta \Psi_m$ in adherent cell cultures. Due to its solubility in the mitochondrial matrix and IM (because of its hydrophobic character), TMRM readily distributes across the IMM and accumulates in the mitochondrial matrix in inverse proportion to $\Delta \Psi_m$ according to the Nernst equation (because of its positive charge). Its positive charge allows a higher accumulation into more negatively charged or polarized mitochondria, whereas depolarized mitochondria will accumulate less TMRM (Perry et al., 2011). The $\Delta \Psi_m$-dependent distribution of TMRM in the mitochondrial matrix can be optically detected by fluorescence microscopy, confocal or multiphoton microscopy, flow cytometry, or by use of a fluorescent plate reader (Perry et al., 2011). Whilst these fluorimetric approaches do not quantify $\Delta \Psi_m$, they each can give a very useful measure of the relative degree of mitochondrial polarisation. A further advantage of using TMRM is that, when compared with other cationic fluorescence dyes such as tetramethylrhodamine ethyl ester (TMRE) or rhodamine 123, TMRM was shown to have the least effect in suppressing respiration when used at low concentrations (Scaduto & Grotyohann, 1999), thus making TMRM a very useful marker of the extent of polarisation of neuronal mitochondria (Beart et al., 2007; Diwakarla et al., 2009b). This study employed confocal live-cell imaging to monitor the distribution of TMRM in CGCs treated with OXPHOS inhibitors in order to examine the loss of $\Delta \Psi_m$ due to inhibitory actions of OXPHOS inhibitors. The advantage of live-cell imaging of live neurones comes from the observation of dynamic processes of living cells in real time while avoiding possible artefacts during the sample processing (Salipalli et al., 2014).
4.1.3.3. Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using Seahorse XF24 analyzer

While measurement of the cellular ATP level reflects a downstream effect of mitochondrial dysfunction, determination of the rate at which oxygen is consumed by intact neurones over time allows a more direct insight of mitochondrial bioenergetics. The Seahorse XF24 analyser (Seahorse Bioscience, North Billerica, MA) is a powerful tool that allows direct measurement of the two major energy-producing pathways, mitochondrial OXPHOS and glycolysis. The analyser allows precise and rapid measurements of the rate of change of analytes, such as oxygen and H⁺ dissolved in the media immediately surrounding neurones which are cultured and maintained in Seahorse cell culture microplates. The non-invasive detection of changes in oxygen or H⁺ content that occurs during mitochondrial respiration and glycolysis allows real-time measurements of mitochondrial bioenergetics under pharmacological inhibition of OXPHOS complexes at the chosen concentrations of inhibitors. This real-time approach allows measurement of physiologically more relevant respiratory parameters of living neurones compared to the experiments involving isolated mitochondria.

The Seahorse XF analyzer allows simultaneously recording of mitochondrial OXPHOS (oxygen consumption rate; OCR) and glycolytic activities (extracellular acidification rate; ECAR due to production of lactic acid as end product of glycolytic metabolism, see below) while successive addition of three different metabolic inhibitors, Oli, FCCP and Rot/AA, are added directly to cells. The changes in oxygen content in the media are measured as an index of respiration during OXPHOS, while the measurement of a net production of H⁺ allows determination of glycolytic activity as H⁺ is extruded into the extracellular medium when glucose is converted to lactate during glycolysis. Each of the metabolic inhibitors injected from the analyser targets specific component of the ETC (F₁Fo ATP synthase, membrane permeability, and complex I/III). Consequently, the key parameters of mitochondrial respiration, such as basal respiration, ATP production via ETC, H⁺ leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration are revealed (Fig. 4.6) (Brand & Nicholls, 2011).
Figure 4.6. Protocol for assessing mitochondrial function and respiration parameters in CGCs using the Seahorse XF24 analyzer

(A) Measurement of mitochondrial function in intact CGCs begins with treatment with the mitochondrial respiratory complex inhibitors for 4 h or 24 h outside the Seahorse XF24 instrument. CGCs were washed free of the inhibitors, and OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were directly monitored over time after successive injection of Oli, FCCP and Rot/AA. (B) Schematic OCR profile generated in the Seahorse XF24 mitochondrial stress analysis. Seahorse Mito Stress Test assay allowed measurement of respiration parameters, which were further analyzed to provide detailed information of bioenergetic dysfunction caused by the stressors. A single experiment is shown where values are from 3-5 replicate wells.
Pretreatment with mitochondrial respiratory complex inhibitors

4 h or 24 h

Begin experiment. Cells washed free of drugs

Measure basal OCR

Inject Oli

Measure OCR due to H⁺ Leak

Inject FCCP

Measure maximal OCR

Inject Rot/AA

Measure non-mitochondrial respiration

(A)

(B) OCR vs TIME (Avg)

OCR (pH/moles/min)

1300

1100

900

700

500

300

100

-100

10 20 30 40 50 60 70 80 90 100 110 120 TIME (min)

Oligomycin, FCCP, Rotenone

Basal Resp., ATP turnover, H⁺ Leak, Respiratory Capacity, Non-mitochondrial respiration
Basal respiration is a baseline oxygen consumption reading for CGCs, which is an index of resting cellular energy demand. Alteration in basal respiration suggests an altered ATP demand in cells or dysfunction of mitochondrial ATP synthesis (Brand & Nicholls, 2011). Basal OCR of cells is measured during the first 15 min of assay to establish the baseline OCR, followed by a sudden reduction in OCR in response to addition of Oli, whose inhibition correlates with the ATP production via $F_1F_0$ ATP synthase. Addition of Oli causes a transient increase in the amount of $H^+$ in IMS by blocking $H^+$ movement through the $F_1F_0$ ATP synthase. Injection of FCCP releases the increased $H^+$ gradient in IMS across the IMM, inducing a non-specific $H^+$ movement into the mitochondrial matrix. This process mimics extreme high energy demand of cells by allowing uninhibited electron flow through the ETC, which subsequently allows measurement of maximal rate of $O_2$ consumption. Also ETC is completely uncoupled from the generation of ATP via $F_1F_0$ ATP synthase during this process. Maximal OCR induced by FCCP allows determination of spare respiratory capacity, which is a measure of the mitochondrial energy reserve (Hill et al., 2009). The spare respiratory capacity is obtained by calculating the difference between maximal respiration and basal respiration. Recent studies with primary neuronal cultures have highlighted the role of spare respiratory capacity as an important index of predicting the ability of cells to overcome stresses such as oxidative stress or inhibition of ETC (Vesce et al., 2005; Yadava & Nicholls, 2007), where the depletion of bioenergetic capacity was an indication of loss of cell’s ability to overcome energy demand and stresses. Addition of Rot and AA blocks $H^+$ translocation by the ETC and rapidly inhibits mitochondrial $O_2$ consumption. Therefore, the remaining OCR measurement subsequent to the addition of Rot and AA determines the oxygen consumed by non-mitochondrial components in cells. Finally, $H^+$ leak through ETC can be determined from the difference between the Oli-dependent OCR (mitochondrial ATP synthesis) and non-mitochondrial OCR (Fig. 4.6A). Together, such measurements allow a construction of bioenergetic profile of cells (Fig. 4.6B), which is further analysed to provide detailed information of bioenergetic status of CGCs. For the purpose of this study, CGCs were pre-treated with OXPHOS inhibitors for 4 h and 24 h and the bioenergetic dysfunction induced by drug treatment was determined by the Seahorse XF24 analyzer.
4.1.4. Aims for Chapter 4

The focus of this part of the thesis is therefore to utilize the above mentioned techniques to perform strategic analyses to document the adverse effects of the specific OXPHOS inhibitors on mitochondrial bioenergetics. The specific aims were to analyse the cytosolic ATP levels, $\Delta \Psi_m$ and OCR after treating CGCs with specific OXPHOS inhibitors to extend the insights into the cytotoxic effects of the inhibitors which were investigated in the previous chapter, therefore to gain mechanistic information on how OXPHOS inhibitors affect mitochondrial bioenergetics. Furthermore, these strategic analyses will provide a platform on which the next chapters of the thesis will be assembled to further investigate quality control of dysfunctional mitochondria.
4.2. Materials and Methods

4.2.1. Primary CGC Culture, Growth and Maintenance

Primary cultures of CGCs were prepared from 6-8 day-old SWISS White mice according to Section 2.2.2. Briefly, cerebella were dissected in ice-cold isolating solution (HBSS pH 7.4 containing 3 mg/ml BSA, 1.16 mM MgSO4, 1 mM sodium pyruvate, 10 mM HEPES, 7.6 mM D-Glucose) and the meninges were carefully removed. The isolated cerebella were placed in fresh ice-cold isolating solution and they were chemically and mechanically dissociated and centrifuged. The cells were resuspended in NBM containing B-27 supplement (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum and seeded in tissue culture plates, pre-coated with poly-D-lysine (50 µg/ml). Cells for measurement of cellular ATP were seeded in 24-well plates (0.35 x 10^6 cells/well), while CGCs for monitoring of ΔΨ_m were seeded in Falcon 6-well plate (2 x 10^6 cells/well) which was designed to fit Zeiss confocal microscopy. For experiments performed in the Seahorse XF24 Extracellular Flux Analyzer, cells were seeded in XF24 V7 microplates (Seahorse Bioscience) at a density of 0.1 x 10^6 cells/well according to the optimal cell seeding density optimization assay (Appendix 1.1). Neurones were left to grow in NBM containing B-27 components (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum for 24 h in a humidified incubator (5 % CO_2, 8.5 % O_2 and N_2 at 37°C). Medium was fully replaced by NBM containing aphidicolin (1 µg/ml) to restrain non-neuronal cell proliferation (Diwakarla et al., 2009b; Giardina et al., 1998), and final concentration of 3 % (v/v) B-27 supplement. Half medium changes were conducted at 4 DIV and included aphidicolin (1 µg/ml) and 3 % of B-27 supplement and all experiments were performed at 7 DIV.
4.2.2. Measurement of Cellular ATP using Luciferase-Luciferin Fluorescence

The ATP assay employed in this thesis has been adapted from the methods previously published by Donnelly et al. (2012).

4.2.2.1. Cell lysis and extraction of ATP

Cells were plated and grown in triplicate in 24-well plates and treated with drugs for 4 and 24 h according to Section 2.2.2 and 3.2.3.1. After drug treatment, media containing drugs were removed by washing cells twice with 500 µl sterile ice-cold PBS (pH 7.4), and cells were lysed with 100 µl of Phosphosafe Extraction Buffer (PhosphoSafe™ Extraction Reagent; Novagen®, EMD Millipore, Billerica, MA) for 5 min at room temperature with gentle shaking. Phosphosafe Extraction Buffer provides efficient lysis of cells while preserving the phosphorylation state of molecules by four different phosphatase inhibitors (sodium fluoride, sodium vanadate, β-glycerophosphate, and sodium pyrophosphate). After lysis, the cellular lysate was gently scraped from the wells and transferred to ice-cold eppendorf tubes and centrifuged at 16,000 x g for 5 min at 4°C. The supernatant was kept on ice until further ATP analysis.

4.2.2.2. ATP standards and measurement of luminescence

ATP standards were made fresh for each experiment by dissolving ATP in sterile mQH₂O in concentrations ranging from 300 pM to 100 nM. Standards were stored in ice until further analyses. Triplicates of 50 µl of ATP standards, sterile mQH₂O in which ATP standards were made (blank for ATP) and Phosphosafe Extraction Buffer (blank for cell lysates) were added in sterile, black walled with clear bottomed 96-well plates which were designed for luminescence reading. 50 µl of centrifuged supernatant of
cell lysates were also transferred to sterile opaque clear bottomed 96-well plates and stored in ice until ready for luminescence reading.

The luciferase-luciferin reagent (FLAA, Sigma Aldrich) was made as advised by the product provider. The assay mix containing luciferase-luciferin was diluted using Dilution buffer provided in the kit and stored in -20°C for further use. The reagent was thawed in ice and placed at room temperature before the reagent was added to the samples, as ATP measurements at high temperatures leads to loss of luciferase-luciferin activities (Crouch et al., 1993). 50 µl of luciferase-luciferin reagent was rapidly added to samples by Eppendorf combitips and the plate was gently swirled and put into the luminometer. The plate was allowed to stand for 3 min in dark at room temperature and relative luminescence unit (RLU) signal was measured for 6 secs per sample. Standard curves were constructed using GraphPad Prism v.4.0. (San Diego, CA, USA) by generating linear regression curve of RLU and ATP standard solutions with known concentration, and ATP levels from samples were determined by interpolation from the standard curves. The raw data were converted into percentage of control to allow analyses between different drug treatments over time. Statistical analysis was performed by repeated measures two-way ANOVA, followed by Bonferroni’s post hoc test. Data represent mean values for 3 samples ± SEM of 3 independent experiments and are expressed as a percentage of the control.

4.2.3. Determination of $\Delta \Psi_m$

TMRM is a cell permeable cationic fluorescence dye that is widely used to quantitate the $\Delta \Psi_m$ across the inner mitochondrial membrane by fluorescence imaging (Scaduto & Grotyohann, 1999). Due to its solubility in the mitochondrial matrix and inner membrane, it readily distributes across the inner membrane and accumulates in the matrix in proportion to $\Delta \Psi_m$ because of its positive charge. Upon accumulation, TMRM exhibits fluorescence in functional mitochondria (excitation 548 nm, emission 573 nm), but fluorescence quickly dissipates once $\Delta \Psi_m$ is lost (Beart et al., 2007; Diwakarla et al., 2018).
2009b; Higgins et al., 2009; Higgins et al., 2012; Ward et al., 2007), allowing simple ratio fluorescence techniques for measurement of $\Delta \Psi_m$. When compared with other cationic fluorescence dyes such as tetramethylrhodamine ethyl ester (TMRE) or rhodamine 123, TMRM was shown to have the least effect in suppressing respiration when used at low concentrations (Scaduto & Grotyohann, 1999). Measurement of the changes in $\Delta \Psi_m$ in live neurones with TMRM was described previously (Beart et al., 2007). Neurones grown on Falcon® 6-well plates at a density of $2 \times 10^6$/well were initially washed twice with MEM without phenol red containing KCl (25 mM) and loaded with TMRM (150 nM) for 15 min at 37°C prior to drug treatment. Media in each well was replaced by drugs containing 50 nM of TMRM and live cell imaging was carried out over time. To induce complete depolarization of mitochondrial membrane potential, a protonophore (CCCP; 10 µM) (Lim et al., 2001; Minamikawa et al., 1999) was added to cells as a positive control. Cells were kept at 37°C in dark at all times.

4.2.3.1. Confocal live cell imaging of $\Delta \Psi_m$

TMRM-labelled live neurones were analysed using an inverted confocal microscope (Zeiss LSM 510 Pascal, Carl Zeiss, Oberkochen, Germany) fitted with 37°C, 5 % CO$_2$ humidified stage incubator. Live cell imaging of neurones was undertaken throughout the time-course of the drug treatment with parallel vehicle controls at 543 nm with a 40x lens. The instrumental settings of laser power, gain and brightness were initially adjusted by using the control neurones which possessed the highest TMRM fluorescence to ensure strong fluorescence intensity with non-saturation, and the settings were kept identical throughout the imaging procedure of drug-treated samples. The fluorescence intensity of unstained neurones was considered as an area devoid of fluorophore, and the fluorescence value was used as a background signal to be subtracted from the fluorescence intensity obtained from samples. The observed fluorescence intensity of test samples was analysed relative to the fluorescence intensity of control sample. Control experiments indicated that TMRM fluorescence remained stable for up to 4 h of observation. Real-time measurements were made at 0.5, 1 and 4 h when plates were removed from the humidified CO$_2$/N$_2$ incubator (5 % CO$_2$, 8.5 % O$_2$, 37°C) and fluorescence intensity was captured at three random fields from the sample well.
Measurements were made over a period of 10 min and then plates immediately returned to the CO\textsubscript{2}/N\textsubscript{2} incubator.

4.2.3.1. Data analysis of TMRM images

The mean fluorescence intensities across populations of vehicle control and treated neurones were obtained by using LSM 5 Image Browser provided by Zeiss. Three fields of the well were randomly imaged, and the fluorescence intensity of each image was divided by the total number of neurones present in the imaging field. Each treatment group involved the imaging of at least 120 neurones in each of duplicate wells. The mean fluorescence intensity of unstained neurones was used as a background and its mean value was subtracted from those obtained for control and drug-treated group. The adjusted values of fluorescence intensities of drug-treated groups were expressed as a percentage of control (expressed as 100 %). Values were expressed as the mean ± SEM and are from n=3 independent experiments. Histograms showing the changes in $\Delta\Psi_m$ over the time-course of drug treatment were analysed and generated by GraphPad Prism v.4.0. (San Diego, CA, USA). Two-way ANOVA followed by Bonferroni post hoc test was performed to determine significant differences between groups and among treatments, and a value of $P < 0.05$ was considered statistically significant.

4.2.4. Measurement of Mitochondrial Respiration with Seahorse XF24

Measurements of the rate of oxygen consumption (OCR) and extracellular acidification (ECAR) in adherent intact primary neuronal cultures were performed using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA), and are indicative of cellular oxidative and glycolytic activity, respectively.
4.2.4.1. Preparation of neurones in XF24 cell culture plate for Mito Stress assay

XF24 V7 Cell Culture Microplates (24 wells) (Seahorse Bioscience) were pre-coated with poly-D-lysine (50 µg/ml) for 2 h or overnight in 37°C incubator. The plate was washed once with sterile mQH$_2$O and left to dry in sterile laminar flow hood before plating cultured neurones at a density of 100,000 cells/well. The four corners of the plate were deliberately left blank to serve as background correction wells as suggested by the supplier. Cells were allowed to grow in NBM containing 10 % FBS, 25 mM KCl, 500 µM L-glutamine, and 100 U/ml penicillin-streptomycin for 24 h and subsequently changed to fresh NBM (final 3 % of B-27 supplement) at 1 DIV and a half-media change was performed at 4 DIV. Experiments were performed at 7 DIV.

4.2.4.2. Drug treatment of neurones and measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

MEM containing 25 mM KCl were pre-warmed in humidified incubator (5 % CO$_2$, 8.5 % O$_2$ and N$_2$ at 37°C) before diluting drugs of interest to appropriate concentrations. The MEM-drug solution was allowed to equilibrate in humidified incubator (5 % CO$_2$, 8.5 % O$_2$ and N$_2$ at 37°C) for further 30 min. Neurones were exposed to MEM (control) and MEM containing Rot 10 nM and 30 nM, 3-NP 100 µM and 300 µM, AA 10 nM and 30 nM and KCN 100 µM and 300 µM, with full media changes with unbuffered serum-free Seahorse assay media (KCl 25 mM) after 4 h and 24 h incubation. The plates were transferred to Seahorse XF24 analyzer for subsequent analyses of OCR and ECAR.

4.2.4.3. Preparation and calibration of XF24 Seahorse analyzer

The instrument measures the rate of change of pH in the media immediately surrounding intact neurones, thus it was crucial to perform all measurements in unbuffered Seahorse assay media (Seahorse Bioscience) supplemented with 0.2 mM
sodium pyruvate, 25 mM glucose and 25 mM KCl. All solutions were strictly adjusted to pH 7.4 on the day of assay. On the day before the planned experiment, XF24 Sensor Cartridges were placed on top of a Seahorse Bioscience 24-well plate containing XF24 Calibrant pH 7.4 solution and were left to hydrate overnight at 37°C without CO₂. The instrument was left on overnight to stabilize at 37°C. On the day of experiment, unbuffered Seahorse assay media was warmed to 37°C and the cells were checked under the microscope to ensure confluency and even seeding. The media from each well was removed, leaving 50 µl behind to prevent cells from drying. Cells were rinsed by adding 1 ml of warm unbuffered Seahorse assay media which was removed again by pipetting, leaving 50 µl behind to prevent cells from drying, and 625 µl of warm unbuffered DMEM was added to achieve final volume of 675 µl/well. The plate was left in a 37°C incubator without CO₂ for 60 min as de-gassing procedure before loading plate in XF24 instrument for OCR/ECAR measurements.

Four corner wells of the plate were deliberately left blank to use as background correction wells. In order to determine the bioenergetic profile of vehicle control and drug-treated neurones, four different metabolic drugs were loaded into four ports of the sensor cartridge, port A, B, C and D were sequentially injected into each well (75 µl/well). The four metabolic reagents, Oli (Sigma Aldrich), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma Aldrich), Rot (Sigma Aldrich) and AA (Sigma Aldrich), were pre-warmed to 37°C prior to loading into the sensor cartridge. The final concentration of each reagent was Oli (1 µM), FCCP (2.5 µM) and Rot/AA (both 2 µM), when drug concentration response titration was performed to determine the optimum concentration that yield the maximal effect for the subsequent OCR/ECAR measurements (Appendix 1). Because the reagents were to be injected sequentially into the well, they were diluted in unbuffered Seahorse assay media in 10 times the final concentration of Oli (port A), 11 times concentration of FCCP (port B), 12 times concentration of Rot (port C) and 13 times concentration of AA (port D).
4.2.4.4. Measurement of OCR/ECAR of intact neurones

Prior to OCR/ECAR measurement, the sensor cartridges were placed into the instrument for calibration. After the calibration process, sensor cartridges were replaced by a cell plate which was incubated in a 37°C incubator without CO\textsubscript{2} for an hour. The plate went through 4 consecutive mixed and waiting cycles to allow oxygen and pH levels of the medium to reach equilibrium. The first three sets of OCR (measurement of oxygen concentration change)/ECAR (measurement of pH change) measurement were performed to establish baseline rate and the medium was gently mixed again waiting for the oxygen tension and pH in the medium to be restored to normal. After the baseline measurement, the first metabolic reagent, Oli (1 µM), was injected into the plate via port A and the plate went through another mix and wait cycle before reading the OCR/ECAR measurement for 3 min. The mix, wait and measurement cycle was repeated 3 times before the reagent in port B, FCCP (2.5 µM), was injected into the plate. Injection of Rot (2 µM) and AA (2 µM) in port C and D followed sequentially after 3 sets of mix, wait and measurement cycles of post-injection of FCCP.

4.2.4.5. Analyses of OCR/ECAR values

All Seahorse experiments were performed once, and respiration and acidification rates are presented as the mean ± SEM (n = 3-5 wells per treatment group). Statistically significant differences between treatment groups were identified using one way ANOVA with Bonferroni post hoc test by GraphPad Prism v.4.0. (San Diego, CA, USA).
4.3. Results

4.3.1. Bioluminometric Method for Measurement of ATP Levels in CGCs

4.3.1.1. Optimization and determination of luciferase-luciferin assay mix concentration with regards to sensitivity required

The luciferase-based ATP assay (ATP Bioluminescent Assay Kit; Sigma Aldrich, St. Louis, MO, USA) is effective for determination of ATP concentrations ranging from $2 \times 10^{-12}$ to $2 \times 10^{-9}$ M in the sample. The luciferase-luciferin assay mix should be diluted depending on the amount of ATP present in the sample. Generally, a higher concentration of luciferase-luciferin assay mix solution gives greater sensitivity for the detection of low amounts of ATP. However, a larger amount of oxyluciferin produced as an end product could cause product inhibition of the reaction and obscure variations in ATP concentration (Lemasters & Hackenbrock, 1979). Therefore, it was necessary to determine the appropriate concentration of luciferase-luciferin reagent so that adequate sensitivity ensued in the assay procedure.

All steps of the ATP assay were performed under sterile conditions to remove the likelihood of microbial contamination which might contribute to variations in ATP content in the samples. New ATP standard curve was generated freshly on the day for each experiment. CGCs were grown in 24-well plate and were processed according to Section in 4.2.2. CGCs were lysed with an extraction buffer that contained phosphatase inhibitors (PhosphoSafe™ Extraction Reagent; Novagen®, EMD Millipore, Billerica, MA). Lysates were transferred to a sterile opaque 96-well plate where the luciferase-luciferin assay mix was added directly. Bioluminescence was recorded by luminometer.
Luciferase-luciferin assay mix was diluted 10- and 25-fold with the dilution buffer, as recommended by the provider. ATP standard solutions at various concentrations ranging from 0.1 nM to 100 nM were made on the day of experiment and were kept on ice for stability (stable for up to 8 h). ATP standard solutions were added to a sterile opaque 96-well plate, to which three different dilutions of assay mix were directly added. Sterile mQH$_2$O in which ATP solutions were made was used as a blank. Luminescence was read using a luminometer as described in Section 4.2.2.2, and the corresponding values were corrected by subtracting the blank values. Fig. 4.7A represents a graph showing relative luminescence units (RLU) plotted against the amount of ATP in the sample. The best fit linear regression lines for all three dilutions produced linear relationship with $R^2$ values of 0.99 (0.9984 for undiluted; 0.9945 for 10-fold dilution; 0.9944 for 25-fold dilution), indicating all three dilutions of assay mix were suitable for use as standard curves. As expected, the undiluted luciferase-luciferin assay mix, which contained the most concentrated amount of luciferase-luciferin, produced a greater amount of luminescence compared to 10- and 25- fold dilution assay mix. The sensitivity of the 25-fold dilution of the assay mix decreased at the lowest concentration of ATP standard (0.1 nM), whereas 10-fold diluted assay mix resulted in higher amount of light and was found to be sufficient to detect ATP in the range of 100 nM and 1 nM. Given the amount of ATP detected from untreated CGCs was on average 54 µM (Fig. 4.7C), 10-fold dilution was selected for use for the rest of the ATP measurements. The mean of slope value of best fit curve of standard curves generated from eight independent experiments of 10-fold diluted reagent was 0.90, with SEM of 0.0098 (Fig. 4.7B). Such small values of SEM confirmed the stability of the luciferase-luciferin reagent across multiple independent experiments, and the reproducibility of the assay.

4.3.1.2. Time- and insult-dependent reduction in ATP level in CGCs

CGCs grown in 24-well plate were treated with OXPHOS inhibitors for 4 h and 24 h. CGCs were also treated with STS and H$_2$O$_2$ as reference insults. Samples were lysed with an extraction buffer and processed according to Section 4.2.2. Bioluminescence was recorded by a luminometer and ATP levels from samples were determined by
Figure 4.7. Optimization of ATP assay mix containing luciferin-luciferase and ATP calibration curve

(A) Various dilutions of luciferin-luciferase ATP assay mix was added to ATP solution (10^{-7} and 10^{-10} M) dissolved in MQH_{2}O (presented in log unit). The intensity of light produced was determined by luminometer and presented in log of relative luminescence unit (RLU). (B) New ATP calibration curve was determined for every experiment. ATP dilutions were made fresh on the day of experiment. Data shows a representative standard curve with a mean of three samples of single experiment. Three seconds of shaking after of luciferin-luciferase reagent injection and six second RLU signal integration time were used. All standard curves produced a linear relationship with R^2 0.998 ± 0.0004. The slope of standard curves from 8 independent experiments produced a mean of 0.90 with a low SEM value (0.0098), indicating stability or the assay reagent and reproducibility of the assay. (C) Amount of ATP in untreated CGCs was measured by luciferin-luciferase ATP assay. Data represent mean ± SEM of four independent samples from single experiment.
<table>
<thead>
<tr>
<th>Sample number</th>
<th>Amount of ATP (M)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>$5.7 \times 10^{-8}$</td>
</tr>
<tr>
<td>2</td>
<td>$5.6 \times 10^{-8}$</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>$5.2 \times 10^{-8}$</td>
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<tr>
<td>Average</td>
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### Table

<table>
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<tr>
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<th>Slope</th>
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<tbody>
<tr>
<td>Mean</td>
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<td>0.998</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
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<td>0.00041</td>
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interpolation from the standard curves. Data were presented as the relative percentage of ATP levels compared with control (untreated) cells.

CGCs treated with STS displayed time-dependent reductions in ATP level, although increasing concentration of STS failed to lead to a greater reduction in ATP level (Fig. 4.8). Data from STS treatment of CGCs for 4 h suggested a small reduction in ATP level, which became statistically significant with a longer period of STS treatment (24 h; 100 nM, 35 ± 15 % of control, P < 0.001; 250 nM, 34 ± 7.4 % of control, P < 0.001). The notable reduction of ATP shown at 24 h incubation, but not at 4 h with STS treatment, is likely to reflect the relatively slow nature of STS-induced apoptotic cell death (Collins et al., 1997; Diwakarla et al., 2009b) which involves ATP-requiring processes such as caspase activation, hydrolysis of macromolecules, chromatin condensation and formation of apoptotic bodies (Barros et al., 2003; Hu et al., 1999; Kass et al., 1996; Li et al., 1997; Nicotera et al., 1998). The repeated measures two-way ANOVA confirmed statistically significant reduction of ATP levels between 4 h and 24 h for both concentrations of STS (P < 0.05 for STS 100 nM; P < 0.01 for STS 250 nM).

Concentration-dependent decreases in ATP levels were observed with H$_2$O$_2$ treatment (Fig. 4.8), where treatment with 30 µM H$_2$O$_2$ failed to induce a significant reduction in the ATP level. Given 30 µM H$_2$O$_2$ was sufficient to cause significant loss of cell viability at 4 h and 24 h (Fig. 3.5), such insignificant reduction in cellular ATP under 30 µM H$_2$O$_2$ treatment suggested H$_2$O$_2$-induced cellular injury does not directly cause bioenergetic dysfunction. Oxidative injury at higher concentration of H$_2$O$_2$ (50 µM) caused a rapid decrease in the cellular ATP level (4 h, 54 ± 21 % of control, P < 0.01; 24 h, 9.6 ± 4.9 % of control, P < 0.0001), which is a phenomenon also observed in other cell types undergoing H$_2$O$_2$-induced necrotic cell death (Saito Y et al., 2006). The repeated measures two-way ANOVA again confirmed a statistically significant time-dependent reduction of ATP levels between 4 h and 24 h for 50 µM H$_2$O$_2$ (P < 0.01) (Fig. 4.8).

The OXPHOS inhibitors also caused reductions of ATP, and their effects generally displayed concentration-dependency (Fig. 4.9). Rot at lower concentration (10 nM) induced small reductions in cellular ATP over 4 h and 24 h which were statistically
insignificant. However, significant reductions in ATP were observed with the treatment of CGCs with higher concentrations of Rot (30 nM) after 4 h (44 ± 14 % of control, P < 0.05) and 24 h incubation (19 ± 6.1 % of control, P < 0.0001), indicating Rot was causing significant bioenergetic dysfunction at higher concentrations. 3-NP induced slow but significant reductions in cellular ATP after 24 h incubation with both 100 µM and 300 µM (100 µM, 20 ± 3.3 % of control, P < 0.01; 6.7 ± 0.7 % of control, P < 0.0001, respectively). This trend for reduction was consistent with the time-dependent reduction of cell viability for 3-NP treated CGCs (Fig. 3.11), and effect which corresponds to the MTT reduction by succinate dehydrogenase activity (Lobner, 2000; Slater et al., 1963). Treatment with AA displayed some variability in the reductions of ATP. Thus, AA displayed concentration-dependent reduction in ATP level, where a lower concentration (10 nM) caused small reduction in ATP level at 4 and 24 h. However, significant reduction in cellular ATP was only observed when CGCs were treated with the higher concentration for 24 h (30 nM, 33 ± 11 % of control, P < 0.01). Similarly, KCN treatment also showed some variability in the reductions of ATP level. KCN at the lower concentration failed to induce significant reduction in cellular ATP, an observation consistent with the MTT cell viability data from Chapter 3 where a small deduction in cell viability was observed under KCN 100 µM treatment. Even at a higher concentration of KCN, the slight reduction in cellular ATP was statistically insignificant (Fig. 4.9). Likewise, Oli-induced inhibition of complex V failed to induce a significant reduction of ATP for 4 h and 24 h incubations at a lower concentration (0.1 µM), and the apparent reduction in ATP with Oli 1 µM was statistically insignificant.

Overall, repeated measures two-way ANOVA revealed that there were significant differences in ATP levels between treatments of different drugs (P < 0.0001). Note that the highly reproducible quantitation of the control neuronal level of cellular ATP attests to the reliability of the assay (see Fig. 4.7C). Additionally, drug-treated cells showed a corresponding lack of variance, hence a high degree of reproducibility in the changes observed, where ATP reductions were large.
Figure 4.8. Time- and insult-dependent reduction in ATP level in CGCs treated with STS and H$_2$O$_2$

The level of ATP of CGCs treated with various concentrations of STS and H$_2$O$_2$ for 4 h and 24 h was measured using the luciferase-based bioluminescence assay kit. CGCs showed time- and concentration-dependent reduction in ATP level. ATP levels were determined from luminescence readings which were interpolated from linear standard curves. The raw data were converted into percentage of control to allow analyses between different drug treatments over time. Data represent mean values for 3 samples ± SEM of 3 independent experiments and are expressed as a percentage of the control. * P < 0.05; ** P < 0.01; # P < 0.001; ## P < 0.0001 compared to control by repeated measures two-way ANOVA, followed by Bonferroni's post hoc test. Repeated measures two-way ANOVA revealed significant differences of ATP levels between 4 h and 24 h treatment and drugs (P < 0.001).
Figure 4.9. Time- and insult-dependent reduction in ATP level in CGCs treated with OXPHOS inhibitors

The level of ATP of CGCs treated with various concentrations of OXPHOS inhibitors for 4 h and 24 h was measured using the luciferase-based bioluminescence assay kit. ATP levels were determined from luminescence readings which were interpolated from linear standard curves. The raw data were converted into percentage of control to allow analyses between different drug treatments over time. Data represent mean values for 3 samples ± SEM of 4 - 5 independent experiments and are expressed as a percentage of the control.

* P < 0.05; ** P < 0.01; ## P < 0.0001 compared to control by repeated measures two-way ANOVA, followed by Bonferroni's post hoc test. Repeated measures two-way ANOVA revealed significant differences of ATP levels between drug treatments (P < 0.0001).
4.3.2. Monitoring Depolarization of Mitochondrial Membrane Potential by tetramethylrhodamine methylester (TMRM)

4.3.2.1. Optimization of live-cell imaging of TMRM fluorescence

Monitoring of $\Delta \Psi_m$ is widely used to examine the mitochondrial function within cells. Changes in $\Delta \Psi_m$ in neurones under treatment of OXPHOS inhibitors were examined using live cell confocal imaging by monitoring the retention of TMRM fluorescence within mitochondria as an indicative of the bioenergetic status of CGCs. Fluorescence microscopy imaging is an essential tool that allows identification of subcellular components; however autofluorescence of samples can be a problem as it may produce a background signal and interfere with detection of specific fluorescent signals. All media used in CGC culture contain phenol-red, which is used as a pH indicator of the media. Although insignificant, phenol-red was identified as a fluorescent constituent that may account for the cellular fluorescence (Benson et al., 1979). Therefore, autofluorescence of CGCs in phenol-free media was imaged as a control. CGCs were grown under the standard conditions for all TMRM imaging experiments as described in Section 4.2.3. Cells at 7 DIV were rinsed twice with phenol-red free media to remove any phenol-red in the media before being subjected imaging using Zeiss confocal live-cell microscopy. Cells were imaged with 40X objective lens without loading of the TMRM dye and corresponding bright field images were also taken. As shown in Fig. 4.10A, no autofluorescence was detected from CGCs, confirming phenol-red free media would not interfere with fluorescence for the subsequent TMRM imaging.

Neurones are vulnerable to oxidative stress and as routinely used herein were grown in the presence of N₂ gas to maintain low level of oxygen in the ambient air (Section 2.2.2). However, the gas composition of the microscope incubator chamber lacked N₂ gas supply. In order to reduce disturbance to the cultured cells, cells were transferred back and forth between N₂ incubator and the microscopy chamber during the whole course of imaging. Although the time taken out of the N₂ incubator was < 10 min,
it was necessary to confirm the process of transferring cells between incubators was not affecting cell viability, which might eventually cause the loss of \( \Delta \Psi_m \). Therefore, control cells were also set up and left in the \( \text{N}_2 \) incubator throughout the experiment in phenol-red free media containing TMRM, and their TMRM fluorescence was imaged at the end of the experiment. Compared to the “incubator” control CGCs which were kept in the incubator for 4 h until the whole imaging processes were carried out (Fig. 4.10C), the “vehicle” control CGCs that were frequently transferred between incubators for 4 h still retained their TMRM fluorescence (Fig. 4.10B), indicating the frequent transfer between incubators did not affect TMRM fluorescence.

4.3.2.2. Qualitative measurement of \( \Delta \Psi_m \) in CGC culture using fluorescence imaging of TMRM dye

CGCs were grown and TMRM dye and drugs were delivered to cells as described (Section 4.2.3). Based upon preliminary observations, live-cell images were captured of three random fields per well at 0.5 h, 1 h and 4 h after the addition of drugs. The intensity of TMRM was maintained in control neurones showing bright red fluorescence, and threads of mitochondria were clearly visible in neuritic networks and in cytoplasm around the nuclei, which was manifested as a clear void (Fig. 4.11, Fig. 4.12). CCCP-treated neurones showed a total loss of fluorescence, indicating complete dissipation of TMRM due to loss of \( \Delta \Psi_m \). As early as 0.5 h after addition of drugs, Rot 10 nM and KCN 100 \( \mu \text{M} \) the majority of cells imaged had lost TMRM fluorescence and only a few cells retained dim fluorescence, indicating these drugs at low concentrations caused substantial loss of \( \Delta \Psi_m \) at this early timepoint (Fig. 4.11). Interestingly, most of the neuritic networks had lost the TMRM fluorescence while some cell bodies still retained the TMRM fluorescence, suggesting Rot- and KCN-induced inhibition of complex I and IV effectively caused mitochondrial dysfunction in the neuritic segments of neurones (Fig. 4.11, Fig. 4.12). CGCs with AA 10 nM treatment showed signs of relatively low levels of fluorescence loss by 0.5 h and the majority of fluorescence were retained in cell bodies and slightly diminished fluorescence on the neuritic networks (Fig. 4.12). In contrast, TMRM fluorescence appeared relatively unchanged in CGCs treated with 3-NP 300 \( \mu \text{M} \) (Fig. 4.11), Oli 1 \( \mu \text{M} \) and \( \text{H}_2\text{O}_2 \) 30 \( \mu \text{M} \) at 0.5 h and 1 h (Fig. 4.13, Fig. 4.15), indicating 3-NP, Oli and \( \text{H}_2\text{O}_2 \) had a slower effect on \( \Delta \Psi_m \). The loss of TMRM fluorescence was
Figure 4.10. Validation of TMRM fluorescence in CGC culture

Control experiments were performed to validate TMRM fluorescence in CGCs at 7 DIV. Cells were incubated in (A) MEM (no loading dye) and (B) MEM containing 50 nM TMRM and TMRM fluorescence was monitored by live-cell imaging. CGCs incubated in MEM (A) showed no fluorescence, while those loaded with TMRM (B) showed bright red fluorescence. (C) CGCs were loaded with TMRM and left in the N₂ incubator throughout the experiment. TMRM fluorescence was imaged at the end of the experiment. Compared to the control CGCs (C), the vehicle control CGCs (B) that were transferred between incubators still retained their TMRM fluorescence, indicating the frequent transfer between incubators did not affect TMRM fluorescence. Scale bar = 20 µm.
No TMRM

TMRM loaded

Incubator Control
Figure 4.11. Depolarization of mitochondria during treatment of CGCs with mitochondrial respiratory complex inhibitors for 0.5 h

TMRM fluorescence was monitored by confocal live cell imaging at 0.5 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of mitochondrial respiratory complex inhibitors, Rot 10 nM, 3-NP 300 µM, AA 10 nM and KCN 100 µM. CCCP 10 µM treatment was applied to untreated neurones loaded with TMRM as a positive control for complete depolarization of ΔΨm. Brightfield images are included to visualize neurones in the field. Scale bar = 20 µm.
Figure 4.12. TMRM fluorescence imaging revealed mitochondria in the neuritic processes were the first segment of neurones to lose $\Delta \psi_m$, followed by the cell bodies

TMRM fluorescence was monitored by confocal live cell imaging at 0.5 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of mitochondrial respiratory complex inhibitors, Rot 10 nM, 3-NP 300 µM, AA 10 nM, KCN 100 µM and Oli 1 µM. White arrows indicate treads of mitochondria in neuritic networks and yellow arrows indicate mitochondria around nuclei.
Control

Rot 10 nM

3-NP 300 µM

AA 10 nM

KCN 100 µM

Oli 1 µM

TMRM

Brightfield
TMRM fluorescence was monitored by confocal live cell imaging at 0.5 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of Oli 1 µM and H₂O₂ 30 µM. CCCP 10 µM treatment was applied to untreated neurones loaded with TMRM as a positive control for complete depolarization of ΔΨ₉₅. Brightfield images are included to visualize neurones in the field. Scale bar = 20 µm.
TMRM Bright field Overlay

Control

Oli 1 μM

H$_2$O$_2$ 30 μM

CCCP 10 μM
continuously observed over the time-course of 1 h and 4 h for Rot 10 nM, AA 10 nM and KCN 100 µM (Fig. 4.14, Fig. 4.16). The fluorescence intensity was notably reduced for 3-NP (Fig. 4.16) and H₂O₂-treated neurones (Fig. 4.17) after 4 h of drug-incubation, although TMRM fluorescence in Oli-treated neurones still retained in high intensity at 4 h (Fig. 4.17).

4.3.2.3. Quantitative measurement of ΔΨₘ in CGC culture using fluorescence imaging of TMRM dye

The significant loss of ΔΨₘ induced by inhibition of mitochondrial respiratory complex I, III and IV was reflected in the quantitative analysis of TMRM fluorescence intensity (Fig. 4.18). The fluorescence intensity values from CCCP-treated neurones were taken as background intensity and these values were subtracted from those obtained for control and drug-treated groups of neurones. The adjusted values were expressed as a percentage of corrected control, which was designated to be 100 % fluorescence. TMRM fluorescence was efficiently dissipated in neurones treated with CCCP which rapidly depolarized ΔΨₘ as early as 0.5 h after exposure to CCCP (P < 0.001; 0.5 h 27 ± 14 % of control, 1 h 32 ± 16 % of control; P < 0.0001; 4 h 16 ± 6.9 % of control) indicating loss of ΔΨₘ by an uncoupling action of CCCP (Fig. 4.18). The failure of complete loss of TMRM fluorescence under CCCP treatment is due to one instance when CCCP was not added enough, however almost complete depolarization of ΔΨₘ was observed in other independent experiments. Significant decreases in ΔΨₘ were most apparent in Rot 10 nM and KCN 100 µM-treated neurones as early as 0.5 h after exposure to drugs (P < 0.0001; 13 ± 6.8 % of control and 5.7 ± 1.8 % of control, respectively). Rot and KCN reproducibly showed significant reductions in ΔΨₘ at 1 h (P < 0.0001; 11 ± 6.8 % of control and 7.2 ± 2.7 % of control, respectively) and 4 h (P < 0.001; 0.5 ± 0.3 % of control and 2.5 ± 1.5 % of control, respectively). These data indicate that inhibition of complex I and IV by the chosen pharmacological agents could effectively induce depolarization of ΔΨₘ. However, 3-NP 300 µM failed to induce statistically significant reduction in ΔΨₘ in contrast to the rapid effects noted above, but there was some evidence of a slow loss of fluorescence during the time-course treatment (0.5 h, 95 ± 15 % of control; 1 h, 90 ± 14 % of control; 4 h, 57 ± 9.2 % of control) (Fig. 4.18). The
repeated measures two-way ANOVA revealed the reduction in TMRM fluorescence intensity over time was statistically significant between 0.5 h - 1 h and 0.5 h - 4 h timepoints ($P < 0.01$). Similarly, AA 10 nM showed a slower reduction in $\Delta \Psi_m$ over 0.5 h, 1 h and 4 h (63 ± 20 % of control; 41 ± 22 % of control, $P < 0.05$; 28 ± 21 % of control, $P < 0.001$, respectively) (Fig. 4.18). Oli 1 µM-treated neurones failed to show significant reduction in TMRM fluorescence intensity over the time-course of drug treatment (0.5 h, 92 ± 9.0 % of control; 1 h, 84 ± 12 % of control; 4 h, 83 ± 6.8 % of control), which could be due to the accumulation of $H^+$ is IMS due to the blockage of $H^+$ pathway at complex V (Devenish et al., 2008; Jonckheere et al., 2012). On the other hand, $H_2O_2$ 30 µM caused a slow, but significant reduction in $\Delta \Psi_m$ after 4 h of treatment (0.5 h, 66 ± 10 % of control; 1 h, 50 ± 11 % of control; 4 h, 35 ± 10 % of control, $P < 0.01$) (Fig. 4.18). The repeated measures two-way ANOVA revealed that there were significant differences between drug treatments ($P < 0.0001$) over the course of time period ($P = 0.0001$).

Overall, of the respiratory chain enzyme inhibitors, Rot and KCN caused the most significant depolarization of the $\Delta \Psi_m$ followed by AA, while 3-NP only showed mild reduction in TMRM fluorescence intensity over the time-course of treatment.

4.3.3. Bioenergetic Profile of CGCs Assessed by Seahorse XF24 Analyzer

To investigate the persistent effects of respiratory chain inhibitors on mitochondrial bioenergetics, CGCs were exposed to drugs for 4 h and 24 h and the consequent alterations of bioenergetic parameters were determined using a Seahorse XF24 analyzer. Optimization assays were run prior to subsequent Seahorse Mito Stress Test assays in order to determine the appropriate density of cells/well and working concentrations of Oli, FCCP in CGC cultures (Appendix 1.1 and 1.2). Control experiments that measured the effects of solvents such as DMSO and NaOH, in which drugs were dissolved, on cellular OCR, are described in Appendix 1.3. At 7 DIV CGCs were treated with mitochondrial respiratory inhibitors for 4 h and 24 h outside the instrument, washed and changed to XF
Figure 4.14. Depolarization of mitochondria during treatment of CGCs with mitochondrial respiratory complex inhibitors for 1 h

TMRM fluorescence was monitored by confocal live cell imaging at 1 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of mitochondrial respiratory complex inhibitors, Rot 10 nM, 3-NP 300 µM, AA 10 nM and KCN 100 µM. CCCP 10 µM treatment was applied to untreated neurones loaded with TMRM as a positive control for complete depolarization of $\Delta \psi_m$. Brightfield images are included to visualize neurones in the field. Scale bar = 20 µm.
Figure 4.15. Depolarization of mitochondria during treatment of CGCs with complex V inhibitor, Oli, and oxidative stressor, H₂O₂, for 1 h

TMRM fluorescence was monitored by confocal live cell imaging at 1 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of Oli 1 µM and H₂O₂ 30 µM. CCCP 10 µM treatment was applied to untreated neurones loaded with TMRM as a positive control for complete depolarization of ΔΨₘ. Brightfield images are included to visualize neurones in the field. Scale bar = 20 µm.
Figure 4.16. Depolarization of mitochondria during treatment of CGCs with mitochondrial respiratory complex inhibitors for 4 h

TMRM fluorescence was monitored by confocal live cell imaging at 4 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of mitochondrial respiratory complex inhibitors, Rot 10 nM, 3-NP 300 µM, AA 10 nM and KCN 100 µM. CCCP 10 µM treatment was applied to untreated neurones loaded with TMRM as a positive control for complete depolarization of $\Delta \psi_m$. Brightfield images are included to visualize neurones in the field. Scale bar = 20 µm.
TMRM Bright field Overlay

Control

Rot 10 nM

3-NP 300 µM

AA 10 nM

KCN 100 µM

CCCP 10 µM
Figure 4.17. Depolarization of mitochondria during treatment of CGCs with complex V inhibitor, Oli, and oxidative stressor, H₂O₂, for 4 h

TMRM fluorescence was monitored by confocal live cell imaging at 4 h post-injury. CGCs were loaded with TMRM in the absence of treatment (control) and in the presence of Oli 1 µM and H₂O₂ 30 µM. CCCP 10 µM treatment was applied to untreated neurones loaded with TMRM as a positive control for complete depolarization of ΔΨ MatDialogue. Brightfield images are included to visualize neurones in the field. Scale bar = 20 µm.
CCCP 10 µM

H₂O₂ 30 µM

Oli 1 µM

Control

TMRM

Bright field

Overlay
Figure 4.18. Quantitative analysis of intracellular TMRM fluorescence intensity in neurones treated with OXPHOS inhibitors and H₂O₂ at 0.5 h, 1 h and 4 h treatment

TMRM fluorescence is expressed as percentage of control (designated as having 100% fluorescence). Overall fluorescence was determined by combined total pixel intensity across a given field divided by the number of cells present in the field (determined from the brightfield image). The fluorescence intensity values for treated neurones were normalized to the fluorescence intensity/cell of untreated neurones at the corresponding time and all values have been corrected for residual TMRM fluorescence after CCCP treatment. The failure of complete loss of TMRM fluorescence under CCCP treatment. The failure of complete loss of TMRM fluorescence under CCCP treatment shown in this graph is due to one instance when CCCP was not added enough. All other experiments showed a complete loss of TMRM fluorescence under CCCP treatment. Values are mean ± SEM of 3-5 independent experiments and 150-250 cells were counted per treatment at each time point. Symbols indicate significant loss of TMRM fluorescence at the indicated time of treatment relative to control (* P < 0.05; ** P < 0.01; # P < 0.001 and ## P < 0.0001 compared to control by repeated measures two-way ANOVA, followed by Bonferroni's post hoc test). Repeated measures two-way ANOVA revealed significant differences of TMRM levels between duration of the treatments (P = 0.0001) and different drugs (P < 0.0001).
assay medium for further analyses. CGCs were left in a non-CO\(_2\) incubator at 37ºC for 1 h for cells to pre-equilibrate with the assay medium. Detailed assessment of mitochondrial function using Seahorse XF24 analyzer is described in Section 4.2.4. After the basal OCR had been established, sequential injections of Oli (1 µM), FCCP (2.5 µM) and Rot/AA (both 2 µM) were performed. Three OCR and ECAR readings were taken after automated injection of each inhibitor and the changes in OCR and ECAR readings in response to inhibitors were automatically recorded and calculated by Seahorse XF24 software (Seahorse Bioscience, Billerica, MA, USA) to determine the key parameters of mitochondrial respiration (Fig. 4.6). The biochemical basis of the general profiles of the Seahorse data from this approach has been considered above in Section 4.1.3.3. The rate of oxygen consumption per min was presented as pmol/min, and the area under the curve (AUC) was calculated as a measure of the total increase or decrease in OXPHOS activity during a given period of time (AUC; pmol). The purpose of this bioenergetic assessment was to observe the magnitude of bioenergetic damage induced by the OXPHOS inhibitors and attention was particularly given to the changes in the key bioenergetic parameters, basal level of OCR and FCCP-induced OCR, which represent the level of oxygen consumed at rest and the maximum capacity at which the neurones are able to perform cellular respiration, respectively.

4.3.3.1. Bioenergetic profiles of CGCs in response to 4 h incubation with mitochondrial respiratory inhibitors

Fig. 4.19A illustrates direct OCR measurements of CGCs which were pretreated with MEM (untreated control), Rot 10 nM, 3-NP 100 µM or KCN 100 µM for 4 h. Based on the measurements taken in Fig. 4.19A, individual bioenergetic parameters such as basal, ATP-linked, H\(^+\) leak, FCCP-induced respiration, and non-mitochondrial OCR were calculated (Fig. 4.19B). As expected, control CGCs showed a standard bioenergetic profile in response to sequential injection of metabolic inhibitors (Fig. 4.19A). Baseline OCR was established during the first 15 min followed by an injection of Oli 1 µM which caused a significant decrease in OCR. Here, the difference between basal OCR and Oli-induced OCR was taken as the amount of oxygen consumed due to mitochondrial OXPHOS (ATP-coupled OCR). Almost 80 % of oxygen consumed in control CGCs accounted for mitochondrial ATP synthesis (77 ± 3.7 % of control basal respiration).
Addition of FCCP stimulated maximal respiration in control CGCs (180 ± 23 % of control basal respiration). Subsequent addition of complex I and III inhibitors, Rot 2 µM and AA 2 µM, respectively, caused a rapid drop in OCR, indicating oxygen consumption due to OXPHOS was effectively inhibited by Rot and AA.

In contrast to control CGCs, 4 h drug-treated CGCs displayed altered bioenergetic profiles. Significant reductions in basal respiration were observed in all drug-treated CGCs; Rot 10 nM (80 ± 7.6 % of control, P < 0.05), 3-NP 100 µM (74 ± 1.6 % of control, P < 0.01) and KCN 100 µM (83 ± 3.7 % of control, P < 0.05) (Fig. 4.19B). CGCs exposed to Rot 10 nM for 4 h showed a significantly reduced utilization of OCR for production of ATP via F\textsubscript{1}F\textsubscript{0} ATPase (76 ± 8.9 % of control; P < 0.05). Cells treated with the lower concentrations of 3-NP (100 µM) and KCN (100 µM) showed small non-significant reduction in OCR for mitochondrial ATP synthesis (83 ± 2.2 % of control and 87 ± 5.0 % of control, respectively), indicating OXPHOS was still functional in these cells (Fig. 4.19B). Exposure to Rot 10 nM for 4 h led to a significant reduction in FCCP-induced respiration (68 ± 5.2 % of control, P < 0.05). Surprisingly, CGCs pretreated with 3-NP 100 µM for 4 h showed almost 80 % reduction in FCCP-induced respiration (19 ± 1.5 % of control, P < 0.0001) (Fig. 4.19B). KCN 100 µM also produced an apparent reduction in FCCP-induced respiration which was statistically insignificant (82 ± 8.6 % of control). Lastly, Rot 10 nM treated CGCs showed reduced non-mitochondrial OCR (33 ± 9.4 % of control; P < 0.01), suggesting non-mitochondrial cellular metabolism was also affected subsequent to 4 h incubation with Rot 10 nM.

Treatment with mitochondrial inhibitors Rot, 3-NP and KCN at higher concentrations for 4 h also produced similar patterns of change of bioenergetic profiles compared to CGCs exposed to lower concentrations. Control CGCs produced a standard Seahorse bioenergetic profile graph showing a stable basal OCR followed by a sudden drop of OCR subsequent to Oli injection, which indicated 82 ± 3.3 % of oxygen consumed at basal level was utilized to generate ATP via complex V of OXPHOS. A rapid increase in maximal respiratory rate in response to FCCP (205 ± 12 % of control basal respiration) and a second drop in OCR due to Rot and AA injection (Fig. 4.20A) were also observed. The baseline OCR established by CGCs pretreated with Rot 30 nM, 3-NP 300 µM or KCN 300 µM for 4 h demonstrated a reduced consumption of oxygen at
(A) CGCs were grown and treated with MEM (untreated control), Rot 10 nM, 3-NP 100 µM and KCN 100 µM for 4 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO₂ incubator for 1 h to allow cells to pre-equilibrate with the assay media. Real time measurement of OCR was performed using Seahorse XF24 analyzer. Oli (1 µM; A), FCCP (2.5 µM; B) and Rot/AA (2 µM/2 µM; C) were sequentially injected as indicated. (B) The individual bioenergetic parameters, basal, ATP-linked, H⁺ leak, FCCP-induced OCR and non-mitochondrial OCR were calculated based on the measurements taken in (A). Data are mean ± SEM, n = 3-5 wells per group. Symbols indicate a significant reduction in OCR (* P < 0.05; ** P < 0.01, ## P < 0.0001) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
Figure 4.20. Cellular bioenergetics of CGCs treated with MEM, Rot 30 nM, 3-NP 300 µM and KCN 300 µM for 4 h

(A) CGCs were grown and treated with MEM (untreated control), Rot 30 nM, 3-NP 300 µM and KCN 300 µM for 4 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO₂ incubator for 1 h to allow cells to pre-equilibrate with the assay media. Real time measurement of OCR was performed using Seahorse XF24 analyzer. Oli (1 µM; A), FCCP (2.5 µM; B) and Rot/AA (2 µM/2 µM; C) were sequentially injected as indicated. (B) The individual bioenergetic parameters, basal, ATP-linked, H⁺ leak, FCCP-induced OCR and non-mitochondrial OCR were calculated based on the measurements taken in (A). Data are mean ± SEM, n = 3-5 wells per group. Symbols indicate a significant reduction in OCR (* P < 0.05; ** P < 0.01; # P < 0.001; ## P < 0.0001) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
basal level, suggesting these drugs effectively caused alteration in energy metabolism in CGCs. KCN 300 µM in particular, caused the greatest reduction in basal OCR (43 ± 3.6 % of control; P < 0.001) compared to Rot 30 nM (70 ± 4.1 % of control; P < 0.05) and 3-NP 300 µM (73 ± 5.8 % of control; P < 0.05) (Fig. 4.20B). As expected, all OCR measurements of CGCs dropped after the injection of Oli and further analyses revealed a significant impairment in the production of ATP via Oli-sensitive F₁F₀ ATP synthase in CGCs treated with Rot 30 nM (67 ± 5.4 % of control; P < 0.01), 3-NP 300 µM (63 ± 5.4 % of control; P < 0.01) and KCN 300 µM (38 ± 13 % of control; P < 0.001) (Fig. 4.20B). All three drugs induced a reduced capacity to produce FCCP-induced respiration compared to control, where 3-NP produced the largest reduction of FCCP-induced respiratory values (14 ± 1.4 % of control; P < 0.0001) compared to Rot 30 nM (47 ± 2.3 % of control; P < 0.0001) and KCN 300 µM (36 ± 13 % of control; P < 0.0001) (Fig. 4.20B).

Fig. 4.21 shows bioenergetic profiles of CGCs subjected to a 4 h exposure to AA 10 nM and 30 nM. Control CGCs displayed a typical bioenergetic profile except the FCCP-induced maximal respiration was lower than what was observed in control cells in other plates (90 ± 13 % of basal respiration), indicating the cells for this particular Seahorse plate were under stress (Fig. 4.21A). Nonetheless, the reductions in basal respiration and ATP-linked OCR of AA 30 nM-treated CGCs (78 ± 1.3 % of control and 77 ± 2.0 % of control, respectively) indicates AA 30 nM incubation for 4 h was successful in inducing significant energetic stress on cells (Fig. 4.21B). In contrast, AA 10 nM failed to induce any significant inhibitory effect on OCR in any of the bioenergetic parameters.

Overall, the bioenergetic profiles indicate CGCs treated with all mitochondrial respiratory inhibitors for 4 h, except AA 10 nM, sustained varying levels of mitochondrial inhibition as indicated by the reduction of basal OCR and maximal OCR levels.
4.3.3.2. Bioenergetic profiles of CGCs in response to 24 h incubation with mitochondrial respiratory inhibitors

Similar to bioenergetic profiles shown by CGCs exposed to drugs for 4 h, notable alterations of bioenergetic parameters were clearly observed also in CGCs after 24 h drug treatment. Control CGCs incubated in MEM showed a steady basal OCR and a rapid drop in OCR subsequent to injection of Oli 1 µM, indicating 84 ± 4.2 % of control basal respiration accounted for mitochondrial ATP-production (Fig. 4.22A). The peak given by FCCP-mediated maximal respiration was reduced in control CGCs for 24 h (63 ± 2.9 % of control basal respiration) (Fig. 4.22A) as compared to the 4 h CGC control (180 ± 23 % of control basal respiration) (Fig. 4.19A). However, the control CGCs still produced a standard profile of bioenergetic responses subsequent to the addition of drugs (Fig. 4.22A). Similar to 4 h drug-treated neurones, significant reductions in basal respiration were apparent in cells that were exposed to Rot 10 nM (72 ± 2.8 % of control, P < 0.05) and 3-NP 100 µM (31 ± 2.4 % of control, P < 0.05) for 24 h (Fig. 4.22A). The most striking result was again found with CGCs pretreated with 3-NP 100 µM, which were unable to drive FCCP-induced respiration, indicating the mitochondrial function was significantly damaged. In contrast, KCN 100 µM and AA 10 nM failed to cause any significant reductions in OCR for both basal respiration and FCCP-induced respiration as compared to control (Fig. 4.22B and Fig. 4.24B, respectively). Interestingly, the OCR related to the non-mitochondrial-mediated oxidation was significantly reduced for CGCs treated with Rot, 3-NP and KCN at their low concentrations, indicating other parts of cellular metabolism were also affected by the inhibitory effects of these drugs (Fig. 4.22B).

Exposure to all drugs for 24 h at higher concentrations had increased detrimental effects on all aspects of mitochondrial bioenergetics. Control CGCs treated with 24 h showed reduced FCCP-induced maximal respiration compared to those treated with 4 h, however, they still exhibited the standard responses to a serial addition of drugs (Fig. 4.23A). The most significant bioenergetic dysfunction was found in CGCs treated with 3-NP 300 µM and KCN 300 µM. The basal respiration were greatly reduced by 3-NP 300 µM (15 ± 0.7 % of control, P < 0.0001) and KCN 300 µM (3.8 ± 3.8 % of control, P < 0.05) and significantly low amount of oxygen was consumed to produce ATP via
Figure 4.21. Cellular bioenergetics of CGCs treated with MEM, AA 10 nM and AA 30 nM for 4 h

(A) CGCs were grown and treated with MEM (untreated control), AA 10 nM, and AA 30 nM for 4 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO₂ incubator for 1 h to allow cells to pre-equilibrate with the assay media. Real time measurement of OCR was performed using Seahorse XF24 analyzer. Oli (1 µM; A), FCCP (2.5 µM; B) and Rot/AA (2 µM/2 µM; C) were sequentially injected as indicated. (B) The individual bioenergetic parameters, basal, ATP-linked, H⁺ leak, FCCP-induced OCR and non-mitochondrial OCR were calculated based on the measurements taken in (A). Data are mean ± SEM, n = 3-5 wells per group. Symbols indicate a significant reduction in OCR (* P < 0.05; ** P < 0.01) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
Figure 4.22. Cellular bioenergetics of CGCs treated with MEM, Rot 10 nM, 3-NP 100 µM and KCN 100 µM for 24 h

(A) CGCs were grown and treated with MEM (untreated control), Rot 10 nM, 3-NP 100 µM and KCN 100 µM for 24 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO₂ incubator for 1 h to allow cells to pre-equilibrate with the assay media. Real time measurement of OCR was performed using Seahorse XF24 analyzer. Oli (1 µM; A), FCCP (2.5 µM; B) and Rot/AA (2 µM/2 µM; C) were sequentially injected as indicated. (B) The individual bioenergetic parameters, basal, ATP-linked, H⁺ leak, FCCP-induced OCR and non-mitochondrial OCR were calculated based on the measurements taken in (A). Data are mean ± SEM, n = 3-5 wells per group. Symbols indicate a significant reduction in OCR (* P < 0.05; ** P < 0.01; # P < 0.001; ## P < 0.0001) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
(A) Graph showing OCR over time with different treatments labeled A, B, and C.

(B) Bar graph showing AUC OCR with various conditions: Basal, ATP, H^+ Leak, FCCP-OCR, Non-mito, with different treatments indicated by colors and symbols.
Figure 4.23. Cellular bioenergetics of CGCs treated with MEM, Rot 30 nM, 3-NP 300 µM and KCN 300 µM for 24 h

(A) CGCs were grown and treated with MEM (untreated control), Rot 30 nM, 3-NP 300 µM and KCN 300 µM for 24 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO2 incubator for 1 h to allow cells to pre-equilibrate with the assay media. Real time measurement of OCR was performed using Seahorse XF24 analyzer. Oli (1 µM; A), FCCP (2.5 µM; B) and Rot/AA (2 µM/2 µM; C) were sequentially injected as indicated. (B) The individual bioenergetic parameters, basal, ATP-linked, H+ leak, FCCP-induced OCR and non-mitochondrial OCR were calculated based on the measurements taken in (A). Data are mean ± SEM, n = 3-5 wells per group. Symbols indicate a significant reduction in OCR (* P < 0.05; ** P < 0.01; # P < 0.001; ## P < 0.0001) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
complex V after treatment with 3-NP 300 µM and KCN 300 µM (7.9 ± 1.7 % of control, P < 0.0001 and 0.4 ± 4.7 % of control, P < 0.0001, respectively) (Fig. 4.23B). These results indicate neurones exposed to these two drugs for 24 h sustained substantial mitochondrial damage and were unable to perform OXPHOS. Their responses to FCCP-induced respiration were also significantly reduced (3-NP 300 µM, 6.6 ± 0.6 % of control, P < 0.0001 and KCN 300 µM, 2.8 ± 2.2 % of control, P < 0.0001), further showing dysfunction of mitochondria (Fig. 4.23B). Rot 30 nM also caused significant mitochondrial damage as indicated by a markedly reduced OCR for basal respiration (62 ± 3.9 % of control, P < 0.0001), OCR linked to ATP production via complex V (62 ± 5.0 % of control, P < 0.001) and FCCP-induced respiration (29 ± 2.2 % of control, P < 0.0001) (Fig. 4.23A and Fig. 4.23B). AA 30 nM also exhibited significant reduction in basal OCR (22 ± 5.7 % of control, P < 0.0001), ATP linked OCR (20 ± 6.2 % of control, P < 0.0001) and FCCP-induced OCR (19 ± 1.5 % of control, P < 0.0001) (Fig. 4.24B). However, 24 h treatment AA 10 nM failed to cause any significant bioenergetic dysfunction in CGCs.

4.3.3.3. Measurement of basal Extracellular Acidification Rate (ECAR) in CGCs pretreated with OXPHOS inhibitors

The glycolytic activity of neurones in response to OXPHOS inhibition was measured by monitoring the changes in pH due to the generation of lactic acid. The rate of changes in the concentration of H⁺ in the media (ECAR; mpH/min) was directly measured by Seahorse XF24 analyzer concurrently with the measurement of OCR. The results are presented as an area under the curve (AUC ECAR; mpH) which represents a measure of the total increase or decrease in glycolytic activity during a given period of time. As shown in Fig. 4.25, glycolytic activity was significantly increased in CGCs pretreated with Rot 30 nM for 4 h (197 ± 18 % of control, P < 0.05). However, all other 4 h drug treatments failed to show any significant increase in glycolytic activity at basal level. On the contrary, pretreatment with OXPHOS inhibitors for 24 h, especially higher concentrations of 3-NP, AA and KCN, significantly reduced glycolysis (3-NP 300 µM, 11 ± 21 % of control, P < 0.05; AA 30 nM, 21 ± 10 % of control, P < 0.01; KCN 300 µM, -6.0 ± 9.2 % of control, P < 0.05), indicating that energy metabolism was substantially
disabled (Fig. 4.26). CGCs pretreated with Rot 30 nM for 24 h showed a change in glycolytic activity (137 ± 19 % of control), but this was not statistically significant.

4.3.3.4. Visualization of the neuronal energetic response to OXPHOS inhibitors

Fig. 4.27 illustrates the XF phenogram plotted to illustrate the relative metabolic state of cells at rest. CGCs were exposed to low and high concentrations of OXPHOS inhibitors for 4 and 24 h and basal OCR and ECAR were measured simultaneously. The relative basal ECAR/OCR values subsequent to exposure to OXPHOS inhibitors were plotted in a phenogram and arbitrary lines were drawn to indicate the shift in metabolism of CGCs relative to population of neurones treated with other drugs. In general, drug treatment resulted in decreases in OCR levels (x-axis), indicating metabolic dysfunction of CGCs which were complemented with a slight increase in ECAR in compensation for OXPHOS dysfunction in some cases. After 4 h of drug treatment, control CGCs showed a normal basal level of metabolism that almost exclusively represented oxidative metabolism rather than glycolytic metabolism, in agreement with previously reports (Ames, 2000; Erecinska et al., 2004; Mayevsky & Chance, 1975; Toescu et al., 2000). Exposure to drugs at various concentrations caused neurones to shift to less oxidative metabolism. The neurones treated with Rot showed a slight increase in glycolytic energy production as a compensatory response to inhibition of OXPHOS. Conversely, AA caused a reduction in energy metabolism without relying on glycolytic activity, and KCN 300 µM resulted in significant drop in oxidative activity as shown by its location in the damaged quadrant of the graph (Fig. 4.27). The metabolic shift of CGCs treated with drugs after 24 h of drug treatment also showed an alteration in energy metabolism in both OXPHOS and glycolysis. A shift to reduced oxidative metabolism was evident for most drugs except CGCs treated with AA 10 nM. All drugs at high concentration induced significant inhibition of respiration of CGCs. Interestingly, glycolytic activity still remained in Rot 30 nM-treated CGCs even when oxidative metabolism was abolished.
Figure 4.24. Cellular bioenergetics of CGCs treated with MEM, AA 10 nM and AA 30 nM for 24 h

(A) CGCs were grown and treated with MEM (untreated control), AA 10 nM, and AA 30 nM for 24 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO₂ incubator for 1 h to allow cells to pre-equilibrate with the assay media. Real time measurement of OCR was performed using Seahorse XF24 analyzer. Oli (1 µM; A), FCCP (2.5 µM; B) and Rot/AA (2 µM/2 µM; C) were sequentially injected as indicated. The individual bioenergetic parameters, basal, ATP-linked, H⁺ leak, FCCP-induced OCR and non-mitochondrial OCR were calculated based on the measurements taken in (A). Data are mean ± SEM, n = 3-5 wells per group. Symbols indicate a significant reduction in OCR (** P < 0.01; ## P < 0.0001) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
Glycolytic activity (ECAR) of CGCs was assessed by measuring the rate of changes in the concentration of H\(^+\) in the media in response to pretreatment with OXPHOS inhibitors for 4 h. The results are presented as an area under the curve (AUC ECAR (mpH)) which represents a measure of the total increase or decrease in glycolytic activity during a given period of time. CGCs were grown and treated with MEM (untreated control), Rot 10 nM and 30 nM, 3-NP 100 µM and 300 µM, AA 10 nM and 30 nM and KCN 100 µM and 300 µM for 4 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO\(_2\) incubator for 1 h to allow cells to pre-equilibrate with the assay media. The amount of H\(^+\) produced at basal level was measured using Seahorse XF24 analyzer. Data are mean ± SEM, n = 3-5 wells per group. Symbol indicates a significant reduction in ECAR (* P < 0.05) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
(A) AUC ECAR (mPH)

(B) AUC ECAR (mPH)

(C) AUC ECAR (mPH)
Glycolytic activity (ECAR) of CGCs was assessed by measuring the alteration of the level of $H^+$ in the media in response to pretreatment with OXPHOS inhibitors for 24 h. CGCs were grown and treated with MEM (untreated control), Rot 10 nM and 30 nM, 3-NP 100 µM and 300 µM, AA 10 nM and 30 nM and KCN 100 µM and 300 µM for 4 h at DIV 7. CGCs were washed free of the inhibitors and stored in a CO₂ incubator for 1 h to allow cells to pre-equilibrate with the assay media. The amount of $H^+$ produced at basal level was measured using Seahorse XF24 analyzer. Data are mean ± SEM, $n = 3-5$ wells per group. Symbol indicates a significant reduction in ECAR (* $P < 0.05$, ** $P < 0.01$) compared to control from one-way ANOVA, followed by Dunnett’s post hoc test.
Figure 4.27. Seahorse XF24 phenogram visualizes the shift in metabolic profiles of CGCs in response to exposure to OXPHOS inhibitors

The XF phenogram was plotted to illustrate the relative metabolic state of cells at rest. CGCs were exposed to low and high concentrations of OXPHOS inhibitors for 4 and 24 h and basal OCR and ECAR were measured simultaneously. The relative basal ECAR/OCR values subsequent to exposure to OXPHOS inhibitors were plotted in a phenogram and arbitrary lines were drawn (dashed lines) to indicate the shift in metabolism of CGCs relative to the population of neurones treated with other drugs. The graph is divided into four quadrants, high metabolism, oxidative, glycolytic and low metabolism (mean ± SEM, n = 3-5). CGCs exposed to OXPHOS inhibitors for 4 h showed a slight increase in ECAR in some drug treated CGCs as a compensative response to inhibition of OXPHOS. However, 24 h exposure to OXPHOS inhibitors, especially at higher concentrations, produced a significant shift of metabolism to the low metabolism quadrant.
4.4. Discussion

Research described in this chapter undertook a strategic analysis of the functional aspects of mitochondria, in order to fully define the adverse effects on mitochondrial bioenergetics of the specific OXPHOS inhibitors that were examined in Chapter 3. Rot, 3-NP, AA and KCN are well known inhibitors of OXPHOS, and they have been extensively utilized in the generation of models mimicking diseases such as PD, HD and acute hypoxia (Aizenman et al., 2000; Cannon et al., 2009; Choi & Han, 2002; Jeong et al., 2003; Ramaswamy et al., 2007). In order to determine the degree of dysfunction induced by these inhibitors in primary cultures of CGCs, several different experimental approaches were employed.

4.4.1. Bioenergetic Dysfunction induced by STS and H$_2$O$_2$ and Correlation with results from Chapter 3

Prior to the pharmacological assessment of inhibition of OXPHOS, STS and H$_2$O$_2$ were used as reference insults (cf. Chapter 3) to provide “benchmark” of the extent of changes in cellular ATP in CGCs. Time-dependent reductions in ATP levels were observed in STS-treated neurones where a slow depletion of ATP occurred over 24 h. Only a small reduction of ATP was displayed by neurones after 4 h exposure to STS 100 nM and 250 nM; however, almost 65% reduction of ATP was found after 24 h incubation with STS. The delayed reduction in cellular ATP level presumably reflects the slow processes of cell death that require ATP, such as caspase activation, chromatic condensation and formation of apoptotic bodies (Collins et al., 1997; Diwakarla et al., 2009b). In comparison, CGCs treated with 30 µM H$_2$O$_2$ failed to display a significant reduction in ATP at both 4 h and 24 h. Combined with the cellular viability result which displayed significant death of CGCs occurring under 30 µM H$_2$O$_2$ treatment (Fig. 3.5), insignificant reduction in ATP level suggested that the injury mediated by 30 µM H$_2$O$_2$
did not directly involve bioenergetic dysfunction in mitochondria. This notion was further supported by the observation of slow but significant dissipation of $\Delta \Psi_m$ in CGCs treated with 30 µM H$_2$O$_2$ for 0.5-4 h (Fig. 4.18), even though CGCs were clearly undergoing PCD with significant reduction in overall cell viability when exposed to 30 µM H$_2$O$_2$ for 4 h (Fig. 3.5). The unaltered ATP level displayed by CGCs treated with 30 µM H$_2$O$_2$ and the absence of necrotic-like morphologies indicated that CGCs were undergoing a non-necrotic, slow form of PCD, as rapid depletion of ATP would indicate progression of PCD towards necrosis in the apoptosis-necrosis continuum (Nagley et al., 2010; Skulachev, 2006). These results are in agreement with those from Chapter 3 which revealed a slow form of cell death in CGCs treated with 30 µM H$_2$O$_2$.

Thus, the methods used in Chapter 3 and the associated results were validated which revealed a slow form of cellular injury. Treatment of cells with higher concentrations of H$_2$O$_2$ is known to potentially recruit components of necrotic cell death (Diwakarla et al., 2009b). Indeed, CGCs treated with a higher concentration of H$_2$O$_2$ (50 µM, 24 h) underwent a substantial reduction of ATP levels, suggesting the cells maybe undergoing cell death involving a mix of regulated and unregulated necrosis (Higgins et al., 2009). A further detailed analysis of cell death modalities in CGCs subjected to insult with STS and various concentrations of H$_2$O$_2$ is beyond the scope of this thesis.

**4.4.2. Bioenergetic Dysfunction induced by OXPHOS Inhibitors: Insights from Measurement of ATP**

The first assessment of mitochondrial bioenergetic dysfunction was the estimation of respiratory inhibition by measuring the resultant level of cellular ATP after exposing neurones to each OXPHOS inhibitor. Dysfunction of bioenergetics was reflected by the concentration-dependent reductions in the level of cellular ATP induced by all OXPHOS inhibitors. Rot and AA showed greater depletion of cellular ATP at higher concentrations, confirming that these drugs were capable of causing bioenergetic dysfunction. These
observations of significant reductions in cellular ATP were consistent with the increases in the number of neurones with permeabilized membranes indicated by PI uptake (Fig. 3.13), further emphasizing the importance of energy availability on cellular survival. On the contrary, a slow time-dependent reduction of ATP was displayed by CGCs that were exposed to 3-NP. A short period incubation in 3-NP failed to induce a significant drop in cellular ATP levels, whereas longer exposure induced a significant depletion of ATP, suggesting that these drugs may elicit modes of inhibition that occur more slowly.

A strong correlation between the depletion of ATP and the ability of Rot to kill neurones in rodent brain has already been well documented by Höllerhage and colleagues, who suggested the reductions in ATP level were one of the main determinants of neurotoxicity (Hollerhage et al., 2009). As expected, mitochondrial dysfunction in concentration-dependent manner was found when CGCs exposed to Rot. Rot 10 nM had a little effect on the reduction of cellular ATP, whereas treatment with Rot 30 nM resulted in ~80 % reductions in cellular ATP levels, demonstrating the significant inhibitory effect of Rot on mitochondrial respiration of CGCs. Such small increase in Rot concentration from 10 nM to 30 nM which resulted in significant inhibition of cellular ATP level again highlighted the potency of this drug on neurones (Table 3.1). In contrast to Rot-treated neurones, CGCs treated with 3-NP displayed time-dependent reductions in cellular ATP. Both 100 µM and 300 µM 3-NP failed to induce significant depletion of cellular ATP after 4 h of exposure, whereas increasing exposure time to 24 h resulted in ~80 % and 94 % (% of control) reductions of cellular ATP, respectively. Such slow, but extensive depletion of cellular ATP as a consequence of the inhibition of complex II reflects the strategic involvement of complex II in energy metabolism as succinate dehydrogenase in the TCA cycle. Succinate dehydrogenase is the only enzyme that is a component of both the TCA cycle and ETC, where oxidation of succinate to fumarate is joined by a simultaneous transfer of electrons to ubiquinone (Huang & Millar, 2013). Given the central role of complex II in energy metabolism via TCA cycle and ETC, its inhibition inevitably results in overall ATP depletion and mitochondrial dysfunction. Interestingly, the 3-NP-induced trend of slow time-dependent depletion of ATP was comparable to the cell viability MTT result shown in Chapter 3, where cell death by both 100 µM and 300 µM of 3-NP induced a slow and steady cell death, similar to what was observed in the reduction of cellular ATP. Indeed, the link between energy depletion and neurotoxicity of 3-NP was demonstrated in rat striatal neurones where the detrimental
effects exerted by 3-NP was closely related to the exhaustion of energy via blocking of energy metabolism (Diwakarla et al., 2009a; Tsai et al., 1997).

The inhibitory actions of AA on bioenergetics of CGCs were also confirmed by concentration-dependent generation of energy deficits. A significant reduction of the level of cellular ATP by ~50% and 70% was observed in response to AA 30 nM over 4 h and 24 h incubation, although AA 10 nM failed to cause significant ATP reduction. This concentration-dependent reduction in cellular ATP was consistent with the increase in PI-positive CGCs shown in Chapter 3, indicating a close relationship between energy availability and cellular survival. Treatment of CGCs with KCN also revealed a slight reduction in the level of cellular ATP, although to a lesser extent compared to the other OXPHOS inhibitors. A rapid and significant cell death in CGCs exposed to KCN 300 µM (Chapter 3 MTT assay results) combined with the substantial level of cellular ATP maintained after 24 h exposure to KCN 300 µM, follow the same trend that was also observed in H2O2 (30 µM) treated neurones. The absence of morphological features of necrosis and the maintenance of cellular ATP after a longer period of treatment with KCN confirmed the absence of necrotic cell death in CGCs treated with KCN, as discussed in Chapter 3.

CGCs were found not to be significantly depleted of ATP after Oli treated at the two concentrations used here (0.1 µM and 1 µM). Some suggestion of depletion of ATP levels at the higher concentration and longer time point (24 h) was observed (Fig. 4.9). This is consistent with the evidence of cellular injury under these more extreme conditions as judged by the cell viability and morphological studies from Chapter 3. At lower concentrations (at shorter time point), there seems to be no appreciable depletion of cellular ATP nor cellular injury. Presumably, under these less extreme conditions for Oli treatment cells are unable to generate mitochondrial ATP due to the block on ATP synthase but this may be compensated by elevated glycolytic ATP production. It would have been interesting to examine more closely the effects of Oli in the Seahorse XF24 Analyser. This experiment was not carried out here primarily because of the availability of the instrument during the late experimental phase of my research program. Interestingly, as shown in data sections of this thesis, Oli showed little evidence of induction of mitophagy (Chapter 5), except perhaps for the 24 h treatment at 1 µM (Fig. 245.)
In terms of induction of general autophagy, Oli showed no effects in this work (Chapter 6).

Overall, the measurement of cellular ATP provided an insight over an overall energy metabolism in neurones; however, due to the variability and some insignificant results observed from AA and KCN-treated CGCs, it was pertinent to employ a technique that allowed more specific observations of mitochondrial bioenergetics. Hence, the measurement of $\Delta \Psi_m$ via fluorescence imaging of TMRM was performed.

### 4.4.3. Bioenergetic Dysfunction induced by OXPHOS Inhibitors: Insights from Real-Time Measurements of $\Delta \Psi_m$

Measurement of the polarized state of mitochondrial membrane ($\Delta \Psi_m$) was employed to gain additional insights into mitochondrial dysfunction. The validity of using live-cell imaging of TMRM-loaded neurones to quantitatively measure $\Delta \Psi_m$ was proven when $\Delta \Psi_m$ of CGCs treated with Oli 1 µM did not show significant reduction of $\Delta \Psi_m$ even after 4 h. Previous literature has shown that slight hyperpolarization of $\Delta \Psi_m$ was induced when Oli was administered to cell lines, as well as primary CGCs, by inhibiting $\text{H}^+$ re-entry into the mitochondrial matrix via complex V (Kalbacova et al., 2003; Nicholls & Ward, 2000), which explains the sustained $\Delta \Psi_m$ of CGCs treated with Oli in this study.

The most compelling evidence of bioenergetic dysfunction in CGCs caused by Rot was provided by the rapid dissipation of $\Delta \Psi_m$ after 0.5 h treatment with Rot 10 nM. Since $\Delta \Psi_m$ is the primary bioenergetic parameter that controls mitochondrial ATP synthesis, $\text{Ca}^{2+}$ sequestration and the generation of ROS (Nicholls & Ward, 2000), the rapid depolarization of $\Delta \Psi_m$ clearly indicated dysfunctional mitochondrial bioenergetics due to Rot 10 nM. In fact, complex I-dependent electron flux appears to be the most sensitive
process in predating $\Delta \Psi_m$ reduction (Kalbacova et al., 2003), supporting the results observed in this study. The evidence of bioenergetic dysfunction in CGCs was similarly found when CGC cultures were treated with KCN 100 $\mu$M where an early dissipation of $\Delta \Psi_m$ was displayed similar to that observed from Rot-treated CGCs. Depolarization of IMM subsequent to KCN treatment was expected, as KCN is known to play an inhibitory role in suppressing electron transfer within the complex IV, which inhibits $\text{H}^+$ translocation into the mitochondrial IMS. Interestingly, although significant reduction of cellular ATP was not observed in CGCs with Rot 10 nM and KCN 100 $\mu$M treatment, rapid depolarization of $\Delta \Psi_m$ after 0.5 h exposure to Rot 10 nM and KCN 100 $\mu$M indicated that inhibitory role of Rot and KCN at their lower concentrations acted successfully upon complexes I and IV, respectively. Although CGCs treated with AA 10 nM displayed a relatively slower dissipation of $\Delta \Psi_m$ compared to Rot or KCN, AA 10 nM-induced depolarization of $\Delta \Psi_m$ also preceded apparent signs of reduction of cellular ATP level like that of Rot- and KCN-treated neurones. These results indicate that depolarization of $\Delta \Psi_m$ occurred as an early step involved in mitochondrial injury, an event which consequently induced decline of the proton motive force to ultimately cause termination of ATP synthesis, which reflects the relatively slower reduction of cellular ATP (Nicholls & Budd, 2000). Although the fine dissection of molecular pathways of Rot-, AA- and KCN-induced injury is beyond the topic of this thesis, it is still clear that lower concentrations of Rot (10 nM), AA (10 nM) and KCN (100 $\mu$M) induced mitochondrial dysfunction where $\Delta \Psi_m$ dissipation occurred prior to depletion of cellular ATP.

The most unexpected result was from the observations of slow dissipation of $\Delta \Psi_m$ in CGCs exposed to 3-NP 300 $\mu$M. Exposure to 3-NP 300 $\mu$M for 0.5 h did not induce significant differences in $\Delta \Psi_m$, as was the case with 1 h and 4 h treatments. The images in Fig. 4.16 certainly suggest that TMRM fluorescence was reduced at 4 h, however this change failed to achieve statistical significance. The statistical analysis performed by repeated measures two-way ANOVA revealed significant differences in TMRM fluorescence from 3-NP-treated CGCs between the timepoints 0.5 h, 1 h and 4 h, suggesting observations at longer time intervals may have shown significant reduction in $\Delta \Psi_m$ for the slower acting drug, 3-NP. However, due to the limitation of the instruments that were employed to determine $\Delta \Psi_m$ from live neurones, extended timepoints were not studied because the TMRM fluorescence in controls appeared to decrease at 4 h (Fig.
4.16) relative to earlier timepoints (Fig. 4.11). Although TMRM is known to have the least inhibitory effect on ETC in comparison to the other dyes, such as Rhod123 or TMRE (Scaduto & Grotyohann, 1999), some inhibition effect may have led to the loss of TMRM fluorescence in neurones. In agreement to the current result shown by 3-NP treatment, a similar pattern of delayed depolarization of $\Delta\Psi_m$ was also observed in other studies where primary hippocampal neurones and cortical neurones were employed to investigate neurotoxicity of 3-NP (Lee et al., 2002; Nasr et al., 2003). The relatively slow depolarization of $\Delta\Psi_m$ induced by 3-NP highlights the distinctive injury pathway induced by 3-NP compared to the other ETC inhibitors. The careful dissection of how inhibition of complex II by 3-NP leads to slower depolarization of $\Delta\Psi_m$ is not the theme of this chapter, however it is important to highlight the metabolic changes induced by the inhibition of complex II by 3-NP. As a complex that directly links cellular metabolism to respiration (Iverson et al., 2012; Wojtovich et al., 2013), functional complex II is also involved in the generation of redox cofactors such as NADH and FADH$_2$ during TCA cycle, which eventually transfer electrons to complex I and II, respectively. Therefore, inhibition of complex II by 3-NP, not only inhibits ETC by stopping the transfer of electrons to ubiquinone to complex III, but also suppresses TCA cycle. Ultimately, alteration of redox balances of NAD$^+/\text{NADH}$ (which will also suppress the complex I, NADH dehydrogenase) and FAD$^+/\text{FADH}_2$ results in overall metabolic inhibition. The slow, but almost complete loss of cellular ATP level, could also be explained by this irreversible inhibition of complex II by 3-NP followed by complete termination of succinate oxidation and TCA cycle. Treatment with 30 µM H$_2$O$_2$, whose mode of action does not directly cause mitochondrial respiration, also showed a slow dissipation of $\Delta\Psi_m$, emphasizing the distinctive features of complex II inhibition as compared to inhibition of other ETC complexes.
4.4.4. Bioenergetic Dysfunction induced by OXPHOS Inhibitors: Insights from Real-Time Measurements of Bioenergetic Parameters

The level of cellular ATP and polarized state of $\Delta \Psi_m$ provided some general insight into mitochondrial bioenergetics. In order to gain more direct and incisive assessment of mitochondrial bioenergetic dysfunction caused by all concentrations of ETC inhibitors, measurement of OCR and ECAR with a Seahorse XF24 analyzer was employed to fully examine metabolic datasets of all concentrations of drugs.

Utilization of Seahorse XF24 analyzer over traditional methods of measuring mitochondrial bioenergetics (i.e. Clarke electrode apparatus) has the advantage of simultaneous real-time measurement of two major energy producing pathways, OXPHOS and glycolysis. As the Seahorse instrument measures bioenergetic parameters of intact neurones that were grown on multiwell microplates, sample preparation is relatively easy and allows higher throughput by using multiple samples. For the purpose of this chapter, the Seahorse XF24 analyzer was used to determine a complete mitochondrial bioenergetic profile by measuring the metabolic changes that occurred in CGCs in response to OXPHOS inhibition. As the instrument measures the rate of change of analytes dissolved in the cell culture media (i.e. oxygen and $H^+$ levels), a change of cell media to unbuffered Seahorse assay media containing 25 mM KCl, and the de-gassing procedure were critical before measuring bioenergetic profiles of CGCs. The de-gassing procedure was performed in a CO$_2$-free 37°C incubator for 1 h before the Mito Stress assay was performed to allow for the removal of CO$_2$ absorbed by the plastic plate. Such residual CO$_2$ is a potential source of error in ECAR measurements, due to the formation of carbonic acid which may alter the pH of media. It was noted that cells preincubated in MEM for 24 h showed a lower respiration rate (after FCCP injection) than those preincubated for 4 h (as seen in “control” data of Fig. 4.22, 4.23 and 4.24). This is presumably due to the general vulnerability of CGCs to oxidative stress. This effect could have been exacerbated by the prolonged incubation in the minimal conditions of MEM for 24 h (albeit in low O$_2$) followed by the exposure to higher O$_2$ in the CO$_2$-free 37°C incubator (to deplete CO$_2$ from plastic as above). However, control CGCs still displayed considerable responses to all metabolic inhibitors during the assay which were
comparable to the drug-treated CGCs on the same plate, therefore the results were still considered as valid.

Although the inhibitory actions and cytotoxicity of ETC inhibitors have been documented in other experimental, including non-neuronal studies, detailed profiling of mitochondrial bioenergetics in response to direct inhibition of ETC by specific inhibitors has not been performed especially in primary neurones. Datasets obtained with the Seahorse XF24 analyzer provided a detailed insight in how mitochondrial bioenergetic parameters were affected by the inhibitory actions of the metabolic inhibitors, and the current study revealed that ETC inhibitors impaired key elements of cellular bioenergetics. All ETC inhibitors effected concentration- and time-dependent reductions of OCR of varying magnitudes, confirming their role in causing bioenergetic dysfunction of mitochondria. The ECAR remained unaltered for most of drugs, except Rot 30 nM which reflects the critical role played by complex I in energy metabolism.

Interestingly, although treating CGCs with Rot 10 nM failed to display an appreciable reduction in ATP level, they possessed substantial inhibition of mitochondrial respiration as indicated by the reductions in cellular oxygen consumption at basal level (Fig. 4.19B, Fig. 4.22B). Basal OCR is an index of energy demand of the cell at rest, but when it is presented from drug-treated cells, basal OCR indicates the compromised energy metabolism of the cell at rest. Reduction in basal OCR after exposure to Rot 10 nM confirmed the inhibitory role on Rot in mitochondrial respiration and its impact could persist on ETC beyond its removal. A significant reduction of OCR related to ATP synthesis via $F_1F_0$ ATP synthase further confirmed that Rot 10 nM was capable of causing a diminished level of OXPHOS. Mitochondrial dysfunction was strongly suggested from the evidence of the reduced ability to produce FCCP-induced maximal respiration, indicating that CGCs exposed to Rot 10 nM had lost the capacity to respond to increased energy demand. Therefore, rapid depolarization of $\Delta \Psi_m$ and the significant inhibitory effect on oxygen consumption during respiration clearly supported the notion that bioenergetic dysfunction was caused by Rot 10 nM. Such significant dysfunction caused by Rot could be explained by the involvement of complex I in mitochondrial metabolism as a NADH dehydrogenase. The inhibition of complex I Rot is likely to prevent the oxidation of NADH to NAD$^+$ to an extent that causes significant
accumulation of NADH, altering the redox balance due to a reduction of the NAD⁺/NADH ratio (Grad et al., 2005; Munnich & Rustin, 2001). Therefore, an overall metabolic dysfunction could result as a consequence of inhibitory effects of excess NADH induced by Rot on glycolysis and TCA cycle in CGCs. Thus, combined with the cytotoxic profiles of Rot-induced cell death that were observed in Chapter 3 by the MTT cell viability assay and the morphological changes observed by phase contrast microscopy, Rot at its low concentration was considered to be sufficient to cause dysfunction of mitochondria and cellular injury, so Rot 10 nM was chosen for use in subsequent experiments.

The most severe mitochondrial bioenergetic dysfunction was displayed by CGCs incubated with 3-NP. Previous literature has already revealed the irreversible inhibitory role of 3-NP on complex II on TCA cycle, followed by depression of ATP levels and impaired oxidative energy metabolism in animal models of HD (Beal et al., 1993; Brouillet & Hantraye, 1995; Brouillet et al., 1993; Gould & Gustine, 1982). In agreement with these publications, exposure of CGCs to both 100 µM and 300 µM of 3-NP for 4 h severely lowered oxygen consumption at basal level and FCCP-induced maximal respiration, indicating mitochondrial respiratory capacity had been exhausted leading 3-NP-treated CGCs to become highly susceptible to bioenergetic crisis. Such significant bioenergetic dysfunction again highlighted the critical role of complex II in overall energy metabolism of neurones. These data complement the ATP results which showed significant depletion of cellular ATP subsequent to 3-NP administration. Guided by the observations from slow depolarization of ΔΨm and the morphological studies where 3-NP 100 µM-treated CGCs still retained largely uninjured morphologies (Chapter 3), 3-NP at 300 µM was chosen for the subsequent experiments.

Similarly, the measurement of OCR after exposure to AA and KCN revealed concentration-dependent inhibitory effects on mitochondrial respiration, wherein higher concentration of AA and KCN caused substantial bioenergetic dysfunction as reflected by significant reduction of OCR in the key bioenergetic parameters such as basal OCR, ATP-linked OCR and FCCP-induced OCR. These results were expected, given the unique nature of complex III as an intermediary complex whose inhibition by AA results in a complete shutdown of respiration by full blockade of electron transfer to cytochrome...
c to the final complex of ETC, complex IV (Ward et al., 2000; Xia et al., 1997). This finding was consistent with the ATP results which showed significant reduction of cellular ATP subsequent to AA 30 nM administration for 24 h. The significant inhibition of basal, ATP-linked and maximal oxygen consumption by KCN 300 µM also confirmed its inhibitory role of complex IV. Binding of KCN at the metal centres of the oxygen reduction site of complex IV eventually prevents the binding and reduction of oxygen (Way, 1984), which effectively blocks the amount of oxygen consumed by all facets of bioenergetics as presented in this study. Interestingly, the inhibitory action of AA 30 nM and KCN 300 µM on oxygen consumption did not show a correlation with the level of cellular ATP measured by luciferase-luciferin bioluminescence assay (Fig. 4.9). The slow reduction of cellular ATP measured from CGCs treated with higher concentrations of AA and KCN could be perhaps due to the nature of the level of ATP as a late index of mitochondrial bioenergetics when compared to the measurement of ΔΨ_m and real-time observation of OCR. Given the aims of Chapter 3 and 4 of this thesis were to find the concentrations of drugs that would lead to a slow progression of cell death rather than rapid disintegration of cells, AA 30 nM and KCN 300 µM successfully induced mitochondrial dysfunction in all bioenergetic parameters. However the significantly reduced glycolytic activity subsequent to AA 10 nM and KCN 300 µM treatment (Fig. 4.25, Fig. 4.26), indicated significant overall metabolic dysfunction and suggested appreciable toxicity. The morphological observations from Chapter 3 phase contrast microscopy also suggested that AA and KCN at their high concentrations displayed high toxicity in CGCs. Although cellular ATP level and OCR remained relatively high in CGCs treated with the lower concentrations of AA and KCN, rapid and significant dissipation of ΔΨ_m induced by AA 10 nM and KCN 100 µM was considered sufficient to cause bioenergetic dysfunction of mitochondria. Therefore, AA 10 nM and KCN 100 µM were chosen for the subsequent experiments for this thesis.

There seems to be a disparity between loss of ΔΨ_m in presence of AA 10 nM or KCN 100 µM and relatively high OCR measurements under these treatment conditions. First, there may have been some “washout” of these inhibitors during the 1 h incubation of cells in (drug-free) unbuffered Seahorse assay media before Mito Stress assays. Second, ΔΨ_m may decline due to primary inhibition of respiratory enzymes (and consequent loss of proton pumping across the IMM), but ΔΨ_m could also be dissipated due to mitochondrial damage (permeabilization of IMM). Under the latter condition, if
respiration (measured by oxygen consumption) is not otherwise inhibited, it may rise substantially (as is the case until FCCP). The relationship between loss of $\Delta \Psi_m$ and reduced OCR may not therefore always hold strongly.

4.4.5. Concluding Remarks

In conclusion, the aim of this chapter was achieved in that all inhibitors of OXPHOS were shown to induce mitochondrial dysfunction, although they were found to display different modes of action in bringing about the mitochondrial injury. Cellular level of ATP, polarization of $\Delta \Psi_m$ and OCR were taken as the indices of mitochondrial function, and each inhibitor led to bioenergetic dysfunction to varying extents. Collapse of $\Delta \Psi_m$ was also apparent when the drugs, except for 3-NP, were added to CGC culture, although at the most extended time interval employed (4 h) there was certainly a suggestion of altered TMRM fluorescence for 3-NP-treated CGCs. The detailed dissection of bioenergetics achieved using the Seahorse technology demonstrates the power of the metabolic analyses which allowed examination of multiple components relevant to mitochondrial function and the concentration-dependent actions of insults on OXPHOS and glycolysis. Thus, in seeking to fully explore all facets of mitochondrial metabolism, effects of both low and high concentrations of insults were studied by Seahorse XF24 analyzer. The use of both lower and higher concentrations of all drugs permitted insights pertinent to the well documented insult-dependency of injury and differential patterns of recruitment of cell death mechanisms (Diwakarla et al., 2009a; Diwakarla et al., 2009b; Higgins et al., 2009; Nagley et al., 2010).

Given the important roles of mitochondria in neuronal homeostasis, cellular mitochondrial quality control plays an important role in efficiently repairing or removing dysfunctional molecular components of mitochondria, or the organelle themselves, to ensure cellular viability. Narendra and colleagues have shown that dissipation of $\Delta \Psi_m$ was the trigger of clearance of mitochondria via autophagic degradation, also known as mitophagy (Narendra et al., 2008), and this notion was further supported by various
studies performed in Drosophila, yeast and mammalian cell lines. The question arising from the present findings was if dissipation of \( \Delta \Psi_m \) and/or compromised mitochondrial metabolism caused by specific OXPHOS inhibitors would also recruit mitophagy in primary neurones. Thus, having successful established of primary CGC cultures and achieved mechanistic dissection of the bioenergetic dysfunction induced by OXPHOS inhibitors, the recruitment of mitochondrial quality control in terms of mitophagy in primary neurones will be investigated in the following chapter.
CHAPTER FIVE

Evidence for Neuronal Mitophagy
Subsequent to Bioenergetic Dysfunction
5.1. Introduction

In Chapter 4, the adverse effects of OXPHOS inhibitors on bioenergetics were confirmed by strategic analyses of mitochondrial metabolic function. As covered in the General Introduction, mitochondrial function and metabolism are closely related to neuronal survival due to the essential role of mitochondria in energy production. Mitochondria produce nearly 90% of ATP in neurones (Toescu et al., 2000) and the distribution of mitochondria in subcellular compartments of neurones is dependent on the site-specific energy demand (Scheffler, 2011). The presynaptic and postsynaptic endings, where the highest energy consumption occurs in neurones, are the good examples of how energy demand determines the distribution of local mitochondria (Morris & Hollenbeck, 1993). High metabolic activities and ongoing neuronal activities lead to an accumulation of ROS and influx of Ca\textsuperscript{2+} making neuronal mitochondria particularly prone to damage. Additionally, neuronal mitochondria have a longer half-life than those of other post-mitotic tissues (Menzies & Gold, 1971; Miwa et al., 2008; O'Toole et al., 2008) and the post-mitotic nature of neurones only allows a limited regenerative capacity. Therefore, efficient clearance of damaged mitochondria from the site is essential for neuronal function.

Neurones employ a number of mechanisms to control mitochondrial quality, through which permanent degradation of damaged mitochondria is facilitated via lysosomal-mediated autophagic degradation, also known as mitophagy (Baker et al., 2014). An emerging literature points to defective mitochondrial quality control as a defining factor for neuropathology (Amadoro et al., 2014; Osellame & Duchen, 2014), and the dysfunction of mitochondria have become a convergence point for neurodegeneration in conditions such as PD, HD, ALS and AD (Beal, 2005). The importance of mitophagy was further highlighted when genetic screens of early onset PD patients revealed mutations of proteins involved in mitochondrial quality control, namely Parkin (Kitada et al., 1998) and PINK1 (Valente et al., 2004). PINK1 has been identified as an upstream effector of mitophagy induction, which occurs through PINK1-dependent recruitment of Parkin in damaged mitochondria, followed by a recruitment of autophagic machinery to facilitate lysosome-mediated degradation (Narendra & Youle, 2011).
Further findings in the cases of HD, ALS and AD all showed mutations of other key molecules which assist progression of mitophagy, such as Mfn2, Drp1 or valosin-containing protein (Chaturvedi & Flint Beal, 2013; Johnson et al., 2010; Koppers et al., 2012). All this evidence suggests that defects in mitochondrial quality control may have a causative role in the pathogenesis of neurodegeneration.

5.1.1. PINK1/Parkin-Mediated Mitophagy in Primary Neurones

The initiation of PINK1/Parkin-mediated mitophagy involves a selective recognition of damaged mitochondria by PINK1. The sequence analyses of PINK1 revealed a mitochondrial targeting sequence at the N-terminus, indicating a close association of PINK1 with mitochondria (Valente et al., 2004). Selective recognition of damaged mitochondria by PINK1 is generally believed to be initiated by the dissipation of $\Delta \Psi_m$, followed by a failure of voltage-dependent cleavage of PINK1 by PARL, thereby allowing an accumulation of PINK1 on the OMM (Fig. 5.1) (Ashrafi & Schwarz, 2013). Stabilized PINK1 is a prerequisite to induce translocation of Parkin to depolarized mitochondria (Koyano et al., 2013; Matsuda et al., 2010), after which Parkin mediates the formation of poly-ubiquitin chains on its substrates on the OMM. Ubiquitinated mitochondria and ULK1 complexes accumulate at the autophagic recognition site (Narendra & Youle, 2011), where they are recognized by the autophagy adaptor proteins such as p62/SQSTM1 (sequestosome-1). Consequently, ubiquitinated mitochondria are linked to the autophagy machinery via LC3-PE, allowing formation of double-membrane autophagosomes around mitochondria. Ultimately, autophagosomes containing mitochondria are delivered to lysosomes for degradation (Ashrafi & Schwarz, 2013; Son et al., 2012) (Fig. 5.2).

The critical role of mitophagy and its molecular pathways have been heavily investigated, mostly in Drosophila, yeast and immortalized mammalian cell lines, where the loss of $\Delta \Psi_m$ and damaging ROS have been widely known as triggers of mitophagy (Frank et al., 2012; Kim et al., 2007; Narendra et al., 2010). Inhibitors of mitochondrial
In healthy mitochondria, PINK1 is imported to the IMM through the TOM/TIM complexes. The TIM complex-associated protease, mitochondrial MPP, cleaves PINK1 mitochondrial targeting sequence (MTS) on the inner face of the outer membrane (OM) of mitochondria. PINK1 is also cleaved by the IMM presenilin-associated rhomboid-like protease (PARL) located on the outer face of inner membrane (IM) of mitochondria, followed by proteolytic degradation. In damaged mitochondria, loss of $\Delta \psi_m$ prevents the import of PINK1, leading to the accumulation of unprocessed PINK1 on the OM surface. Here, it associates with the TOM complex, and recruits cytosolic Parkin to damaged mitochondria for ubiquitination, which ultimately leads to mitophagy (adapted from Ashrafi & Schwarz, 2013).
Figure 5.2. Current model of PINK1/Parkin-mediated mitophagy

PINK1 is normally maintained at low levels on mitochondria by voltage-dependent proteolysis, which is mediated by the mitochondrial presenilin-associated rhomboid-like protein (PARL) present in the IMM. When the $\Delta \psi_m$ is dissipated by mitochondrial damage, PINK1 accumulates as a 63 kDa full length form in the outer mitochondrial membrane (OMM) (detailed in Fig. 5.1). Accumulation of PINK1 on mitochondria subsequently recruits Parkin to ubiquitinate VDAC and/or mitofusin on the OMM, which becomes a target for mitophagy. The ubiquitin-binding adaptor p62 recruits ubiquitinated cargo into autophagosomes by binding to LC3 (adapted from Son et al., 2012).
respiratory chain complexes have also been employed to induce mitophagy in yeast or cell lines, such as the complex I inhibitor, Rot (Chu et al., 2013), the complex III inhibitor, AA (McLelland et al., 2014), and the ATP synthase inhibitor, Oli (Bhatia-Kissova & Camougrand, 2013). However, the current literature is relatively sparse on the involvement of mitophagy in primary neurones. There are profound differences between primary neurones and mammalian cell lines particularly in their metabolic preference for OXPHOS over glycolysis and their limited ability to switch to glycolysis in times of metabolic stress (Almeida et al., 2001; Almeida et al., 2004). Therefore, removal of the whole network of mitochondria subsequent to global mitochondrial damage is very undesirable in primary neurones, which are thus quite likely to possess different regulation of mitophagy compared to the cell lines. To date, little is known about the molecular regulation and triggers of mitophagic pathway in neurones - indeed recent studies based in primary neurones have shown quite contradictory results (Cai et al., 2012; Van Laar et al., 2011). Although the existence of PINK1-dependent recruitment of Parkin to mitochondria was shown in some studies (Cai et al., 2012; Joselin et al., 2012; McCoy et al., 2014), the same mitophagy trigger has failed to induce a similar pattern of Parkin recruitment in other studies (Rakovic et al., 2013; Van Laar et al., 2011). Careful comparison between these studies suggested that the expression of PINK1/Parkin pathway in primary neurones could be dependent on the culture conditions of neurones, such as the presence of antioxidants in the culturing media or the severity of injury (Grenier et al., 2013). These findings further highlight the complexity of neuronal mitophagy. Therefore, a study focusing on post-mitotic neuronal mitophagy is critical.

5.1.2. Salient Aspects of Monitoring Mitophagy

Mitophagy is a highly complex and dynamic cellular process. Despite the considerable advances in our knowledge of molecular pathways involved in mitophagy, there is no overall agreement on the best methods for monitoring mitophagy, and both biochemical and morphological observations are often integrated to investigate mitophagy. Determination of sequestration of mitochondria in double-membrane autophagosomes during the early phase of mitophagy can be achieved by an
ultrastructural analysis of cells, using transmission electron microscopy (TEM) following exposure to mitophagic stimuli in cells. Biochemical approaches are most often used to visualize colocalization of mitochondria in autophagosomes, using immunocytochemical labelling of LC3 or transfection of cells with GFP-LC3, with concurrent staining of mitochondrial proteins such as VDAC2 (voltage-dependent anion channel 2), TOMM20 (translocase of outer mitochondrial membrane 20 homolog (Yeast)) or components of ETC complexes. Monitoring of PINK1 and Parkin antibodies using fluorescence microscopy can also confirm the endogenous levels of PINK1 and Parkin and their distribution in cells. These qualitative approaches of monitoring mitophagy can be complemented with quantitative approaches using ratiometric analysis of pH-sensitive fluorescent proteins targeting mitochondria, such as mt-Keima (Katayama et al., 2011) and mt-Rosella (Rosado et al., 2008). mt-Rosella in particular, has been developed to monitor acidification of mitochondria during processes of mitophagy by monitoring the appearance and intensity of a pH-sensitive GFP, superecliptic pHluorin, to quantify formation of autolysosomes containing mitochondria (Rosado et al., 2008). In these fluorescence-based approaches, the key is to use the red fluorescence of one component of the reporter (pH-insensitive) alongside the pH-sensitive green fluorescence of the other element of the reporter. Ratiometric quantification of green/red fluorescence therefore determines the extent to which the intramitochondrial reporter is in an acidic environment.

5.1.3. Aims for Chapter 5

In this chapter, the research seeks to document the progression of mitophagy in primary neurones. Based on the strategic analyses of bioenergetic dysfunction caused by OXPHOS inhibitors (Chapters 3 and 4), the possible triggers of mitophagy in primary neurones will be examined. Methodologically, this study employed an immunocytochemical approach to examine the stabilization of the upstream marker of mitophagy, PINK1, in mitochondria following bioenergetic dysfunction. Downstream assessment of the mitophagic cascade was also undertaken using the dual-colour biosensor, mt-Rosella, to observe mitochondrial acidification and transit to lysosomal vesicles in bioenergetically compromised neurones.
5.2. Methods

5.2.1. Primary Cerebellar Granule Cell Cultures

Primary cultures of CGCs were prepared from 6-8 day-old SWISS White mice according to Section 2.2.2. Briefly, cerebella were dissected in ice-cold isolating solution (HBSS pH 7.4 containing 3 mg/ml BSA, 1.16 mM MgSO4, 1 mM sodium pyruvate, 10 mM HEPES, 7.6 mM D-Glucose) and the meninges were carefully removed. The isolated cerebella were placed in fresh ice cold isolating solution and they were chemically and mechanically dissociated and centrifuged. The cells were resuspended in NBM containing B-27 supplement (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum and seeded in tissue culture plates, pre-coated with poly-D-lysine (50 µg/ml). CGCs for Western immunoblotting for PINK1 were seeded in 6-well plates (2 x 10^6 cells/well) and CGCs prepared for immunocytochemistry for PINK1 were seeded in 48-well plates (0.2 x 10^6 cells/well). CGCs for transfection and imaging of mt-Rosella were seeded in 24-well plates (0.35 x 10^6 cells/well). Neurones were left to grow in NBM containing B-27 components (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum for 24 h in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C). Medium was fully replaced by NBM containing aphidicolin (1 µg/ml) to restrain non-neuronal cell proliferation (Diwakarla et al., 2009b; Giardina et al., 1998), and final concentration of 3 % (v/v) B-27 supplement. Half medium changes were conducted at 4 DIV and included aphidicolin (1 µg/ml) and 3 % of B-27 supplement and all experiments were performed at 7 DIV.
5.2.2. Drug Treatment

Drug treatments were performed as described in Section 3.2.3.1. Briefly, drugs were prepared fresh on the day of experiment except for Oli (dissolved in ethanol and stored in -20°C), and the stock solutions of Rot, 3-NP, AA, KCN and Oli were diluted in MEM containing 25 mM K⁺ to make the chosen concentrations of drugs based on preliminary experiments in Chapter 3 and 4. All drugs were kept in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C) for 1 h to allow the drug solutions to be warmed and equilibrated. For drug treatment, growth medium was removed and replaced by MEM containing drugs with a full medium change. Neuronal injury with drugs was induced for various time points during incubation in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C).

5.2.3. Western Immunoblotting for PINK1

Protein extraction and determination of lysate concentrations were carried out as described in Section 2.2.5. Briefly, CGCs were rinsed once with ice-cold PBS and lysed using RIPA buffer containing protease inhibitor cocktail (dissolved according to the manufacturer’s instruction). Cells were shaken at 150 rpm on ice for 1 h and samples were centrifuged at 14,000 x g at 4°C for 5 min. The supernatant was transferred to Eppendorf tubes and stored at -20°C until ready for use. Protein levels were determined using the commercially available DC Bio-Rad Protein Assay Kit (Sydney, Australia).

SDS-PAGE was performed to visualize the endogenous level of PINK1 in CGCs as described in Section 2.2.5. Briefly, equal amounts of protein (~10 µg) were prepared in loading buffer and loaded onto 8 % SDS-polyacrylamide separating gel. Proteins were electrophoresed at 100 V for 1.5 h at room temperature. Proteins were transferred to PVDF membrane and blocked with 5 % (w/v) skim milk in TBS for 1 h at room
temperature. The membrane was incubated with 1 % (w/v) skim milk in TBS containing PINK1 primary antibody (anti-rabbit, 3 µg/ml) (A kind gift from Dr Janetta G. Culvenor from University of Melbourne; George et al., 2010) overnight at 4°C. Membranes were washed three times with TBST, and subsequently incubated for 1 h at room temperature on a rocking platform with IRDye-conjugated secondary antibodies (1:10,000, Li-Cor, Lincoln, NE, USA). Membranes were washed three times with TBST and infrared fluorescence was detected by Odyssey Classic infrared imaging system. The membrane was re-probed using primary antibodies specific for β-actin (1:5,000) in 1% (w/v) skim milk in TBS. Data were analysed according to Section 2.2.5.4.

5.2.4. Immunocytochemical Staining for PINK1

Cultures were grown in 48 well plates and fixed with 300 µl of pre-warmed 4 % paraformaldehyde solution pH 7.4 (4 g of paraformaldehyde dissolved in 100 ml PBS; 137 mM NaCl, 50 mM Na₃HPO₄, 50 mM NaH₂PO₄, pH 7.4) for 10 min at room temperature. Cells were washed three times with pre-warmed PBS, and cells were permeabilized for 10 min with 0.3 % (v/v) TX-100 in PBS. Cells were briefly washed with PBS and blocking with 1 % BSA dissolved in PBS for 1 h at room temperature to prevent non-specific binding of the antibody. After blocking, cells were incubated with primary PINK1 antibody (anti-rabbit, 3 µg/ml) diluted in PBS containing 1 % BSA overnight at 4°C. The primary antibody was aspirated and cells were washed with PBS three times and incubated with goat anti-rabbit Alexa 488 secondary antibody (1:200) diluted in PBS containing 1 % BSA for 3 h at room temperature in dark. After the incubation, the secondary antibody was removed and cells were washed three times with PBS. Images from random fields were taken with an Olympus 1x71 inverted fluorescence microscope, which was connected to the Olympus C-5050 digital camera. A targeted series of experiments with double immunolabelling was performed with an anti-SDHA mouse monoclonal antibody to label mitochondrial complex II (Succinate dehydrogenase complex, subunit A) (1:200) where the secondary antibody was goat anti-mouse Alexa 488 (1:200). PINK1 primary antibody was also employed where the secondary antibody was goat anti-rabbit Alexa 568 (1:200).
5.2.4.1. Data analyses of immunofluorescence staining of PINK1

The differential distribution of PINK1 immunofluorescence displayed in CGCs was semi-quantitatively analyzed by assigning neurones into three distinct populations: diffuse labelling, redistributed PINK1, and cells undergoing PCD which showed no PINK1 fluorescence. Analyses were performed on raw counts which were then expressed as a percentage of the total population of cells counted where typically 250-500 cells were scored in each of duplicate wells per experiment condition. Data represent the mean ± SEM of two independent experiments. Symbols indicate significant differences in distribution of PINK1 relative to control were analyzed by two-way ANOVA, followed by Bonferroni's post hoc test using GraphPad Prism, v.4.0. (San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

5.2.5. Monitoring Mitophagy using mt-Rosella Biosensor

Fluorescent proteins are structurally stable molecules whose relative resistance to proteolysis and extremes of pH in acidic vacuoles make them favourable for the design of reporter molecules involving acidic vacuoles. The fluorescent proteins provide a persistent signal in acidic vesicles, allowing the monitoring of the late stage of autophagy during which the specific cellular organelles, such as mitochondria, are delivered to acidic lysosomes (Rosado et al., 2008). mt-Rosella is a genetically encoded, dual-colour emission biosensor which is comprised of mitochondrial target sequence, red fluorescent protein (RFP) and green fluorescent protein (GFP) linked by a 9 amino acid linker (Fig. 5.3A) (Mijaljica et al., 2011; Rosado et al., 2008). The sequence for a RFP encodes for pH-insensitive fast-maturing variant of the RFP, DsRed.T3 (maximal excitation/emission wavelength at 560 nm/587 nm), whereas the sequence for a GFP encodes for pH-sensitive mutants of GFP, superecliptic pHluorin (maximal excitation/emission wavelength at 488 nm/508 nm). The pH difference found between cytoplasm and lysosomes underlies the operation of mt-Rosella biosensor, where fluorescence of pHluorin is gradually lost in response to the acidification of the surrounding and becomes almost negligible at the
Figure 5.3. Schematic representation of mt-Rosella fluorescent biosensor, a reporter molecule for monitoring mitophagy

(A) mt-Rosella construct is comprised of mitochondrial target sequence linked to two fluorophores, relatively pH-stable RFP, DsRed.T3, and the pH-sensitive GFP, pHluorin. The response of the Rosella pH-biosensor relies on the different sensitivity of DsRed.T3 and pHluorin to pH. The fluorescence emission of pHluorin is almost negligible under the acidic condition (pH of ~5.0-5.5) in which the pH-stable DsRed.T3 still shows fluorescence emission. The differences in physiological pH between the vacuole (pH ~5.0-5.5) and mitochondria (pH ~8.2) in living cells allow the identification of mitochondrial localization in lysosomal vesicles (adapted from Mijaljica D et al., 2011).

(B) Fluorescence excitation spectra of GFP eclips pHluorin. pH-sensitive pHluorin gradually loses its fluorescence as pH is lowered, until at pH values of 6.0 (adapted from Miesenböck et al., 1998).
(A) Emission Intensity at 508 nm

High pH (Mitochondrial pH=8.2)

Low pH (Vascular pH=5.5)

(B) Emission Intensity at 508 nm vs. Excitation wavelength (nm)
vacuolar pH of 6.0 due to its pH-sensitive property (Fig. 5.3B) (Mahon, 2011; Miesenbock et al., 1998). On the contrary, DsRed.T3 possesses a more photostable property with respect to changes in pH, and its red-shifted fluorescence provides a better separation from the emission peaks of GFP. Together with its high quantum yield, DsRed.T3 is considered as an ideal candidate for multicolour experiments involving GFP fluorescence (Baird et al., 2000; Bevis & Glick, 2002; Muller-Taubenberger & Anderson, 2007).

Based on these chemical properties, an attachment of specific targeting sequence to cellular components to mt-Rosella construct allows a specific monitoring of autophagic delivery of cellular components to acidic lysosomal compartments. Here mt-Rosella had been engineered to localize to the mitochondrial matrix by fusion with COX VIII pre-sequence peptide as a mitochondrial targeting sequence (presented as mt-Rosella) to allow monitoring of mitochondrial delivery to the acidic compartments of lysosomes. The red fluorescence provided information about the position of mitochondria within the cell, while the loss of green fluorescence identified delivery of mitochondria into acidic vesicles for degradation (Rosado et al., 2008; Sargsyan et al., 2015). Therefore, the reduction in green fluorescence was interpreted as the transit of mt-Rosella-tagged mitochondria to acidic lysosomal vesicles.

5.2.5.1. Transfection of CGCs with mt-Rosella biosensor using Lipofectamine 2000

CGCs were transfected using a cationic lipid mediated transfer of DNA into cells where DNA is encapsulated by lipidic vesicles and fused with the plasma membrane to allow entry of the DNA. Lipofectamine 2000 Transfection Reagent (Invitrogen) was used according to the manufacturer’s instruction.

CGCs were seeded in 24-well plates (0.35 x 10^6 cells/well) pre-coated with poly-D-lysine (50 μg/ml per well) and cells were grown according to Section 2.2.2. At 6 DIV, the growth medium in which CGCs were maintained was gently removed and set aside in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C) for a later use. CGCs were
gently washed twice with pre-warmed serum-, antibiotic-free Opti-MEM containing 25 mM K⁺ to remove any traces of penicillin-streptomycin in the culture. The transfection DNA-Lipofectamine complex was prepared according to the manufacturer's protocol using a ratio of 3 µl of Lipofectamine 2000 Transfection Reagent for every 1 µg of total DNA transfected. DNA-Lipofectamine complex was mixed and incubated for 20 min at room temperature. Preliminary experiments have shown that higher amount of DNA decreased the viability of cells (Appendix 2), therefore 0.4 µg of DNA/well was used for all transfection procedures. The DNA-Lipofectamine complex was gently added to CGCs and cells were left in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C) for 1 h. Transfection was stopped by gently removing and washing cells with pre-warmed serum-, antibiotic-free Opti-MEM containing 25 mM K⁺ to remove the DNA-Lipofectamine complex and the medium was fully replaced by growth medium in which the neurones were previously maintained. Cells were left in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C) for 24 h and drug treatment with appropriate vehicle controls was carried out according to Section 2.2.3 and 3.2.3.1. Subsequent to drug treatment, images of transfected neurones were captured with Olympus 1x71 inverted fluorescence microscope, which was connected to the Olympus C-5050 digital camera.

5.2.5.2. Data analyses of mt-Rosella fluorescence signals

The integrated intensity of green/red fluorescence from each neurone was quantified using ImageJ software (obtained from the NIH web site: http://rsb.info.nih.gov.ij). The images were converted into 8 bit pictures and appropriate threshold was applied the same in all pictures being analysed. Raw values of integrated intensity of green/red fluorescence from 17-43 neurones per experiment condition collected over 4 wells were presented as the ratio of the control cells. Data were given as mean ± SEM from two independent experiments in quadruplicate wells in each experiment. P < 0.05 was considered statistically significant based on unpaired t-test using GraphPad Prism software, v.4.0. (San Diego, CA, USA).
5.3. Results

5.3.1. Localization of Endogenous PINK1 in CGC Culture

5.3.1.1. Detection of PINK1 in CGCs by Western immunoblotting

PINK1 encodes a 581 amino acid protein with a predicted molecular weight of 62.8 kDa, showing 95% sequence similarity between human and mouse (Nakajima et al., 2003; Unoki & Nakamura, 2001). PINK1 is normally present in three isoforms, either unprocessed long (~63 kDa) precursor protein or a mature form of short proteins (~54 and ~45 kDa), which are the cleaved form after being imported into mitochondria (Becker et al., 2012; Beilina et al., 2005; Lin & Kang, 2008; Matsuda et al., 2010; Silvestri et al., 2005; Takatori et al., 2008; Zhou et al., 2008). Before proceeding to investigations of cellular localization of PINK1 in CGCs, it was important to confirm the expression of endogenous PINK1 in CGC culture. Here the antibody employed was an affinity-purified rabbit PINK1 antibody raised against the C-terminal domain of human PINK1 (amino acids 511–526) (George et al., 2010), which has previously been used to detect the expression of PINK1 in mouse brain by Western immunoblotting technique (George et al., 2010). Thus, the expression of PINK1 was investigated using Western immunoblotting technique. Multiple bands were observed when untreated CGC lysate was immunoblotted with PINK1 antibody on 8% SDS-PAGE gel, where three bands corresponding to ~63 kDa, ~54 kDa and ~45 kDa were clearly visible, confirming the endogenous PINK1 was present in CGC cultures (Fig. 5.4).

5.3.1.2. Detection of PINK1 distribution in CGCs by immunofluorescence

Since the presence of endogenous PINK1 was confirmed by Western immunoblotting technique, the next critical step was to examine the subcellular
distribution of PINK1 in cultured neurones. Previous studies of PINK1 have indicated that PINK1 resides in cytoplasm in addition to mitochondria (Takatori et al., 2008). In agreement with the Western immunoblotting experiment, immunocytochemical staining using the affinity-purified rabbit PINK1 antibody visualized endogenous PINK1 in CGCs by fluorescence microscopy. Interestingly, the distribution of PINK1 in untreated neurones varied in that the majority of cells displayed diffuse staining across the whole cell body whilst a lesser proportion of cells showed concentrated localization mainly in the cytosol, leaving the nuclei areas as a clear void (Fig. 5.5; Control panels). This differential pattern of PINK1 staining was more prominent in CGCs exposed to OXPHOS inhibitors (Fig. 5.5 and Fig. 5.6). Fluorescence imaging of neurones revealed that 4 h exposure to Rot 10 nM, 3-NP 300 µM, AA 10 nM, KCN 100 µM and Oli 1 µM, displayed a relatively higher proportion of PINK1 immunolabelling that was redistributed around the nuclei, giving a clear void in the nuclear region.

In order to investigate this phenomenon further, a semi-quantitative analyses of PINK1 distribution in CGCs was performed by scoring and grouping neurones into three distinct populations: cells showing diffuse distribution across the cell body, cells manifesting redistribution of PINK1 revealing a noticeable void in the nuclear region, and cells undergoing PCD that failed to show distribution of PINK1 (Fig. 5.7). The percentage of cells displaying different distribution of PINK1 for each drug-treated group was presented as proportion of the total number of neurones counted per drug treatment.

Initial analyses focused on neurones treated with Rot 10 nM and 3-NP 300 µM. Results indicated that 53 ± 1.0 % of control CGCs showed diffuse distribution of PINK1 while 40 ± 3.5 % of the whole population accounted for cells displaying PINK1 localized around the nuclei (Fig. 5.7A). The proportion of control CGCs with diffuse staining was higher than CGCs displaying redistribution of PINK1, although the difference was not statistically significant. Compared to control, CGCs treated with Rot 10 nM and 3-NP 300 µM showed a significantly reduced proportion of CGCs displaying diffuse staining (37 ± 6.1 % of whole population, P < 0.05 compared to control; 34 ± 3.1 % of whole population, P < 0.05 compared to control, respectively) and significantly increased proportion of cells with redistributed PINK1 staining around the nuclei (55 ± 3.8 % of whole population, P < 0.05 compared to control; 59 ± 1.6 % of whole population, P <
Figure 5.4. Endogenous PINK1 is expressed in lysates of untreated CGCs

Lysates of untreated CGCs at 7 DIV were prepared for Western immunoblotting where the PINK1 antibody recognized an unprocessed long protein (~63 kDa) and processed short proteins (~54 kDa, ~45 kDa) of endogenous PINK1. Affinity-purified rabbit PINK1 antibody was raised to the C-terminal domain of human PINK1 (amino acids 511–526) (George et al., 2010).
Weight Marker CGC 20 µg

100 75 50 42

~63 kDa
~54 kDa
~45 kDa

PINK1

β-actin
Figure 5.5. Effect of OXPHOS inhibition on PINK1 distribution following 4 h exposure to Rot 10 nM or 3-NP 300 µM

PINK1 immunofluorescence (green) and Hoechst 33342 (blue) in control CGCs following a 4 h treatment with Rot 10 nM and 3-NP 300 µM. Cells were immunolabelled with antibodies specific for PINK1, and with Hoechst 33342 dye to visualize nuclei. Neurones that possessed a pattern of diffuse cytosolic staining of PINK1 are indicated with yellow arrows and neurones displaying redistributed PINK1 around the nuclei are indicated by white arrows. Red arrows indicate nuclei of cells undergoing PCD and were unable to show distribution of PINK1. Scale bar = 20 µm.
Figure 5.6. Effect of OXPHOS inhibition on PINK1 distribution following 4 h exposure to AA 10 nM, KCN 300 µM or Oli 1 µM

PINK1 immunofluorescence in control CGCs following a 4 h treatment with AA 10nM, KCN 300 µM or Oli 1 µM. Cells were immunolabelled with antibodies specific for PINK1, and with Hoescht 33342 dye to visualize nuclei. Neurones that possessed a pattern of diffuse cytosolic staining of PINK1 are indicated with yellow arrows and neurones displaying redistributed PINK1 around the nuclei are indicated by white arrows. Scale bar = 20 µm.
The differential distribution of PINK1 immunofluorescence displayed in CGCs treated with (A) Rot 10 nM, 3-NP 300 µM, (B) AA 10 nM, KCN 300 µM and Oli 1 µM for 4 h was quantitatively analyzed by assigning neurones three distinct populations: diffuse labelling, redistributed PINK1, and cells undergoing PCD. Analyses were performed on raw counts which were then expressed as a percentage of the total population of cells counted (total 250-500 cells were counted in each experimental condition). Data represent the mean ± SEM of two independent experiments from duplicate wells. Symbols indicate significant differences in distribution of PINK1 relative to control (* P < 0.05, ** P < 0.01, and ## P < 0.0001 compared to control by two-way ANOVA, followed by Bonferroni's post hoc test).
(A) Oli 1 µM
KCN 100 µM
AA 10 nM
Control
3-NP 300 µM

(B) Control
AA 10 nM
KCN 100 µM
Oli 1 µM
0.05 compared to control, respectively). Two-way ANOVA revealed the population of neurones treated with Rot 10 nM and 3-NP 300 µM represented a significantly increased population of CGCs displaying redistributed PINK1 relative to diffuse stained CGCs (P < 0.05 and P < 0.01, respectively) (Fig. 5.7A).

CGCs treated with AA 10 nM, KCN 100 µM and Oli 1 µM also displayed similar patterns of PINK1 fluorescence (Fig. 5.7B). Again, a higher proportion of control CGCs exhibited diffusely distributed PINK1 (61 ± 0.3 % of whole population) relative to the redistributed pattern (31 ± 0.3 % of whole population), a difference that was statistically significant (P < 0.0001). Drug treatment with for AA 10 nM, KCN 100 µM and Oli 1 µM induced significant reduction in the proportion of neurones scored for diffuse staining compared to control (49 ± 4.5 % of whole population, P < 0.05 compared to control; 47 ± 3.5 % of whole population, P < 0.01 compared to control; 51 ± 0.3 % of whole population, P < 0.05 compared to control, respectively). Furthermore, a significantly increased proportion of cells was determined to possess the redistributed pattern of PINK1 relative to control following exposure to AA 10 nM, and KCN 100 µM (41 ± 4.5 % of whole population, P < 0.05 compared to control; 46 ± 2.3 % of whole population, P < 0.001 compared to control, respectively), a phenomenon also observed in Rot 10 nM and 3-NP 300 µM treatment. There was a slight increase in proportion of neurones with the pattern of PINK1 redistribution in Oli 1 µM-treated cells, which however failed to reach a statistical significance (40 ± 1.7 % of whole population).

The PINK1 distribution patterns were compared within the groups of neurones treated with each OXPHOS inhibitor. The exposure to Rot 10 nM and 3-NP 300 µM induced a significant shift of PINK1 distribution from being diffuse to redistributed, whereas AA 10 nM, KCN 100 µM and Oli 1 µM treatment failed to shift the distribution pattern from diffuse to redistributed, although the population displaying the pattern of redistribution was higher than that observed in control CGCs (Fig. 5.7A & Fig. 5.7B). These results suggest that Rot and 3-NP may have been more effective in inducing the redistribution of PINK1 around the nuclei from the cytosol.
5.3.1.3. Confocal imaging using PINK1 and complex II antibodies in CGCs exposed to OXPHOS inhibitors suggest PINK1 mobilization on mitochondria

In order to determine if the pattern of PINK1 redistribution induced by exposure to OXPHOS inhibitors was co-localized with mitochondria, double immunocytochemistry was performed using antibodies of mitochondrial marker, complex II (via Alexa 488), and PINK1 (via Alexa 568). CGCs were exposed to Rot 10 nM or 3-NP 300 μM for 4 h. Complex II immunostaining revealed a cytoplasmic distribution of mitochondria around the nuclei and on neuritic processes (Fig. 5.8), which was similar to the observed localization of PINK1 upon insult. As expected, PINK1 immunolabelling in control CGCs displayed predominantly diffuse staining of PINK1 in the cytosol. In agreement with the single immunofluorescence study in Section 5.3.1.2, Rot 10 nM and 3-NP 300 μM treatments both led to an increased proportion of cells with redistribution of PINK1 around the nuclei, whose localization was similar to mitochondrial location within the cytosol. These results are in agreement with literature that has specifically shown mitochondrial localization of PINK1 upon depolarization of $\Delta \Psi_m$ (Kawajiri et al., 2010; Matsuda et al., 2010; Narendra et al., 2010), suggesting PINK1 redistribution in CGCs was on mitochondria upon bioenergetic insult.

5.3.2. Quantification of Mitophagy by Fluorescence Ratio Analysis using mt-Rosella Reporter

To investigate the recruitment of mitophagy in bioenergetically compromised neurones, CGCs were transiently transfected with mt-Rosella. The utility of mt-Rosella has already been demonstrated in yeast (Saccharomyces cerevisiae) and mammalian cells (HeLa cells), in which transfection with mt-Rosella resulted in specific labelling of mitochondria, accompanied with the loss of green fluorescence in low pH media and autophagy-induced conditions without affecting the red fluorescence (Mijaljica et al., 2011; Rosado et al., 2008; Sargsyan et al., 2015). Furthermore, the expression of mt-
Figure 5.8. Distribution of PINK1 and mitochondrial protein, complex II, following 4 h exposure to Rot 10 nM or 3-NP 300 µM

CGCs were exposed to Rot 10 nM or 3-NP 300 µM for 4 h and double immunolabelled with mitochondrial marker, complex II (via Alexa 488), and PINK1 (via Alexa 568). Nuclei were stained with Hoechst 33342. Yellow arrows indicate CGCs displaying diffuse staining of PINK1 in the cytosol and white arrows indicate redistributed PINK1 around the nuclei co-localized with mitochondria. Scale bar = 10 µm.
Rot 10 nM

3-NP 300 µM
Rosella was reported to have no effect on the extent of mitophagy or autophagy compared to the control transfection (Sargsyan et al., 2015).

As expected, both red and green fluorescence were expressed in control cells. A strong signal for red fluorescence was observed in all morphological components such as the cell body, dendritic and axonal processes of CGCs (Fig. 5.9; Control panels). The strongest fluorescence was observed in the cell body around nuclei, illustrating localization of mitochondria at high density. Red fluorescence was also detected in the neuritic processes and exhibited thread-like mitochondrial localization showing the distribution of mitochondria in all neuritic processes, thus illustrating the complex neuritic tree of CGCs. These features of morphological and cellular distribution of mitochondria observed here are consistent with the study done by Skulachev and colleagues who showed diffuse ethyl rhodamine staining of mitochondria in the cell body due to a larger amount of mitochondria and much thicker layer of cytosol, and long filamentous mitochondria in other parts of human fibroblast (Skulachev et al., 2004). The green fluorescence was also expressed in control neurones, however the intensity of green fluorescence was less than the red fluorescence in all imaged neurones. Nonetheless, the mitochondrial distribution was again demonstrated in mostly the cell body and also in all parts of neuritic processes (Fig. 5.9; Control panels).

Similarly, a strong red fluorescence emission (587 nm) was again displayed in the cell body, dendritic and axonal processes of CGCs exposed to drugs for 24 h, particularly in the cell body (Fig. 5.9, see also Fig. 5.11 below). In contrast to control neurones, mitochondria in drug-treated CGCs generally showed significant morphological changes from longer thread-like structures to shorter and granular forms. The disappearance of green fluorescence was most prominently observed in CGCs treated with Rot 10 nM or 3-NP 300 µM (Fig. 5.9). The disappearance of green fluorescence was clearly observable in distal parts of the neuritic processes, while the cell body still retained strong green fluorescence, suggesting mitochondria in the distal processes of neurones had entered an acidic environment and were subjected to mitophagy in response to bioenergetic dysfunction (Fig. 5.10). Similarly, neurones treated with KCN 100 µM and Oli 1 µM also showed a reduction in green fluorescence (Fig. 5.11). Surprisingly, green fluorescence from CCCP 3 µM treated CGCs showed a strong emission (508 nm) in all parts of
neuronal structure, indicating mitochondria failed to localize to an acidic environment after CCCP-induced injury (Fig. 5.11). This result was unexpected as depolarization of $\Delta\Psi_m$ induced by protonophores, such as CCCP, is regarded as a common trigger of mitophagy in mammalian non-neuronal cells, which would potentially lead to the loss of green fluorescence due to induction of mitophagy (Matsuda et al., 2010; Narendra et al., 2010). The special case of CCCP is considered below in the discussion (Section 5.4.4). Similar result was also displayed by CGCs treated with AA 10 nM, which failed to show loss of green fluorescence (Fig. 5.9), but the explanation is not the same as for CCCP.

For a better understanding of the induction of neuronal mitophagy, the ratio of green and red fluorescence intensity from control and drug-treated CGCs was quantitatively analysed (Fig. 5.12). The ratio of green/red fluorescence of drug-treated CGCs was presented as percentages of the green/red fluorescence ratio determined for control CGCs. As expected from the fluorescence images, neurones exposed to Rot 10 nM showed a significant decrease in green/red fluorescence ratio compared control (72 ± 8.6 % of control, P < 0.05) (Fig. 5.12), indicating that a significant population of mitochondria had entered the acidic vesicles for degradation. Similarly, 3-NP 300 µM treated CGCs also showed a significant reduction in green fluorescence compared to the control cells (68 ± 8.0 % of control, P < 0.01), suggesting mitophagy was induced due to bioenergetic dysfunction by 3-NP 300 µM (Fig. 5.12). Exposure to KCN 100 µM and Oli 1 µM failed to induce a significant reduction in green/red fluorescence ratio. On the contrary, AA 10 nM and CCCP 3 µM treatment appeared to increase the green/red fluorescence ratio as observed from the microscopic fluorescence images, although the increase observed in CCCP-treated neurones was not statistically significant (115 ± 5.2 % of control, P < 0.05; 119 ± 12 % of control, respectively). The unusual outcome for CCCP could be due to the CCCP-mediated dissipation of lysosomal pH, which will be discussed in Section 5.4.4.

Since the reduction in green fluorescence was mostly observed in neuritic processes, green/red fluorescence ratio was measured again excluding the cell body, in order to confirm if the loss of green fluorescence due to mitophagy occurred in the distal parts of neurones (Fig. 5.13). Again, neurones that were exposed to Rot 10 nM and 3-NP 300 µM displayed a significant loss of green fluorescence reflected by a reduction in the
Figure 5.9. Acidification of neuronal mitochondria was monitored by transiently transfecting CGCs with mt-Rosella followed by 24 h exposure to inhibitors of complex I, II and III

Representative fluorescence micrographs of CGCs transiently transfected with mt-Rosella. CGCs were subsequently exposed to MEM, Rot 10 nM, 3-NP 300 µM and AA 10 nM for 24 h and the red and green fluorescence from neurones was concurrently monitored. The red fluorescence (DsRed.T3) provided information about the position of mitochondria within the cell while the loss of green fluorescence (pHluorin) identified delivery of mitochondria into acidic vesicle. Scale bar = 20 µm.
Rot 10 nM

Control

DsRed.T3

pHluorin

Rot 10 nM

3-NP 300 µM

AA 10 nM
Figure 5.10. Mitophagy is revealed by disappearance of green fluorescence in distal neuronal axons of CGCs treated with Rot 10 nM and 3-NP 300 µM for 24 h

Disappearance of green fluorescence (pHluorin) in CGCs treated with complex I and II inhibitors was clearly observable in distal parts of the neuritic processes compared to the red fluorescence (DsRed.T3) (inset). Scale bar = 20 µm.
Figure 5.11. Acidification of neuronal mitochondria was monitored by transiently transfecting CGCs with mt-Rosella followed by 24 h exposure to inhibitors of complex IV, V and CCCP

Representative fluorescence micrographs of CGCs transiently transfected with mt-Rosella. CGCs were subsequently exposed to MEM, KCN 100 µM, Oli 1 µM and CCCP 3 µM for 24 h and the red and green fluorescence from neurones was concurrently monitored. The red fluorescence (DsRed.T3) provided information about the position of mitochondria within the cell while the loss of green fluorescence (pHluorin) identified delivery of mitochondria into acidic vesicle. Scale bar = 20 µm.
Figure 5.12. Quantification of mitophagy by fluorescence ratio analysis (green/red) of neurones transfected with mt-Rosella biosensor following 24 h exposure to OXPHOS inhibitors and CCCP

Green/red fluorescence ratio of mt-Rosella transfected neurones was measured from CGCs exposed to Rot 10 nM, 3-NP 300 μM, AA 10 nM, KCN 100 μM, Oli 1 μM and CCCP 3 μM for 24 h. Data represent mean ± SEM of ratios for n = 17-43 cells per condition. * P < 0.05, * < P 0.01, based on unpaired t-test.
Figure 5.13. Quantification of mitophagy by fluorescence ratio analysis (green/red) of neuritic processes of CGCs transfected with mt-Rosella biosensor following 24 h exposure to OXPHOS inhibitors and CCCP

Green/red fluorescence ratio of mt-Rosella transfected neurones was measured from neuritic processes of CGCs exposed to Rot 10 nM, 3-NP 300 µM, AA 10 nM, KCN 100 µM, Oli 1 µM and CCCP 3 µM for 24 h. Data represent mean ± SEM of ratios for n = 17-43 cells per condition. * P < 0.05, * < P 0.01, based on unpaired t-test.
green/red fluorescence ratio (68 ± 5.8 % of control, P < 0.05; 57 ± 5.7 % of control, P < 0.05, respectively), indicating mitochondria in neuritic processes entered acidic lysosomal vesicles for degradation pathway. Minor decreases in green/red fluorescence ratio after exposure to KCN 100 µM and Oli 1 µM in neuritic processes were not statistically significant. Slight increase in green fluorescence was again displayed by CGCs treated with AA 10 nM and CCCP 3 µM.
5.4. Discussion

The identification of genetic mutations involved in mitochondrial quality control from genetic studies of the early-onset recessive PD patients has led to a wider understanding on the pathogenic mechanisms underlying neurodegenerative diseases (Andres-Mateos et al., 2007; Bonifati et al., 2003; Kitada et al., 1998; Valente et al., 2001). The failure of clearance of dysfunctional mitochondrial via mitophagy and its detrimental consequences have been suggested as one of the main pathological event of neurodegeneration (Ashrafi & Schwarz, 2013). An increasing body of literature has dissected the molecular mechanisms of PINK1- and Parkin-dependent mitophagy. However, most of the current research has employed non-neuronal cells such as Drosophila, yeast or immortalized cell lines and not primary neurones. It is of interest that some studies have shown that primary cultured neurones have failed to display the PINK1- and Parkin-mediated mitophagic mechanisms under the same trigger, loss of ΔΨ_m, commonly used for non-neuronal or immortalized cell lines (Rakovic et al., 2013; Van Laar et al., 2011), raising the need for detailed prescriptive dissection of the involvement of these processes in neurones. Therefore, this chapter set out to examine the possible triggers of PINK1-dependent mitophagy in primary CGC culture, and to determine if mitophagy is involved in bioenergetically impaired, degenerating neurones.

5.4.1. Expression and Distribution of PINK1 in CGCs

In order to investigate the involvement of PINK1-mediated neuronal mitophagy, the endogenous level of PINK1 in neurones in primary CGC cultures was first determined by Western immunoblotting. PINK1 is reported to be present in multiple fragments in human and rodent brains (Beilina et al., 2005; Gandhi et al., 2006; George et al., 2010), a full-length protein (~63 kDa), and smaller fragments (~54 and ~45 kDa) (Lin & Kang, 2008; Takatori et al., 2008). The smaller fragments of PINK1 result from mitochondrial import and cleavage of the N-terminus by PARL (Beilina et al., 2005;
Gandhi et al., 2006). In agreement with these reports, Western immunoblotting of CGCs using PINK1 antibody (as described above) detected multiple fragments of PINK1, ~63, ~54 and ~45 kDa, confirming that endogenous PINK1 was present in CGC cultures and that the PINK1 antibody used for this study was able to detect endogenous PINK1. Since the PINK1 antibody was raised to the C-terminal domain of human PINK1 (George et al., 2010), all isoforms of PINK1 including the full length and two N-terminally cleaved fragments were recognized and showed multiple bands on Western immunoblotting.

Previous studies have reported the localization of endogenous PINK1 to be in both mitochondria and in the cytosol, in human and mammalian cell lines (Haque et al., 2008; Jin et al., 2010; Takatori et al., 2008). Full length PINK1 was considered to be constitutively localized in mitochondria due to its mitochondrial targeting sequence, however a substantial amount of endogenous PINK1 was also present in the cytosol in skin fibroblasts obtained from a PD patient and in mammalian cell lines (Haque et al., 2008). Immunocytochemistry using the PINK1 antibody revealed the distribution of PINK1 in two main patterns: diffuse cytosolic distribution, and mitochondrial localization around nuclei. The diffuse pattern of PINK1 labelling in the cell body is likely to represent the cytosolic localization of full-length and fragmented PINK1 proteins, whereas the concentrated fluorescence of PINK1 around nuclei may represent the accumulation of full length PINK1 on the surface of depolarized mitochondria (Jin et al., 2010). In line with the pattern of PINK1 distribution observed in CGC cultures, some studies have reported relatively higher expression of cleaved, mature forms of PINK1 in the cytosol in normal cells in which the cleaved PINK1 fragments were subject to rapid degradation by the proteasome (Beilina et al., 2005; Lin & Kang, 2008; Takatori et al., 2008). Immunocytochemistry using a complex II antibody revealed the localization of mitochondria around the nuclei, supporting the concept that the mobilization of PINK1 subsequent to OXPHOS inhibition could reflect mitochondrial localization. Therefore, the pattern of diffuse staining of PINK1 across the whole soma was considered to represent the distribution of all fragments of PINK1 in normal cells, whereas the concentrated fluorescence in the cell body with a clear void around the nuclei was interpreted as the stabilization of PINK1 on mitochondria. The failure to cleave full-length PINK1 in depolarized mitochondria may subsequently lead to a smaller proportion of cleaved forms of PINK1 fragments, explaining the more clearly visible void in the nuclei area (Jin et al., 2010).
5.4.2. Inhibition of OXPHOS Increased Patterns of PINK1 Redistribution in Bioenergetically Compromised CGCs

The majority of the current literature on PINK1- and Parkin-dependent mitophagy has employed global depolarization of $\Delta\Psi_m$ using protonophores as a trigger of mitophagy, wherein immortalized cell lines responded with an accumulation of PINK1 on the OMM as part of initiating mitophagy via a Parkin-dependent mechanism (Matsuda et al., 2010; Narendra et al., 2008). As reported in Chapter 4, the inhibitors of OXPHOS such as Rot, AA and KCN produced a rapid and significant dissipation of $\Delta\Psi_m$, while 3-NP treatment induced a much slower dissipation of $\Delta\Psi_m$ which may have reached a statistical significance if cells had been incubated for longer time period. In contrast, 4 h exposure to Oli failed to effect a significant reduction in $\Delta\Psi_m$. In the current study, the effects of OXPHOS inhibition on the pattern of PINK1 redistribution were investigated by observing if the $\Delta\Psi_m$ depolarization induced by inhibitors of OXPHOS could recruit PINK1 to neuronal mitochondria. An increased proportion of cells showing patterns of PINK1 redistribution was also found for CGCs treated with Rot, 3-NP, AA and KCN, indicating the injuries caused by the ETC inhibitors were sufficient to recruit PINK1 to mitochondria in primary CGCs. In contrast, CGCs treated with Oli failed to show statistically significant mobilization of PINK1. In this context, combined with the observations from Chapter 4, this distribution pattern of PINK1 in CGCs suggests the depolarization of $\Delta\Psi_m$ induced by inhibition of ETC complexes could induce stabilization of PINK1 on the mitochondrial surface in primary neurones. Interestingly, all inhibitors of ETC at their effective concentration were able to mobilize PINK1 to mitochondria following 4 h treatment, when a substantial level of cellular ATP was relatively unaffected subsequent to the 4 h drug treatment (Chapter 4). These results suggest the reduced cellular ATP level may not be a trigger of mitophagy in primary neurones.
5.4.3. Use of pH-sensitive Dual Fluorescence Reporter Revealed Signs of Mitophagy in Primary Neurones

The immunocytochemical studies using the PINK1 antibody focused on the expression and distribution of endogenous PINK1 mainly in the cellular soma. Given neurones have a highly polarized architecture with heterogeneous functions and varying energy demand in each structural compartment (Ashrafi & Schwarz, 2013; Brand & Nicholls, 2011; Hill et al., 2009), the process of mitophagy needed to be investigated in all structures including the soma, dendritic regions and axons. Transfection of CGCs with the dual-fluorescence mitophagy reporter, mt-Rosella, allowed observations of mitophagy from the whole neuronal perspective. The biochemical assembly of mt-Rosella biosensor reports the cellular location of mitochondria by its pH-insensitive RFP (DsRed.T3), while the pH-sensitive GFP (pHluorin) reports the pH of the surround by loss of its fluorescence at a lower pH (< 6.0). Therefore, monitoring the changes in green and red fluorescence allows identification of mitochondrial distribution and whether they have entered acidic lysosomal vacuoles for degradation (Rosado et al., 2008). The function of mt-Rosella biosensor as a mitophagy reporter has been demonstrated in yeast cells where the reporter successfully followed autophagic engulfment of mitochondria followed by an observation of the abolition of green fluorescence upon lysosomal entry (Rosado et al., 2008). The proteolytic cleavage of the linker between RFP and GFP of mt-Rosella was reported to be minor after transfection. However, the time taken for the maximal expression of both DsRed.T3 and pHluorin was delayed in primary neurones where differential maturation rates of the fluorescence proteins were observed after transfection into CGCs. Thus, the temporal expression of both fluorescence proteins was monitored over time (18, 20, 24, 48, 72 h post-transfection, data not shown) to determine an appropriate post-transfection timepoint when relatively equal fluorescence was expressed by both fluorescent proteins (24 h post-transfection). After exposure to OXPHOS inhibitors, the overall green fluorescence was diminished compared to the red fluorescence even in the vehicle control CGCs. Any possible interfering condition that may have affected the level of green fluorescence was ruled out by performing quantitative analyses and expressing data as a percentage of control.
One of the most noticeable changes found in neurones exposed to OXPHOS inhibitors was the change in the shape of mitochondria from thread-like filamentous shape to short granular forms which was reported to occur via fusion and fission processes in response to metabolic stresses (Chen & Chan, 2009; Westermann, 2010; Westermann, 2008; Youle & van der Bliek, 2012). The determination of the ratio of green/red fluorescence from neurones treated with ETC inhibitors provided novel insights into whether mitochondrial depolarization and stabilization of PINK1 on mitochondria were sufficient to trigger mitophagy. CGCs treated with the complex I and II inhibitors, Rot and 3-NP, respectively, showed significantly reduced green/red fluorescence ratios, indicating Rot and 3-NP treatment induced lysosomal entry of mitochondria. On the contrary, CGCs treated with AA, KCN and Oli failed to show a significant reduction in green fluorescence, indicating treatment with these drugs failed to recruit mitochondria into acidic lysosomes for mitophagy. These results were interesting particularly with AA- and KCN-treated CGCs, as AA and KCN induced a rapid depolarization of $\Delta\Psi_m$ (Chapter 4), and effected a significant redistribution of PINK1. The absence of signs of mitophagy following exposure to AA and KCN suggests depolarization of $\Delta\Psi_m$ and PINK1 recruitment may not be sufficient triggers to initiate PINK1-dependent mitophagy in primary neurones. A similar result was also shown in a study where widespread mitochondrial depolarization induced by CCCP in primary cortical neurones failed to trigger PINK1-Parkin-mediated mitophagy (Van Laar et al., 2011). Thus, recruitment of PINK1 following depolarization $\Delta\Psi_m$ may not be sufficient to initiate the progression into mitophagy and primary neurones may require other triggers to overcome a relatively high threshold to upregulate mitophagy. This scenario is not surprising given the critical roles of mitochondria in post-mitotic neurones where a mass degradation of mitochondria may be undesirable and inhibited due to their high dependence on mitochondrial OXPHOS and Ca$^{2+}$ buffering capacity (Schwarz, 2013).

Observations of fluorescent images of mt-Rosella-transfected neurones revealed the signs of mitophagy at the extreme ends of the neuritic processes. Quantititative analyses further confirmed that significantly increased proportion of mitochondria in neuritic processes of CGCs had entered acidic environment under treatment with Rot and 3-NP. Furthermore, the presence of red fluorescence, but disappearance of green fluorescence at the distal tips of neurites suggested an existence of local mitophagy in neurites. The recruitment of local mitophagy in primary neurones was also demonstrated
by Ashrafi and colleagues (2014) who observed the formation of autophagosomes in axons at the site of mitochondrial damage in cultured hippocampal neurones. They also reported the presence of significant population of lysosomes in the axons which would facilitate local engulfment of mitochondria and entry into the proteosomal cascade (Ashrafi et al., 2014). Local mitophagy is highly relevant to current knowledge on the pattern of degenerating neurones in PD patients, where the first signs of disease process begins in the distal axon and proceeds in retrograde manner (Cheng et al., 2010). Due to the low resolution power of the fluorescence microscopy, further work with a higher resolution and magnification for finer observations is required to confirm the progression of local mitophagy in the neuritic ends of CGCs.

Interestingly, bioenergetic dysfunction caused by inhibition of complex III, IV and V again failed to initiate progression into mitophagy even in the neuritic processes of CGCs. Following the earlier observations on mitochondrial bioenergetics using Seahorse XF24 analyzer (Chapter 4) where Rot and 3-NP had caused significant dysfunction in bioenergetic events, while effects of AA and KCN were less effective on CGCs, one could hypothesise that the disturbance in the overall energy metabolism could play a significant role as a trigger of mitophagy in primary neurones. Complex I (NADH dehydrogenase) and II (succinate dehydrogenase) are the only complexes directly involved in TCA cycle and regulating the redox balance of coenzymes such as NADH and FADH$_2$ (Scheffler, 2011). The redox balance of NAD$^+$ and NADH, in particular, plays an important role as an immediate controlling factor of the overall glucose metabolism via glycolysis and the TCA cycle (Scheffler, 2011), and require a steady supply of NAD$^+$ to allow a forward reaction. Therefore, NADH must be continuously oxidized back to NAD$^+$ via oxidation steps in complex I (Fig. 5.14). The failure of the redox balance of NAD$^+$ and NADH due to inhibition of complex I results in an accumulation of NADH above the threshold, which strongly inhibits metabolic reactions catalysed by pyruvate dehydrogenase and $\alpha$-ketoglutarate dehydrogenase, eventually inhibiting the whole TCA cycle (Scheffler, 2011). Similarly, 3-NP as an irreversible inhibitor of complex II, succinate dehydrogenase, of the TCA cycle, alters the redox balance of FAD$^+$/FADH$_2$ ratio. As a result, the overall TCA cycle is blocked due to the inhibition of reactions that are catalysed by succinate dehydrogenase (Scheffler, 2011) (Fig. 5.15). More precise determination of dysfunction of which components of bioenergetic and metabolic steps triggered mitophagy would require a further
investigation, however, the current study suggests involvement of TCA cycle and/or the regulation redox balance could be strong triggers of mitophagy in primary neurones.

5.4.4. Use of CCCP as a Trigger of Mitophagy in Primary Neurones

CCCP is the most commonly employed reagent for triggering mitophagy in current research field (Chan et al., 2011; Narendra et al., 2008; Narendra et al., 2010; Tanaka et al., 2010). In the present work on primary neurones, the proposition was tested using the mt-Rosella reporter, whether CCCP-mediated depolarization of IMM was also a trigger of mitophagy in CGCs. However, as explained below, it is not possible to use this reporter when CCCP is the administered “mitophagic trigger”.

Surprisingly, in contrast to neurones treated with Rot or 3-NP, neurones exposed to CCCP showed a higher fluorescence in green compared to red fluorescence. This suggests, somewhat counterintuitively, that the pH of the mitochondrial milieu may have increased and caused fluorescence of pHluorin protein to emit an intense green fluorescence in response to the increased pH. CCCP is a nonspecific chemical protonophore which can produce a global depolarization of all organelles possessing membrane potential primarily generated by proton flux, such as mitochondria and lysosomes. Hence lysosomes with a low pH lose their acidity due to the CCCP-induced free flow of H⁺ across the membrane and consequent loss of their degrading properties. Such interference of CCCP with lysosomal function has in fact been reported by Padman and colleagues (2013). The use of CCCP therefore equilibrated the pH across all intracellular compartments with the extracellular medium, causing dissipation of the pH gradient of the lysosomal membrane, preventing lysosomal acidification and subsequent lysosomal degradation (Padman et al., 2013). Even though mitochondria may have been launched along the mitophagy pathway by CCCP, using the mt-Rosella reporter and their delivery to lysosomes is impossible to monitor in the presence of CCCP.
The redox balance of NAD\(^+\) and NADH plays an important role as an immediate controlling factor of the overall glucose metabolism via glycolysis and the TCA cycle. The final accounting of all the reactions involved in TCA cycle, with the inclusion of the pyruvate dehydrogenase reactions, requires a steady supply of NAD\(^+\) to allow forward reaction. Therefore, NADH must be continuously oxidized back to NAD\(^+\) via oxidation steps in complex I of the ETC (Scheffler, 2011).
Pyruvate + Pi + GDP + 4NAD\(^+\) + FAD \rightarrow 3\text{CO}_2 + 4\text{NADH} + \text{FADH}_2 + \text{GTP}
Pyruvate (Pyr) supplied by glycolysis is oxidized by the mitochondrial TCA cycle. Regulation of cellular redox balance is achieved by exporting excess NADH and FADH₂ as electrons shuttle to complex I and II of the ETC, respectively, where NADH and FADH₂ are continuously oxidized back to NAD⁺ and FAD, respectively. Increased level of cellular NADH negatively regulates reactions in TCA cycle. The failure of the redox balance of NAD⁺ and NADH due to inhibition of complex I, and FADH₂ and FAD by inhibition of complex II lead to an inhibition of metabolic reactions in the TCA cycle (adapted from Scheffler, 2011).


5.4.5. Concluding Remarks

The effects of OXPHOS inhibitors on the stabilization of PINK1 in mitochondria and acidification of mitochondria were observed in primary CGC culture using fluorescent imaging approaches. Whilst depolarization of ∆Ψ<sub>m</sub> was sufficient to induce redistribution of PINK1, the trigger was suggested to be under the threshold for recruitment of mitophagy. Agents such as AA and KCN that led to a rapid depolarization of ∆Ψ<sub>m</sub> failed to show signs of mitophagy in CGCs. However an application of 3-NP, which induced relatively slower loss of ∆Ψ<sub>m</sub>, showed significant signs of mitophagy. These results demonstrate that ∆Ψ<sub>m</sub> loss is not a necessary condition for induction of mitophagy in primary neurones. It is premature to propose specific mechanism for induction of mitophagy on the basis of the present findings. It is likely that elements of metabolic dysfunction are involved in triggering mitophagy. Some relevant aspects will be considered in more detail in Chapter 7 General Discussion.

The mitophagic cascade is initiated by the recruitment of PINK1 to mitochondria, and completed by the recruitment of general autophagic and lysosomal machineries. Therefore, the following chapter will examine the recruitment of general autophagic mechanisms in CGCs to complement the initiation of mitophagic cascade observed in this chapter.
CHAPTER SIX

Recruitment of General Autophagy
In Primary Neurones Subsequent to
Bioenergetic Dysfunction
6.1. Introduction

6.1.1. Regulation of Autophagy in Mammalian Cells

Autophagy is an intracellular degradation system through which cytoplasmic components are degraded via a lysosomal-mediated process to provide substrates for energy generation or protein synthesis (Cuervo, 2004; Klionsky, 2007; Levine & Klionsky, 2004; Mizushima & Klionsky, 2007; Shintani & Klionsky, 2004). Despite the simplicity of this definition, great complexity of autophagy regulation has been revealed in mammalian cells where the molecular signalling pathways were found to be cell-type and context-specific (Chu, 2006). In general, autophagy is regulated by the master regulator, mTOR, which controls cell growth and protein synthesis in response to nutrient and energy availability within the cell (Wullschleger et al., 2006). The rapamycin-sensitive mTORC1 is involved in the negative regulation of autophagy whose inhibition by deprivation of growth factors (e.g. insulin) or nutrients (e.g. amino acids), ROS and pharmacological agents such as RAP can induce catabolic processes of autophagy (Fleming et al., 2011; Hall, 2008; Liu et al., 2008; Meley et al., 2006) (Fig. 6.1). Furthermore, the activation of upstream component AMPK by global depletion of ATP due to glucose starvation or mitochondrial dysfunction, can also induce autophagy via subsequent activation and inactivation of its downstream regulators of autophagy, TSC1/2 and mTOR (Dunlop & Tee, 2013; Hay & Sonenberg, 2004; Zhao & Klionsky, 2011). Autophagy can also be activated via mTOR-independent pathway by compounds such as ceramide, trehalose and sphingosine, or intracellular levels of cAMP and Ca^{2+} levels, but their mechanisms of autophagosome biogenesis still remain elusive (Ravikumar et al., 2009; Zhang et al., 2007).

Autophagy is regarded as a vital system for neuronal survival as it is the only mechanism through which organelles are turned over in long-lived cells such as neurones
Neurones differ from most other types of cells as they depend almost exclusively on glucose to generate energy and provide carbons for protein synthesis, and the utilization of glucose in neurones is relatively independent of insulin in contrast to most other cell types in which insulin serves as a main signal of autophagy induction (Belanger et al., 2011; Chu, 2006). Furthermore, in contrast to other types of tissues, induction of autophagy was not observed in the brain during starvation (Mizushima et al., 2004), indicating regulation of autophagy is organ specific and thus mechanisms of autophagy in neurones may differ from other cells. Despite the difficulty of detection of constitutively active autophagy in healthy neurones due to the rapid clearance of autophagosomes in the CNS (Boland et al., 2008; Boland & Nixon, 2006), studies of autophagic mechanisms in neurones indicated that autophagy could be activated in an injury-dependent manner in which autophagy served as an alternative death executing mechanism (PCD-Type II) or neuroprotective mechanism (Higgins et al., 2011; Tsujimoto & Shimizu, 2005; Xue et al., 1999). Furthermore, components of autophagy contribute to the formation of autophagosomes around PINK1/Parkin-primed mitochondria to initiate the removal process during the later stage of mitophagy where dysfunctional mitochondria are specifically recognized by the general autophagic machinery, engulfed by double-membrane autophagosomes and delivered to lysosomes for complete degradation (Ding, 2010; Geisler et al., 2010).

6.1.1.1. Steps involved in autophagic flux and markers of autophagy

Inhibition of mTOR lifts the negative regulation on autophagic pathway, which occurs through sequential steps including biogenesis of autophagosomes, maturation of autophagosomes into autolysosomes, degradation of the cytoplasmic constituents and utilization of degradation products (Mizushima, 2007). The initiation step involves autophagosome formation via involvement of ULK1 complexes and multiple Atg proteins (see Section 1.5.2). The assembly of the phagophore and elongation of the double-membrane is mediated by the activation of PI3K-III, Vps34 complex and a combined action of the Atg12-Atg5-Atg16L and Atg8-PE conjugation complexes (Fleming et al., 2011; Ravikumar et al., 2009) (Fig. 6.2). Atg 8 is also known as LC3, a soluble protein with a molecular mass of approximately 18 kDa. A cytosolic form of LC3
Autophagy is negatively regulated by mTOR, a serine/threonine kinase, via the formation of the mTORC1 complex. This complex is inhibited by specific inhibitor, rapamycin (RAP), or other factors such as starvation, ROS formation, DNA damage or deprivation of trophic factors which induce autophagy. Upstream of mTOR, the pathway can be manipulated by activation of AMPK by ATP depletion or mitochondrial generated ROS, which activates TSC1/2 and thereby inhibits mTOR to activate autophagy. In addition, dual PI3K and mTOR inhibitors that specifically target class I PI3 kinases can also exert an autophagy-inducing effect (adapted from Fleming et al., 2011).
Low energy (AMP/ATP ratio)
Low insulin
Low amino acids

Rapamycin (RAP)
Starvation
ROS
DNA damage
Deprivation of trophic factors

Low ATP
Glucose
ROS

Low energy (AMP/ATP ratio)
Low insulin
Low amino acids
In mammalian cells, the ULK complex is responsible for initiation of autophagy, in response to certain signals. The formation of double-membrane autophagosome requires class III PI3K activity of the Vps34 complex. Elongation of the phagophore involves C-terminal cleavage of proLC3 by Atg4, resulting in LC3-I. LC3-I is lipidated to the form LC3-II, and added to the elongating membrane with the Atg12–Atg5–Atg16L complex via Atg7-Atg3 conjugation system. The transition from LC3-I to LC3-II is detected by Western immunoblotting technique and the detection of LC3-II on the blot is considered as an upregulation of autophagy. The membrane grows to enwrap a portion of the cytosol, forming an autophagosome. In the final step of the process, lysosomes fuse with the autophagosome, releasing lysosomal hydrolases into the interior, resulting in degradation of the vesicle contents. Detection using the autofluorescent molecule, monodansylcadaverine (MDC), which specifically accumulates in the acidic autolysosomes, can also indicate an upregulation of autophagic flux (adapted from Fleming et al., 2011).
(LC3-I) is conjugated to PE to form LC3-PE conjugate (LC3-II), which is recruited to autophagosomal double-membranes during the phagophore elongation phase (Tanida et al., 2008). As the phagophore elongates to form an autophagosome, a portion of cytoplasm containing proteins or specific organelles is engulfed into the autophagosomes, forming a characteristic double-membrane vesicle containing cytoplasmic constituents destined to be degraded in lysosomes (Ravikumar et al., 2009). LC3-II remains as a main constituent of the inner- and outer-autophagosomal membranes until the autophagosome is fully formed. LC3-II on the outer membrane is recycled after delipidation by Atg4, while LC3-II located on the inner membrane is subsequently degraded following fusion with lysosomes (Mizushima et al., 2010). The autophagic pathway is completed by the degradation of cargo within autolysosomes, and subsequent recycling of the resultant molecules for energy production or synthesis of new macromolecules (Klionsky et al., 2008). The dynamic processes involving initiation and maturation of the autophagosomes, and subsequent fusion with lysosomes for cargo degradation are collectively termed autophagic flux (Mizushima et al., 2010).

As autophagy is a tightly regulated process that occurs in distinctive steps, monitoring of autophagic flux is made possible by assessment of the specific biochemical characteristics of each step of the autophagic flux (Mizushima, 2007). Due to the specific property of LC3-II, which specifically associates with autophagosomes, the detection of LC3-II has been widely used as a reliable marker for monitoring the upregulation of autophagic activity (Klionsky et al., 2008; Tanida et al., 2008). Thus, the detection of an increased level of LC3-II via Western immunoblotting technique can be interpreted as an increased formation of autophagosomes (Klionsky et al., 2008) (Fig. 6.2). The use of LC3-II as a maker of the early stage of autophagy needs to be complemented by assays that assess the later steps of autophagy to estimate the overall autophagic flux. Therefore, assays involving acidotropic dyes, which accumulate in the late-stage autophagic compartments, are preferred to complement the data obtained from the early stage markers. Monodansylcadaverine (MDC) is an autofluorescent acidotropic dye which accumulates in the late-stage autophagic compartments, particularly in autolysosomes (Klionsky et al., 2012) (Fig. 6.2). Monitoring of the pattern of MDC labelling under fluorescence microscopy provides an insight on the later phase of autophagic flux.
6.1.1.2. Specific recruitment of general autophagic machinery to dysfunctional mitochondria for mediation of the later stages of mitophagy

Mitophagy involves distinctive steps that are guided by molecular interactions of various proteins, including the autophagic machinery. The intermediate step that leads to an engulfment of mitochondria in double-membrane autophagosomes involves the recruitment of general autophagic machineries with proximity to the PINK1/Parkin-primed mitochondria, which are eventually degraded (Geisler et al., 2010). More specifically, subsequent to PINK1/Parkin-mediated recognition of dysfunctional mitochondria and ubiquitination of OMM proteins, the general autophagic machinery is recruited to mitochondria via autophagic adaptor proteins, such as p62/SQSTM1, to complete the lysosomal degradation of mitochondria (Chan et al., 2011; Gegg et al., 2010; Geisler et al., 2010; Matsumoto et al., 2015; Poole et al., 2010; Tanaka et al., 2010; Yoshii et al., 2011) (Fig. 6.3). The adaptor proteins contain a LC3-interacting region (LIR) allowing an interaction with LC3 proteins for efficient incorporation of damaged mitochondria into autophagosomes (Ichimura et al., 2008; Pankiv et al., 2007). Therefore, autophagosomes containing damaged mitochondria fuse with lysosomes at the later stage of mitophagy for complete degradation of mitochondria (Ding, 2010; Geisler et al., 2010; Johansen & Lamark, 2011; Yan et al., 2013) (Fig. 6.3).

6.1.2. Aims for Chapter 6

As shown in Chapter 5, Rot- and 3-NP-induced bioenergetic dysfunction of mitochondria led to induction of mitophagic-related processes in primary CGCs. The aim of Chapter 6 was to evaluate recruitment of general autophagy components in CGCs by employing markers of autophagic flux in response to the same induction of bioenergetic dysfunction using OXPHOS inhibitors (Chapter 5). The early stage of autophagic flux was monitored using antibodies to the main component of autophagosomes, LC3, whose detected level of intensity by Western immunoblotting reveals the upregulation of
Depolarized mitochondria are marked with a polyubiquitin chain that is conjugated by specific ubiquitin E3 ligases including Parkin. Unphosphorylated p62 proteins, which exist abundantly in cytoplasm, bind to the K63-linked polyubiquitin-conjugated mitochondria. Optineurin (OPTN) recruits TANK-binding kinase 1 (TBK1) to the ubiquitin-coated waste, and then TBK1 gets activated through trans- autophosphorylation. The active TBK1 phosphorylates p62 at S403, and the S403-phosphorylated p62 can tether phagophores to the autophagic cargo, allowing efficient autophagosome development which fuse with lysosomes at the later stage of mitophagy (adapted from Matsumoto et al., 2015).
autophagosomal formation. Monitoring of the late-phase of autophagic flux was performed by employing the autofluorescent acidotropic dye MDC, which accumulates mainly in acidic autolysosomes and therefore reports the increased fusion activities of autophagosome to lysosomes, to complement the results obtained from the LC3 Western immunoblotting data.
6.2. Methods

6.2.1. Primary CGC Culture, Growth and Maintenance

Primary cultures of CGCs were prepared from 6-8 day-old Swiss White mice according to Section 2.2.2. Briefly, cerebella were dissected in ice-cold isolating solution (HBSS pH 7.4 containing 3 mg/ml BSA, 1.16 mM MgSO4, 1 mM sodium pyruvate, 10 mM HEPES, 7.6 mM D-Glucose) and the meninges were carefully removed. The isolated cerebella were placed in fresh ice cold isolating solution and they were chemically and mechanically dissociated and centrifuged. The cells were resuspended in NBM containing B-27 supplement (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum. CGCs for Western immunoblotting for LC3 were seeded in 6-well plates (2 x 10^6 cells/well) and CGCs prepared for fluorescence study for MDC were seeded in 48-well plates (0.2 x 10^6 cells/well). All multi-well plates were pre-coated with poly-D-lysine (50 µg/ml). Neurones were left to grow in NBM containing B-27 components (2 % v/v), 25 mM KCl, 500 µM L-glutamine, 100 U/ml penicillin-streptomycin and 10 % fetal bovine serum for 24 h in a humidified incubator (5 % CO2, 8.5 % O2 and N2 at 37°C). Medium was fully replaced by NBM containing aphidicolin (1 µg/ml) to restrain non-neuronal cell proliferation (Diwakarla et al., 2009b; Giardina et al., 1998), and final concentration of 3 % (v/v) B-27 supplement. Half medium changes were conducted at 4 DIV and included aphidicolin (1 µg/ml) and 3 % of B-27 supplement and all experiments were performed at 7 DIV.
6.2.2. Visualization of Autophagic Vesicles using Monodansylcadaverine (MDC)

6.2.2.1. Exposure of CGC cultures to insults

Cells were plated and grown in 48-well plates and treated with drugs for 4 h according to the Section 2.2.2 and 3.2.3.1. Briefly, cells were exposed to MEM containing specific concentrations of insults, RAP 20 µM, Rot 10 nM, 3-NP 300 µM, AA 10 nM, KCN 100 µM or CCCP 10 µM for 4 h (Table 6.1). Controls were also included using MEM containing 25 mM of KCl, and cells were left in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C) for the appropriate time intervals.

6.2.2.2. Monitoring of Autophagic Flux using MDC

The stock solution of MDC was made fresh for all experiments according to the manufacturer’s recommendation. At the cessation of drug treatment, medium was replaced by pre-warmed sterile PBS (pH 7.4) containing 50 µM MDC and cells were incubated for 10 min in a humidified incubator (5 % CO₂, 8.5 % O₂ and N₂ at 37°C) (Biederbick et al., 1995; Munafo & Colombo, 2001). After incubation, cells were washed four times with sterile pre-warmed PBS. Preliminary experiments revealed an extreme light sensitivity of MDC, which was photobleached quickly (not shown), therefore all procedures involving MDC were performed in the dark. Cells were observed under a fluorescence microscope (excitation wavelength 380 nm, emission filter 525 nm) and pictures were taken immediately.
6.2.2.3. Semi-quantitative analyses of MDC-labelled CGCs for determination of late-stage autophagic flux

The autophagic index was determined by counting cells based on their patterns of MDC-labelling. Phase contrast images were concurrently taken to count the total number of neurones present in the area of interest. The MDC-labelled cells were manually counted and scored into three different groups, those displaying a diffuse fluorescence in the cytoplasm with a minor number of labelled puncta, cells displaying intense granular structures in the cytoplasm, and the group of neurones which were not labelled with MDC, presumably going through PCD. The experiment was repeated twice, and the percentage of the total number of neurones counted displaying diffuse or granular labelling of MDC for each image presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's post hoc test using GraphPad Prism v.4.0. (San Diego, CA, USA) to determine significant differences between groups and among treatments. A P value < 0.05 was considered statistically significant.
6.3. Results

6.3.1. The Endogenous Level of Autophagy in Primary CGCs

6.3.1.1. Detection of the endogenous level of autophagy in CGCs by Western immunoblotting

Previous studies have already shown the presence of autophagic machineries in rodent and human brain samples, and the conversion process of LC3-I to LC3-II was shown to be tissue- and cell type-specific (Klionsky et al., 2012; Mizushima, 2004; Tanida et al., 2005). Therefore, an essential baseline observation was required to confirm the presence of endogenous autophagy in primary cultured CGCs. LC3-I was reported to be more labile than LC3-II especially during the processing of samples in SDS-containing sample buffer and prone to degradation during freezing-thawing processes (Klionsky et al., 2012), therefore, the level of LC3-II was compared with the housekeeping protein, β-actin, rather than determining the ratio of LC3-II to LC3-I. In this study, increased intensity, as detected by Western immunoblotting, of a band at 16 kDa was considered as indicative of the initiation or upregulation of autophagy arising from the lipidation of LC3-I to LC3-II.

Hence, CGCs were exposed to a well-known autophagy inducer, RAP, in an attempt to observe the upregulation of mTORC1-mediated autophagy. The concentration of RAP used for this study (20 µM) was determined from MTT cell viability assays and concentration-dependent morphological changes of neurones observed using phase contrast microscopy (Appendix 3). CGCs were treated with RAP 20 µM for 1, 2, 4, 6, 8, 12 and 24 h, and the subsequent level of LC3-II was investigated using Western
immunoblotting. PVDF membrane was probed with an anti-LC3 antibody which recognized the soluble form of LC3 (LC3-I; 18 kDa) and the membrane-bound form of LC3 (LC3-II; 16 kDa) (Fig. 6.4). Immunoblotting of the untreated CGC lysates revealed two faint bands at the molecular weights of 18 and 16 kDa, equivalent to LC3-I and LC3-II, respectively. In contrast, exposure of CGCs to RAP 20 µM for 1-24 h produced a time-dependent increase in the intensity of the LC3-II band (Fig. 6.4). The increase in the LC3-II band appeared to be maintained even up to the longest time of 24 h (Fig. 6.4). The intensity of the LC3-I band increased slightly after 1 and 2 h of drug exposure, however its intensity did not further increase in the remaining drug-treated CGC samples. The increased LC3-II band intensity at 16 kDa indicated the presence of mTOR-dependent autophagic machinery in primary CGCs cultures which was upregulated by RAP treatment.

6.3.1.2. Injury-dependent involvement of autophagy in primary CGCs

The physiological functions of autophagy as a cell death mechanism contributing to the PCD type II autophagic cell death, or as a protective mechanism via autophagic clearance of damaged organelles/misfolded protein aggregates in neurodegenerative diseases have been a topic of much investigation (Higgins et al., 2011; Komatsu et al., 2006; Nixon et al., 2005; Ravikumar et al., 2002). The injury-dependent roles of autophagy have been reported in primary neurones as either an alternative executor of cell death in response to the inhibition of PCD type I, apoptotic cell death, or as a neuroprotective mechanism that prevented death of neurones under oxidative stress (Higgins et al., 2011). In order to investigate and determine if autophagic activities in CGCs can also be upregulated in response to different injuries, CGCs were exposed to RAP 20 µM (6 h), STS 250 nM (24 h) and H$_2$O$_2$ 30 µM (12 h) and the cell lysates collected after drug treatment. The duration of drug treatment chosen to induce neuronal injury was determined from observations from phase contrast microscopy and cell viability MTT assays (RAP, Appendix 3; STS and H$_2$O$_2$, Chapter 3). Immunoblotting of the cell lysates of untreated CGCs revealed two bands equivalent to LC3-I and LC3-II at 18 and 16 kDa, respectively (Fig. 6.5). As expected, RAP-treated CGCs displayed an increased intensity of LC3-II band, which was also displayed by CGCs treated with STS.
Figure 6.4. Upregulation of endogenous level of LC3-II in CGCs in response to RAP

Representative immunoblot for detection of LC3-I and LC3-II in cell lysates isolated from CGCs exposed to RAP 20 µM for 1, 2, 4, 6, 8, 12 and 24 h. The anti-LC3 antibody recognized both soluble form of LC3 (LC3-I; 18 kDa) and membrane-bound form of LC3 (LC3-II; 16 kDa). Increases in LC3-II band intensity were interpreted as an increased formation of autophagosomes and autolysosomes during autophagy. RAP 20 µM induced time-dependent upregulation of autophagy in CGCs as indicated by an increased density of LC3-II bands at 16 kDa. Anti-β-actin (42 kDa) was employed to ensure equal loading of samples. Data are from single experiment.
Figure 6.5. Increased autophagic flux was displayed in CGCs exposed to RAP, STS and H$_2$O$_2$

(A) Representative Western immunoblot showing detection of LC3-I and LC3-II in cell lysates isolated from CGCs treated with MEM (Control; Cont) or MEM containing RAP 20 µM (6 h), STS 250 nM (24 h) or H$_2$O$_2$ 30 µM (12 h). Anti-LC3 antibody recognized both conformational variants of LC3, LC3-I (18 kDa) and LC3-II (16 kDa), from CGC lysates. Anti-β-actin (42 kDa) was used to ensure equal loading of samples. The blot is a representative of four independent experiment which showed similar results. (B) Quantitative analysis of fold increase of LC3-II relative to β-actin. Data represent the mean ± SEM of four independent experiments. Data failed to reach significance (P < 0.05) because of variable findings with STS. (C) Due to the large SEM, normal distribution was not assumed for these data, therefore nonparametric Kruskal-Wallis analysis was used with the Dunn’s test for post hoc comparisons. However statistical significance was not found for the results.
(A) Western blot analysis showing the expression of LC3-I, LC3-II, and β-actin. The proteins were stained with antibodies specific for each marker.

(B) Bar graph showing the fold increase of LC3-II relative to control. The fold increase is calculated as the ratio of the mean intensity of the band in each condition to the mean intensity of the band in the control condition. The values are normalized to the control and represented as mean ± SEM. The fold increase in LC3-II expression is significantly increased in the RAP and STS groups compared to the control.

(C) Ranks of the LC3-II expression in different conditions. The ranks are calculated using the Wilcoxon rank-sum test. The ranks are significantly different between the control and treated groups.
A minor increase in the level of LC3-II was also observed following H$_2$O$_2$ treatment.

The conversion of LC3-I to LC3-II, which occurs during autophagic vesicle formation, was quantified to confirm an increased autophagic activity. Changes in LC3-II levels were determined by quantifying LC3-II relative to β-actin by densitometry. RAP produced reproducible increases in LC3-II across all independent experiments (2.9 ± 0.4 fold increase relative to β-actin) (Fig. 6.5B). Although LC3-II was elevated in each experiment with STS (3.1 ± 1.5 fold increase relative to β-actin) the data showed appreciable variability and prevented the changes reaching statistical significance. Due to the large SEM detected in the STS data, normal distribution was not assumed for these data; therefore nonparametric Kruskal-Wallis analysis was performed with the Dunn’s test for post hoc comparisons. However statistical significance was not found across the various experimental groups. There were relatively minor changes observed in LC3-II expression with H$_2$O$_2$ (1.4 ± 0.47 fold increase relative to β-actin). Despite the variability in the measured increases in LC3-II band intensity, the results confirmed that autophagic mechanisms in CGC culture systems could be stimulated and manipulated in response to various drugs as previously observed in other studies.

6.3.2. Upregulation of Autophagy in Bioenergetically Compromised CGCs

6.3.2.1. Increased autophagic activity was observed in CGCs subjected to bioenergetic dysfunction induced by ETC inhibitors.

Following the observations from Chapter 5 where significant mitophagic activity was observed in response to Rot- and 3-NP-induced bioenergetic dysfunction, the later
stage of mitophagy, which involves general autophagic machineries, was investigated by monitoring the conversion of LC3-I to LC3-II after drug treatment. CGCs at DIV7 were exposed to lower and higher concentrations of ETC inhibitors (Rot 10 nM & 30 nM; 3-NP 100 µM & 300 µM; AA 10 nM & 30 nM and KCN 100 µM & 300 µM) for 4 h and 24 h, and the subsequent lipidation of LC3-I to LC3-II was monitored by Western immunoblotting. The results showed that there was very minor or no apparent increases in LC3-II level after 4 h incubation with ETC inhibitors (Fig. 6.6A). At the longer treatment time, the control cell lysates showed a small increase in LC3-II perhaps attributable to some minor decreases in cellular viability in the presence of the less supportive MEM medium. 24 h exposure to drugs generally increased levels of LC3-II, notably with treatments at the higher concentrations of Rot, 3-NP and KCN, which all induced prominent increases in LC3-II levels (Fig. 6.6B, Fig. 6.7). Both high and low concentrations of AA failed to induce increases in LC3-II intensity (Fig. 6.6B). These results indicate that the formation of autophagosomes increased to varying degrees in response to the different ETC inhibitors, reflecting the differential upregulation of autophagy under bioenergetic dysfunction. The immunoblots were re-probed with anti-β-actin antibody and densitometry was performed for quantitative analysis. The fold increase of LC3-II intensity relative to β-actin was compared to the level of LC3-II in control (Fig. 6.7). There was a significant time-dependent change across drug treatments (P < 0.0001) with overall expression of LC3-II being increased at 24 h relative to observations at 4 h. A significant increase in autophagic activity was observed in CGCs treated with 3-NP 300 µM for 24 h (2.9 ± 1.1 fold increase relative to β-actin), which was significantly higher than the level of LC3-II observed at 4 h (P < 0.01). For all other treatments, although overall data indicated population differences after 4 h versus 24 h treatment, individual differences were not found. Thus the level of LC3-II was elevated after 24 h drug treatment in each experiment, but variability observed from some results prevented the increase in LC3-II level from reaching statistical significance compared to control when analysed by two-way ANOVA and Bonferroni's post hoc test.
Figure 6.6. Autophagic flux was increased in CGCs exposed to high and low concentrations of Rot, 3-NP, AA and KCN for 4 h and 24 h

Representative Western immunoblots showing detection of LC3-I and LC3-II in cell lysates isolated from CGCs treated with MEM (Control; Cont) or MEM containing Rot 10 nM, 30 nM, 3-NP 100 µM, 300 µM, AA 10 nM, 30 nM or KCN 100 µM, 300 µM for (A) 4 h and (B) 24 h. Anti-LC3 antibody recognized both conformational variants of LC3, LC3-I (18 kDa) and LC3-II (16 kDa), from CGC lysates. Longer exposure to drugs induced an increased conversion of LC3-I to LC3-II, particularly after Rot 30 nM, 3-NP 300 µM and KCN 300 µM treatment. Anti-β-actin (42 kDa) was used to ensure equal loading of samples. Data are representative of 3-4 independent experiments.
(A) Cont

<table>
<thead>
<tr>
<th></th>
<th>Rot</th>
<th>3-NP</th>
<th>AA</th>
<th>KCN</th>
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<tr>
<td>10 nM</td>
<td>30 nM</td>
<td>100 µM</td>
<td>300 µM</td>
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LC3-I 18 kDa

LC3-II 16 kDa

β-actin 42 kDa

(B) Cont

<table>
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<tr>
<th></th>
<th>Rot</th>
<th>3-NP</th>
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<td>10 nM</td>
<td>30 nM</td>
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LC3-I 18 kDa

LC3-II 16 kDa

β-actin 42 kDa
Figure 6.7. Autophagic flux was increased in CGCs exposed to high and low concentrations of Rot, 3-NP, AA and KCN for 4 h and 24 h

Quantitative analysis of increases of LC3-II relative to β-actin. Data represent the mean ± SEM of 3-4 independent experiments. Two-way ANOVA followed by Bonferroni's post hoc test revealed a significant time-dependent change across drug treatments (## P < 0.0001) with overall expression of LC3-II being increased at 24 h relative to observations at 4 h. Treatment with 3-NP 300 µM for 24 h (**) P < 0.01) was significantly higher than the level of LC3-II observed at 4 h.
6.3.2.2. Inhibition of complex V, $F_1F_0$ ATP synthase, by Oli failed to induce autophagic activity in CGCs.

Oli is an inhibitor of $H^+$ translocation in $F_1F_0$ ATP synthase, complex V, and its application significantly reduces electron flow through the ETC (Devenish et al., 2000), resulting in the failure of mitochondrial ATP generation. ATP deprivation can be responsible for various manifestations of death. Cells may overcome ATP depletion by autophagy (Baehrecke, 2003) and the expression of general autophagy in response to the blocking mitochondrial ATP generation was investigated. Due to the lack of data showing a concentration-dependent response for Oli-induced injury in CGCs, three concentrations of Oli (1, 0.3 and 0.1 µM), were chosen based on the time-dependent reduction in cell viability in response to Oli (Chapter 3), reduction in ATP level (Chapter 4) and results of experiments using the Seahorse XF24 (Chapter 5). Although the drop of cellular ATP level did not reach statistical significance after 24 h treatment with Oli 1 µM (Fig. 4.9), the time-dependent cell viability results showed ~70 % reduction in cell viability when CGCs were exposed to Oli at 1 µM for 24 h (Fig. 3.12). Furthermore, the concentration of Oli used for the Seahorse XF24 assay experiments was 1 µM (refer to Section 4.3.3.1), which caused almost 80 % inhibition of mitochondrial respiration at rest (Fig. 4.19A). Therefore, Oli at 1 µM was chosen as the highest concentration to be used for the subsequent experiments.

Following exposure of CGCs to different concentrations of Oli for 4 and 24 h there were no apparent signs of upregulation of autophagy as reflected by formation of LC3-II (Fig. 6.8). Quantitative analysis using densitometry showed Oli failed to induce significant time- or concentration-dependent effects on autophagic flux compared to control (Fig. 6.9). These results were unexpected because the increase in ratio of AMP/ATP is detected by AMPK which triggers autophagy in mammalian cells (Mihaylova & Shaw, 2011). Future experiments involving the blockage of autophagosome-lysosome fusion step by increasing the pH of lysosomes or inhibition of lysosome-mediated proteolysis could be performed to allow accumulation of the cargo in a recognizable early state to observe the autophagic processes (Klionsky et al., 2012).
6.3.2.3. Treatment with CCCP and autophagic activity in CGCs.

As mentioned earlier in the Chapter 5, CCCP has been widely used as a trigger of mitophagy. However in this study, monitoring of mitophagy using the Rosella construct showed CCCP failed to detect mitophagy in primary neurones. In order to investigate the previous observations relative to mitophagy, the late stage of mitophagy was further investigated by monitoring the formation of LC3-II using Western immunoblotting. CCCP 10 µM was previously used in the study of $\Delta W_m$ (Chapter 4), lower concentrations were also employed due to the cytotoxicity of CCCP. Exposure to CCCP at 1 µM, 3 µM and 10 µM for 0.5 h and 4 h induced only minor changes in formation of LC3-II in CGCs (Fig. 6.10). Whilst densitometry of the blots detected changes in the level of LC3-II (Fig. 6.11), these were inconsistent with post hoc analyses demonstrating an absence of concentration- and time-dependent effects.

6.3.3. Monitoring Formation of Autophagic Vesicles by Monodansylcadaverine (MDC)

Monodansylcadaverine (MDC) is an autofluorescent molecule which is known to accumulate in acidic vesicles, in particularly in autolysosomes, due to its interaction with membrane lipids of autophagic vesicles (Biederbick et al., 1995; Munafo & Colombo, 2001; Niemann et al., 2000). Therefore, the majority of vesicles labelled with MDC are considered as the autolysomes, which form during the later stages of autophagic flux, but not the initial autophagic vesicle such as the early autophagosomes (Klionsky et al., 2012). To provide further insight into the changes of the later stage of autophagic flux in response to inhibition of ETC, MDC dye was employed to label the autophagic vesicles and the level of labelling monitored in bioenergetically compromised CGCs.
Figure 6.8. Autophagic flux was not affected by 4 h and 24 h treatment with Oli in primary CGCs

Representative Western immunoblot showing detection of LC3-I and LC3-II in CGCs treated with Oli 1 μM, 0.3 μM and 0.1 μM for 4 h and 24 h. CGC lysates were subjected to Western immunoblot analysis using anti-LC3 or anti-β-actin antibodies. Anti-LC3 antibody recognized both conformational variants of LC3, LC3-I (18 kDa) and LC3-II (16 kDa). Although strong LC3-I bands were detected for all drug-concentrations, there was no apparent increase in LC3-II bands. Blot is a representative of 3 independent experiments which detected similar results. Anti-β-actin (42 kDa) was employed to ensure equal loading of samples.
Figure 6.9. Quantitative analysis of increases of LC3-II showed autophagic flux was unaffected in CGCs exposed to Oli for 4 h and 24 h

Expression of LC3-II was quantified using densitometry analysis by determining LC3-II expression relative to that of β-actin. Data represent the mean ± SEM of three independent experiments. Two-way ANOVA revealed Oli failed to induce significant time- and concentration-dependent effects on autophagic flux compared to control.
Figure 6.10. Exposure to CCCP for 0.5 h and 4 h failed to induce upregulation of autophagic flux in CGCs

Representative Western immunoblot showing detection of LC3-I and LC3-II in CGC lysates exposed to CCCP 1 µM, 3 µM and 10 µM for 0.5 h and 4 h. The anti-LC3 antibody recognized both conformational variants of LC3, LC3-I (18 kDa) and LC3-II (16 kDa), from CGC lysates. Anti-β-actin (42 kDa) was employed to ensure equal loading of samples. Strong LC3-I bands were detected throughout the concentration range, however there was no apparent increase in LC3-II bands. Blot is a representative of 4 independent experiment where very similar results were obtained.
Figure 6.11. Autophagic flux in CGCs exposed to CCCP for 0.5 h and 4 h

Expression of LC3-II was quantified using densitometry analysis by determining LC3-II expression relative to that of β-actin. Data represent the mean ± SEM of four independent experiments. Two-way ANOVA revealed an overall effect of CCCP concentration (* P < 0.05), but post hoc analyses failed to show individual time- and concentration-dependent effects on autophagic flux compared to control.
Fold increase of LC3-II relative to control

Control 1 μM 3 μM 10 μM

0.5 h 4 h

*
6.3.3.1. Qualitative observation of autophagic vesicles in CGCs exposed to an autophagy inducer, RAP, using fluorescence imaging of MDC

Before proceeding to the observation of autophagic flux induced by OXPHOS inhibitors, CGCs were first exposed to the autophagy inducer, RAP, to observe and confirm the pattern of MDC labelling in response to a known inducer of autophagy. CGCs were exposed to RAP 20 µM for 4 h, followed by 10 min incubation with MDC 50 µM at 37°C (Munafo & Colombo, 2001). The accumulation of MDC in acidic vesicles was immediately visualized using fluorescence microscopy. Under these conditions MDC labelled all neurones except a few cells undergoing PCD, which displayed fragmented morphology when observed by phase contrast microscopy. Differential patterns of MDC labelling were displayed by CGCs (Fig. 6.12), where the majority of control neurones displayed a diffuse fluorescence in the cytoplasm with a minor number of puncta showing labelling (Fig. 6.12A), whereas following RAP treatment an increased number of neurones exhibited intense granular structures in the cytoplasm presumably representing MDC-labelled autophagic vesicles (Fig. 6.12B). These results indicated that RAP-induced autophagy was detectable by the increase in MDC-labelled puncta. In addition, intensely fluorescent puncta were also observed in the neuritic processes in both control and RAP-treated neurones, indicating the presence of autophagic vesicles.

6.3.3.2. Qualitative observation of autophagic vesicles in CGCs exposed to ETC inhibitors using fluorescence imaging of MDC

Further investigations were performed to observe changes in autophagic flux in response to 4 h exposure to inhibitors of ETC and CCCP. MDC labelling in CGCs was visualized using fluorescence microscopy, and three major patterns of MDC labelling were displayed by neuronal populations: diffuse cytosolic fluorescence, bright granular structures, and cells apparently undergoing PCD which showed no labelling. Examples of diffuse and punctate labelling can be found in micrographs of vehicle and drug-treated cells shown in Fig. 6.13. The majority of control neurones exhibited a diffuse pattern of fluorescence in the cytoplasm with occasional examples of granular labelling (Fig. 6.12A).
By comparison, CGCs exposed to ETC inhibitors, Rot 10 nM, 3-NP 300 µM, AA 10 nM and KCN 100 µM showed an increased proportion of neurones displaying intensely fluorescent puncta, indicative of an increased presence of acidic vesicles in neurones (Fig. 6.13B-E). However, CGCs exposed to CCCP 10 µM either showed no fluorescence or diffuse fluorescence labelling by MDC in the cytoplasm (Fig. 6.13F).

6.3.3.3. Semi-quantitative observation of autophagic vesicles in CGCs exposed to ETC inhibitors using fluorescence imaging of MDC

In order to confirm the microscopic observations which showed an increased labelling of autophagic vesicles in response to 4 h exposure to ETC inhibitors, semi-quantitative analysis of MDC distribution in CGCs was performed by scoring and grouping neurones into three distinct populations: cells showing diffuse fluorescence of MDC, punctate labelling in the cytosol, and cells apparently undergoing PCD that were unlabelled with MDC (Fig. 6.14). Phase contrast images were taken concurrently and were used for counting the total number of neurones in the image. The percentage of cells displaying different labelling of MDC for each drug-treated group was presented as proportion of the total number of neurones counted per drug treatment.

Analyses indicated that 68 ± 2.4 % of control CGCs showed diffuse labelling of MDC, while 24 ± 0.3 % of cells displayed an increased granular labelling of MDC (Fig. 6.14). Compared to control, CGCs treated with Rot 10 nM showed a significantly reduced proportion of neurones displaying diffuse labelling of MDC (38 ± 2.6 %; P < 0.0001 compared to control), while there was a significantly increased number of neurones containing intense punctate labelling (47 ± 2.3 %; P < 0.01 compared to control). As expected, 4 h exposure to 3-NP 300 µM also resulted in the reduction in proportion of cells with diffuse MDC labelling (43 ± 5.3 %; P < 0.001 compared to control), concomitant with an increase in neurones with punctate labelling (50 ± 3.3 %; P < 0.001 compared to control). Interestingly, the proportion of cells exposed to AA 10 nM and KCN 100 µM for 4 h also showed an increased population of neurones displaying bright granular labelling for MDC (47 ± 8.2 % and 55 ± 0.6 %, respectively; P < 0.01 compared
Figure 6.12. Detection of autophagic vesicles in CGCs exposed to RAP for 4 h with monodansylcadaverine (MDC) histochemistry

CGCs at DIV7 were exposed to (A) MEM (control) or (B) RAP (20 µM) for 4 h and medium was removed and cells were washed three times with pre-warmed PBS. 0.5 µM MDC was loaded into cells by incubation for 10 min at 37°C. Accumulation of MDC in autolysosomes in CGCs was visualized using fluorescence microscopy (right panels) and phase contrast (left panels). Neurones that displayed a pattern of diffuse cytosolic fluorescence of MDC are indicated with yellow arrows and neurones displaying bright puncta were indicated by white arrows. Scale bar = 20 µm. The upper insets show 40% magnification with respect to main image.
Figure 6.13. Detection of autophagic vesicles in CGCs exposed to Rot, 3-NP, AA, KCN or CCCP for 4 h with monodansyleadaverine (MDC) histochemistry

CGCs at DIV7 were exposed to (A) MEM (control), (B) Rot 10 nM, (C) 3-NP 300 µM, (D) AA 10 nM, (E) KCN 100 µM or (F) CCCP 10 µM for 4 h and medium was removed and cells were washed three times with pre-warmed PBS. 0.5 µM MDC was loaded into cells by incubation for 10 min at 37°C. Accumulation of MDC in autolysosomes in CGCs was visualized using fluorescence microscopy (right panel) and phase contrast (left panel). Neurones that had a pattern of diffuse cytosolic fluorescence of MDC were indicated with yellow arrows and neurones displaying bright puncta were indicated by white arrows. Scale bar = 20 µm. The upper insets show 40% magnification with respect to main image.
Figure 6.14. Semi-quantitative analysis of autophagic flux monitored by MDC histochemistry observed in CGCs exposed to Rot, 3-NP, AA and KCN for 4 h

Semi-quantitative analysis of the changes in the pattern of labelling by MDC. Data represent the mean ± SEM of two independent experiments where in each experiment typically 350-450 cells were scored over two wells for the differential patterns of MDC labelling. Neurones were ranked as displaying no, diffuse or punctate patterns of MDC labelling. Symbols indicate significant increase in proportion of cells with punctate labelling (** P < 0.01 compared to control, # P < 0.001), analysed by two-way ANOVA, followed by Bonferroni’s post hoc test). The pattern of diffuse labelling was decreased by cell treatments (### P < 0.0001).
to control), indicating an increased autophagic flux in neurones in response to AA and KCN treatment. The increase in number of MDC-positive neurones indicates an upregulation of autophagic flux in response to bioenergetic dysfunction induced by ETC inhibitors.
6.4. Discussion

Autophagy is a major cellular catabolic process that involves sequential steps involving the sequestration and transport of macromolecules and organelles to the lysosomes for degradation (Levine & Kroemer, 2008; Xie & Klionsky, 2007). The steps of autophagy are complex and its activation involves a wide range of stimuli via mTOR-dependent and -independent pathways (Ravikumar et al., 2009). Furthermore, activation of autophagy was also demonstrated in injury-dependent manner in mammalian neurones (Higgins et al., 2011). In particular, the involvement of autophagic machineries in mediating the delivery of dysfunctional mitochondria to lysosomes during the later stages of mitophagy was demonstrated in mammalian cells (Geisler et al., 2010). The work reported in this chapter was aimed at providing evidence regarding the upregulation of general autophagy in response to bioenergetic dysfunction induced by exposure to OXPHOS inhibitors.

6.4.1. General Autophagy in CGCs

Autophagy has been found to be operative in virtually all cell types and the pathway in mammalian cells has been well-characterized (Johansen & Lamark, 2011). However, the CNS appears to have a different pattern of autophagic regulation and shows little evidence of basal autophagy under normal conditions due to a rapid and tight regulation of autophagic flux (Nixon et al., 2005). Neuronal autophagy actively occurs constitutively and is considered to be an important neuroprotective mechanism by efficiently removing damaged proteins or organelles which otherwise may cause neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). In order to investigate neuronal autophagy, the endogenous level of autophagy in primary CGC culture was investigated as an initial step. Thus CGCs were exposed to RAP, which exerts an
inhibitory effect on mTORC1, to activate general autophagy (Yang & Guan, 2007; Zoncu et al., 2011). In agreement with this notion, a time-dependent increase in the level of the marker protein of autophagosomes, LC3-II, was detected by Western immunoblotting in response to treatment with RAP 20 µM, indicating the endogenous autophagic pathway could be upregulated by RAP-mediated inhibition of mTORC1. Further experiments using STS and H2O2 confirmed that autophagy in CGCs could also be regulated in injury-dependent manner, which was in agreement with previous studies that showed injury-dependent involvement of autophagy in primary cortical neurones (Higgins et al., 2011; Higgins et al., 2012). Although STS treatment induced autophagy in CGCs, oxidative stress exerted by H2O2 treatment failed to induce a significant upregulation of autophagy in CGCs, whereas previous work from our laboratory in cortical neurones showed both insults induced autophagy, suggesting neuronal autophagy maybe both cell-, insult- and concentration-dependent (Higgins et al., 2011).

6.4.2. Upregulation of Autophagosome Formation in CGCs in response to Bioenergetic Dysfunction

The changes in autophagic flux in response to bioenergetic dysfunction were investigated in primary neurones after exposure to ETC inhibitors for 4 and 24 h. Increased intensity of LC3-II detected using Western immunoblotting is widely accepted to represent the induction of the autophagosome formation during the early stage of autophagy (Klionsky et al., 2007). Here, Western immunoblotting results revealed time- and concentration-dependent upregulation of general autophagy in neurones subjected to bioenergetic dysfunction. Statistical analysis revealed a statistically significant increase in LC3-II levels subsequent to 24 h exposure to all ETC inhibitors compared to the observations made after 4 h treatment, indicating autophagy was significantly upregulated when CGCs were subjected to prolonged bioenergetic dysfunction. Notably, exposure to 3-NP 300 µM for 24 h exhibited a significant increase in LC3-II level, an observation in agreement with the induction of mitophagy in neurones exposed to 3-NP (Chapter 5), suggesting an upregulation and recruitment of autophagy as part of the mitophagic...
process. Furthermore, the 24 h exposure of CGCs to Rot 10 nM and 30 nM also showed a trend for increased autophagosome levels, also consistent with the mitophagy observed in response to Rot 10 nM treatment (Chapter 5). Other studies employing primary cortical neurones or SH-SY5Y neuroblastoma cell lines also have reported increased autophagy and mitophagy when cells were exposed to Rot or 3-NP at concentrations that caused ~40-50% of cell death (Chu et al., 2013; Mader et al., 2012; Solesio et al., 2013), which is a similar condition to that used in the current study. The increased level of LC3-II may represent increased recruitment of autophagic machineries to compromised mitochondria as an intermediate step during mitophagy. Further morphological studies allowing observations of the contents of autophagosomes that were formed in response to prolonged Rot or 3-NP treatments would complement the results reported in the current study.

A concentration-dependent increase in the level of autophagy was also observed in CGCs exposed to AA and KCN. Although the lower concentrations of AA and KCN failed to induce autophagy, Western immunoblots of CGCs exposed to the higher concentrations showed increased levels of LC3-II after 24 h drug treatment, indicating the prolonged exposure of neurones to AA and KCN at higher concentrations triggered autophagy. There is only a sparse literature investigating the effects of AA and KCN on autophagy, and one study in yeast revealed that non-lethal concentrations of AA and KCN triggered non-specific autophagy, but infrequent mitophagy (Deffieu et al., 2013), which is similar to the results observed in this study. Furthermore, the level of cytochrome b reduction as a result of ETC inhibition was suggested as the first signal in the pathway leading to the induction of autophagy in yeast (Deffieu et al., 2013). The exact mechanisms of autophagic recruitment consequent to AA- and KCN-mediated mitochondrial injury require further investigation.

Despite its significant inhibition effect on OCR, Oli 1 µM failed to induce upregulation of autophagy. This result was unexpected as Oli is known to activate AMPK, which works as an upstream trigger of general autophagy (Gleason et al., 2007). Since neurones are suggested to have a higher threshold for executing autophagy (Van Laar et al., 2011), the concentration of Oli used here may not have reached the threshold for
triggering the autophagic cascade, and it is considered that neurones are less susceptible to the occurrence of autophagy (Amadoro et al., 2014).

Due to the variability (between experiments) observed in the quantitative evaluation of general autophagy by LC3-II detection, statistical analyses failed to detect significant differences in some cases although there were apparent differences inspected by visual observation. Further insight could be obtained using another marker for autophagy induction, such as p62. p62 serves to link ubiquitinated proteins or organelles to the autophagic machinery via its direct interaction with LC3 proteins, and its degradation is dependent on autophagy (Bjørkøy et al., 2009; Geisler et al., 2010). Such experiments could complement the MDC data reported here, but were not undertaken in this work.

6.4.3. Increased numbers of Autolysosomes in response to Bioenergetic Dysfunction further indicates increased Autophagic Flux

Observation of LC3-II using Western immunoblot showed an increase in autophagy in CGCs, and these sets of data were confirmed by monitoring the accumulation of MDC in acidic vesicles in response to ETC inhibition (Klionsky et al., 2012). The majority of vesicles labelled by MDC represent autolysosomes, however some vesicles such as acidic endosomes and lysosomes are also documented to be labelled by MDC (Klionsky et al., 2012). The purpose of monitoring the accumulation of MDC was to observe the overall changes in proteasomal activities during the late stage of autophagy, hence the data were collected by semi-quantification to analyse the overall changes in the proportion of neurones showing MDC-positive cells in response to pharmacological inhibition of ETC. An increased number of neurones positive for MDC-labelled vesicles was observed in CGCs exposed to all ETC inhibitors for 4 h, indicating late-stage autophagy was upregulated in response to the direct inhibition of OXPHOS by inhibitors as early as 4 h.
The MDC labelling results suggest that not only Rot and 3-NP were involved in inducing general autophagy at the extended bioenergetic dysfunction, but also during the early hours of mitochondrial injury, suggesting a rapid response of autophagic upregulation in response to altered energy balance. In addition, an increased level of MDC-positive neurones subsequent to 4 h exposure to AA 10 nM and KCN 100 µM indicate the late stage of autophagic flux was significantly enhanced in response to bioenergetic dysfunction caused by AA and KCN at their low concentrations despite the lack of LC3-II level detected from Western immunoblotting data.

The early observation of increased autophagic flux might be explained by an upregulation of autophagy via AMPK regulation of mTOR-mediated autophagy. AMPK is as an upstream regulator of mTOR-mediated autophagy, and may play a role in upregulating general autophagy due to global energy depletion arising from direct inhibition of respiration. ATP depletion due to mitochondrial dysfunction may activate AMPK, which in turn activates TSC1/2 complexes, thereby inhibits mTOR, or activates ULK1 to stimulate autophagy (Dunlop & Tee, 2013; Zhao & Klionsky, 2011). Neurones are highly metabolic cells which need a constant supply of energy, hence it is pertinent to suggest that recognition of change in the AMP/ATP ratio may lead to an induction of a defensive mechanism against the reduction in energy level that involves induction of autophagy. Hence, although the cellular ATP levels were not significantly altered after 4 h exposure to Rot 10 nM, 3-NP 300 µM, AA 10 nM and KCN 100 µM (Chapter 4), autophagy may have been upregulated to resist the fluctuation in the energy level. In fact, constitutive activity of autophagy has been previously reported in rodent brain irrespective of nutrient conditions (Mizushima, 2004). Similar results were found in fibroblasts of patients with OXPHOS defects, where mitophagy was not observed, but bulk autophagy was clearly present (Moran et al., 2014), supporting the results presented herein.

Lastly, the different results found between MDC-labelling and the lack of detectable LC3-II bands after 4 h drug treatment could be due to the efficient clearance of autophagosomes by their rapid fusion with lysosomes for total degradation, as previously reported by other studies involving brain tissue (Boland et al., 2008; Boland & Nixon, 2006). Therefore, despite an early accumulation of autophagolysosomes, the low levels of
LC3-II observed in CGCs may represent the rapid progression of autophagy. Further studies using agents such as bafilomycin A1 or vinblastine, which block fusion of autophagosomes with lysosomes, or NH$_4$Cl which neutralizes the acidic compartment of lysosomes and prevents degradation of autophagosomes, would allow more incisive observations of early-stage autophagic flux (Klionsky et al., 2012).

### 6.4.4. Concluding Remarks

Bioenergetic dysfunction induced by direct inhibition of ETC complexes induced an upregulation of general autophagy, irrespective of the induction of mitophagy. Increased autophagic flux as indicated by an increase in MDC-positive neurones at 4 h of drug treatment indicated an early upregulation of autophagy in response to mitochondrial injury, suggesting the neuroprotective role of autophagy in sustaining the sufficient level of ATP for neuronal activities. As expected, general autophagy was upregulated subsequent to Rot and 3-NP treatment, which may represent its recruitment as part of the mitophagy processes. Exposure to complex III and IV inhibitors, AA and KCN, was sufficient to trigger general autophagy, possibly mediated by AMPK-mTOR pathways, which are triggered by the changes in cellular AMP/ATP ratio.
CHAPTER SEVEN

General Discussion
7.1. General Discussion

7.1.1. Overview of the Thesis

Mitochondria are essential organelles of the eukaryotic cell and play a key role in the generation of ATP via OXPHOS, regulation of cytosolic Ca\(^{2+}\) levels and other important metabolic pathways such as the TCA cycle (Beal, 2005; Howell et al., 2003; Scheffler, 2011). They also play decisive roles in the induction of specific death responses during PCD where the engagement of mitochondrial signalling influences the resultant pattern of cell death (Higgins et al., 2010; Nagley et al., 2010). The CNS has a high demand for energy to maintain the signalling activities of neurones which have an absolute dependence on mitochondria for generation of ATP via OXPHOS and buffering of intracellular Ca\(^{2+}\) (Ames, 2000; Erecinska et al., 2004; Mayevsky & Chance, 1975; Obel et al., 2012; Toescu et al., 2000). Given the high reliance of neurones on proper mitochondrial function, and their inherent post-mitotic and non-proliferative nature which limits their regenerative capacity, it is not surprising to find dysfunctional mitochondria as a convergence point for neurodegeneration. Indeed failures of mitochondrial quality control systems have been reported in numerous neuropathologies and especially in PD patients (Hara et al., 2006; Komatsu et al., 2006; Levine & Kroemer, 2008; Narendra et al., 2008).

Several mechanisms of cellular quality control of mitochondria have been documented, including mitophagy which involves the specific recognition of dysfunctional mitochondria and their subsequent delivery to lysosomes for eventual complete degradation. Loss of \(\Delta\Psi_m\) is routinely accepted as the trigger of mitophagy in mammalian cell lines, but in reality little is known about the molecular regulation and triggers of the mitophagic pathway in primary neurones. Recent studies employing primary neurones have delivered quite contradictory results, revealing the complexity of
neuronal mitophagy as compared to cell lines and non-neuronal cells (Cai et al., 2012; Van Laar et al., 2011). Despite $\Delta \Psi_m$ being widely accepted as the trigger of mitophagy in mammalian cells, studies using primary neurones have shown not only a loss of $\Delta \Psi_m$, but other factors such as the culture conditions or the presence of antioxidants can influence the triggering of neuronal mitophagy (Cai et al., 2012; Joselin et al., 2012; McCoy et al., 2014; Rakovic et al., 2013; Van Laar et al., 2011). These findings further highlight the complexity of neuronal mitophagy and emphasise the critical need for careful studies focusing on all aspects of its regulation.

In this study, attention was centred on the triggering of neuronal mitophagy with the focus being a detailed documentation of the bioenergetic dysfunction of mitochondrial OXPHOS by specific inhibitors together with strategic investigations of its subsequent induction of mitophagy. The primary cultures of CGCs employed not only represent an homogenous population closely resembling neurones in vivo (Kramer & Minichiello, 2010), but also the culture conditions obviate many of the confounding issues (see above) (Brewer & Cotman, 1989; Carroll et al., 1998; Cheung et al., 1998a; Diwakarla et al., 2009b; Giardina & Beart, 2001), making this system an ideal one for elucidating the molecular aspects of neuronal mitophagy. The work described herein demonstrated that $\Delta \Psi_m$ may not be a necessary trigger of neuronal mitophagy as the inhibition of mitochondrial OXPHOS complexes I and II induced significant recruitment of neuronal mitophagy, whereas the inhibition of complexes III and IV failed to induce neuronal mitophagy despite the rapid dissipation of the $\Delta \Psi_m$. Common themes that emerged as a consequence of complex I and II inhibition were alterations of the redox balance and the TCA cycle, raising the possibility that dysfunction in these components of metabolism could serve as the triggers of neuronal mitophagy. As already stated, inhibition of complexes III and IV failed to induce mitophagy, although signs of recruitment of general autophagy were present under these conditions. This study represents a timely contribution to the ongoing discussions about the triggers of neuronal mitophagy, and provides evidence for a determinant role of bioenergetic dysfunction in neuronal mitophagy (Table 7.1).
Table 7.1. Summary of bioenergetic injuries caused by inhibition of mitochondrial ETC and the subsequent recruitment of mitophagy and general autophagy

CGCs were exposed to drugs reported to inhibit mitochondrial ETC complexes I-IV (Rot, 3-NP, AA and KCN, respectively). The magnitude of bioenergetic injury was determined by the measurements of cellular ATP, OCR and $\Delta \psi_m$. Subsequent induction of mitophagy was monitored using PINK1 immunocytochemistry and mitochondrial targeted biosensor, Rosella. Significant PINK1 redistribution was observed at 4 h in CGCs exposed to all inhibitors, however mitophagy was only observed with dysfunction of complexes I and II. Signs of general autophagy induction were observed with inhibition of all ETC complexes as monitored using LC3-II levels and accumulation of MDC in acidic vesicles. The concentrations of each inhibitor used for monitoring bioenergetic indices and the subsequent observation of mitophagy (PINK1 and Rosella) and increases in acidic autophagic vesicles during the later stages of the degradation process (MDC) were as follows: Rot 10 nM, 3-NP 300 µM, AA 10 nM and KCN 100 µM. ATP and LC3-II data were gathered with higher concentrations of ETC inhibitors. Symbols represent extensive (↑↑↑, ↓↓↓), partial (↑↑, ↓↓), minor (↑, ↓) and no actions (-) in the relevant index.
<table>
<thead>
<tr>
<th></th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex III</th>
<th>Complex IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular [ATP]</td>
<td>↓ ↓ ↓</td>
<td>↓ ↓ ↓</td>
<td>↓ ↓</td>
<td>-</td>
</tr>
<tr>
<td>OCR</td>
<td>↓ ↓ ↓</td>
<td>↓ ↓ ↓</td>
<td>-</td>
<td>↓</td>
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<tr>
<td>Loss $\Delta \Psi_m$</td>
<td>↓ ↓ ↓</td>
<td>-</td>
<td>↓ ↓ ↓ ↓</td>
<td>↓ ↓ ↓ ↓</td>
</tr>
<tr>
<td>Mitochondrial Stabilization of PINK1</td>
<td>↑ ↑ ↑</td>
<td>↑ ↑ ↑</td>
<td>↑ ↑</td>
<td>↑ ↑ ↑</td>
</tr>
<tr>
<td>Mitophagy (Rosella)</td>
<td>↑ ↑ ↑</td>
<td>↑ ↑ ↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Autophagy (LC3-II)</td>
<td>↑ ↑ ↑</td>
<td>↑ ↑ ↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Autolysosomes (MDC)</td>
<td>↑ ↑ ↑</td>
<td>↑ ↑ ↑</td>
<td>↑ ↑</td>
<td>↑ ↑</td>
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</tbody>
</table>

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7.1.2. Triggering of Mitophagy: Is $\Delta \Psi_m$ Loss a Necessary and/or Sufficient Condition for Mitophagy?

One of the major goals of this thesis was to provide insights into the initiation of neuronal mitophagy. In order to determine the extent of activation of mitophagy in primary neurones, direct inhibition of the core machinery of ATP generation was performed using specific inhibitors of OXPHOS complexes. Documentation of the consequent bioenergetic dysfunction was followed in detail using parameters of bioenergetics such as cellular ATP, OCR and monitoring of $\Delta \Psi_m$ in Chapter 4 (Table 7.1).

Complex I and II are critically involved in the cellular metabolism via the regulation of redox balance of NAD$^+$/NADH and the TCA cycle (Scheffler, 2011), so their likelihood of playing a strategic role in triggering mitophagy is hardly surprising. Complex I inhibition was induced by employing Rot which effected a rapid depolarization of $\Delta \Psi_m$ accompanied by a significant reduction in basal OCR, clearly establishing malfunction of mitochondria (Table 7.1). Application of the inhibitor of complex II 3-NP produced a much slower reduction of $\Delta \Psi_m$ in treated neurones, accompanied by a severe dysfunction of OXPHOS as shown by the reduction in basal OCR and the failure to produce maximal respiratory OCR (Chapter 4). Exposure to both Rot and 3-NP for 24 h induced significant mitophagic degradation in CGCs indicating the dysfunction caused by these drugs was sufficient to recruit mitophagy in primary neurones as shown in Chapter 5. CGCs exposed to the inhibitors of complex III and IV, AA and KCN respectively, displayed prominent mitochondrial dysfunction with rapid reduction of $\Delta \Psi_m$ and minor effects on OCR, indicating the injury pathways mediated by AA and KCN occurred with an early dissipation of $\Delta \Psi_m$ (Chapter 4). However, 24 h exposure to AA and KCN failed to induce mitophagy, indicating the depolarization of $\Delta \Psi_m$ may not be a necessary or sufficient condition for mitophagy in neurones (Chapter 5) (Table 7.1). Thus these incisive data on the rapid depolarization of $\Delta \Psi_m$ and recruitment of mitophagy identify a clear difference between earlier work in non-neuronal cells relative to neurones.
This notion was further explained by work with CCCP, a protonophore that induces global dissipation of proton gradients across the biomembranes in cells. CCCP has been widely accepted as a common trigger of mitophagy in studies employing non-neuronal cells or mammalian cell lines, in which the CCCP-mediated dissipation of $\Delta \Psi_m$ was shown to effectively cause PINK1-dependent mitophagy (Narendra et al., 2008; Narendra et al., 2010). However, despite the rapid and significant loss of $\Delta \Psi_m$, it was not possible to monitor the progress of mitophagy using the mt-Rosella reporter in the presence of CCCP (Chapter 5). Further experiments using alternative approaches to analysing mitophagy would be useful to answer the question of whether CCCP actually induces mitophagy in CGCs. Such studies could, in principle, involve immunocytochemical tests of PINK1 and Parkin localization. However, I found (data not shown) that CCCP-treated cells were not amenable to the requisite immunocytochemical analysis using PINK1 antibodies, as the cells underwent degradative changes after exposure to CCCP.

In this context, a study of mitophagy involving primary cortical and striatal/midbrain neurones documented that CCCP treatment failed to increase Parkin localization to mitochondria and colocalization of mitochondria with GFP-tagged LC3 upon mitochondrial depolarization (Van Laar et al., 2011), suggesting depolarization of $\Delta \Psi_m$ may not play an important role as a trigger of mitophagy in primary neurones.

In general, due to the high dependence of neurones on mitochondria, a higher threshold may be required for triggering mitophagy that involves metabolic dysfunction. Possibly depolarization of mitochondria (loss of $\Delta \Psi_m$) would need additional upstream triggers for progression to mitophagy. The high resistance of primary neurones to mitophagy was suggested by Van Laar and colleagues (2011) who showed prevention of mitophagy when HeLa cells, which usually display robust mitophagy upon depolarization of $\Delta \Psi_m$ (Ding et al., 2010; Narendra et al., 2008; Narendra et al., 2010; Van Laar et al., 2011), were forced into dependence on mitochondrial OXPHOS rather than glycolysis as their main pathway of energy generation. This evidence highlights the absolute dependence of neurones on mitochondria and OXPHOS may result in a higher threshold to elicit mitophagy and a requirement for triggers other than the loss of $\Delta \Psi_m$. Interestingly, cardiolipin, which appears to be externalized to OMM following mitochondrial
depolarization (Chu et al., 2013), has recently been suggested to act as an alternative mitophagy activation signal although further work is needed on its precise role in neurones.

7.1.3. Downstream of PINK1 Redistribution: Cessation of Proteolytic Process During Mitophagy

Stabilization of PINK1 in the OMM following depolarization is a key event in the initial steps of mitophagy. Parkin is subsequently recruited to mitochondria and initiates the selective recognition of dysfunctional mitochondria by ubiquitylating the OMM proteins prior to their delivery to lysosomess for complete degradation (Jin & Youle, 2012; Narendra et al., 2008). Mobilization of cytoplasmic PINK1 was observed in CGCs in response to the inhibitors of ETC complexes I-IV, which was strongly suggestive of stabilization of PINK1 in mitochondria. Interestingly, even though PINK1 redistribution was displayed in all cases, progression into mitophagy was only observed as a result of the inhibition of complex I and II by Rot and 3-NP, respectively, whereas AA- and KCN-induced redistribution of PINK1 on mitochondria failed to proceed to mitophagy (Chapter 5). This phenomenon brings attention to the sufficiency of mitochondrial localization of PINK1 as a trigger for mitophagy in neurones, and whether PINK1 possesses additional cellular functions outside PINK1/Parkin-mediated mitophagy upon recruitment to mitochondria. The role of PINK1 in promoting mitophagy has been a topic of great interest, however there is also an additional body of evidence demonstrating a dual role of PINK1 as a suppressor of mitophagy (Gegg et al., 2009; Morais et al., 2014; Steer et al., 2015), by which it is suggested to regulate mitochondrial biogenesis via complexes I and IV and trigger pro-survival pathways under conditions where mitochondria have incurred less severe injuries. Perhaps the bioenergetic dysfunction induced by AA and KCN may have been below the threshold for PINK1 triggering mitophagy (Table 7.1) and thus the turnover of mitochondria may have been suppressed in the experiments reported in this study. Mechanisms underpinning the role of PINK1 as a switch that determines the progression to either PINK1/Parkin-mediated mitophagy or
the upregulation of pro-survival mechanisms and suppression of mitophagy, are under active investigation and still remain to be fully elucidated (Dagda et al., 2014; Fedorowicz et al., 2014; Liu et al., 2011; Murata et al., 2011). In addition, the deubiquitination processes of mitochondria by deubiquitylases in OMM such as ubiquitin-specific protease 30 (USP30), should also be taken into consideration as they have been reported to antagonize the mass degradation of mitochondria and prevent PINK1/Parkin-mediated mitophagy (Bingol et al., 2014; Cunningham et al., 2015; Nakamura & Hirose, 2008). However, the pathways by which these deubiquitylases might contribute to mitophagy are still uncertain. Due to the wide spectrum of cellular functions regulated by PINK1, further work involving the direct downstream effector protein, Parkin (Kane et al., 2014; Narendra et al., 2008; Narendra et al., 2010), would provide useful insights here specific to the investigation of neuronal mitophagy.

7.1.4. Limitations of Current Studies and Possible Future Approaches

Neurones have a highly polarised morphology where each compartment has varying energy demands and physiological function, and therefore require efficient distribution of mitochondria to distal areas where energy is in high demand, such as synaptic terminals, active growth cones and axonal branches (Morris & Hollenbeck, 1993; Ruthel & Hollenbeck, 2003). Since neurones are post-mitotic cells, aged and damaged mitochondria need to be efficiently removed and replenished by healthy mitochondria at the distal ends of neurones (Sheng, 2014). Site-specific local mitophagy has been suggested by recent studies where continuous generation of autophagosomes and local mitophagy of dysfunctional mitochondria were observed in axons and axon tips in primary dorsal root ganglion and hippocampal neuronal cultures (Ashrafi et al., 2014; Maday & Holzbaur, 2014). Here, monitoring of mitophagy was achieved using the dual-fluorescence biosensor, Rosella, with the goal being to observe mitophagy in all structural components of neurones. In agreement with the above mentioned studies, the use of the Rosella biosensor reported the entrance of mitochondria into acidic lysosomal
compartment, indicative of mitophagy, mainly in the distal ends of axons. Due to the limitations of instrument availability, higher resolution fluorescent imaging was not achieved in this study. Therefore, the use of live-imaging confocal microscopy, equipped with a chamber containing a specific gas mixture for neurones intended to reduce oxidative stress, would be able to provide more detailed insights on which sub-area of neurones is more susceptible to mitophagy. Real-time imaging would add to the ‘snapshots’ provided by assays conducted here at two time points of 4 and 24 h post-treatment allowing a full dissection of the temporal recruitment of mitophagy in response to bioenergetic dysfunction.

Furthermore, use of other inhibitors of complex I-IV would complement the results observed in this thesis. Thus, for example, despite the well known neurotoxicity of Rot, its off-target activities have been documented in studies where the structure and function of the centrosome with consequent induction of cytoskeleton disturbances and inhibition of actin dynamics through modifications of Rho-GTPase activity were reported (Diaz-Corrales et al., 2005; Sanchez et al., 2008). In addition, the differential effect of inhibitors of complex III on induction of autophagy has been also reported in yeast and mammalian cell lines. AA and myxothiazol, which bind to complex III at different sites (refer to Chapter 4, Fig. 4.3), showed contradictory results with respect to autophagy induction where autophagy was produced under exposure to AA, while myxothiazol was ineffective (Deffieu et al., 2013; Ma et al., 2011). The different levels of reduced cytochrome b consequent to AA- or myxothiazol-mediated inhibition were suggested as the defining factor for autophagy induction, which further emphasizes the complexity of its regulation. Therefore, using other inhibitors of ETC complexes may offer new insights into neuronal mitophagy and autophagy additional to those reported here.

7.1.5. Concluding Remarks

Appropriate operation of mitochondrial quality control is critical to post-mitotic neurones as mitochondrial function is closely associated with neuronal viability.
Dysfunctions of mitochondria have been a major theme in many neurodegenerative diseases, thus understanding the mechanisms of neuronal mitochondrial quality control relevant to mitophagy is of key importance. The findings of this thesis support the previous evidence on the complexity of mitophagy in neurones as opposed to non-neuronal cells or cell lines, and also revealed the close link between bioenergetic dysfunction of complex I and II of the OXPHOS system and the clearance of neuronal mitochondria via autophagy. Although induction of mitophagy is critical for clearance of dysfunctional mitochondria, it is poorly understood whether mitophagy contributes to neurodegeneration via excessive clearance of mitochondria. Finally, it needs to be emphasized that the present investigations looks at the recruitment of mitophagy in the context of mitochondrial dysfunction and neuronal injury. Thus this work is relevant to the critical roles of complexes I and II in brain pathologies such as PD and HD. Therefore, these mitochondrial ETC complexes could represent viable drug targets in crippling neurological conditions.
APPENDICES
Appendix 1.1. Determination of optimum seeding density of CGCs in XF24 V7 cell culture microplates

The purpose of the cell titration assay was to choose the most appropriate density of cells that gave the baseline rate which was sufficiently above the background noise and also gave robust respond to the uncoupling agent, FCCP, by producing robust maximal OCR. CGCs were seeded at various densities of 50,000, 100,000, 120,000 and 150,000 cells/well while leaving the four corners of the plate blank (media only) to serve as background correction wells. The basal rate of OCR was measured for the first 13 minutes of assay and 1 µM FCCP was injected (line A) to induce maximal respiration. Values are mean ± SEM of 5 wells. The basal OCR for 100,000 and 120,000 cells/well provided an optimal baseline OCR and demonstrated a robust response to FCCP. Hence, 100,000 cells/well was chosen for the optimum density of cell seeding for the subsequent Seahorse Mito Stress assays.
Background

50,000 cells/well
100,000 cells/well
120,000 cells/well
150,000 cells/well

OCR (pMole/min)

Time (min)

150,000 cells/well
120,000 cells/well
100,000 cells/well
50,000 cells/well
Background
Appendix 1.2. Determination of optimum concentrations of Oli and FCCP for subsequent Seahorse XF24 analyses

The optimal concentrations of Oli and FCCP which would achieve maximal inhibition and maximal increase of OCR, respectively, were determined via titration experiments. Titration of these drugs was important, especially with FCCP which can diminish responses of OCR at high concentrations. The optimization assay was done by performing three basal OCR measurements followed by injection of (A) Oli or (B) FCCP at a range of concentrations. Values are mean ± SEM of 5 wells. The maximum inhibition of OCR was induced in CGCs by Oli 1 µM while the maximum increase in OCR was induced by FCCP 2.5 µM, therefore these two concentrations were employed for CGCs for all other subsequent Seahorse XF24 experiments.
Appendix 1.3. Basal OCR measurements from CGCs exposed to controls containing vehicle, DMSO and NaOH, in which Rot, 3-NP and AA were dissolved revealed that DMSO and NaOH did not significantly affect cellular OCR

The effects of solvents in which the drugs were prepared, DMSO and NaOH, on cellular OCR were investigated by measuring basal OCR of CGCs exposed to vehicle controls. Due to the nature of Seahorse XF24 analyses, the portion of CGCs assigned to be the control were exposed to MEM without vehicles. Therefore, any possible effects of the DMSO or NaOH on cellular OCR were further examined to confirm the bioenergetic dysfunctions observed from drug treatment were due to the inhibitory actions of OXPHOS complex inhibitors. CGCs were exposed to the equivalent amount of DMSO and NaOH which were present in drugs used in other experiments. The concentration of DMSO used for preparing (A) Rot 10 nM, 30 nM and AA 10 nM and NaOH for preparing (B) 3-NP 100 µM, 300 µM and DMSO for AA 30 nM showed that DMSO and NaOH did not significantly affect the cellular OCR compared to control CGCs which were exposed to MEM only. Values are mean ± SEM of 5 wells.
Appendix 2. Optimization of transfection conditions for Rosella biosensor to achieve the best transfection efficiency and optimal neuronal viability

The ratio of the amount of DNA being delivered over the amount of transfection reagent (Lipofectamine 2000, Invitrogen) was optimized in order to achieve the most efficient transfection with the maximal viability of neurones. CGCs at 6 DIV were subjected for transfection. A 1:3 ratio of DNA to Lipofectamine reagent was used throughout the transfection experiments. Phase contrast images were taken 24 h after transfection with (A) control (no DNA), (B) 0.4 µg, (C) 0.6 µg and (D) 0.8 µg of DNA with a DNA:Lipofectamine complex ratio of 1:3, where CGCs transfected with 0.4 µg of DNA displayed the least cytotoxicity. (E) represents the total number of cells expressing Rosella biosensor 16-48 h post-transfection where green and red fluorescence were observed under fluorescence microscopy. Values are from two replicate wells. Together, transfection with 0.4 µg of DNA at 1:3 DNA:Lipofectamine ratio induced the least cytotoxicity and high number of neurones expressing the DNA when observed at 24 h of post-transfection. Therefore these conditions were chosen for future experiments. Scale bar = 20 µm
<table>
<thead>
<tr>
<th>DNA:Lipo Ratio</th>
<th>0.4 µg</th>
<th>0.6 µg</th>
<th>0.8 µg</th>
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<tr>
<td>16 h</td>
<td>4</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>1:2</td>
<td>12</td>
<td>22</td>
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</tr>
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<td>1:3</td>
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<tr>
<td>24 h</td>
<td>9</td>
<td>13</td>
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<td>1:2</td>
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<td>1:3</td>
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<td>48 h</td>
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<td>82</td>
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<td>1:3</td>
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Appendix 3. Concentration-response curves for cell viability of CGCs treated with RAP, and morphological changes of CGCs at various concentrations

(A) CGCs were treated with various concentrations of RAP for 24 h. Cells showed concentration-dependent decreases in cell viability. Data represent mean ± SEM from quadruplicate determinations in 3 independent experiments. The average IC$_{50}$ of RAP on CGCs was 56 µM. Phase contrast images of representative fields of cells were taken after treatment with various concentrations approximating the IC$_{50}$ of RAP for 24 h: (B) vehicle control, (C) 20 µM, (C) 30 µM and (D) 50 µM. Neurones showed significant disintegration in morphology when treated with RAP 30 and 50 µM, therefore all drug treatments of RAP employed 20 µM. Scale bar = 50 µm.


cytochrome bc1 with bound substrate and inhibitors at the Qi site. *Biochemistry* **42**(30): 9067-9080.


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