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Analysis of Mitochondrial Proteomes and Supercomplex Structures in Brain Tissue

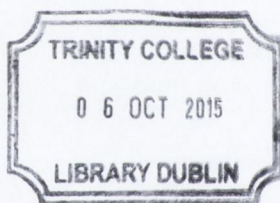
By

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**A dissertation submitted to the University of Dublin
in candidature for the degree of Doctor of Philosophy**

**School of Biochemistry and Immunology
Trinity College
Dublin**

2015

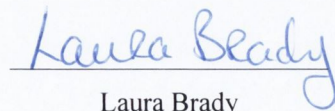


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Laura Brady

2015

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Summary

Mitochondria play a complex multifactorial role in the control of cell bioenergetics. A primary function of mitochondria is the production of adenosine 5'-triphosphate (ATP) by the process of oxidative phosphorylation that takes place in the mitochondrial inner membrane and any malfunction of this metabolic cascade can severely affect the cell. Consequently, given the brains high-energy requirements, any decline in brain respiratory chain enzyme complexes activity could have a significant effect on brain function. Based on their cellular localization, mitochondria from brain can be divided into two major populations. These include synaptic mitochondria (SM), which are predominantly neuronal and presynaptic in origin and non-synaptic mitochondria (NSM), which originate from glia and non-synaptic regions of the neuron. This study characterises the differences between synaptic and non-synaptic mitochondria and investigates age-dependent variation in mitochondrial proteomes and supercomplex structures. The abundance of OXPHOS supercomplexes, which are natural assemblies of the respiratory complexes I, III, and IV that form supramolecular stoichiometric entities, such as $I+III_2+IV_{1-4}$, were found to differ between synaptic and non-synaptic mitochondria. Supercomplex assemblies of $I+III_2+IV_{1-4}$ were more numerous in non-synaptic mitochondria than synaptic mitochondria. In the brains from aged rats, a loss of the $I+III_2+IV_1$ assembly was found in both synaptic and non-synaptic mitochondria compared to mitochondria from young animal brains. This also correlated with experiments that showed an alteration of the mitochondrial proteomes in the brains of aged rats. The use of quantitative proteomic techniques (iTRAQ labeling) showed substantial differences in the proteomes of synaptic and non-synaptic mitochondria from young adult, middle aged and old rats. A set of interesting proteins identified as specific to synaptic mitochondria were found to vary in abundance in the different age groups. Preliminary experiments into the study of respirosomes following reconstitution of mitochondria into a lipidic cubic mesophase suggest a new technique for studying mitochondrial function and structure has been generated. In conclusion, cellular environment, different metabolic needs and age may drive diversity in supercomplex assembly and proteomic structures between synaptic and non-synaptic mitochondria, allowing the respiratory chain to accommodate the ATP demands of the cell. The specific changes in synaptic protein expression may be necessary for the maintenance of ATP production required specifically at the synapse despite age associated insult.

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Abbreviations

2-DE	Two dimensional gel electrophoresis
AAC	ATP/ADP carrier protein
ACAD9	Acyl CoA dehydrogenase member 9
ACADL	Acyl CoA dehydrogenase long chain
AD	Alzheimer's Disease
ADP	Adenosine diphosphate
ADT	Adenine nucleotide translocator
ADOA	Autosomal dominant optic atrophy
amu	Atomic mass units
ANOVA	Analysis of variance
Anti A	Antimycin A
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma-2
BH	Beef heart
BN-PAGE	Blue native gel electrophoresis
BSA	Bovine serum albumin
BTHS	Barth's syndrome
cAMP	Cyclic adenosine monophosphate
CK	Creatine kinase
CL	Cardiolipin
CMP	Crude mitochondrial pellet
CNP	Cyclic nucleotide 3'-phosphodiesterase
CN-PAGE	Clear native gel electrophoresis
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
CoQ	Coenzyme Q
CoQH ₂	Coenzyme Q, reduced
COX	Cytochrome c oxidase
DAB	Diaminobenzidine tetrahydrochloride
DAP3	Death associated protein 3

DAVID	Database for Annotation, Visualisation and Integrated Discovery
DDM	n-Dodecyl β -D-maltoside
DNA	Deoxyribonucleic acid
DNM	Dynamin
2,4-DNP	2,4 Dinitrophenol
DOC	Deoxycholate
DRP	Dynamin related protein 1
ECHM	Enoyl CoA hydratase
EM	Electron microscopy
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH ₂	Flavin adenine dinucleotide (reduced)
FAO	Fatty acid oxidation
FASP	Filter aided sample preparation
FCC	Flux control coefficient
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBAS	Glioblastoma amplified sequence
GDAP1	Ganglioside induced differentiation protein 1
GK	Glycerol Kinase
GO	Gene ontology
GOT2	Aspartate aminotransferase
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrogen chloride
HIG1A	Hypoxia inducing domain family member 1A
HIG2A	Hypoxia inducing domain family member 2A
hrCNE	High resolution clear native electrophoresis
HRP	Horseradish peroxidase
Hsp60	Heat shock protein 60
IMM	Inner mitochondrial membrane
IMS	Inner membrane space
iTRAQ	Isobaric tag for relative and absolute quantitation
KCl	Potassium chloride
KCN	Potassium cyanide

KPB1	Phosphorylase kinase B
kV	Kilovolt
LC/MS	Liquid chromatography/mass spectrometry
LDH	Lactate dehydrogenase
MAG	Monoacylglycerol
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
Mbp	Myelin basic protein
MDH	Malate dehydrogenase
Mfn	Mitofusin
MO	Monooelein
MPP	Mitochondrial processing peptidase
MRC	Mitochondrial respiratory control
mtDNA	Mitochondrial deoxyribonucleic acid
MPTP	mitochondrial permeability transition pore
NADH	Nicotinamide adenine di nucleotide, reduced
NSM	Non-synaptic mitochondria
O ₂ ^{-•}	Superoxide anion
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PD	Parkinson's Disease
PDH	Pyruvate dehydrogenase
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PHB	Prohibitin
PKA	Protein kinase A
PMF	Proton motive force
PLP	Proteolipid protein
POS	Polygraphic oxygen sensor
PRDX	Peroxisredoxin

PS	Phosphatidylserine
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
Rcf-1	Respiratory complex factor 1
Rcf-2	Respiratory complex factor 2
RCR	Respiratory control ratio
RNA	Ribonucleic acid
RPM	Revolutions per minute
SBG	Serva Blue G
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SMP	Submitochondrial particle
SUOX	Sulphite oxidase
TCA	Tricarboxylic acid cycle
TEAB	Tetraethylammonium bromide
THTR	Thiosulfate sulfurtransferase
ROS	Reactive oxygen species
SM	Synaptic mitochondria
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
Tfam	Mitochondrial transcription factor A
TMPD	Tetramethyl-p-phenylenediamine
VDAC	Voltage dependent anion channel

CHAPTER 1

GENERAL INTRODUCTION

1.1 Brain Physiology

The brain is a complex organ that serves as the center of the nervous system in all vertebrate and most invertebrate animals. It constantly receives and interprets nerve signals from the body and responds based on this information. Different parts of the brain control movement, speech, emotions, consciousness and internal body functions, such as heart rate, breathing and body temperature. Every creative thought, feeling, and plan is developed in the brain. The brains of all species are composed primarily of two broad classes of cells: neurons and glial cells. The central nervous system (CNS) comprises a network of approximately 10^{12} neurons plus 10-fold more glial cells (Hubel, 1979). Four types of glial cells exist: astrocytes, oligodendrocytes, microglia, and ependymal cells. These glial cells perform a number of critical functions, including structural support, metabolic support, insulation, guidance of development and mediate synapse formation and function (Clarke and Barres, 2013). Neurons, however, are usually considered the most important cells in the brain. *They* perform all of the communication and processing within the brain. In a typical human, the cerebral cortex (the largest part) is estimated to contain 15–33 billion neurons each connected by synapses to several thousand other neurons (Pelvig et al., 2008). These nerve cells possess unique architecture for integrating and transmitting information. A typical neuron possesses a cell body (soma), dendrites, and an axon (Figure 1.1). Dense dendritic arboizations such allow the cell to receive numerous inputs. They send these signals by means of an axon, which is a thin protoplasmic fiber that extends from the cell body and projects, usually with numerous branches, to other areas, sometimes nearby, sometimes in distant parts of the brain or body. Axons transmit signals to other neurons by means of specialized junctions called synapses. Synapses are the key functional elements of the brain as they are the points at which communication occurs. There is roughly 1000-10,000 synapses per neuron (Pelvig et al., 2008). Neurotransmitters are the chemical medium through which signals flow from one neuron to the next at chemical synapses. Since neurons require large amounts of energy for the firing of action potential, neurotransmission, and other processes, the energy producing organelle, mitochondria, play a pivotal role in optimal brain function. Parts of the neuron, such as at the nerve terminal, have a higher energy demand and accordingly, have higher density of mitochondria (Shepherd and Harris, 1998). High levels of ATP are required at the synapse for maintaining Ca^{2+} homeostasis (Sheng and Cai, 2012),

maintenance of membrane potential and vesicle mobilization (Murthy and De Camilli, 2003).

1.2 Mitochondria: An Introduction

Mitochondria are highly dynamic organelles that play pivotal roles in a diverse set of essential cellular functions. These include regulatory pathways of intermediate metabolism, steroid metabolism, amino acid biosynthesis, fatty acid oxidation, and apoptosis. Other functions include ion homeostasis, cell signaling as well as the biosynthesis of iron-sulphur clusters, heme, and further prosthetic groups. However, a major function of mitochondria is production of adenosine 5'-triphosphate (ATP), the key energy source of the cell. Each day, the human body consumes a quantity of ATP that almost equals its own weight and the main organelle responsible for satisfying this huge demand is the mitochondria (Nabi, 2011). As a result these organelles are often referred to as the 'power house' of eukaryotic cells.

The term 'mitochondria' was first coined in 1898 by German biologist Carl Benda due to their appearance under the microscope. The term is derived from the Greek *mitos*, meaning 'thread' and *knondrion*, the diminutive of *knondros*, meaning 'granule' (Benda, 1898). Mitochondria are membrane-bound organelles that are located in the cytosol of eukaryotic cells (Henze and Martin, 2003). They contain two membranes, a smooth outer membrane and a folded inner membrane, separated by a narrow intermembrane space. The inner membrane forms numerous folds termed cristae, which extend into the inner cavity of the organelle, the matrix. Each of these compartments has distinct functional roles. The matrix contains the mitochondrial genetic system as well as enzymes of the citric acid cycle, which are responsible for the central reactions of oxidative metabolism. The mitochondrial inner membrane is a phospholipid bilayer that contains proteins, which are involved in oxidative phosphorylation as well as in the transport of metabolites between the cytosol and mitochondria. Thus, the inner mitochondrial membrane represents the principal site of ATP generation. This critical role is reflected in the structure of the inner membrane. Firstly, the cristae provide an increased surface area in which the process of oxidative phosphorylation (see Chapter 1, section 1.6.2) can take place. The number of cristae can vary with the respiratory activity of the particular type of cell (Fawcett, 1981, Benard et al., 2006). In addition,

the topology of the inner mitochondrial membrane can affect mitochondrial function by influencing the diffusion dynamics of metabolites and freely diffusing proteins (Mannella, 2008). It is impermeable to most ions and small molecules, which is another critical property to maintaining the proton gradient that drives oxidative phosphorylation (Cooper, 2000). The outer membrane on the other hand is freely permeable to small molecules. It contains proteins called porins, which form channels that allow the free diffusion of molecules up to 10-kDa. Consequently, the composition of the intermembrane space is similar to that of the cytosol with respect to ions and small molecules (Alberts, 1994).

It is believed that mitochondria evolved from ancient alpha bacterium through the development of a symbiotic relationship in which they lived within larger proto-eukaryotic cells (Lang et al., 1999, Gray et al., 1999). Over the billions of years of their symbiotic relationship with eukaryotic hosts, mitochondria have maintained their double membrane structure, circular plasmid DNA, RNA and protein synthesis systems.

1.3 The Mitochondrial Genome

Reminiscent of their origin, mitochondria are the only organelle to have their own genome. mtDNA in mammals is a circular molecule that encodes 37 genes that generate 2 rRNAs, 22 tRNAs, and 13 polypeptides. All 13 polypeptides are components of the oxidative phosphorylation system. They are encoded using a non-standard genetic code, which requires its own translational machinery separate from that of the nucleus. Mitochondrial DNA is densely packed into nucleotids, each containing as few as 1-2 mtDNA molecules (Kukat et al., 2011). Due to these distinct characteristics of mitochondria and the uniparental mechanism of inheritance (through the maternal line), dividing cells have the extra challenge of segregating mitochondria effectively. This is achieved through remodelling where mitochondria can fuse with each other and divide within the cell (see Chapter 1, section 1.4). There is a constant cycle of fusion, where two mitochondria join to form a singular mitochondrion, and fission, in which singular mitochondrion divide into two mitochondria. The balance of this process allows for the maintenance of mtDNA within the cell and provides a good mechanism for mitochondrial distribution during cell division (Mattson et al., 2008).

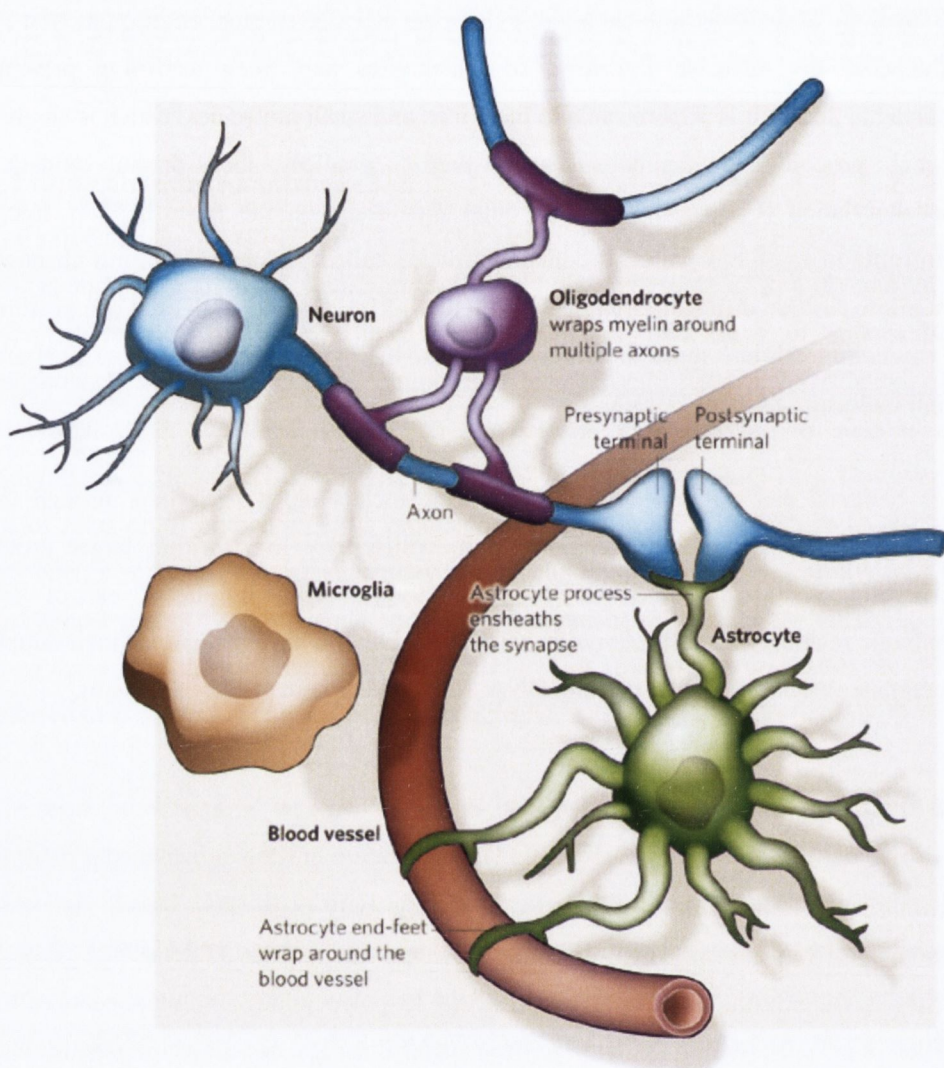


Figure 1.1 The brain is made up of two types of cells: neurons and glial cells. Neurons (blue) typically consist of dendrites that receive information, a cell body, and an axon that is used to transmit information throughout the nervous system. Different types of glia interact with neurons and the surrounding blood vessels. Oligodendrocytes (purple) wrap myelin around axons to speed up neuronal transmission. Astrocytes (green) extend processes that ensheath blood vessels and synapses. Microglia (brown) keep the brain under surveillance for damage or infection. Image taken from Waagerpeterson et al., 2006.

1.4 Mitochondrial Dynamics

Changes in mitochondrial architecture, morphology and their movement along the cytoskeleton, via fission and fusion events, have become collectively known as mitochondrial dynamics (Liesa et al., 2009, Chen et al., 2005). When identified over 120 years ago, mitochondria were first described as a collection of free-floating individual vesicles existing in hundreds of copies remaining freely in the cytosol (Benda, 1898). However, with the development of cell culture and fluorescence microscopy, along with mitochondrial specific fluorescent probes, a more accurate, three-dimensional view of the mitochondria in living cells from various tissues has now been established (Griparic and van der Bliek, 2001, Bereiter-Hahn and Voth, 1994). In these studies, the mitochondria appear more like a wide network of long tubules rather than a collection of small individual vesicles. These highly complex and dynamic organelles can exist in both tubular and punctuated forms within the cell as a part of an interconnected reticulum, owing to the continuous fission and fusion events governing their shape (Sukhorukov et al., 2012). Mitochondria also make use of microtubules and actin filaments to redistribute and relocate. This is especially true with respect to axonal transport in neurons (Cai et al., 2011).

Through the process of fusion, a single organelle can combine with another to form a larger mitochondrion. This process also allows a mitochondrion to integrate with the larger reticulum and become involved in cellular processes occurring in cellular regions distal to its own location (Sukhorukov et al., 2012). Through fission, a tubular mitochondrion of 2-25 μm in length can divide into two or more smaller organelles of $\sim 0.5\mu\text{m}$ or larger. These continuous cycles of fusion and fission, and the balance between these two events is understood to determine the organization, shape and size of the organelle (Chen et al., 2005).

1.4.1 Mitochondrial Fusion

Mitochondrial fusion promotes exchange of mtDNA and other vital components such as metabolites and enzymes, thus reinvigorating and extending the mitochondrial network. Fused mitochondria are preferred when optimal mitochondrial function is needed. Thus, fused mitochondrial networks are frequently found in respiratory active cells. Fusion events have been best characterized in yeast mitochondria (Bleazard et al., 1999,

Nunnari et al., 1997, Hermann et al., 1998), but mammalian equivalents to some of the proteins machinery involved have been identified. In mammals, the proteins involved in mitochondrial fusion include dynamin-related proteins mitofusion 1 and mitofusion 2 (Mfn1 and Mfn2), localized to the mitochondrial outer membrane, and the dynamin related GTPase protein optical atrophy 1 (OPA1) in the mitochondrial inner membrane.

The mitofusions are human orthologs of the Fuzzy onion protein found in *Drosophila melanogaster* and yeast (Hales and Fuller, 1997). It was named fuzzy onions for the onion-like and fuzzy appearance of unfused mitochondria in electron micrographs of these mutants. Mfn1 and Mfn2 are widely expressed and have overlapping but not completely redundant roles in the fusion process. Both are anchored to the outer mitochondrial membrane through their C-terminal membrane-binding domain. Their N-terminal GTPase domain is present in the cytoplasm (Rojo et al., 2002). Mfn2 has an additional Ras-binding domain that is lacking in Mfn1 and displays a surprising level of functional pleiotropy (de Brito and Scorrano, 2008a, de Brito and Scorrano, 2008b). In its simplest terms, fusion begins with the tethering and fusion of these N-terminal domains to opposing mitochondrial membranes, (Chen et al., 2003) docking and fusion of the inner mitochondrial membranes with subsequent mixing of intramitochondrial components (Ishihara et al., 2004). OPA1 is another protein identified through human genetic studies that is required for inner membrane fusion and remodeling of the cristae structure (Frezza et al., 2006). OPA1 is the mammalian ortholog of Mgm1, which was found to be essential for mitochondrial fusion in yeast (Olichon et al., 2002, Song et al., 2009). A mutation in the gene encoding for OPA1 leads to an optic neuropathy, autosomal dominant optic atrophy (ADOA), in which retinal ganglion cells degenerate and cause atrophy of the optic nerve (Delettre et al., 2000).

Decrease in fusion events leads to mitochondrial fragmentation due to the increased fission events. Disruption of mitochondrial fusion results in mitochondrial dysfunction and loss of respiratory capacity both in yeast (Rapaport et al., 1998, Hermann et al., 1998) and in mammalian cells (Chen et al., 2005, Chen et al., 2003, Santos et al., 2014, Simcox et al., 2013).

1.4.2 Mitochondrial Fission

Mitochondrial fission facilitates the redistribution of mitochondria and in the case of neurons, transport mitochondria from the cell body, along the axon, to the synapse. Fission also allows the partitioning and inheritance of the organelles during cell division, release of cytochrome c and other intermembrane space proteins during apoptosis (Detmer and Chan, 2007, Westermann, 2010). In mammals, the proteins involved in mitochondrial fission are dynamin-related protein 1 (Drp1) and fission protein (Fis1). During mitochondrial fission, Drp1, which normally exists as either cytosolic dimers or tetramers, is recruited to mitochondrial surfaces where it associates with Fis1 and assembles into large oligomers as foci that serve as potential scission sites for fission events (Smirnova et al., 2001). These oligomers then wrap around the mitochondrial tubule and constrict and sever the mitochondrial membrane following conformation change by GTP hydrolysis (Smirnova et al., 2001). There are conflicting reports on the role Fis1 plays in the fission process and may act as an anchor for Drp1. The current consensus seems to be that while Fis1 may be involved in mammalian mitochondrial fission, it may not be an essential component and may even be dispensable in some cells (Otera and Mihara, 2011). The fission event also requires input from many other accessory proteins, including mitochondrial protein of 18 kDa (MTP18) (Tondera et al., 2005), endophilin B1 (Takahashi et al., 2005), ganglioside-induced differentiation protein 1 (GDAP1) (Niemann et al., 2005) and death-associated protein 3 (DAP3) (Mukamel and Kimchi, 2004).

Decrease in fission events generates long and highly interconnected mitochondria (Sesaki and Jensen, 1999). The absence of fission in neurons results in mitochondrial accumulation in the cell body with no transport of mitochondria to the synapse. This leads to neurite death and as a result neurons would no longer be able to communicate with each other via the release of neurotransmitters (Chen and Chan, 2009).

1.4.3 Function of Mitochondrial Dynamics

It should be noted that mitochondria cannot be created *de novo*. Therefore one of the major functions of mitochondrial dynamics is the distribution of this organelle amongst dividing cells (Palmer et al., 2011). Accordingly, DRP1, Mfn1 and 2 and OPA1 have all been shown to possess essential roles in development (Chen et al., 2003, Ishihara et al.,

2004, Chen et al., 2005). But the importance of mitochondrial dynamics extends far beyond this function.

Fusion and fission events are essential in the maintenance and sharing of mitochondrial DNA and the regulation of apoptosis. Proper mitochondrial dynamics are important for the accurate segregation and transmission of mtDNA during mitosis. Although not fully understood, it has been proposed that the exchange of mitochondrial contents by the fusion process enables complementation of mutant mtDNA in heteroplasmic cells. Introduction of wild type mtDNA would dilute out levels of mutant mtDNA molecules and prevent their accumulation and ultimately reaching a critical threshold, whereby respiratory dysfunction occur (Sato et al., 2009). The maintenance of mtDNA is crucial for cellular function, as it encodes proteins required for oxidative phosphorylation and ATP synthesis (Wallace and Fan, 2010). Therefore, it is likely that alterations in the expression levels of both fusion and fission proteins would affect the mitotic segregation of mutant and wild type mtDNA, and consequently play a pivotal role in the proliferation of dysfunctional mitochondria that is observed with aging and mitochondrial disease. The involvement of increased mitochondrial fission events has been identified in some neurodegenerative diseases such as Alzheimer's disease (AD) (Wang et al., 2009, Calkins et al., 2011) and Parkinson's disease (Kamp et al., 2010, Gegg et al., 2010).

The constant renewal of mitochondria is crucial for maintaining healthy mitochondria with age. Accurate cellular turnover requires the coordination of mitochondrial biogenesis and autophagy (Twig et al., 2008). Mitochondrial fission followed by selective fusion provides a mechanism to segregate damaged and dysfunctional mitochondria and permit their degradation by autophagy. In the long term, this mechanism contributes to the maintenance of a healthy mitochondrial population and maintenance of bioenergetic capacity. The capacity for mitochondrial biogenesis diminishes with age and this is an important factor in the mitochondrial dysfunction that is associated with the aging process (Sugiyama et al., 1993).

Both OPA1 (see Chapter 1, section 1.9.4) and Mfn2 are particularly interesting as they exhibit roles outside of fusion. They represent attractive candidates as integral mitochondrial proteins that mediate the interplay between dynamics and other important

functions, particularly bioenergetics. Mfn2 expression is crucial in mitochondrial metabolism through the maintenance of the mitochondrial network architecture. It has been reported to have role in regulating OXPHOS expression through signals that are independent of its role as a mitochondrial fusion protein (Pich et al., 2005). Mutations in Mfn2 in cells can disrupt several cellular processes regulating oxidative metabolism, cell cycle progression and cell death (Chen et al., 2005, Bach et al., 2003, Pich et al., 2005). Drp1, Mfn1 and Mfn2 all undergo posttranslational modifications, which might intervene in the modulation of mitochondrial dynamics and the subsequent control of OXPHOS capacity (Ishihara et al., 2004, Karbowski et al., 2007, Chen et al., 2003). However, the impact of these changes on energy production has yet to be fully elucidated.

1.5 The Mitochondrial Proteome

Mitochondria represent an extremely protein-rich organelle (Douce, 1985). They also have the highest proportion of membrane proteins of all examined mammalian membrane systems (Cooper, 2000). It has been estimated by electron microscopy that OXPHOS protein complexes alone represent 80% of the total intrinsic mass of the mitochondrial inner membranes (Harmon and Crane, 1974, Harmon et al., 1974).

In order to gain deeper understanding of the multiple functions of this organelle, efforts have been made to define the mitochondrial proteome (Da Cruz et al., 2005, Meisinger et al., 2008). In mammals, mitochondria may contain up to 1,500 proteins different proteins (Taylor et al., 2003a, Taylor et al., 2003b). Using highly purified mitochondria and quantitative two-dimensional gels, approximately 1000 distinct proteins have been identified (Hunzinger et al., 2006, Lopez et al., 2000) but the others lack experimental validation (Pagliarini et al., 2008, Cotter et al., 2004). Interestingly, the genome of alpha-proteobacteria, the closest living relatives of modern day mitochondria, encode a total of ~ 1000 distinct protein products (Andersson et al., 1998). Altogether, these estimates suggest that the mitochondrial proteome consists of around 1000-1500 distinct proteins.

Several databases currently exist that integrate the increasing body of proteomic data on the composition of mitochondria in many organisms. However, defining the mitochondrial proteome is proving challenging. Firstly, no single targeting sequence directs import into the organelle. Secondly, some proteins are of very low abundance while other components may only be expressed in specific development stages or cell types (Calvo and Mootha, 2010). In addition, the problem of false positive or negative attribution of mitochondrial localization is aggravated by the fact that at least one-tenth of the mitochondrial proteins exhibit a dual cellular localization (Sickmann et al., 2003). Also, considering the various posttranslational modifications occurring, the actual number of functionally and structurally different protein species in mitochondria may be higher. As a result no one method can identify all mitochondrial proteins successfully. However, through the combination of high throughput and traditional biochemical techniques, identification of a complete mitochondrial proteome is imminent. The next challenge is to understand how these proteins function together in pathways and complexes.

1.6 Mitochondrial Bioenergetics: An Overview

Bioenergetics is the broad term given to the reactions that are concerned with energy flow through systems. Mitochondria harbor sophisticated enzymatic machinery that employs molecular oxygen to complete the oxidation of fats, proteins and sugars to carbon dioxide and water, finally transforming the released energy into ATP (Nabi, 2011). Depending on the tissue, mitochondria are responsible for the production of up to 80-90% of cellular ATP (Stanley et al., 2005, Mathews CK, 1995). Their double-membrane structure facilitates intra-organelle compartmentalisation with high functional specificity and the integration of multiple metabolic pathways (Figure 1.2). Through the oxidation of amino acids, β -oxidation of fatty acids and the tricarboxylic acid cycle (TCA), mitochondria can generate reduced forms of nicotinamide adenine dinucleotide and flavin adenine dinucleotide (NADH and FADH₂, respectively). These reduced equivalents are used as electron donors to the electron transport chain (ETC) (Figure 1.3). The mitochondrial respiratory chain consists of four electron-transporting complexes embedded in the mitochondrial inner membrane, known as the NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol-cytochrome c reductase (complex III) and cytochrome oxidase (complex IV).

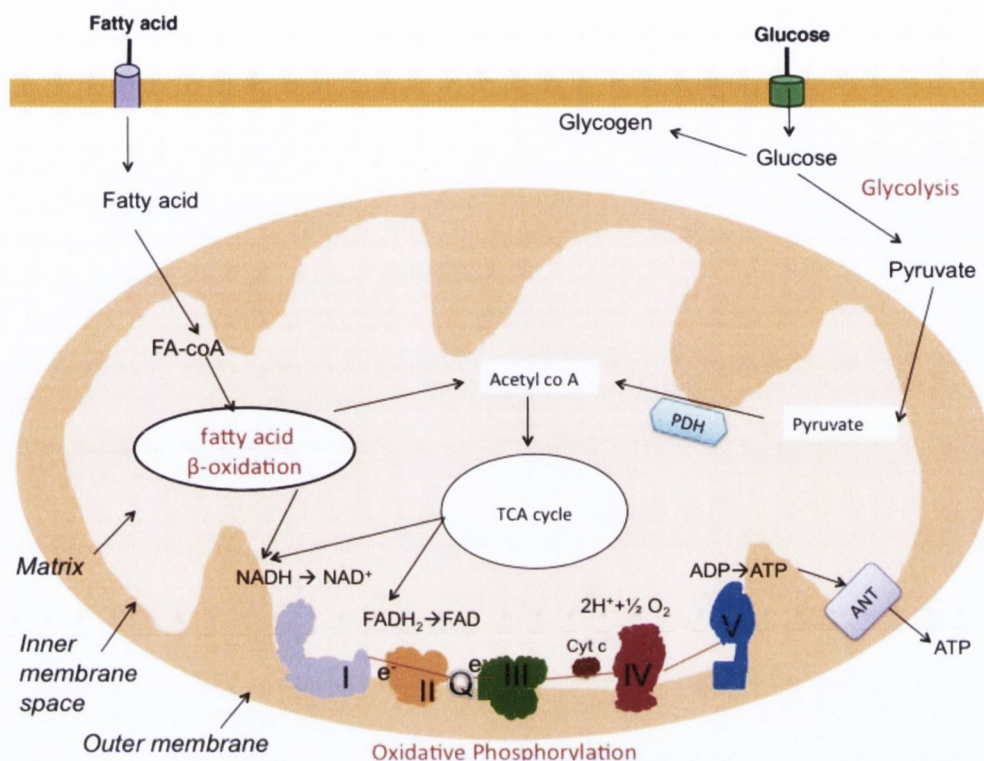


Figure 1.2 Main pathways of cellular and mitochondrial energy metabolism. The two main metabolic pathways (Glycolysis and Oxidative Phosphorylation) are linked by the enzyme complex pyruvate dehydrogenase (PDH). In simple terms, enzymatic reactions degrade carbohydrates, fats and proteins into smaller molecules such as pyruvate, fatty acids and amino acids, respectively. Mitochondria further metabolise these into NADH and/or FADH₂, through β-oxidation and the TCA cycle. These reduced equivalents can be oxidized by the electron transport chain. While the electrons are transferred to molecular oxygen and water is produced at complex IV, protons are translocated from the mitochondrial matrix through complexes I, III & IV into the intermembrane space, thus creating a protonmotive force. It is this protonmotive force that drives ATP synthesis, through ATP-synthase, a process known as oxidative phosphorylation.

Each of the OXPHOS complexes consists of multiple polypeptide subunits encoded by either nuclear or mitochondrial DNA (mtDNA), except for complex II, which is exclusively encoded by the nuclear genome. The correct biosynthesis of the OXPHOS complexes is thus a highly intricate and regulated process that requires the concerted action of the two cellular genomes. According to the chemiosmotic theory, the free energy liberated from the passage of electrons from each enzyme of the ETC is harnessed by complexes I, III and IV to translocate protons (H^+) from the mitochondrial matrix into the intermembrane space (IMS) against their concentration gradient, thus establishing a protonmotive force (PMF). While the electrons are transferred to molecular oxygen and water is produced at complex IV, it is this PMF that ultimately drives ATP synthesis through another inner membrane complex, F_1F_0 -ATP-Synthase (complex V) (Mitchell and Moyle, 1968). While not part of the ETC, ATP synthase is coupled to the ETC. The energy generated by the PMF powers rotation of the ATP synthase; this rotation is used to combine adenosine diphosphate (ADP) with inorganic phosphate (P_i) to form ATP. Newly formed ATP is translocated to the cytosol by the adenine nucleotide translocase (ANT), an inner membrane antiporter that exchanges ATP for ADP.

1.6.1 The Electron Transport Chain

The ETC is composed of four enzymatic complexes embedded in the mitochondrial inner membrane (Figure 1.3). Two mobile redox-active molecules aid electron transfer between complexes; ubiquinone (coenzyme Q) and cytochrome c. Ubiquinone is a free diffusible electron carrier within the inner mitochondrial membrane that transports pairs of electrons from complexes I and II to complex III. Cytochrome c is a highly conserved, water-soluble molecule (~12 kDa) located in the intermembrane space and transfers electrons from complex III to complex IV. Electron shuttling within individual complexes is facilitated by different redox-centers; iron-sulfur clusters, cytochromes and Cu^{2+} . The energy release by this electron flow creates a proton concentration gradient across the membrane. It is this proton gradient that drives ATP synthesis through Complex V (Mitchell and Moyle, 1968).

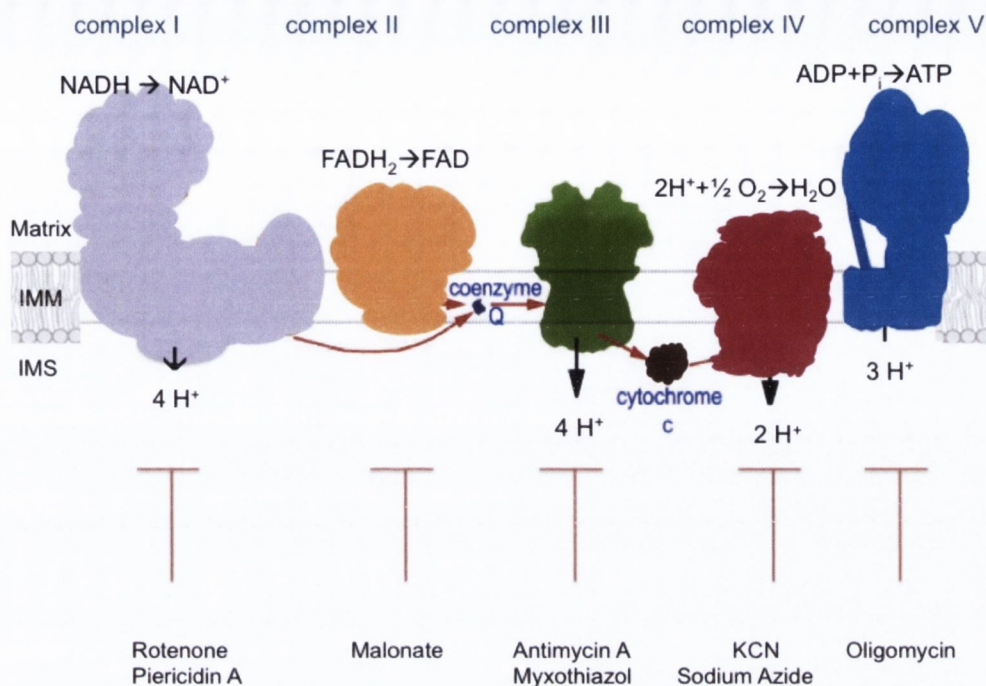


Figure 1.3 The electron transport chain complexes and ATP synthase (complex V)

The electron transport chain is composed of four enzymatic complexes embedded in the mitochondrial inner membrane. Two mobile redox-active molecules aid electron transfer between complexes; ubiquinone (coenzyme Q) and cytochrome c. While the electrons are transferred to molecular oxygen and water is produced at complex IV, the generated proton motive force drives ATP synthesis through complex V. The position of the matrix (M), the intermembrane space (IMS) and cristae of inner membrane (IMM) have been indicated. The image is a representative of the well-known ‘fluid model’ where respiratory chain complexes are independent entities that freely diffuse within the inner mitochondrial membrane and electron transfer between them is supported by their random collision. Electron transport can be inhibited at complex I with rotenone or piericidin A (Okun et al., 1999). Complex II can be inhibited by malonate (Dervartanian and Veeger, 1964), while antimycin A and myxothiazol inhibit complex III (von Jagow and Engel, 1981). Azide and potassium cyanide (KCN) inhibit complex IV (Grant et al., 1997) while finally ATP synthase can be inhibited by oligomycin (Galante et al., 1979).

Complex I (EC 1.6.5.3; NADH dehydrogenase) (~1000 kDa), the first and largest enzyme of the ETC, is comprised of 45 subunits that assemble into a hydrophobic membrane-spanning area and a perpendicular hydrophilic matrix-facing arm. Due to the large size, L-shaped symmetry, membrane bound location and multi component structure, human complex I has yet to be crystallised for X-ray based structure determination. However, complex I from bovine heart (BH) mitochondria (which has been used as a model for the human enzyme) (Shimada et al., 2014, Carroll et al., 2006, Carroll et al., 2003, Hirst et al., 2003, Vinothkumar et al., 2014), and recently, bacterial complex I (Baradaran et al., 2013) have been characterized extensively. Both enzymes contain 45 subunits encoded by both nDNA and mtDNA and nine redox cofactors (a flavin mononucleotide and eight iron-sulphur clusters) (Hirst et al., 2003).

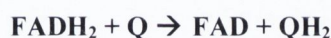
Fourteen subunits are the core subunits that are conserved in all complex I enzymes. These catalyse the energy transducing reactions such as NADH oxidation, ubiquinone reduction and proton translocation (Baradaran et al., 2013). Seven of these core subunits are encoded by nDNA and harbor a flavin mononucleotide to oxidise NADH, FeS clusters for electron transfer and the ubiquinone site. The remaining seven core subunits are encoded by mtDNA and contain four antiporter like domains for proton translocation. The cohort of 30 supernumerary subunits has been accumulated through evolution. These may have function in assembly, regulation, stability or protection against oxidative stress. Recently, cryo-EM studies of complex I purified from bovine heart mitochondria identified 18 of these supernumerary subunits, of which 14 were assigned and modeled (Vinothkumar et al., 2014). The structure and exact position of the remainder supernumerary subunits remain unknown.

Within complex I, electrons from oxidized NADH are transferred to a non-covalently bound flavin mononucleotide (FMN) and then through a chain of several iron-sulphur (Fe-S) clusters to the mobile ubiquinone (Q), a quinone derivative with a hydrophobic isoprenoid tail. Reduction of the ubiquinone induces conformational changes in the membrane arm, which results in the translocation of four protons across the membrane (Baradaran et al., 2013). For every electron pair transported by complex I, four protons are translocated from the matrix to the IMS. Q and QH₂ refer to the oxidized and reduced forms of ubiquinone, respectively.

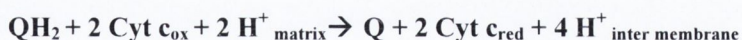
The overall reaction catalysed by complex I can be described as follows:



Complex II (EC 1.3.5.1, succinate:ubiquinone oxidoreductase) (~ 130 kDa) is bound to the mitochondrial inner membrane on the matrix side and it is the only complex that participates in both the electron transport chain and the citric acid cycle. In the citric acid cycle complex II converts succinate to fumarate with the concomitant reduction of FAD to FADH₂. In the electron transport chain, FADH₂ is subsequently oxidized, releasing electrons that are transferred to ubiquinone, reducing it to ubiquinol. Ubiquinol then transfers electrons to complex III.

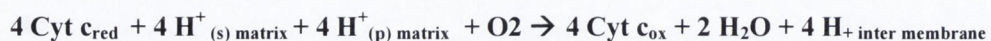


Complex III (EC 1.10.2.2; cytochrome bc₁) (~300 kDa) is known to exist in the membrane as a dimer and catalyses the transfer of electrons from ubiquinol to cytochrome c in the electron transport chain through a process known as the Q cycle. Mammalian complex III consists of 11 subunits, the main three of which contain a b-type cytochromes, a c₁ cytochrome and an iron-sulphur center. Cytochrome b has two heme groups B_H and B_L. B_H is located near the Q_n site (also known as the Q_i site) of the Q cycle and B_L is found near the Q_p site (also known as Q_o site) of the Q cycle. Ubiquinol undergoes two cycles of reoxidation, the net result being the reduction of two cytochrome c proteins (one electron each) and the translocation of four protons for every ubiquinol oxidized. Complex III does not function as a proton pump *per se* (as with complex I and IV). It instead facilitates the redox channeling of ubiquinol, which in turn facilitates the translocation of protons across the inner membrane. The reaction is summarised as follows:



Complex IV (EC 1.9.3.1: cytochrome c oxidase) (~200 kDa) is the terminal enzyme for the transported electrons and the site at which they are used to reduce molecular oxygen to water. This enzyme consists of 13 subunits, three of which are encoded by the mitochondrial genome. It catalyses the transfer of electrons from reduced cytochrome c (ferrocytochrome c) to molecular oxygen. It contains four catalytic centers – two heme groups, heme a and heme a₃, and two copper ions, Cu_A and Cu_B. Four electrons from four individual cytochrome c proteins are transferred to the O₂ reduction site (heme a₃/

Cu_B) via Cu_A and heme a. One molecular oxygen molecule is reduced to two water molecules. As this occurs, protons are translocated through the membrane. There are two pathways of proton transfer: the K-pathway and the D-pathway. The reason for the existence for the two proton pumping pathways is still unclear at present. For each reaction cycle where two molecules of water are produced four H⁺ are pumped from the matrix, therefore in each complete cycle complex IV removes eight H⁺ from the matrix, fully summarised as follows:



1.6.2 Oxidative Phosphorylation

At the start of their journey through the ETC, electrons have very high energy. However, as they pass along the ETC, they lose this energy in a controlled manner. This released energy is used at complex I, III and IV to expel protons from the matrix into the inner membrane space, creating the proton motive force (PMF). The PMF (alternatively called an electrochemical gradient, Δp) is generated by the active pumping of protons across the inner membrane reaches a maximal value of ~ 180-220 mV (Brand and Nicholls, 2011) and confers a net negative charge to the mitochondrial matrix. It comprises two elements: an electric potential ($\Delta\Psi$) and a chemical potential ($\Delta\mu_{\text{H}^{+}}$). At physiological conditions, mitochondria can alternate between two energy-production states: state 4, in which respiration is slow and there is little ATP production and state 3, in which respiration is fast and ATP is produced (creating a lower mitochondrial membrane potential ($\Delta\Psi_{\text{m}}$)).

Discharge of the PMF is an exergonic process, a property exploited by ATP synthase to generate ATP. This type of ATP synthesis is referred to as oxidative phosphorylation. In addition to its role in ATP synthesis, the PMF is used for many other energy dependent processes such as mitochondrial Ca²⁺ uptake (Ohsumi and Anraku, 1983), ATP/ADP exchange by the adenine nucleotide translocator (ANT) (Klingenberg, 2008) and action of mitochondrial antiporters (O'Rourke, 2007).

Complex V (3.6.3.14: ATP synthase) (~600 kDa) exploits the energy produced by proton translocation to generate ATP. ATP synthase is both ubiquitous and unique; it is one of the most abundant and highly conserved proteins across species but also one of only two proteins known to use a rotary motion as a step of catalysis (the other being

the bacterial flagellum) (Noji et al., 1997, Yoshida et al., 2001). The mechanism of ATP synthesis by this enzyme is called the 'binding change' mechanism proposed by Paul Boyer (Kayalar et al., 1977). ATP synthase is composed of a hydrophobic transmembrane portion (F_o) and a hydrophilic matrix portion (F_1), connected by a central rotor). As protons pass through F_o , a central set of F_o subunits rotate, leading to the rotation of the central rotor portion. This causes conformational changes in the β subunits of F_1 , which leads to the sequential binding of ADP + P_i , ATP synthesis and release of ATP.

Although it has not yet been definitively quantified, it is estimated that the synthesis of one ATP molecule requires the passage of four protons through F_o (Yoshida et al., 2001). Therefore, electrons from each NADH molecule can form a total of 3ATP while FADH can produce a total of 2ATP. As a result, the 10 NADH molecules (from glycolysis and Krebs) and the 2 FADH₂ can form a total of 34 ATP from this electron transport chain. This means that combined with the Krebs cycle and glycolysis, the efficiency for the ETC is about 65%, as compared to only 3.5% efficiency for glycolysis alone.

1.6.3 Assessing Mitochondrial Respiration Experimentally

The degree of coupling between ATP synthesis and respiration can be experimentally quantified in isolated mitochondria and intact cells by defining the ratio of oxygen consumption during state 3 and state 4 respiration, called the 'respiratory control ratio' (RCR) (Brand and Nicholls, 2011). A high RCR indicates a high degree of coupling and a low level of 'proton leak', i.e., the re-entry of protons to the matrix through routes alternative to ATP synthase. In vivo, facilitated proton leak is regulated through the action of specific inner mitochondrial membrane proteins known as uncoupling protein (UCPs), primarily UCP1 (Nicholls and Lindberg, 1973, Nicholls, 1977). Uncoupling protein 1 is most highly expressed in brown adipose tissue (BAT) of mammals and human infants, and its transport of protons across the inner membrane induces mitochondrial uncoupling and the dissipation of the energy generated by electron transport as heat (Nicholls and Rial, 1999). This forms the molecular basis of non-shivering thermogenesis in mammals (Nedergaard et al., 2001).

Electron transport and oxidative phosphorylation can be specifically inhibited using a number of chemical agents targets the individual enzymes of the ETC/OXPHOS

machinery (Figure 1.2). Inhibition of the electron transport chain can have two downstream effects: (1) respiration rates are reduced as there are no electrons available for the reduction of molecular oxygen and (2) the F_1 portion of ATP synthase can consume ATP and turn in reverse, forcing the F_0 to pump protons out of the matrix, thus maintaining membrane potential. Inhibition of complex I with compounds such as rotenone, piericidin A or 1-methyl-4-phenylpyridinium (MPP⁺) blocks the transfer of electrons from CoQ and thus respiration from NAD⁺-linked substrates (Okun et al., 1999). Respiration is still possible through FAD-linked substrates via complex II. Conversely, inhibition of complex II with malonate inhibits respiration through FAD-linked substrates (Dervartanian and Veeger, 1964), but respiration is still possible via NAD⁺-linked substrates. Inhibiting complex III with antimycin A, or myxothiazol, completely blocks respiration from all substrates (von Jagow and Engel, 1981), as does inhibition of complex IV with potassium cyanide (KCN) or sodium azide (Grant et al., 1997). The F_0 region of ATP synthase can be inhibited by oligomycin, which prevents proton translocation in either direction (Galante et al., 1979).

1.7 Mitochondrial Supercomplexes: An Introduction

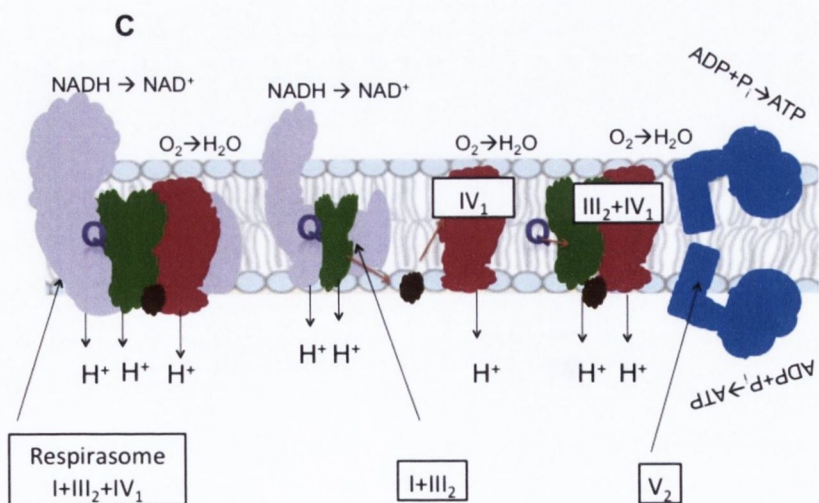
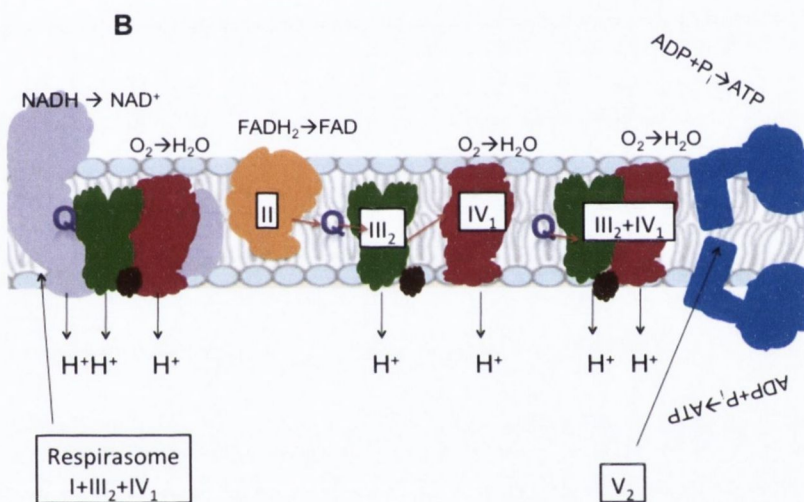
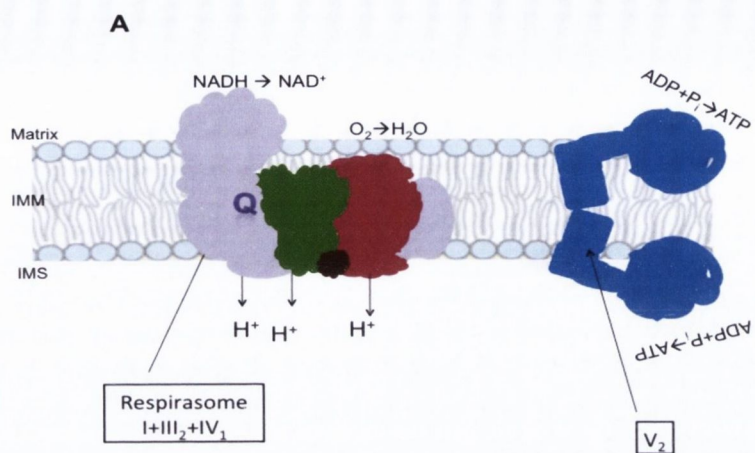
For a long time it was believed that respiratory chain complexes were independent entities that freely diffuse within the inner mitochondrial membrane and that electron transport occurs when the complexes randomly collide (Hackenbrock et al., 1986). This model, the “fluid state” model (Figure 1.2), was widely accepted with its main defense being the isolation of individual respiratory complexes in their active form (Hatefi et al., 1962). However, in the last decade, accumulating data has evidenced the existence of a higher organisation level of respiratory complexes and points toward a different model, the “solid state” model (Figure 1.3A). In fact, individual complexes can interact and associate into so called ‘respirasomes’, supercomplex structures composed of several respiratory complexes of defined stoichiometries. Vast variations in the amount and content of these supercomplex structures have been observed between different cell types. The coexistence of the respirasome unit, composed of at least I+III+IV, together with free complex III₂ and complex IV has been described in bovine heart mitochondria (Althoff et al., 2011, Dudkina et al., 2011, Schafer et al., 2006, Schagger and Pfeiffer, 2001) and yeast mitochondria (Heinemeyer et al., 2007). These results suggest that the

supercomplexes may be in a dynamic equilibrium with the isolated complexes. As a result, it is now accepted, that both organisations (fluid and solid model) can coexist, giving rise to the “dynamic aggregate” or “plasticity model” (Acin-Perez et al., 2008) (Figure 1.3 B+C). This suggests that supercomplex organization is compatible with electron transfer but that electron transfer is still possible in the membrane in the absence of supercomplex assembly. OXPHOS complexes can switch from freely moving to fixed substrates and vice versa in response and adapt to changes in cellular metabolism (Acin-Perez et al., 2008). Therefore, different metabolic needs for different tissue types may dictate the existence of supercomplexes at any time allowing the entire respiratory chain to accommodate the ATP demands of the cell (Vartak et al., 2013).

1.7.1 Discovery of Mitochondrial Supercomplexes

This idea that respiratory enzymes occur in a supramolecular complex has been around for almost 60 years but has not been fully understood or accepted until more recent years. Chance and Williams were the first to propose that the respiratory enzymes existed as a single unit of respiration (Chance and Williams, 1955) and depicted the respiratory chain as a solid state assembly of flavins and cytochromes in a protein matrix. At the time, this proposal was met with great skepticism, as all evidence appeared to contradict the solid-state model. Despite the fact that most reports backed the fluid state model, it did not explain the isolation of complex I+III from bovine heart (Hatefi et al., 1962). This group reconstituted a mixture of complex I and complex III and showed that these reconstituted complexes were stable with enzyme activities similar to rates observed in particles directly isolated from mitochondria (Hatefi et al., 1962). This was one of the earliest reports of evidence that indicated the functional significance of the whole supercomplex assembly. Other early reports of isolated supercomplex assemblies included complex II-III units (Yu et al., 1974), complex III+IV from bacteria (Berry and Trumpower, 1985, Iwasaki et al., 1995) and complex III+IV from yeast (Boumans et al., 1998).

OVERLEAF: Figure 1.3 Plasticity model of the mitochondrial OXPHOS system organization. (A) Schematic representation of “respirasomes” or the solid-state model, in which the membrane complexes are assembled within supramolecular edifices that trap the soluble electron carriers and thereby restrict their diffusion. The shape and color code representing the individual complexes can be seen in A; coenzyme Q is represented as Q and cytochrome c as brown circle. (B) & (C) Plasticity model of the mitochondrial electron transport chain in which complexes I–IV are partly organised into supercomplexes, except for complex II, which feeds electrons via ubiquinone to complex III non-bound to complex I.



A major breakthrough occurred in our understanding of respiratory chain assembly though the introduction of blue native polyacrylamide gel electrophoresis (BN-PAGE) and milder non-ionic detergents used to isolated mitochondrial membrane proteins. This technique was pioneered by Hermann Shägger and colleagues (Arnold et al., 1998, Schagger and Pfeiffer, 2000) and marked the beginning of the study of the higher level of structural organisation of the OXPHOS system. This technique isolates the largest stable protein complexes that can withstand the mild solubilisation, thus allowing the separation of respiratory supercomplexes on gels.

Based on the BN-PAGE technique, the existence of respirasomes was reported for organisms belonging to different kingdoms of eukaryotes. Despite their phylogenetic distances from each other, the presence of supercomplex assemblies is common to them all. Respiratory supercomplexes have been revealed for algae (van Lis et al., 2003), bacteria (Stroh et al., 2004), plant (Eubel et al., 2004), fungi (Dudkina et al., 2005b) and mammalian mitochondria (Schagger et al., 2004, Schagger and Pfeiffer, 2000). Supramolecular organization of OXPHOS systems have been characterized in different mammalian tissues including the rat brain (Dencher et al., 2007, Reifschneider et al., 2006), bovine heart (Schagger and Pfeiffer, 2001, Schagger, 2002, Reifschneider et al., 2006, Krause et al., 2005), liver (Reifschneider et al., 2006, Dani et al., 2010) and kidney (Reifschneider et al., 2006). These supercomplexes separated by BN-PAGE and related techniques are active for their component individual complexes, as shown by in-gel catalytic activity assays (Wittig et al., 2007, Schafer et al., 2006).

1.7.2 Composition of Mitochondrial Supercomplexes

Supercomplexes can be divided into four main groups based on their composition. Complex I, III₂ and IV all interact in various stoichiometries and form three types of supercomplexes: I+III₂, I+III₂+IV_X and III+IV_X (Berry and Trumpower, 1985, Schagger and Pfeiffer, 2001, Stroh et al., 2004, Dudkina et al., 2011). The fourth group is composed of complex V. ATP synthase can form assemblies of dimers and homooligomers. Association of complex II within supercomplex assemblies is still not clear and controversial. A couple of studies show it to associate in small proportions with the supercomplex I+III₂+IV_X (Eubel et al., 2003, Acin-Perez et al., 2008, Muster et al., 2010). Acin-Perez et al, reported complex II to be part of the mouse respirasome, which is able to transfer electrons from succinate (Acin-Perez et al., 2008). However,

the majority of reports in plant and mammalian mitochondria find complex II in a free non-associated form and fail to show structural or functional evidence to suggest its presence in a supercomplex assembly under physiological conditions (Dudkina et al., 2011, Lenaz and Genova, 2010, Quarato et al., 2011, Schafer et al., 2006, Schagger and Pfeiffer, 2001).

The abundance of supercomplexes with varying compositions appears to vary between different organisms. For example, the supercomplex with the composition I+III₂ is most abundant in plants (Eubel et al., 2003), where 90-100% of complex IV is in its monomeric form (Eubel et al., 2004). I+III₂+IV_X supercomplex has been reported to be highest in mammals (Schagger and Pfeiffer, 2000). In bovine supercomplex assemblies it appears likely that complex I is bound to complex III dimers in physiological conditions. Approximately 90% of complex I is associated with a complex III dimer and several units of complex IV to form the two major supercomplexes I+III₂ and I+III₂+IV_X. Finally, in fungi the supercomplex with the composition III+IV_X is most prevalent (Schagger and Pfeiffer, 2000, Heinemeyer et al., 2007).

The largest respiratory chain supercomplex I-III₂-IV_X is one of the most intriguing because it contains all the redox enzymes required for the complete electron transfer from NADH to molecular oxygen. This assembly is referred to as a functional “respirasome”. A recent demonstration on the functional significance of supercomplexes demonstrated that supercomplexes containing complex I+III+IV and I+II+III+IV actively respire (Acin-Perez et al., 2008). This group confirmed the presence of different forms of supercomplexes after solubilisation of mouse liver mitochondria in different detergents and BN-PAGE. The resolved bands on the native gel were identified to contain containing complex I+III+IV and I+II+III+IV. These bands also contain both cytochrome c and ubiquinone. The I+II+III+IV band was excised from the gel and was shown to actively respire in the oxygen electrode in response to both NADH and succinate. The oxygen consumption observed here, fascinatingly demonstrates the presence of all the respiratory components and mobile carriers needed but also demonstrates the proper arrangement into a functional structure.

Complex V exists in large proportions as dimers and homo-oligomers in several organisms and has been intensely studied in yeast and bovine heart (Krause et al., 2005, Dudkina et al., 2006). Complex V as a dimer co-migrates with other supercomplexes but it is unclear whether it is part of the actual assembly. Ribbon like structures composed of ATP synthase dimers can play a major role in shaping the inner mitochondrial membrane because they exert a bending force on the lipid bilayer and induce the formation of tight bends or ridges, which can extend for several hundred nanometers in the cristae membrane. Destabilisation of the ATP synthase dimerization coincides with a collapse of oligomeric architecture resulting in mitochondrial morphologies in the form of onion-like structures (Velours et al., 2009). Cryo-electron tomography identified long rows of complex V dimers located exclusively on the tightly curved cristae edges. On the other hand, supercomplex assemblies locate primarily in the flat adjacent membrane regions of the cristae. The arrangement of respiratory chain proton pumps on flat cristae membranes and ATP synthase dimer rows along cristae edges is conserved in all species investigated and ensure optimal conditions for ATP synthesis (Davies et al., 2011). Interestingly, a recent investigation into the relationship between respiratory function and mitochondrial ultrastructure provided evidence that cristae shape determines the assembly and stability of supercomplexes and hence mitochondrial respiratory efficiency (Cogliati et al., 2013).

An even higher level in the complexity in the organization state of supercomplexes can exist due to the presence of further interaction partners such as the ADP/ATP carrier (Stuart, 2009), some chaperones for the cytochrome c oxidase assembly (Mick et al., 2007), the carbonic anhydrase subunits that co-assemble with the OXPHOS supercomplexes (Sunderhaus et al., 2006) and even the multifunctional fatty acid β -oxidation complex (Wang et al., 2010).

1.7.3 Structure of Mitochondrial Supercomplexes

Additional evidence for supercomplex organisation was based on single particle electron microscopy 2D projection map analyses from different organisms, such as plant (Dudkina et al., 2005a), bovine (Schafer et al., 2006) and yeast (Heinemeyer et al., 2007). Later, 3D projection maps of bovine mitochondria supercomplexes yielded more insights into relative positions and the interactions of the individual respiratory complexes that take place within the supercomplex (Schafer et al., 2007, Althoff et al.,

2011, Dudkina et al., 2011).

2D electron projection map of the plant supercomplex I+III₂ indicates a specific type of interaction between monomeric complex I and dimeric complex III in a 1:1 ratio. Complex III is laterally attached to the membrane arm of complex I in its concave portion (Dudkina et al., 2005a, Bultema et al., 2009, Peters et al., 2008). This was further validated by the characterization of supercomplexes I+III₂ and I+III₂+IV_X from bovine heart mitochondria (Schafer et al., 2007) where similar observations to Dudkina et al., 2005 were made with regards the interactions in I+III₂. This association may help ubiquinone bind to complex I, which occurs above the junction of the membrane and matrix arm.

EM analysis of III+IV_X in *Saccharomyces cerevisiae* (which lacks complex I) revealed that complex III₂ is flanked from both sides by monomeric complex IV with their convex sides facing the complex III₂ (Heinemeyer et al., 2007). This detailed EM map of the supercomplex III+IV₂ is in agreement with the arrangement of the respiratory complexes revealed by a recent cryo EM and proposed pseudo-atomic model, which was based on the negatively stained projections (Mileykovskaya et al., 2012). Here, the distance between cytochrome c binding sites of complex III and IV is about 6 nm, slightly greater than the shorter 4 nm predicted from the 2D images (Heinemeyer et al., 2007). The recent 3D cryo EM map also demonstrated a closer association between individual complex domains at the aqueous membrane interface and larger spaces between the membrane-embedded domains. It is proposed that these spaces may be filled with lipid molecules (Mileykovskaya et al., 2012).

The structure of the supercomplex I+III₂+IV in bovine was recently determined by both cryo-ET of digitonin-solubilised respirasomes (Dudkina et al., 2011) and cryo-EM of amphipol-solubilised respirasomes (Althoff et al., 2011). Both demonstrate that complex III₂ sits in the arc of the membrane arm of complex I while complex IV is attached to the tip of complex I. Again, the ubiquinone and cytochrome c binding sites of each complex in I+III₂+IV_X appeared to be in proximity to the binding site of the succeeding complex in the respiratory chain. In theory, this would lead to a more efficient electron flow due to direct substrate channeling and reduction in the production of reaction oxygen species.

1.8 Functional Consequences for Mitochondrial Supercomplexes

It is currently unclear whether the assembly of supercomplexes has a catalytic or structural purpose in the inner mitochondrial membrane. To date, four major roles have been attributed to the supercomplex organization of the ETC: (a) increased efficiency of electron flux, through substrate channeling or enhanced catalysis, (b) sequestration of reactive intermediates to prevent generation of ROS, (c) structural stabilization of individual respiratory complexes and (d) adjustment to the metabolic demands of the cell.

1.8.1 Increased efficiency of electron flux, through substrate channeling

Supercomplexes have been proposed to confer a catalytic advantage by channeling of substrates between enzymes in the assemblies. In the respiratory chain all enzymes are linked by the electron exchange. Substrate channeling can be described as the direct transfer of an intermediate between the active sites of two enzymes catalyzing consecutive reactions. With respect to the “fluid” model, each complex is a free and independent entity, and substrates exchange freely between enzymes through common ubiquinone/ubiquinol (Q) and cytochrome c_{ox}/c_{red} (cyt *c*) pools. In contrast, complexes in the supercomplex/respirasome are assembled into units that contain everything required for catalysis. In this latter scenario, substrates are channeled between enzymes within the respirasome and are not exchanged with external pools (Schagger and Pfeiffer, 2001, Acin-Perez et al., 2008). This would mean that inter-complex electron transfer becomes indistinguishable from intra-complex electron transfer so that the so-called mobile intermediates, predicted to exhibit substrate-like behavior in the classic fluid model, are buried in the interface between the two consecutive complexes (Figure 1.4).

One of the earliest reports that show that the supercomplexes are active and utilize channeling of the mobile ubiquinone and cytochrome *c* comes from the study of NADH-cytochrome *c* oxidase and NADH oxidase activities in the respirasome isolated by BN-PAGE from *P. denitrificans*. In fact, ubiquinone was found to be enriched tenfold in the supercomplex (Stroh et al., 2004). However, to date, the most widely accepted evidence in support of substrate channeling comes from flux control analysis.

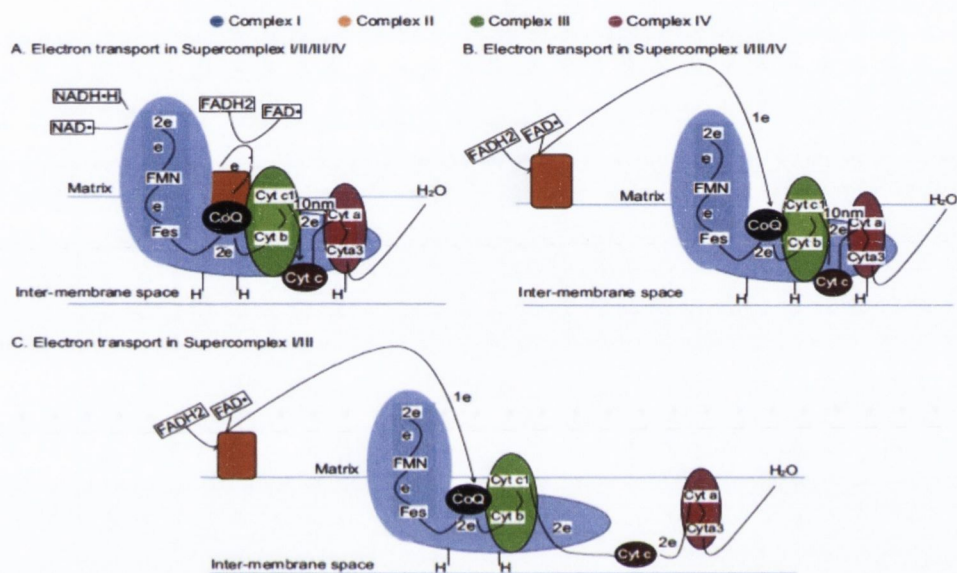


Figure 1.4 Proposed electron transport through different forms of functional supercomplexes. (A) Electron transport through supercomplex I+II+III+IV, **(B)** Electron transport through supercomplex I+II₂+IV₁₋₄ and **(C)** electron transport through supercomplex I-III₂. Illustration taken from (Vartak et al., 2013).

Metabolic flux control analysis allows the quantitative measurement of the control exerted on a pathway by its individual enzyme steps. In the fluid state, the individual steps consist of separate enzymes joined by the diffusion of common intermediates. In this system, the control would be differently exerted by one or more steps in the pathway and the sum of the control coefficients of all steps would approach 1 and not overcome unity. In the situation of a supercomplex assembly, the metabolic pathway would behave as a single enzyme and each component of the catalytic unit would exert the same flux control and the sum of the control coefficients of all steps would be more than 1 and overcome unity (Lenaz and Genova, 2007). Kinetic analysis therefore can assist in distinguishing the channeling in the supercomplex assembly from the random diffusional encounter.

Conflicting evidence to support substrate channeling between bovine complex I and III via ubiquinone currently exists. One of the earliest demonstration of the existence of substrate channeling in supercomplexes was given by kinetic analysis of the pool function of ubiquinone and cytochrome *c* in yeast (Boumans et al., 1998). Mitochondria did not follow 'pool behavior' unless treated with chaotropic reagents that dissociated the individual enzymes from the original single unit respiratory complex. Following on from this, mammalian and plant mitochondrial supercomplexes, detected by BNE were found to be functionally relevant by flux control analysis (Bianchi et al., 2004a, Genova et al., 2008). Bianchi et al. 2004 demonstrated that complex I and complex III in bovine heart submitochondrial particles, both exert rate control on NADH oxidation. This can only be accommodated if they are joined into a single supercomplex that catalyses a single rate-determining step. This study also predicted that cytochrome *c* and ubiquinone pools reside in free form or associate with supercomplexes, depending on the metabolic needs of the mitochondria. A recent study reports the existence of dedicated ubiquinone and cytochrome *c* pools within supercomplex assemblies (Lapuente-Brun et al., 2013). Evidence for channeling between Complexes I and III derived from the demonstration that selective reduction of Complex III under a certain threshold induces a preferential deficiency in the transfer of electrons between Complexes II and III, while the transfer of electrons between Complex I and III remains unaltered (Lapuente-Brun et al., 2013). The partitioning of the ubiquinone pool into supercomplex assemblies was proposed to determine electron transport chain flux. Together, the above studies combined would suggest that the ubiquinone pool is

partitioned such that the ubiquinol produced by complex I is channeled to complex III within supercomplex assemblies, whereas the ubiquinol produced by complex II diffuses into the membrane to access a separate complex III population. In disagreement with these previous reports, a very recent study proposes that ubiquinone exists as a single, common pool in mammalian mitochondria and is exchanged freely between complexes (Blaza et al., 2014). Using both kinetic and spectroscopic experiments this group demonstrate how the metabolic pathways for NADH and succinate oxidation communicate and catalyse via a single, universally accessible ubiquinone/ubiquinol pool that is not partitioned or channeled (Blaza et al., 2014).

Despite conflicting evidence to support substrate channeling between bovine complex I and III via ubiquinone, flux control experiments do not provide evidence for substrate channeling between III and IV via cytochrome *c*. Complex IV in BH submitochondrial particles (SMP) showed weak flux control suggesting that it remains in free form state (Bianchi et al., 2004a). Complex II was fully rate limiting for succinate oxidation, suggesting the absence of substrate channeling towards complexes III and IV via cytochrome *c* (Bianchi et al., 2004a). In addition, a time-resolved kinetic study of the respiratory chain in intact yeast cells concluded that cytochrome *c* is not trapped within supercomplexes and encounters no restriction to its diffusion (Trouillard et al., 2011).

In an attempt to further clarify the role of substrate channeling in supercomplex assemblies, researchers have evaluated the supercomplex structures obtained by electron microscopy. With respect to III₂+IV₂ supercomplex formation identified in yeast, the distance between the cytochrome *c* binding sites of complex III and IV has been estimated to be 4 nm by 2D EM (Heinemeyer et al., 2007) and 6 nm by cryo 3D EM (Mileykovskaya et al., 2012). Given that cytochrome *c* has an approximate diameter of 3.4 nm, there is the potential of direct electron channeling of cytochrome *c* between complex III and IV. Structural analysis of the mammalian I+III₂+IV supercomplex have estimated distances of 130 Å between the ubiquinone binding sites of complexes I and III and 100 Å between the cyt *c* binding sites of complexes III and IV (Althoff et al., 2011, Dudkina et al., 2011). Althoff et al. conclude that formation of the respirasome brings all these sites closer to each other and the short distance by which ubiquinone and cytochrome *c* must travel has been hypothesized to not only speed up the electron transfer process as a whole, but also to minimize loss of electrons during transfer.

Dudkina et al. doubt whether the distances support a kinetic justification for supercomplex formation. In both supercomplex structures cytochrome *c* is clearly visible on the outside of the assembly and the membrane domains of the complexes do not interact directly but are “glued together” by layers of phospholipids; these lipids provide pathways for exchanging Q outside of the supercomplex. A recent interpretation of the structural data on the mammalian I+III₂+IV supercomplex conclude that the structure is consistent with free exchange of both Q and cytochrome *c* (Blaza et al., 2014). In conclusion, the compartmentalization of the Q pool and cytochrome *c* pool remain unclear and under debate (Blaza et al., 2014, Lapuente-Brun et al., 2013).

It is evident that the catalytic role of supercomplexes in substrate channeling is poorly understood and in need of further investigation. On one hand, supercomplex assemblies may be intended to make electron transfer more efficient than if the complexes were randomly distributed in the mitochondrial membrane. However, on evaluating all the current reports available to date, it would suggest that substrate channeling may not be the main reason behind formation of respirasomes.

1.8.2 Sequestration of reactive intermediates to prevent generation of ROS

On the assumption that supercomplexes facilitate electron transfer through substrate channeling, another potential function of these assemblies is to prevent excessive generation of reactive oxygen species (ROS) (see Chapter 1, section 1.11.1). Depending on their concentration, these ROS are either physiological signals essential for the life of the cell or toxic species that damage cell structure and functions (Panov et al., 2007, Seelert et al., 2009). The most harmful of mitochondrial superoxide is generated at complex I and complex III (Finkel and Holbrook, 2000, Lenaz et al., 2006), with complex I being the major contributor (Barrientos and Moraes, 1999). Ubiquinone physiologically interconnects complex I and III by accepting electrons from the former and delivering them to the latter. Therefore substrate channeling of ubiquinone through the assembly of a supercomplex structure would serve to not only increase the speed of electron transfer but also minimize electron leakage (Lenaz and Genova, 2012, Lenaz and Genova, 2007). Limited examples of enhanced ROS generation by dissociation of supramolecular units exist in the literature (Diaz et al., 2012, Rodrigues and Gomes, 2012). It has been proposed that the assembly of high molecular weight supercomplexes act as a way to contain complex I which most likely produces more ROS in its free state

(Lenaz and Genova, 2012). This phenomenon may be a compensatory mechanism to prevent further ROS generation and oxidative damage. The site of electron escape from complex I is still unclear. To date, two sites have been proposed: FMN or the iron sulphur cluster N2. The N2 site may be predominant when complex I is present in a supercomplex assembly. On the other hand, FMN may be predominant following supercomplex dissociation and exposure of the FMN site to oxygen (Porras and Bai, 2015).

In a study by Marazana et al., complex I containing supercomplexes were disrupted using detergents. In a second model, the assembly of supercomplexes was prevented by blocking complex I+III interactions using reconstituted proteoliposomes at a high lipid/protein ratio. The study demonstrated that ROS production at the site of complex I is increased in the absence of supercomplex assembly (Maranzana et al., 2013b). Further, it has been discovered that mtSODs interact directly with the I+III₂+IV_x supercomplex and may protect it from oxidative damage (Suthammarak et al., 2013). A cytochrome b p.278Y to C mutation was shown to lead to an increase in superoxide production and a decrease in the III₂ and III₂+IV_x assembly but coinciding with an increase in the I+III₂+IV_x. Normal activity of complexes I+III and II+III were also measured despite the mutation, suggesting that supercomplexes maintain mitochondrial function through the sequestration of ROS (Ghelli et al., 2013). Together, these studies suggest that supercomplexes maintain mitochondrial function by promoting the sequestration of ROS, as well as the sequestering of vulnerable sites, which protects the complexes from oxidative damage, thus providing a means to regulate ROS levels in the cell (Ghelli et al., 2013).

1.8.3 Structural stabilization of individual respiratory complexes

Another possible function of supercomplexes is a role in the assembly of its components. Analysis of the state of supercomplexes in human patients with an isolated deficiency of complex III (Acin-Perez et al., 2004, Schagger et al., 2004) and in cultured cell models harboring complex III (Acin-Perez et al., 2009a), complex IV (Diaz et al., 2006) or cytochrome c (Vempati et al., 2009) depletion led to the proposal that the formation of respirasomes may be essential for the assembly/stability of Complex I. Mutations in mitochondrial complex III uniquely affect complex I in *Caenorhabditis elegans* (Suthammarak et al., 2010). Cytoplasmic hybrids (cybrids)

made by fusing one cell type with a complex IV deficiency with another cell type with complex III deficiency completely recovered respiration by complementation and this recovery of respiration correlated with the presence of complex I-III-IV supercomplexes (D'Aurelio et al., 2006). It has also been demonstrated that complex I is necessary for the full assembly of the complex III in human cells (Moreno-Lastres et al., 2012, Ugalde et al., 2004). Structural stabilization of a membrane protein complex was also identified as a function of respiratory chain supercomplexes in the cytoplasmic membrane of the possible ancestor of eukaryotic mitochondria, *Paracoccus denitrificans*. Here, the assembly of the respirasome I+III₂+IV₄ was shown to stabilize complex I. A recent study performed on NDUFS4 knockout mice, reported that the absence of NDUFS4 (a gene encoding an accessory subunit of complex I) in different tissues results in decreased activity and stability of complex I. This complex I instability leads to an increased disconnection of electron influx of the NADH dehydrogenase module from the holo-complex, however, the portion of complex I that is still active is bound in the supercomplexes (Calvaruso et al., 2012).

1.8.4 Adjustment to the metabolic needs of the cell

The “plasticity” model highlights the ability of the OXPHOS complexes to switch from freely moving to fixed substrates and vice versa. Through the observations of varying assemblies of complexes and supercomplexes in different cell types, a newly proposed function for supercomplex assembly and disassembly is to respond and adapt to changes in cellular metabolism (Acin-Perez et al., 2008). A recent study of electron flux in the OXPHOS system points to separate pathways in the electron transport chain depending on the presence of the assembly factor Cox7A21 (SCAF1). In the presence of this assembly factor, three complex IV populations were identified. Complex IV can receive electrons from NADH via I+III₂ or I+III₂+IV, from FAD via complex II and single III₂ and IV or III₂+IV, or from both NADH and FAD (Lapiente-Brun et al., 2013). This organization of complexes in the inner mitochondrial membrane would enable the simultaneous oxidation of multiple substrates. In agreement with this, the same group demonstrated that the ratio between electrons coming from NADH and FAD is higher when glucose is the main substrate and lower if fatty-acid oxidation is the main substrate source. These findings suggest that assembly of OXPHOS complexes into supercomplexes is a dynamic process and may have a functional role in the efficient oxidation of available substrates.

A more recent and new function of supercomplex assembly has been put forward. They propose that supercomplexes have evolved in response to pressure to maintain an extremely high protein concentration but avoid nucleation and aggregation. A set of preferred, weak, and reversible intercomplex interactions preclude the formation of irreversible strong interactions (Blaza et al., 2014).

1.9 Mitochondrial Supercomplex Assembly

The mechanism behind the assembly of these supramolecular complexes has still not been fully elucidated. There are currently two theories behind supercomplex assembly (Vartak et al., 2013). The first hypothesizes that there are exclusive assembly factors that assist in the assembly of supercomplexes post individual complex assembly. The second theory hypothesizes that assembly factors are shared between the different enzymes.

Earlier studies reported the presence of a temporal gap between the assembly of individual complexes and supercomplexes (Acin-Perez et al., 2008). This group performed pulse chase experiments that analysed the time course incorporation of the thirteen mtDNA encoded proteins into the respiratory chain complexes and supercomplex assemblies and their results suggested that assembly of supercomplexes originate by the direct association of single pre-assembled complexes.

Other investigations from the study of yeast contradict this and show that the supercomplex I+III₂ was formed before complex I had been had been fully assembled and synthesized (Marques et al., 2007). A later study addressed the assembly of newly imported complex IV subunits in mitochondria from control cells and from cells of patients with defects in complex IV biogenesis (Lazarou et al., 2009). They observed that in the absence of monomeric holo-complex IV, newly imported nDNA-encoded complex IV subunits were preferentially integrated into supercomplexes. This suggested that the formation of supercomplex assemblies does not always require the preassembly of individual holocomplexes, but can also be achieved through the coordinated assembly of partially assembled complexes and free subunits. This latter further addressed by a group that partially depleted control cell lines of OXPHOS complexes

though treatment with doxycycline, a reversible inhibitor of mitochondrial translation (Moreno-Lastres et al., 2012). After removal of the drug, synthesis of mt-DNA-encoded subunits resumed and their time course incorporation into supercomplex assembly intermediates was monitored. This group has proposed a multistep model from supercomplex assembly. The first step involves the assembly of complex III and IV as individual entities until reaching a threshold. At the same time, complex I assembly also starts to take place parallel to complex III and IV assembly as already previously reported (Pello et al., 2008). This parallel assembly of complex I continue until the complex I intermediate of ~830 kDa that lacks the NADH dehydrogenase module and other additional subunits is reached. This pattern of complex I subcomplexes is in agreement with previous reports (Lazarou et al., 2009, Vogel et al., 2007, Ugalde et al., 2004). The Moreno-Lastres group show that the association of complex IV and III, in that order to a partially assembled complex I, was necessary for the incorporation of the remaining complex I subunits (Moreno-Lastres et al., 2012). It has been proposed that this ~830 kDa complex I sub-assembly constitutes the first supercomplex assembly intermediate. It is possible that these remaining subunits (catalytic subunits from the NADH dehydrogenase module) are, in fact, supercomplex assembly factors since complex III and complex IV have already assembled with complex I (Vartak et al., 2013).

Although the assembly of individual respiratory complexes in the inner mitochondrial membrane into distinct supercomplexes is established, the factors required for this assembly are unknown. These assembled respirasomes appear to be very stable as they can be isolated as whole entities without a significant amount of degradation. Therefore, it is believed a role exists for other molecules to act as stabilizing factors. In recent years, there has been a lot of interest and research performed to identify these factors that are responsible for gluing together the respirasome components.

1.9.1 Role of Membrane Lipids

A major determinant of protein organization in biological membranes is the heterogeneous nature of the lipids in the membrane bilayer. Mitochondrial membranes in the brain, for example, contain large amounts of lipids, most of which are composed of phospholipids (Bolanos et al., 1997). Predominant phospholipids in the mitochondrial inner membrane include cardiolipin (CL), phosphatidylcholine (PC),

phosphatidylethanolamine (PE) and lesser amounts of neutral lipids and phosphatidylinositol (PI) (Fleischer et al., 1962).

Cardiolipin (CL), diphosphatidylglycerol, (1,3-bis(sn-3'-phosphatidyl)-sn-glycerol), is an anionic phospholipid of unusual structure and is exclusively localized within the IMM. The unique structure of CL is composed of two phosphatidic acid moieties that connect with a glycerol backbone in the center to form a dimeric structure. It has four alkyl groups and potentially carries two negative charges. As there are four distinct alkyl chains in CL, the potential for complexity of this molecule species is enormous. To date, the presence of CL in the inner mitochondrial membrane has been demonstrated necessary for the regulation of many bioenergetic processes including electron transport, inner membrane supermolecular assembly, anion transport, efficient ATP synthesis, binding of cytochrome c and the function of multiple other mitochondrial inner membrane enzymes (Hoch, 1992, Robinson, 1993, Schlame et al., 2000, Lee, 2004). CL can physically bind complex I, complex III, complex IV and complex V with complex III and complex IV even have up to six binding sites for CL (Fry and Green, 1981, Arnarez et al., 2013a, Arnarez et al., 2013b, Bazan et al., 2013). Having determined that CL specifically binds the ETC complexes, it was also discovered that this phospholipid is necessary for the activity of complex I, complex III and complex IV (Fry and Green, 1981) as well as several mitochondrial carriers (Houtkooper and Vaz, 2008). Reports from different laboratories show that CL is specifically required for electron transfer in complex I of the respiratory chain (Ragan, 1987, Fry and Green, 1981, Ohtsuka et al., 1993, Drose et al., 2002). Further consolidating the evidence that CL is involved in the functioning of complex I, came from findings that show that NAO (nonyl acridine orange), a compound that interacts with CL, inhibits the complex I activity in bovine SMPs and that on the addition of exogenous CL, full inactivation of the enzyme was prevented (Paradies et al., 2002). These results indicate that CL may therefore be necessary for the structural integrity of the complexes and play a functional role in the coupled electron transfer process (Sharpley et al., 2006). In fact, CL is now one of the earliest assembly factors believed to have an important role in supercomplex biogenesis.

CL has been demonstrated to be necessary for the formation of the III₂+IV₂ supercomplex in mitochondria (Zhang et al., 2002, Pfeiffer et al., 2003). Mass

spectrometry analysis of the yeast III₂+IV₂, identified up to fifty molecules of CL per yeast supercomplex (Mileykovskaya et al., 2012). In yeast III₂, CL associates at sites known to interact with complex IV (Heinemeyer et al., 2007). In agreement with this, CL was identified to locate between VIIa and VIIIc subunits of complex IV, which can interact with complex III (Sedlak et al., 2006). The crystal structure of bovine complex IV reveals one CL molecule at subunit III (Shinzawa-Itoh et al., 2007). Coincidentally, the position of this CL is at the interface with complex III (Dudkina et al., 2011). Recently, a molecular dynamics simulation has identified a further three potential sites for CL which can play a role in the assembly of the III₂+IV₁ supercomplex (Arnarez et al., 2013b). One way by which CL stabilizes supercomplex formation is by neutralizing the charge of lysine residues in the interaction domain of complex III with complex IV (Wenz et al., 2009).

Interpretation of the mammalian respirasomes architecture demonstrate that the complexes are in loose association with each other and at some distance within the supercomplex in the lipid bilayer. In these cry-EM maps, the space between the complexes is composed of material with a lower density (Althoff et al., 2011, Dudkina et al., 2011). It likely that these spaces are filled with membrane lipids such as CL. This would likely have a role in keeping the assemblies stable or enhance the diffusion of ubiquinone and cytochrome c. These results strongly indicate that CL plays a central role in the higher order organization of the respiratory chain complexes and may stabilize respiratory supercomplexes as well as the individual complexes. The dependence on CL for supercomplex formation suggests that changes in CL levels resulting from changes in physiological conditions may control the equilibrium between individual respiratory complexes and supercomplexes (Bazan et al., 2013).

There is also a focus on other phospholipids that can bind and stabilize supercomplexes (Wenz et al., 2009). Phosphatidylethanolamine (PE) is also emerging as a potential stabilizing candidate. In contrast to depletion of CL, depletion of PE did not destabilize respiratory chain supercomplexes but favored the formation of larger supercomplexes between the complex III and complex IV in yeast mutants (Bottinger et al., 2012). In conclusion, these studies indicate that lipid composition of the inner mitochondrial membrane is important for the formation and stability of supercomplexes. It is evident there is an intricate relationship between the protein/lipid ratio and supercomplexes. For

supercomplex assembly, it is important that the overall concentration of lipids is low enough to favor protein aggregation. At the same time, the concentration of CL specifically must be high enough to successfully stabilize supercomplexes after their assembly (Porrás and Bai, 2015).

1.9.2 Role of Membrane Potential

Phospholipids composition may change by genetic or dietary reasons and involve a relatively long time-scale. Some biochemical parameters, however, at a shorter time scale may be responsible for regulating supercomplex assembly. One of these is the membrane potential. An *in vivo* study on the control membrane potential exerts on respiration demonstrated that in the uncoupled condition, complex IV has a low reserve capacity and a large control coefficient. This control is lost upon the establishment of an electrochemical potential (Piccoli et al., 2006, Dalmonte et al., 2009). Piccoli et al., suggests that a high membrane potential is indicative of low energy demand in the cell. In response, supercomplexes would disassemble into their individual entities resulting in a decrease in oxygen consumption. In contrast, a low membrane potential is indicative of an increase in the energy demand and ATP is rapidly used. In response, supercomplexes would reassemble to satisfy the requirement for a higher rate of respiration (Piccoli et al., 2006). The composition mitochondrial supercomplexes in plants change in response to pH changes and oxygen availability (Ramírez-Aguilar et al., 2011). Complex I dissociated from I+III+IV supercomplex causing an increase in the III+IV assembly under low mitochondrial pH and hypoxia.

1.9.3 Role of Post Translational Modifications

Another biochemical parameter at a shorter time scale suggested to affect the supermolecular structure of respiratory complexes is the phosphorylation state of the protein subunits of the proteins subunits of the complexes. Post-translational modification (PTM) of proteins is an important process for quickly and transiently modifying the structure of a protein causing changes in enzyme activity as well as interfering or aiding protein-protein interactions. To date no work has specifically analyzed the role of PTM in supercomplex assembly or function. But there is a large body of information on PTMs of individual respiratory complexes and other mitochondrial proteins. Mitochondria contain protein kinases and phosphatases, and

both serine/threonine phosphorylation (Deng et al., 2011, Grimsrud et al., 2012) and tyrosine phosphorylation (Hebert-Chatelain, 2013) of mitochondrial proteins are important in regulation of activity of these organelles. It therefore makes sense that this post-translational event should be considered when exploring the assembly and stability of supercomplexes.

Phosphorylation has been linked to complex I activity as well as its ROS generating capacity (Papa et al., 2008, Bellomo et al., 2006). It is known that phosphorylation by Protein kinase A (PKA) is important in regulating oxidative phosphorylation. Protein kinase A phosphorylates a number of complex I units, including NDSUF7, NDUFA1 and NDUF54 (Raha et al., 2002, De Rasmo et al., 2008). Phosphorylation of these subunits leads to stability of complex I, increased NADH/cytochrome c reductase activity and decreased superoxide formation (Maj et al., 2004). Phosphorylation of the same NDSUF7 by pyruvate dehydrogenase kinase has an opposite effect (Raha et al., 2002). The phosphorylation of complex IV subunits by PKA has been well documented and Phosphorylation sites were found in subunits II, III, IV, Va, Vb, VIa, VIb, VIc, and VIII and COX1 (Lee et al., 2005, Helling et al., 2008). This phenomenon would affect the ability of complex IV to bind with OXPHOS complexes and other proteins (Lee et al., 2005, Rosca et al., 2011). Phosphorylation COXI and COX IVb was shown to increase respiration and reduce ROS (Acin-Perez et al., 2009b).

1.9.4 Role of Other Assembly Factors

Beside lipids as discussed earlier, the cytochrome c oxidase subunit VIIa polypeptide 2-like (Cox7a21) and other non-respiratory chain proteins are shown to play a role in the stability of supercomplexes. The Cox7a21 polypeptide was identified in the respirasome and III+IV₁ supercomplex in mouse fibroblasts, but not in free complexes III or IV (Lapiente-Brun et al., 2013). In the absence of Cox7a21, complex IV does not assemble into these complex IV containing supercomplexes, suggesting it an authentic supercomplex assembly factor. As a result it has now been renamed as supercomplex assembly factor 1 (SCAF1) (Lapiente-Brun et al., 2013)

A recent study identified a cytochrome c oxidase subunit 7a-related protein (COX7RP) as a protein that functions in I+III₂+IV_n supercomplex assembly and is required for full activity of mitochondrial respiration. BNE reveals that COX7RP is a key molecule that

promotes assembly of the III₂+IV_n supercomplex with complex I (Ikeda et al., 2013). Recently it was demonstrated that C11orf83 is a new assembly factor of the bc₁ complex and also a stabilizing factor of the III₂+IV supercomplex and thus is required for proper mitochondrial morphology and function (Desmurs et al., 2015). Stomatin-Like Protein 2 (SLP-2) is another mitochondrial protein that has recently been shown to play a role in the stability and assembly of respiratory chain supercomplexes (Mitsopoulos et al., 2015). Primary T-cells lacking SLP-2 results in decreased amounts of (I+III₂)₁₋₂ and I+III₂+IV₁₋₃ indicating this protein as a key regulator of mitochondrial respiration by assisting in optimal assembly of the supercomplexes.

Two small proteins Rcf-1 (respiratory complex factor 1) and Rcf-2 (respiratory complex factor 2) of 18 and 25 kDa respectively, are critical components of the III-IV supercomplex assembly and stability in yeast (Strogolova et al., 2012, Chen et al., 2012). It is now thought that Rcf-1 belongs to an evolutionary conserved protein family that acts to promote respiratory complex assembly and activity (Chen et al., 2012). This phenomenon was also observed in mammalian mitochondria. Mammalian orthologs have been identified for Rcf-1 (HIG1A and HIG2A). Knockdown of HIG2A causes impaired supercomplex formation (Chen et al., 2012). However, as observed in yeast this protein is required for the assembly of the COX3 subunit into mature complex IV (Strogolova et al., 2012). Therefore, interference with this subunit impairs both complex IV assembly and consequently supercomplex assembly. This questions the authenticity of these factors as unique to supercomplex assembly and should be regarded more as a complex IV assembly factor than a supercomplex assembly factor.

The abundant isoform of the ATP/ADP carrier protein AAC2, facilitates the transport of ATP across the mitochondrial membrane. In yeast, it immunoprecipitates with the III₂+IV₁₋₂ supercomplex and deletion of AAC2 results in decreased levels of this supercomplex (Dienhart and Stuart, 2008). It was also shown that AAC2 requires CL for proper function (Claypool et al., 2008). Due to these observations, AAC is now emerging as a potential player in supercomplex assembly.

Another proposed assembly factor is prohibitin but to date its exact role in supercomplex assembly and stability is not fully understood. Prohibitins (PHB) are a conserved family of proteins present in the mitochondrial inner membrane (Berger and

Yaffe, 1998). PHB1 and PHB2 are most commonly found in mitochondria. Mitochondrial prohibitin complexes control cell proliferation, cristae morphogenesis and the functional integrity of mitochondria (Merkwirth and Langer, 2009). Homomeric PHB2 is important for cytochrome-c oxidase assembly and mitochondrial respiration (Strub et al., 2011). In yeast mitochondria, PHB co migrates with III-IV supercomplex (Marques et al., 2007).

To date, only one protein has been described as a potential player in the reversible regulation of supercomplex association and activity. MCJ/DnaJC15 is a distinct co-chaperone that localises at the mitochondrial inner membrane where it interacts preferentially with Complex I. MCJ impairs the formation of supercomplexes and functions as a negative regulator of the respiratory chain (Hatle et al., 2013).

Mitochondrial dynamics may also play a role in the assembly of supercomplexes. The reorganization of mitochondrial cristae during fusion and fission requires supercomplexes to be relocated in order to maintain proper OXPHOS function. This requires their disassembly and reassembly. A recent investigation into the relationship between respiratory function and mitochondrial ultrastructure in mice suggest that cristae shape determines the assembly and stability of supercomplexes and hence mitochondrial respiratory efficiency (Cogliati et al., 2013). OPA1 has been implicated in maintaining the cristae junctions tight (Frezza et al., 2006). Genetic ablation or overexpression of OPA1 revealed that supercomplex assembly stability and function require an intact cristae structure (Cogliati et al., 2013). Induction of apoptosis promotes the rupture of cristae junctions, which releases cytochrome c to the cytosol, thereby destabilizing supercomplexes (Cogliati et al., 2013). This investigation provides evidence of a direct link between mitochondrial inner membrane organization and incorporation of functional supercomplexes.

1.10 Supercomplexes and Disease

Attention is now being given to the possibility that supercomplexes may have biomedical implications. At present, supercomplex disassembly is not fully understood and it is still unclear whether this is a rapid or slow event in the mitochondria (Muster et al., 2010). However, the loss of supercomplexes and disassembly into individual respiratory complexes may either be an indicator or directly cause pathology. As a result, supercomplex deficiencies have been linked to various disorders. Barth syndrome is the most characterized of these disorders, but supercomplexes have to a lesser extent been implicated in respiratory complex deficiency disorders, neurodegenerative disorders, cancer progression as well as in the aging process.

1.10.1 Cardiovascular Disease

Perhaps the most well known example of pathology directly linked to destabilization of supercomplexes is Barth Syndrome (BTHS). This is an X-linked recessive disorder and is characterized in affected males by cardiomyopathy, neutropenia, cardioskeletal myopathy and abnormal growth. Barth syndrome is caused by mutations in the *Taz1* gene whose product, tafazzin, is an acyltransferase involved in the synthesis of mature CL (Bione et al., 1996). The mitochondria of these patients show a variety of respiratory abnormalities and abnormal cristae formation (Barth et al., 1996). One of the characteristics of tafazzin is its physical association with two distinct respiratory components in the mitochondrial inner membrane, ATP synthase and the adenine nucleotide carrier (ANC). Mutated tafazzin leads to instability and reduced abundance of these assemblies, ultimately resulting in abnormal cristae formation and respiratory defects (Claypool et al., 2008). Barth syndrome is also characterized by decreased level of CL and this aberration is now used as a diagnostic parameter for the identification of individual suffering from Barth Syndrome. Loss of mature CL species in Barth Syndrome results in unstable respiratory chain supercomplexes by weakening the interactions between complex I and complex III, ultimately affecting complex I biogenesis, respiratory activities and subsequent pathology.

The role of the organization of the respiratory chain into supercomplex in experimentally induced heart failure has been strongly promoted. Investigations into canine cardiac mitochondria during heart failure showed a severe reduction of

supercomplex organization, with major decrease in complex I+III+IV supercomplex (Rosca et al., 2008). Later studies ruled out changes in phospholipid composition as the cause for the decline in supercomplexes. Interestingly, complex IV units that were not incorporated into these higher molecular weight assemblies presented with increased threonine phosphorylation. It was postulated that phosphorylation of complex IV subunits decreases oxidative phosphorylation either by limiting the incorporation of the enzyme into supercomplexes or decreasing the stability of the supercomplex assembly (Rosca et al., 2011).

1.10.2 Respiratory Complex Deficiency Disorders

The supercomplex assembly model may explain why certain genetic defects affecting a single complex lead to combined respiratory chain complex deficiencies in patients and animal models. For example, complex IV deficiencies due to mutations in Cox1 and Cox2 lead to pleiotropic complex I defects (D'Aurelio et al., 2006, Moreno-Lastres et al., 2012). The incorporation of these two subunits into a supercomplex assembly takes place prior to the incorporation of the complex I catalytic module subunits, NDUFV1 and NDUF4. The absence of the complex IV subunits affects the assembly and stability of the complex I catalytic subunits within the supercomplex assembly. This has a knock on effect and results in a build up of the inactive ~830 kDa supercomplex assembly intermediate. It also results in the accumulation of a I+III₂ intermediate that is missing a complex I catalytic module. This may explain the decrease in complex IV activity observed in Cox1 and Cox2 mutant cells.

Another study was carried out on two unrelated children with complex III deficiencies (Fernandez-Vizarra et al., 2007). Both children were compound heterozygotes for novel mutations in the human bc1 synthesis like (BCS1L) gene, which encodes an AAA (ATPases associated with a variety of cellular activities) mitochondrial protein putatively involved in both iron homeostasis and complex III assembly. Defective BCS1L leads to the formation of a catalytically inactive, structurally unstable complex III, which may lead to secondary complex I deficiencies. A more recent study on mutations in BCS1L showed similar findings (Moran et al., 2010). Cells from patients with the most severe clinical phenotypes exhibited slow growth rates in glucose medium, variable combined enzyme deficiencies, and assembly defects of respiratory chain complexes I, III, and IV, increased H₂O₂ levels, unbalanced expression of the

cellular antioxidant defenses, and apoptotic cell death. In addition, fragmentation of the mitochondrial networks, and decreased Mfn2 protein levels was observed. It is therefore, quite possible that interference in supercomplex assembly can have detrimental knock on effects.

1.10.3 Neurodegeneration

The role of supercomplex assembly defects in neurodegenerative disease is only in the early stages of investigation. Supercomplex deficiencies in theory will impact tissues more reliant on oxidative phosphorylation than other tissues, which preferentially utilize glycolysis as a means for ATP production (Vartak et al., 2013). Therefore, nervous tissues may be particularly vulnerable and there may well be a connection between supercomplex deficiencies and neurodegenerative disorders.

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor disturbances, appearance of Lewy bodies and dopaminergic neuronal death in the substantia nigra. The resultant dopamine deficiency leads to a movement disorder characterised by classical parkinsonian motor symptoms. These parkinsonian symptoms include bradykinesia, muscular rigidity, rest tremor, and postural and gait impairment (Gibb and Lees, 1988). Mutations in specific genes have been conclusively shown to cause PD. These genes code for alpha-synuclein (SNCA), parkin (PRKN), leucine-rich repeat kinase 2 (LRRK2 or dardarin), PTEN-induced putative kinase 1 (PINK1), DJ-1 and ATP13A2 (Lesage and Brice, 2009).

It would also appear that mitochondrial dysfunction is a factor in the development of the disease of PD. Loss of complex I content and activity accompanied by oxidative stress has been widely reported. Complex I activities were reported to be significantly reduced (in the range of 30%) in post-mortem substantia nigra of PD patients (Schapira, 1993, Schapira et al., 1990, Schapira et al., 1989) and platelets from patients with PD (Parker et al., 1989, Krige et al., 1992, Haas et al., 1995, Benecke et al., 1993). In addition to the substantia nigra, a 30% complex I deficiency has been reported in frontal cortex mitochondrial from PD post mortem brain (Parker et al., 2008, Keeney et al., 2006). The cause and the consequences of complex I deficiency in PD are not well understood. One theory behind the observed reduced complex I function in PD brain mitochondria is oxidation of its catalytic subunits. This complex I auto-oxidation may derive from

mutations in complex I genes in the mitochondrial or nuclear genome, complex I assembly factors or a combination of both (Keeney et al., 2006) and correlates with dysfunction in complex I activity, assembly and/or stability. Parkin can also be linked to mitochondria as it physically interacts with the outer mitochondrial membrane (Darios et al., 2003). A 60% decrease in complex I activity was found both in patients with parkin mutations and sporadic PD patients, whereas complex IV activity was only reduced in sporadic PD patients (Muftuoglu et al., 2004). Cultured fibroblasts from parkin mutant patients showed morphological and functional mitochondrial defects, i.e. a decrease in the membrane potential, complex I activity ATP production and an increase in rotenone-induced mitochondrial fragmentation (Mortiboys et al., 2008). Loss-of-function mutations in the genes encoding PINK1 mediate autosomal recessive PD. Both Parkin and PINK1 may have a possible role in modulating mitochondrial morphology and dynamics (Poole et al., 2008). Fission and fusion events alongside mitophagy are important quality control mechanisms for the cell and defects in these processes have also been linked with Parkinson's disease (Narendra et al., 2008, Vives-Bauza et al., 2010). Given the complex I deficiencies alongside oxidative stress observed, and the role of supercomplexes in complex stabilization and ROS sequestration, it is conceivable that a link exists between supercomplex deficiencies and mitochondrial dysfunction in PD.

1.10.4 Cancer

Mitochondria play essential roles in cancer progression. In cancer, cell metabolism switches towards aerobic glycolysis as an energy source (the Warburg effect) accompanied by a depression in OXPHOS (Ostroukhova et al., 2012, DeBerardinis et al., 2008). The biochemical and molecular mechanisms underlying this metabolic switch are not well understood. Impaired K-ras expression causes mitochondrial dysfunction, leading to decreased respiration, elevated glycolysis, and increased generation of reactive oxygen species (Hu et al., 2012). This coincides with a decrease in complex I content and activity and poor ROS defense mechanisms (Baracca et al., 2010). In response, mitochondria switch towards glycolysis, lactate production and apoptosis that leads to tumor formation and proliferation (Hu et al., 2012). Mutations of the mitochondrial or nuclear DNA that affect components of the mitochondrial OXPHOS system also result in reactive oxygen species (ROS) overproduction and promote tumour cell proliferation. The possible influence of supercomplex organization

on the depression of the OXPHOS system, increased ROS production and tumorigenesis was proposed recently (Gasparre et al., 2013). The authors suggest a deep implication of the supercomplex organization of the respiratory chain as a missing link between oxidative stress, energy failure, and tumorigenesis. They propose that ROS produced in tumorigenesis would alter supercomplex formation, leading primarily to disruption of complex I assembly and activity and thus promoting a second peak of ROS generation, which would amplify the mitochondrial defect (Brandon et al., 2006). Interestingly, modifications in the levels of ROS have been linked to the resistance of cancer stem cells to chemotherapy (Diehn et al., 2009). The OXPHOS system appears to be an attractive drug target in treatment of cancer and mitochondria-associated diseases. Therefore, understanding the supramolecular organisation of the OXPHOS system and its role in functioning of the respiratory chain is essential for the development of such treatment.

1.10.5 Aging

Mitochondrial dysfunction and deficiency of respiratory supercomplexes are correlated to mitochondrial morphology/quality maintenance also in the development of the mitochondrial aging phenotype. Aging-dependent decay of mitochondrial function is directly linked to increased ROS production, but little is known about the molecular mechanisms involved. ROS have a strong influence on the maintenance and performance of supercomplexes, and a growing number of studies show that ROS levels are correlated with a decrease in supercomplex assembly and disease (Maranzana et al., 2013a). Recent hypotheses propose that the decline in mitochondrial function with age is related to the decrease in the levels of high molecular weight supercomplexes in heart (Gomez and Hagen, 2012, Gomez et al., 2009) and rat brain cortex (Frenzel et al., 2010). In mitochondria of rat cortex, there was a 2.4 fold (58%) decline in supercomplexes lacking complex IV, I+III₂. The proportion of supercomplex I+III₂+IV₁ and I+III₂+IV₂ also decreased during aging by about 1.4-fold (31%) and 1.7-fold (41%), respectively. The decline of supercomplex I+III₂+IV₃ was less pronounced. As a consequence, the pronounced decrease of complex-I containing supercomplexes reflects the large, functional decreased of complex I during aging of about 1.6-fold (40%) (Frenzel et al., 2010). In contrast, a study on aging rat skeletal muscle demonstrated an increase in the levels of the highest molecular weight supercomplexes (Lombardi et al., 2009). This may represent a compensatory molecular mechanism that enhances the

catalytic activity of the respirasome by better channeling of fuels and by preventing further ROS generation and oxidative damage.

1.11 Aging

There is no universal definition of the aging process. It can be described as the gradual and progressive structural and functional deterioration of all tissues and organs over time. However, the losses are more marked in the functions that depend on integrated responses of the central nervous system (Gilad and Gilad, 1995). The fact that aging is a universal process would suggest that there is an equally universal mechanism that governs it. Different aging theories have proposed a number of such mechanisms over the years, the mitochondria being a key player.

1.11.1 Reactive Oxygen Species (ROS)

In Harman's original "free radical/mitochondrial theory of aging," which was first proposed in 1978, reactive oxygen species (ROS) generated during aerobic metabolism can damage the mtDNA and proteins, thereby leading to deterioration of cells, tissues and organs and, eventually, the entire body. Aging-dependent decay of mitochondrial function is directly linked to increased ROS production (Lass et al., 1998, Sohal et al., 1994). ROS are free radicals with a single unpaired electron. They are generated during all types of aerobic metabolism and even though they have a half-life only nanoseconds, they can cause damage to virtually every molecule type in the cell, including lipids, proteins, and nucleic acids (Floyd et al., 2001), cumulatively known as oxidative stress. The most common forms of ROS are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$). The superoxide anion can be converted to H_2O_2 by superoxide dismutase, SOD. Although H_2O_2 itself is not a free radical, it can be converted to the highly reactive hydroxyl radical ($\bullet OH$) in the presence of reduced transition metals through the Fenton reaction. $\bullet OH$ is considered to be the most damaging form of ROS (Fridovich, 1997).

Physiological levels of ROS and the exact magnitude of ROS generation are still unclear and continue to be a subject of great debate (Chance et al., 1979, Aguilaniu et

al., 2003). Depending on their concentration, mitochondrial ROS can have beneficial or detrimental effects. In the physiological range, ROS participate in homeostasis as modulators of growth factor signaling (Sundaresan et al., 1995), activators of uncoupling proteins (Echtay et al., 2002) and regulators of mitochondrial biogenesis (Moreno-Loshuertos et al., 2006). In contrast, above the physiological threshold ROS are pathological causing lipid peroxidation, protein oxidation and mitochondrial DNA damage. Hence mitochondria are both producers and targets of ROS. This is the premise of the “free radical/mitochondrial theory of aging”. Accordingly, the vicious cycle of ROS and mitochondrial dysfunction has been extensively studied in models of aging (Bratic and Larsson, 2013, Marchi et al., 2012) and the deleterious effects of free radicals in the aging process have been demonstrated.

Considerable defense mechanisms have evolved to cope with the free radical load produced by mitochondria (Gutteridge and Halliwell, 2000). Among these are antioxidants, both enzymatic and non-enzymatic, which regulate the intra and extracellular steady state free radical content. The primary enzymatic antioxidant of aerobic cells is SOD (Fridovich, 1997). ETC-generated ROS are detoxified through a two-step process. First the $O_2^{\bullet-}$ is converted to H_2O_2 either spontaneously or with the help of SOD. Secondly, H_2O_2 in the mitochondrial matrix is detoxified by peroxiredoxins III and V (PrxIII and PrxV) and by glutathione peroxidase (GPx1) (Rhee et al., 2005). $\bullet OH$ cannot be eliminated by enzymatic antioxidants, instead it can be neutralised by non-enzymatic antioxidants such as vitamins A, C and E. In the aging brain, as well as in the case of several neurodegenerative diseases, there is a decline in the normal antioxidant defense mechanisms, which increases the vulnerability of the brain to the deleterious effects of oxidative damage (Finkel and Holbrook, 2000). This accumulation of free radical induced damage combined with the inability to counterbalance these changes by endogenous anti-oxidant defenses, ultimately results in aging and death

Dysfunctional mitochondria have to be either repaired constantly or degraded in order to prevent cellular damage and maintain mitochondrial homeostasis. The cell has several mechanisms to prevent the accumulation of damaged mitochondria, including mitochondrial fission and fusion cycles (Wang et al., 2009), biogenesis (Boveris and Navarro, 2008), and the selective elimination of damaged mitochondria by autophagy

(Cuervo, 2008). However, disruptions in these processes have also been implicated in the aging process (Gonzalez-Freire et al., 2015, Sandri, 2010, Stauch et al., 2014a). For example, aging is associated with the upregulation of mitochondrial fusion in skeletal muscle, possibly in order to cope with increased levels of mtDNA mutations (Joseph et al., 2013).

1.11.2 Mitochondrial DNA and Aging Process

It is generally believed that ROS generated during oxidative metabolism cause accumulation of mtDNA damage and mutations leading to loss of fidelity in newly synthesized proteins which ultimately impacts on mitochondria physiology, and may lead to premature senescence and aging (Larsson, 2010). The accumulation of aging associated point mutations and deletions in mitochondrial DNA have been reported in a variety of tissues in humans, monkeys and rodents (Corral-Debrinski et al., 1992, Yen et al., 1989, Fayet et al., 2002) leading to a decreased assembly of respiratory chain complexes, promotion of aging phenotypes and consequently premature aging (Chomyn and Attardi, 2003). These studies consistently show that mtDNA mutations (deletions, point mutations, gross DNA rearrangements) increase with age in mammals, especially in post-mitotic highly aerobic tissues such as the brain, heart and skeletal muscle. Less than 5% of the total mtDNA from the skeletal tissues of a 90 year old was still in the form of full length DNA (Linnane et al., 1998) while the bulk of the mtDNA was made up of deletion products mutations. In the heart and brain of slowly aging mammals, lower mtDNA damage has been shown than those who age faster (Barja and Herrero, 2000).

It was once believed that free radicals of mitochondrial origin are among the primary causes of mitochondrial DNA (mtDNA) damage. Several studies have found increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, in mtDNA in the aged brain (Agarwal and Sohal, 1994). This higher susceptibility of mtDNA to oxidative damage was thought to be due to a lack of mtDNA repair mechanisms, a lack of protection by histone proteins, as well as the fact that mtDNA is located close to the inner mitochondrial membrane where ROS are generated (Mecocci et al., 1997). However, these theories are continuously being debated. There is contradicting evidence against lack of mtDNA repair mechanism with reports that the repair of some oxidative DNA lesions is more efficient in mtDNA than

in nDNA (Thorslund et al., 2002). mtDNA with unrepairable damage are degraded and new molecules are synthesised using intact molecules as template. Human mtDNA is packed with TFAM that is important in the maintenance of mtDNA and contradicts the belief that it is susceptible to oxidative damage due to lack of protection (Alam et al., 2003). Also, proximity to the source of ROS, by itself, is insufficient to explain the higher mutation rate of mtDNA. There is no direct connection between increased levels of mtDNA mutations and elevated ROS production. In addition, high levels of 8-OHdG, a marker for oxidative damage, have been found in both nuclear DNA (nDNA) and in mtDNA of the post mortem brains of aged subjects (Mecocci et al., 1993), in rat liver (Anson et al., 1999) and other studies (Lim et al., 2005).

At present, it remains unclear how mtDNA mutations accumulate but evidence continues to emerge supporting a yet to be defined role for mtDNA mutations in aging. Reduced expression of mtDNA as well as elevated levels of mtDNA mutations have cautiously been suggested as possible cause of the decline in OXPHOS. Complex I, alone, contain 13 subunits that are encoded by mtDNA. As the percentage of mutant mtDNA increases, the cellular energy capacity decreases until it falls below the bioenergetic threshold — the minimum energy output necessary for a cell or tissue to function normally. This may in turn lead to exponentially increasing levels of mtDNA damage and oxidative stress in the cell (Barja and Herrero, 2000). The threshold level at which mutated mtDNA impairs respiratory function or create aging phenotypes is still not fully understood. It can depend on the type of mutation and also on the mitotic segregation of wild type and mutated mtDNA molecules between different tissues and even between different cells of the same tissue (Durham et al., 2006).

1.11.3 Energy Metabolism and the Aging Process

Glucose is the main source of energy in the brain. Clinically, age-dependent reduction of glucose utilization was observed in most human brain regions (Petit-Taboue et al., 1998). Similarly, an age-dependent decrease in O₂ uptake was observed in the rodent brain (Navarro and Boveris, 2008). These changes correlate with lower electron transport efficiency, which is a common observation in mammalian mitochondria aging studies. A decline in electron transfer and OXPHOS in aging is well recognized in the aging brain (Beckman and Ames, 1998, Navarro and Boveris, 2007, Nicoletti et al., 1996, Bowling and Beal, 1995).

Activities of complex I, III and IV have been reported to decrease with age in the brain of mice and rats (Lenaz et al., 1997, Benzi et al., 1992, Manczak et al., 2005, Navarro and Boveris, 2004). The decreased activity of brain complex I was observed as a decreased NADH-cytochrome c reductase activity with a simultaneously unchanged succinate-cytochrome c reductase activity in mitochondrial membranes (Navarro and Boveris, 2004, Navarro and Boveris, 2007). Petrosillo et al., 2008 show that the complex activity of complex I was reduced by 30% in brain mitochondrial from 24 month aged rats relative to young animals. These changes were associated with parallel changes in state 3 respiration. H_2O_2 was also significantly increased, while CL a phospholipid required for optimal complex I activity was decreased by 31% in the aged brain (Petrosillo et al., 2008).

The decreased activity of complex IV in aged mammalian brain was observed with the assay of the enzymatic activity in mitochondrial fragments ((Ferrandiz et al., 1994, Navarro and Boveris, 2007) along with the histochemical staining of cytochrome oxidase in human substantia nigra (Itoh et al., 1996) and rat hippocampal synaptic mitochondria (Bertoni-Freddari et al., 2004).

Northern blot analysis revealed an increased expression of mitochondrial-encoded genes in complexes I, III, IV, and V of the respiratory chain in 12- and 18-month-old C57BL6 mice compared to 2-month-old mice, suggesting a compensatory mechanism that allows the production of proteins involved in the electron transport chain that is exhausted in 24-month mice (Manczak et al., 2005). The F_1 -ATPase activity of complex V also decreases with age in the brain due to the nitration of Tyr²⁶⁹ close to the Mg^{++} binding site of the $F1\beta$ subunit (Lam et al., 2009).

Age related reductions in complex II and III activities are less widely reported but have been demonstrated in mouse brain mitochondria (Kwong and Sohal, 2000) and rat brain synaptosomes (Kilbride et al., 2008).

Moreover, under conditions of reduced electron flow through the electron transport chain, a reduction in proton pumping out of the mitochondrial matrix would be expected, resulting in a lower level of membrane potential (Hagen et al., 1998). Decreased membrane potential has been documented in the aged rat brain (LaFrance et al., 2005, Sastre et al., 1998). Mitochondrial active respiration decreases in aging in terms of a marked decline in state 3 respiration (Navarro and Boveris, 2007, Navarro et

al., 2008) as well as increase in state 4 respiration (Lam et al., 2009), which indicate a lower energy transducing efficiency. Age associated decrease in the mitochondrial respiratory control (MRC) capacity in various human tissues such as skeletal muscle (Trounce et al., 1989) and liver (Yen et al., 1989) has also been reported. Respiratory chain capacity is reduced up to 40% in rat liver mitochondria of old animals and a similar reduction observed in the spleen (Stocco and Hutson, 1978) and brain (Petrosillo et al., 2008).

As discussed earlier, components of (ETC) exist as supercomplexes, the ultra-structure of which determines the activity of mitochondrial OXPHOS and, therefore, can also play vital role in mitochondrial phenotype in aging. This has been demonstrated in the aging brain (Frenzel et al., 2010, Seelert et al., 2009).

Besides genetic determinants, decline in OXPHOS activity and the detrimental effects of ROS, age dependent changes in the abundance and posttranslational modification of the mitochondrial proteome, are currently considered as targets and even triggers of aging. In fact, partial loss of the energy transducing capacity documented in mitochondria from aged animals can be attributed to changes in protein expression. At the protein level, numerous alterations in the abundance (Groebe et al., 2007, Gomez et al., 2009, Lombardi et al., 2009, Poon et al., 2006, Stauch et al., 2014a) have been reported. For example with regards brain mitochondria, expression levels of dihydropyrimidinase-like 2 (DRP2), alpha-enolase (ENO1), dynamin-1 (DNM1), and lactate dehydrogenase 2 (LDH2) were significantly increased in the brains of old versus young mice. The specific carbonyl levels of beta-actin (ACTB), glutamine synthase (GS), and neurofilament 66 (NF-66) as well as a novel protein were significantly increased, indicating protein oxidation, in the brains of old versus young mice (Poon et al., 2006). A very recent quantitative proteomics approach allowed for the comparison of protein expression levels in synaptic mitochondria isolated from 5 (mature), 12 (old), and 24 (aged) month old mice. During the process of aging they report that dynamic proteomic alterations occur in synaptic mitochondria (Stauch et al., 2014a).

There is absolutely no doubt that mitochondrial dysfunction remains one of the enduring mainstream theories to explain aging and processes that lead to the development of aging phenotypes. While it is obvious that aging is associated with a decline in mitochondrial function, we must not assume that this is the primary cause.

Mitochondrial dysfunction might be secondary to other mechanisms (Evans, 2004). During aging, deregulation of various signaling pathways implicated in metabolism can induce mitochondrial dysfunction (Houtkooper et al., 2010). Mitochondrial biogenesis is controlled at many levels. Hormones for example not only play a role in cell growth and differentiation but also are regulators of mitochondrial biogenesis. It is therefore important to consider that the observed decline energy metabolism may be the result of other age-related changes.

1.12 Conclusion

In conclusion, changes in mitochondrial function are the result of a very complex hierarchical system of basic cell-aging processes and cell-adaptive responses. Despite the incredible progress made in recent years in this area, there are significant gaps in our knowledge. A better understanding of the mechanisms of all these processes should give valuable insight into the diverse pathways relating to mitochondrial function.

1.13 Aims of Thesis

Mitochondria serve as central hubs of energy conservation in the cell, transforming the energy deriving from oxidation of substrates into the chemical energy required to perform work, establish ion gradients, and carry out biosynthetic reactions. Neurons are highly dependent on the energy provide by mitochondrial respiration. Because of their unique function and bioenergetic requirements, neurons have an uneven cellular distribution of mitochondria. In particular, the synapse relies on high ATP levels for maintenance of membrane potential and maintaining proper Ca^{2+} in the synapse. Mitochondrial dysfunction has been implicated as a key player in aging and age associated diseases. This thesis investigates (i) how ETC complexes are arranged into supercomplexes in mitochondria from different origin in the brain (ii) the nature of the proteome of mitochondria from different origin in the brain as well as how it changes with aging (iii) the potential for utilizing the cubic mesophase as a technology for the study of mitochondrial function. The specific aims are described in relevant chapters.

CHAPTER 2

GENERAL METHODS

2.1 Materials

All reagents were obtained from Sigma-Aldrich Co., Poole, Dorset, UK, unless otherwise stated.

Bovine heart mitochondria was a kind gift from Dr. Oliver Joyce, Trinity College, Dublin.

Thermo Scientific™ Pierce™ Coomassie (Bradford) Protein Assay (Cat no: 11805964) and Thermo Scientific Pierce BCA Protein Assay Kit (Cat No. 23227) were obtained from Thermo Fisher, UK.

Protogel Ultra Pure (30%) 37.5:1 Acrylamide to Bisacrylamide Stabilized Solution was obtained from Bio-Sciences Limited, Dublin, Ireland.

Spectra Multicolor Broad Range Protein ladder was obtained from Thermo Fisher, MA, USA.

pH 3-10, non-linear Ready Strip™ (Cat No. 163-2099) was obtained from Bio-Rad, Fannin, Leopardstown, Dublin 18, Ireland.

Native PAGE™ Bis-Tris Gels 4-12% (Cat No. BN1001) were provided by Biosciences, Dun Laoghaire, Co. Dublin, Ireland.

Serva Dye (Cat No. D-69115) was obtained from Ams Biotechnology, Oxon, OX14 4RX, UK

Protease Inhibitor cocktail (Product No. P9599) was provided by Sigma-Aldrich Co., Poole, Dorset, UK.

Antibodies to the electron transport chain complexes used were as follows; mouse anti-NDUFA9 [EPR13061(B)] antibody, Cat No. ab181381; mouse anti-Ubiquinol-Cytochrome C Reductase Core Protein 1 [16D10AD9AH5] antibody, Cat No. ab110252; mouse anti-Cox 5A [6E9B12D5] antibody, Cat No. ab110262; mouse anti-

ATP5A [15H4C4] antibody, Cat No. ab1478. These were all obtained from Abcam, Cambridge, UK.

Other antibodies used were; Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA, Cat No. 111-035-144); Goat Anti-Mouse (Santa Cruz, Ca, USA, Cat No. SC-2005).

EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL) (Cat No. WBKL S0100) was obtained from Millipore, Cork, Ireland.

Sequencing grade modified Trypsin used for MALDI-TOF (Cat No. V5111) and Trypsin Gold Mass Spectrometry Grade used for iTRAQ (Cat No. V5280) were purchased from Promega, MyBio Ltd., Kilkenny, Ireland.

α -cyano-4-hydroxycinnamic acid and calibrants for the MALDI-TOF were a kind gift from Professor Luke O'Neill, Trinity College, Dublin.

iTRAQ Reagent-8-plex was purchased from Applied Biosystems, Foster City, CA 94494, USA.

Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Cat No. MRCF0R030) and Microcon YM-30 (Cat no. 42410) were obtained from Millipore, Cork, Ireland.

2.2 Preparation of Solutions and Pipetting

Reagents were weighed either on a Mettler College Model analytical balance or on a Mettler K7T top-loading balance for weights above or below 5 g, respectively. All aqueous solutions were prepared using distilled, deionized water from a Millipore Elix Advantage 10 Water Purification System. Gilson pipettes were used to pipette volumes from 1 μ l to 5 ml.

2.3 pH Determination

All solutions were adjusted to the appropriate pH using a Corning pH meter, Model 240, equipped with a Corning general-purpose combination electrode. The pH meter was calibrated daily using BDH standard buffer solutions at pH values of pH 4.0, 7.0 and 10.01. The pH of solutions was adjusted when necessary using solutions of 5 M sodium hydroxide (NaOH), 6 M hydrogen chloride (HCL) or 2 M potassium hydroxide (KOH) as appropriate.

2.4 Centrifugation

A variety of centrifuges were used. For volumes of 2 ml or less, an Eppendorf Benchtop Centrifuge 5415 was used. Various steps in the purification of mitochondria required the use of the following centrifuges: a Sorvall RC-5C centrifuge fitted with a SS34 rotor or SM24 rotor, a Sorvall Discovery 100 ultracentrifuge, fitted with an AH-629 rotor or an AH650 rotor and a benchtop Beckman TL100 ultracentrifuge, fitted with a TL100-A rotor. Centrifuge tubes were balanced before centrifugation and all centrifugation carried out at 4°C, unless otherwise stated. Rotors were pre-cooled to this temperature before use. All g forces quoted refer to the relative centrifugal force at the bottom of the centrifugal tube. Centrifugation time does not include acceleration or deceleration time.

2.5 Experimental Animals

Adult female Wistar rats, weighing approximately 250 g each, were stunned and killed by cervical dislocation. For ageing studies, young (6 months), adult (10 months) and (22 months) male Hanover rats were supplied by Banton and Kingman. All experimental animals were housed in approved facilities at Trinity College, Dublin and maintained by the Comparative Medicine Unit. All procedures and animal handling were in keeping with approved institutional animal care and use guidelines.

2.6 Protein Determination

2.6.1 Bradford Protein Assay

The Thermo Scientific™ Coomassie (Bradford) Protein Assay (Cat no:11805964) is a quick and ready-to-use modification of the Bradford dye binding method (Bradford, 1976), for determining the total concentration of protein in a solution. When Coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595 nm with a concomitant color change from brown to blue. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine.

5 µl of each standard or unknown sample was combined with 250 µl Coomassie Reagent and mixed for 30 seconds. Samples were incubated for 10 minutes at room temperature and absorbance measured at 595 nm using a SpectraMAX PLUS Microplate Spectrophotometer reader. Comparison to a standard curve of a concentration range of bovine serum albumin (BSA) standards (Figure 2.1), taken through the same protocol, provided a relative measurement of protein concentration. All assays were carried out in triplicate.

2.6.2 Bicinchoninic acid (BCA) Protein Assay

The Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Scientific, UK, Cat No. 23227) is a detergent compatible and ready to use modification of the BCA method for determining the total concentration of protein in a solution (Smith et al., 1985). This method combines the reduction of Cu^{2+} to Cu^{+1} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The total protein concentration is exhibited by a color change of the sample solution from green to purple in proportion to a protein concentration, which can then be measured at 562 nm. The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cystine or cysteine, tyrosine, and tryptophan) in the amino acid sequence of the protein. However, unlike the Coomassie dye-binding methods, the universal peptide backbone also contributes to color formation, helping to minimize variability caused by protein compositional differences.

The working reaction was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. 25 µl of each standard or unknown sample replicate was pipetted into a microplate well. 200 µl of the working reaction was added to each well and mixed thoroughly by placing plate on a shaker for 30 seconds. The plate was covered and incubated at 30°C for 30 min. After the plate had cooled to room temperature, the absorbance was measured at 562 nm on a SpectraMAX PLUS Microplate Spectrophotometer reader. Comparison to a standard curve of a concentration range of bovine serum albumin (BSA) standards, taken through the same protocol, provided a relative measurement of protein concentration. All assays were carried out in triplicate.

2.6.3 Markwell Protein Assay

The protein concentration of mitochondrial suspensions was determined by the method of (Markwell et al., 1978). Stock solutions were prepared as follows:

- A: 2% Sodium carbonate in 0.4% NaOH
0.16% Sodium potassium tartrate
1% Sodium dodecyl sulphate
- B: 4% Copper Sulphate
- C: 100 ml solution A : 1 ml solution B – freshly prepared
- D: 1:1 dilution of Folin Ciocalteu's reagent in H₂O

Mitochondrial samples were diluted to a volume of 1 ml with distilled deionized H₂O. 3 ml of solution C was added, mixed and allowed stand at room temperature for 10 min. 0.3 ml of solution D was then added and allowed stand for a further 45 min. Absorbance was read at 660 nm against 1 ml H₂O, taken through the above procedure, as a blank reading. All assays were carried out in triplicate. Protein concentration was calculated from a standard curve using a range 0.05 mg/ml to 0.35 mg/ml, prepared from bovine serum albumin (BSA) and taken through the same procedure.

2.7 Isolation of Brain Mitochondria

2.7.1 Isolation of metabolically active SM and NSM rat brain mitochondria using discontinuous Ficoll/Ficoll gradients.

Synaptic (SM) and non-synaptic mitochondria (NSM) were isolated by a modification of a method by (Lai and Clark, 1979). All steps were carried out at 4°C and the procedure was carried out with minimum delays in order to obtain well-coupled brain mitochondria.

For each preparation four female Wistar rats were killed by cervical dislocation, and the brains extracted and suspended in 40 ml ice-cold isolation buffer (320 mM Sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4). The brains were chopped on ice, with several changes of buffer to remove excess blood. The chopped brains were homogenized by hand using a glass Kontes tissue grinder and centrifuged at 1,050 g for 3 min in a Sorvall RC5C centrifuge fitted with SS34 rotor to remove nuclei and unwanted debris. The supernatant was centrifuged at 10,085 g for 10 min. The resultant crude mitochondrial pellet (CMP) was resuspended in 9 ml isolation buffer and layered on top of a discontinuous Ficoll gradient (Figure 2.1 A), which was centrifuged in a Sorvall Discovery 100 centrifuge, fitted with an AH-629 rotor, at 104,200 g (28k RPM) for 45 min at 4°C.

Following ultracentrifugation, the non-synaptic pellet (NSM) was resuspended in 1 ml ice-cold isolation buffer, homogenized in a Wheaton 2 ml homogenizer and centrifuged at 16,000 g for 5 min. This NSM pellet was resuspended in 1 ml ice-cold isolation medium (0.24 M Mannitol, 60 mM Sucrose, 0.05 mM EDTA K₂ salt, 10 mM Tris-HCL pH 7.4) and protein concentration determined using Coomassie Bradford method as described in Chapter 2, section 2.6.1. Isolated NSM samples were stored at -80°C until required.

The synaptosomal layer was extracted and homogenized by hand in 6 mM Tris-HCL pH 8.1 using a 40 ml glass Kontes tissue grinder. The lysed synaptosomal suspension was centrifuged at 10,085 g for 10 min. The pellet was resuspended in 6 mM Tris-HCL pH 8.1 and the homogenization and centrifugation step repeated. The resultant synaptic mitochondrial pellet was resuspended in 9 ml and layered on top of a second discontinuous Ficoll gradient (Figure 2.1 C), which was centrifuged in a Sorvall

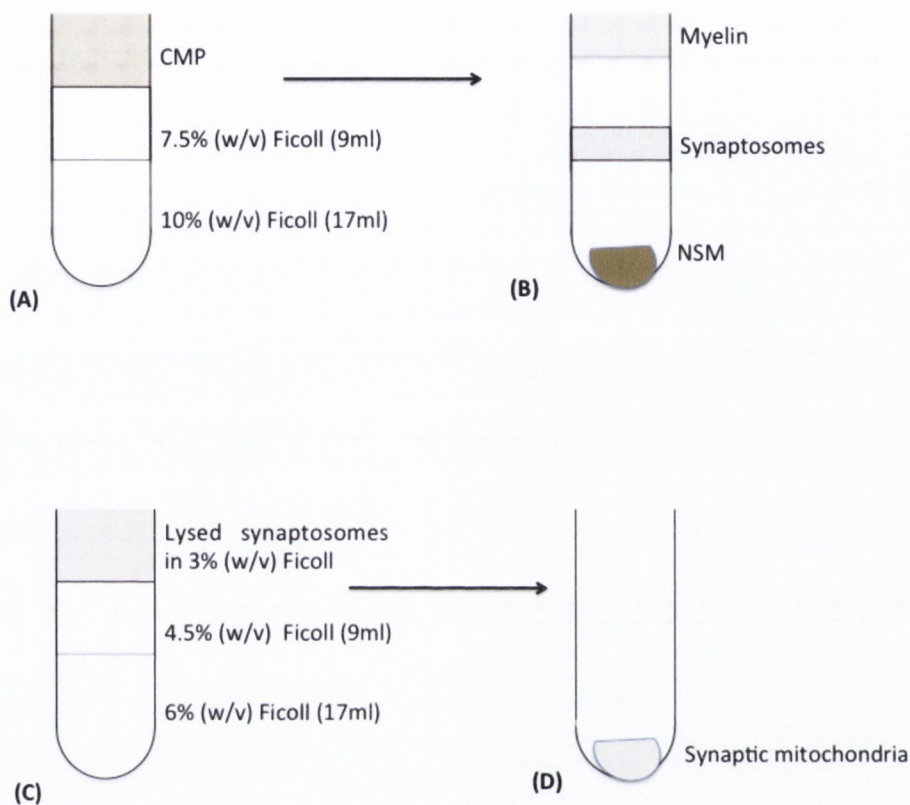


Figure 2.1 The discontinuous Ficoll gradient for the isolation of relatively pure NSM and SM. The crude mitochondrial pellet (CMP) was layered on top of the Ficoll gradient **(A)**. Centrifugation at 28,000 RPM for 45 minutes results in the fractionation of the CMP into myelin, synaptosomes and non-synaptic mitochondria (NSM), as shown in **(B)**. Lysed synaptosomes are layered on top of a second Ficoll gradient **(C)**. Centrifugation at 11,300 g for 30 minutes results in the pelleting of SM below the 6% (w/v) Ficoll solution, as shown in **(D)**.

Discovery 100 centrifuge, fitted with an AH-629 rotor, at 11,300 g for 30 min at 4°C. The pellet below the 6% (w/v) Ficoll medium fraction was washed with ice-cold isolation buffer by gently homogenizing in the all-glass homogenizer. The resultant suspension was centrifuged at 10,085 g for 10 min. The SM pellet was resuspended in 0.5 ml ice-cold isolation buffer and protein concentration determined as described in Chapter 2, section 2.6.1. Isolated samples were stored at -80°C until required.

2.7.2 Isolation of metabolically active SM and NSM rat brain mitochondria using discontinuous Ficoll/Percoll gradients.

Synaptosomes and NSM were prepared as described in Section 2.7.1. However, following the first Ficoll gradient, the NSM pellets was resuspended in 40 ml ice-cold isolation medium (0.24 M Mannitol, 60 mM Sucrose, 0.05 mM EDTA K₂ salt, 10 mM Tris-HCL pH 7.4 and homogenized at 10,085 g for 10 min. The resultant NSM pellet was resuspended in 2.5 ml ice-cold isolation medium.

The synaptosomal layer was extracted and homogenized by hand in 6 mM Tris-HCL pH 8.1 using a 40 ml glass Kontes tissue grinder. The lysed synaptosomal suspension was centrifuged at 10,085 g for 10 min. The pellet was resuspended in 6 mM Tris-HCL pH 8.1 and the homogenization and centrifugation step repeated. The resultant SM pellet was resuspended in 2.5 ml ice-cold isolation medium.

An equal volume of 30% (w/v) Percoll solution (2.5 ml) to each suspension of SM and NSM The mitochondrial/Percoll suspension was then layered on top of a discontinuous Percoll gradient (Figure 2.2 C), which was centrifuged in a Sorvall RC5C, fitted with an SM-24 rotor, at 30,400 g for 10 min.

Following the Percoll gradient, the mitochondrial layer is removed and suspended in 10 ml of ice-cold isolation buffer and washed at 10,085 g for 10 min. Each pellet is resuspended in isolation medium and washed at 20,897 g for 10 min. This step is repeated and mitochondria washed at 14,961 g for 10 min. A final wash at 12,365 g for 10 min is carried out and the SM and NSM pellets are resuspend in 500 µl isolation medium (high sugar content prevents dissociation upon freezing) and protein concentration determined as described in Chapter 2, section 2.6.1. Isolated purified samples were stored at -80°C until required.

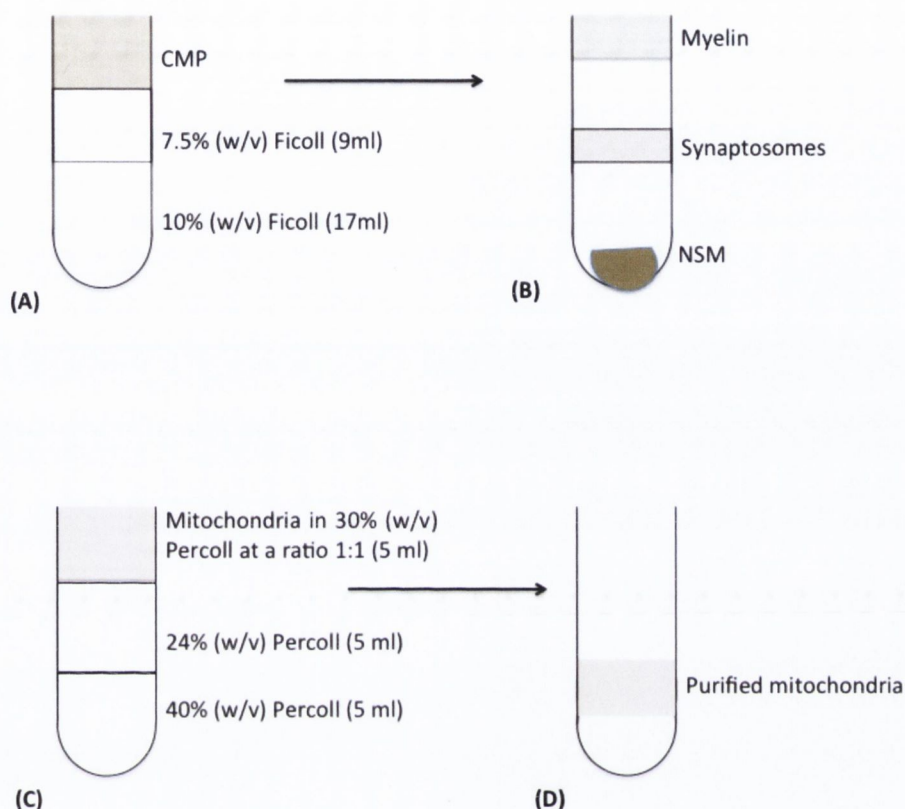


Figure 2.2 Discontinuous Ficoll gradients for the isolation of pure NSM and SM.

The crude mitochondrial pellet (CMP) was layered on top of the Ficoll gradient (A). Centrifuging at 28,000 RPM for 45 minutes results in the fractionation of the CMP into myelin, synaptosomes and non-synaptic mitochondria (NSM), as shown in (B). Crude SM and NSM were mixed with 30% (w/v) Percoll and layered on top of a Percoll gradient (C). Centrifugation at 30,400 g for 10 minutes results in the fractionation of pure mitochondria above 40% (w/v) Ficoll as shown in (D). Individual gradients were carried out for the purification of SM and NSM.

2.8 Denaturing Gel Electrophoresis Techniques

2.8.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the discontinuous buffer system of (Laemmli, 1970). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis is a separation technique based on the movement of charged molecules in an electric field. The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in the separation by size during electrophoresis.

Samples were estimated for protein content and loaded onto 12% polyacrylamide gels in sample buffer (3% (w/v) SDS, 30% (v/v) glycerol, 3% (v/v) 2-mercaptoethanol and 0.005 (w/v) bromophenol blue). Between 10 and 20 µg protein were loaded into each well, using Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher, MA, USA) as a molecular weight marker. Gels were run at constant Amps (20 mA/gel) for 90 min using a Bio-Rad Mini-Protean II Cell apparatus or a Bio-Rad Protean II xi 2D electrophoresis system and a BioRad PowerPac 300.

After separation in SDS-PAGE gels, the proteins were visualised by Coomassie blue staining as described in 2.10.2. A gel imaging system (Alpha Innotech, San Leandro, Ca, USA) was used to digitize gels and were analysed by PDQuest software (Bio-Rad Laboratories).

2.8.2 Two-dimensional Gel Electrophoresis (2-DE)

2-DE may resolve mixtures containing up to 1000 proteins and provide information on their isoelectric point and molecular weight.

500 µg of purified mitochondria was extracted in 2D sample buffer (30 mM Tris-HCL, 7M urea, 2M thiourea, 4% CHAPS (w/v), 40mM DTT, bromophenol blue 0.002% (w/v,) pH 8.5) with ampholytes to a final concentration of 0.2% (v/v). The preparation was centrifuged at 16,000 g for 10 min to avoid loading any insoluble material. The supernatant was collected and applied to a 11cm, pH 3-10, non-linear Ready Strip™ (BIO-RAD, CAT No. 163-2099) IPG strip. Mineral oil was added to prevent dehydration and crystallisation of the urea. The strips were rehydrated overnight at room

temperature. After rehydration was completed, the proteins were resolved according to their isoelectric point using the PROTEAN IEF cell, with the focusing conditions recommended by the manufacturer and shown in Table 2.1.

Linear slope to 500V	1 hour
Constant 500 V	5 hours
Linear slope to 3,500 V	5 hours
Constant 3,500 V	12 hours

Table 2.1 Focussing conditions for 11 cm, pH 3-10, non-linear Ready Strip™

After the first dimension run, the strips were equilibrated with 2% DTT and 2.5% iodoacetamide buffer solutions respectively to reduce and alkylate the proteins. The proteins were separated based on their size on 8% SDS-PAGE. The electrophoretic equipment included a Protean II Multi-cell vertical chamber. No stacking gel was employed. The IPG strips were embedded with 0.5% (w/v) melted agarose prior to the run on the SDS-PAGE slabs. The agarose contained 0.0001% bromophenol blue as a tracking dye. The gels were run at a constant current of 25 mA and maintained at a temperature between 8°C and 12°C.

After separation in SDS-PAGE gels, the proteins were visualised by a double staining procedure: first the Coomassie blue staining as described in Section followed by the silver staining as described (see Chapter 2, section 2.10.3).

The gel images were analysed by PDQuest software (Bio-Rad Laboratories). Spot intensities were quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by total area of all spots. Spots of interest were then excised and analysed using mass spectrometry (MALDI-TOF) as described in Chapter 2, section 2.9.

2.8.3 Tricine-SDS-PAGE

The 2-D BN/Tricine-SDS-PAGE technique is commonly used to analyze the subunit composition of native complexes. Tricine-SDS gels were prepared as described by (Schagger, 2006). Gel lanes were cut out from a first dimension blue native gel (see Chapter 2, section 2.9.3) and treated with 20 mM dithiothreitol (DTT)/1%SDS solution for 15 min. Excess denaturing solution was removed, and the gel lane placed at the usual position between for stacking gels. Spacers were positioned and the second glass plate put on top. A 10% resolving mixture was poured between the plates, leaving a 2 cm gap below the piece of first dimension native gel. A 1 cm layer of 4% stacking solution was overlaid after polymerization of the resolving gel. The gel lane of the first dimension was then fully embedded with a 4% overlay solution containing 10% glycerol and 0.2% SDS. An ATTO AE-6220 gel electrophoresis unit was used and the gel run at 60 V for approximately 50 min until the samples had entered the stacking gel. The voltage was then increased to 180 V. After 3 hours the voltage was increased to 200 V. After separation in SDS-PAGE gels, the proteins were visualised by a double staining procedure: first the SBG blue staining (see Chapter 2, Section 2.7.1) followed by the silver staining as described in Section (see Chapter 2, Section 2.10.3).

2.9 Native Gel Electrophoresis Techniques

2.9.1 Generation of Linear Gradients

One of the applications of gradient gels includes the determination of protein molecular weights and the separation of molecules, which co-migrate on uniform gels. Gradient gels were cast with a higher concentration of acrylamide at the bottom than the top. To generate a linear gradient between two concentrations, equal volumes of solutions of the two concentrations were measured into the two chamber of the gradient maker, (Hoefer SG 50, Figure 2.3). As solution was delivered out of the mixing chamber, an equal volume flowed in from the reservoir chamber where it was rapidly diluted and mixed to uniformity by a magnetic stirring bar. The initial concentration delivered was that of the solution in the mixing chamber, the final concentration was that of the reservoir chamber. For the most consistent delivery of gradients, a peristaltic pump was used.

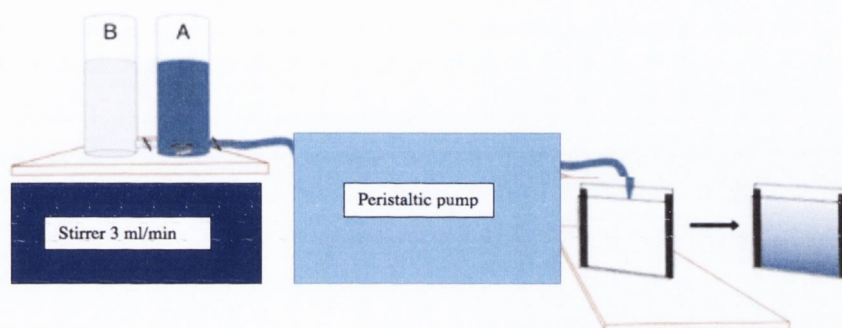


Figure 2.3 Generation of linear gradients for native gel electrophoresis. The gradient maker must be positioned above the gel cassette to encourage flow due to gravity. A gradient maker consisted of two chambers, joined by a narrow connector at their bases, with one container having an additional outlet in the base. As liquid drained from A, it was replaced from B due to the equilibration of hydrodynamic pressure, which kept the levels in A and B equal. A was constantly stirred, which caused the solution draining from A to be progressively diluted with B. After the gradient maker emptied, the outflowing material was essentially 100% B.

2.9.2 Sample Preparation for Blue Native PAGE (BN-PAGE)

Separation of electron transport supercomplexes was performed using BN-PAGE according to the protocol described by (Wittig and Schagger, 2009). Solubilisation with digitonin allows for the separation of ETC supercomplexes.

A digitonin:protein ratio of 10:1 (w/w) was determined to be optimum for the solubilization of SM and NSM membranes but maintaining mitochondrial supercomplexes. For the isolation of individual complexes a maltoside:protein ratio of 1:1 was determined to be optimum for membrane solubilisation. Mitochondrial membranes were solubilized with digitonin in a buffer composed of 1.5 M aminocaproic acid, 100 mM BisTris-HCL, pH 7.0. Following solubilisation, samples were centrifuged at 100,000 g for 10 min at 2°C in a benchtop Beckman TL100 ultracentrifuge, fitted with a TL100-A rotor. Shortly before starting blue native PAGE, Serva Blue G from a 5% stock solution in 500 mM aminocaproic acid was added with a final detergent-to-dye ratio of 8:1 (w/w). Proteins (80-150 µg per lane) were separated on a 4.8-12% gradient gel as described in Chapter 2, section 2.9.3.

2.9.3 Blue Native Page (BN-PAGE)

The polyacrylamide gels were prepared by a modification of a method by (Schagger et al., 1994). Non-linear gradient gels (4.8-12%) were poured using a gradient maker and peristaltic pump as described in Section 2.9.1. A Mini-protean II from BioRad was used for first-dimension gels. The gels were overlaid with a 4% stacking gel. Electrophoresis was carried out using a cathode buffer of 50 mM Tricine, 15 mM BisTris, 0.02% Serva Blue G, pH 7.0 and an anode buffer of 50 mM BisTris-HCL, pH 7.0. It was started at 40 volts for the first 30 min until the samples had entered the stacking gel. The voltage was then increased to 80 volts. Electrophoresis was continued until the dye front reached the end of the gel. When performing western blotting or in-gel activity assays, the cathode buffer was replaced by a second cathode buffer, which contained a 10-fold dilution of Serva Blue G when the dye reached one third of the gel. This ensured that excess dye did not interfere with antibody binding or enzyme activity. Immediately after electrophoresis, the gels were either frozen for use later or fixed and stained with Serva Blue G as described in Chapter 2, section 2.10.1.

2.10 Staining Methods

2.10.1. Serva Blue G staining of Native PAGE gels

Proteins resolved by native PAGE and second-dimension Tricine-SDS and were visualized by staining the gels in Serva blue G according to the method of (Wittig et al., 2006). Gels were fixed for 30 min in a 50% (v/v) methanol/10% (v/v) acetic acid solution, followed by staining in 10% (v/v) acetic acid/0.025% (w/v) Serva blue G for 30 – 60 min. The gels were destained in a 10% (v/v) acetic acid solution that contained a piece of sponge to soak up excess dye.

2.10.2 Coomassie Blue staining of SDS-PAGE gels

Proteins resolved by SDS-PAGE were visualized by staining the gel in Coomassie Brilliant R 0.25% (w/v) solution made in 20% (v/v) methanol and 10% (v/v) acetic acid. Coomassie blue stain was then removed by washing the gel in 20% (v/v) methanol and 10% (v/v) acetic acid.

2.10.3 Silver Staining

Gel was fixed for 30 min in 50% (v/v) methanol/10% (v/v) acetic acid. Gel was then rinsed with water (2 changes, 2 min per wash) and left on a shaking platform for 1 hour in water. The gel was then incubated in sensitizing solution (0.02% (w/v) sodium thiosulphate) for 1 – 2 min. The sensitizing solution was discarded and the gel washed twice with water (10 sec per wash). The gel was incubated in 0.1% (w/v) Silver Nitrate for 30 min at 4°C on a shaking platform. The silver nitrate solution was discarded and the gel washed twice with water (30 sec per wash). The gel was then incubated in the developing solution (0.04% (w/v) formaldehyde, 2% (w/v) sodium carbonate). As soon as the developing solution started to turn yellow, it was discarded and replaced with fresh solution. When the proteins started to appear, the formaldehyde solution was discarded and 1% (v/v) acetic acid added to quench the developing process.

2.11 Western Blotting

Following the electrophoretic separation of proteins as described in Section 2.5.1 and Section 2.6.4 (20 µg of total protein added per lane), a semi-dry transfer technique was employed to transfer the samples onto Immobilon polyvinylidene difluoride (PVDF) membrane, at constant voltage (10 volts) for one hour. The membrane was blocked in 5% BSA in phosphate buffered saline (PBS)/Tween 20 (0.05%) overnight at 4°C and then incubated with the following mouse monoclonal antibodies: anti-NDUFA9 (39 kDa of Complex I); anti-Ubiquinol-Cytochrome C Reductase Core Protein 1 (51 kDa of Complex III); anti-Cox 5A (16.8 kDa of Complex IV); anti-ATP5A (60 kDa of Complex V). A secondary antibody (goat anti-rabbit HRP, Millipore, goat anti-mouse HRP, Sigma, both 1:10,000 dilution) was used to develop the reactions.

Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL) was used to detect the proteins targeted in western blotting by revealing the activity of the secondary antibodies. Chemiluminescence substrates were comprised of a luminol reagent and hydrogen peroxide in a Western blotting reagent kit. Horseradish peroxidase (HRP) enzyme from the secondary antibody facilitated oxidation of the luminol reagent in the presence of hydrogen peroxide, causing activation of the luminol. The end product of this reaction being highly unstable, released energy in the form of photons only at the protein of interest labeled by secondary antibody, enabling visualization. The film was developed using a Fuji X-Ray Film Processor RG II.

2.11.1 Imaging and Analysis of gels

A gel imaging system (Alpha Innotech, San Leandro, Ca, USA) was used to digitize gel and western blot images. The densitometric in-gel quantification of bands was performed using Image J (National Institute of Health).

2.12 Mass Spectrometry

Mass spectrometry was used to identify unknown and differently expressed spots (Figure 2.4).

2.12.1 Band Preparation and Destaining

The proteins spots/bands of interest from a stained 1-D or 2-D polyacrylamide gel were excised manually and placed in a 1.5 ml Eppendorf® Safe-Lock microcentrifuge tube. Gel pieces were equilibrated in 50% acetonitrile/25 mM ammonia bicarbonate pH 8.0 and then dehydrated in 100% acetonitrile. The acetonitrile was aspirated off and the gels dessicated in Speed-Vac at a low temperature for 30 min.

2.12.2 Enzymatic Digestion

Promega sequencing grade Modified Trypsin 10-15 ug/ml in 25 mM ammonia bicarbonate pH 8.0 was used to digest the gel pieces. Gel pieces were rehydrated by adding 15 µl of cold activated trypsin and incubated at room temperature for 30 min. Additional trypsin was added accordingly if there was insufficient volume to cover and swell gel pieces. Samples were incubated at 37°C overnight with shaking.

2.12.3 Extraction of Peptides

Peptides were extracted in 50% acetonitrile (v/v) / 5 % (v/v) trifluoroacetic acid (TFA) for 60 min with gentle agitation. The supernatant was transferred to a second clean tube and the extraction procedure carried out again on the gel piece. The two supernatant extracts were pooled and speed-vac to complete dryness. The dried sample was reconstituted in 50% acetonitrile (v/v) / 0.1% (v/v) trifluoroacetic acid. Samples were then ready for LC/MS or MALDI-TOF.

2.12.4 Assembly of 10 mg/ml solution of alpha-cyano matrix

For all mass spectrometry, the matrix, α -cyano-4-hydroxycinnamic acid (CHCA) was used (Figure 2.5). A 10mg/ml saturated solution of the matrix was solubilised in 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. The calibration standards (Calmix) were diluted in this matrix diluent.

2.12.5 MALDI Preparation

One microlitre of the tryptic peptides were spotted on a Teflon coated 96 well format plate along with equal amount of saturated solution of CHCA as the matrix. One microlitre of calmix/matrix was spotted in adjacent sample positions on the plate. The plate was air dried for a few minutes, until matrix crystals formed.

2.12.6 Peptide Mapping and Protein Identification.

Spectra were acquired using an Applied Biosystems Voyager DE STR workstation operated according to the manufacturer's instructions. Peptide Mass Fingerprints (PMFs) covered the mass range 700-4000 amu, with low mass gate set at 50 Daltons. The accelerating voltage was set to 20 kV, the grid to 76% and the delay time to 120 ns. For each spectrum at least 1000-1200 shots were acquired.

The freeware "Mascot" at <http://www.matrixscience.com> was used to match the obtained masses against the NCBI database. The criteria for search included one possible missing cleavage, possible methionine oxidation and carbamidomethylation of cysteine residues as variable modifications. The maximum error tolerance was set at 50ppm. For each search the MASCOT algorithm calculates a probability score. Protein score greater than 61 ($p < 0.05$) were considered significant and additionally at least 10 peptides match were considered essential for positive protein identification.

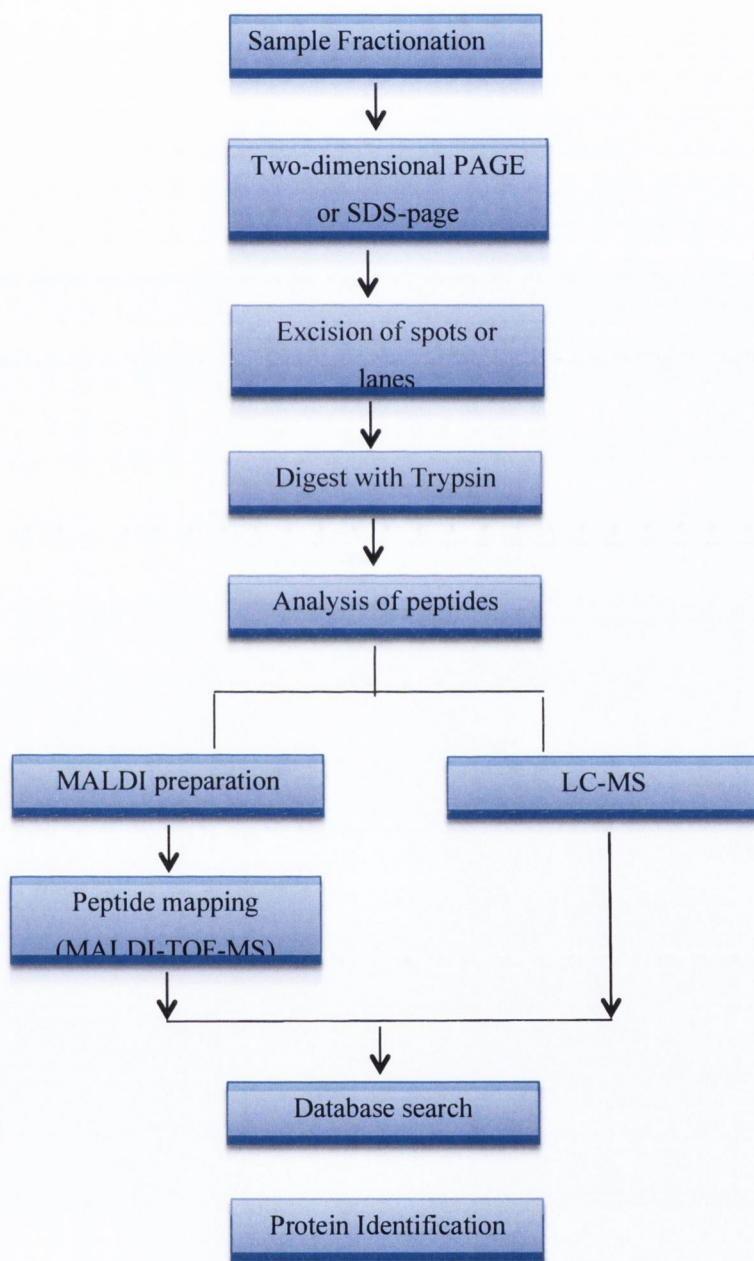


Figure 2. 4 Flow chart for working process for MALDI-TOF

2.13 Isobaric Tag for Relative and Absolute Quantitation (iTRAQ)

The iTRAQ-6-plex kit was used in this study to label all peptides from different brain mitochondrial populations. iTRAQ is a non-gel-based proteomics technique using post-labeling with isotopically labeled tags to quantify proteins from different sources in a single experiment. The method is based on the covalent labeling of the N-terminus and side-chain amines of peptides from protein digestions with tags of varying mass. These individually labeled samples were pooled and usually fractionated by strong cation exchange (SCX), followed by analysis using LC- MS/MS. A database search was then performed using the fragmentation data to identify the labeled peptides and hence the corresponding proteins. The fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they originated. Using this technique, a high diversity of proteins was observed, including acidic, basic, high molecular weight, low molecular weight and even hydrophobic membrane proteins. The high resolution and high sensitivity of this technique ensured that both abundant and scarce proteins are identified.

2.13.1 Filter Aided Sample Preparation (FASP) for LC/MS

Filter-aided sample preparation prepares protein for mass spectrometry. Since the essential steps of the method occur within a filtration device the method has been termed filter aided sample prep (FASP). It combines the advantages of in-gel and in-solution digestion of mass spectrometry-based proteomics.

2.13.2 Lysis and Thiol-reduction

Heating in presence of high concentrations of SDS and reduction of disulfide bridges results in a high extent of lysis. Aliquots (200 µg) of mitochondria prepared as described in Section 2.7.2 were lysed by mixing with 60 µl of lysis buffer (4% (w/v) SDS, 0.1 M DTT, 100 mM Tris pH 7.6) and incubated at 95°C for 5 min. The samples were sonicated to shear DNA and reduce the viscosity of the sample. The lysates were clarified by centrifugation at 16,000 g for 5 min.

2.13.3 Removal of detergent micelles and protein detergent complexes

This is an important step as detergents create noise in the mass spectrometer and contaminate the chromatography. The lysates was transferred to individual Microcon devices YM-10 (Millipore) and mixed with 200 µl of 8 M Urea in 0.1 M Tris-HCL, pH 8.5. The device was centrifuged at 14, 000 g for 15 min at room temperature. The concentrate was diluted with 200 µl of 8 M Urea in 0.1 M Tris-HCL, pH 8.5 and the device centrifuged as in the previous step. The flow-through was discarded from the collection tube.

2.13.4 Alkylation

100 µl of IAA solution (0.05 M iodoacetamide in 8 M urea) was added, mixed for 1 min at 600 rpm and allowed incubate for 20 min at room temperature. Following incubation, filter units were centrifuged at 14,000 g for 10 minutes at room temperature and the filtrate removed. The flow-through was discarded from the collection tube.

2.13.5 Removal of remaining alkylating reagent

200 µl of 8 M Urea was added to the filter unit and centrifuged at 14, 000 g for 15 min. The step was repeated twice.

2.13.6 Removal of Urea and generation of suitable environment for digestion

100 µl of dissolution buffer (0.05 M triethylammonium bicarbonate (TEAB), pH 8.0) was added to the filter unit and centrifuged at 14,000 g for 15 min. This dissolution step was repeated.

2.13.7 Trypsin Digestion

Trypsin was reconstituted in 40 µl 0.05 M TEAB and the proteins in the filter unit digested (enzyme to protein ration of 1:100) in a wet chamber at 37°C overnight. It is important to note that the stock Trypsin Gold 100µg/25µl was prepared in 50 mM acetic acid as ammonia bicarbonate is not compatible with iTRAQ.

Following overnight incubation, filter units were transferred to new collection tubes and centrifuged at 14,000 g for 10 min. 50 µl of 0.05 M TEAB was added and the filter units centrifuged at 14,000g for 10 min. Filters were removed and the filtrate was Speed-Vac for 30 minutes. Each pellet was resuspended in 5 µl of dissolution buffer (0.5 M TEAB).

2.13.8 Labeling of each digest with an iTRAQ Reagent

The iTRAQ reagents (Figure 2.6) were allowed settle to room temperature and 50 µl of HPLC grade isopropanol was added to each reagent. The contents of one iTRAQ reagent vial was added to one sample tube and incubated at room temperature for one hour. The contents of each iTRAQ labeled sample tube were combined into a fresh tube and mixed. This was then aliquoted into 6 tubes, desiccated in Speed-Vac and ready for processing.

2.13.9 Processing of iTRAQ

Pre-fractionation, LC-MS/MS analysis and the bio-informatics tools dealing with searches and statistical analysis were carried out at the Dunn School of Pathology, Oxford University.

Before LC/MS/MS analysis, the sample was prefractionated using strong cation exchange chromatography (SCX), followed by desalting of each SCX fraction. Prefractionation based on SCX resulted in the largest number of identified proteins. The samples were analyzed by LC-MS/MS using 2 hour gradients on a "top 5" PQD fragmentation on a Thermo Orbitrap LCMSMS mass spectrometer. All raw instrument data files were converted to mzXML files using ReaDW. Data files were submitted to the Central Proteomics Facility Pipeline (in-house developed software based on the TPP) for data searching using Mascot, X!Tandem and OMSSA search engines. iProphet statistical data validation and iTRAQ quantitation was done using the LIBRA TPP tool.

2.14 Statistical Analysis

Statistical analysis was carried out using a combination of Microsoft Excel 2003 and Prism (Version 6.0c) computer programme (GraphPad Software Inc). Unpaired t-tests were used to analyse samples from two different groups. One-way ANOVA with uncorrected Fisher's LSD analysis were used to examine results that consisted of more than two groups. All data are presented as mean + SEM unless otherwise stated. A P-value of 0.05 or less was deemed to be statistically significant. The particulars of statistical analyses pertaining to each experiment are described in the relevant results sections.

CHAPTER 3

Supramolecular Organisation of the Respiratory Chain in Synaptic and Non-synaptic Mitochondria

3.1 Introduction

The brain is an organ consisting of a large amount of heterogeneous cells with multiple functional purposes and metabolic characteristics. This is exemplified by the existence of neurons and glial cells that indeed have their own functional properties and metabolic characteristics (Schousboe et al., 1992). The study of primary neurons and astrocytes suggests that metabolic compartmentalization can even exist at the single cell level (Waagepetersen et al., 2002, Waagepetersen et al., 2003, McKenna et al., 2000, Waagepetersen et al., 1998, McKenna, 2003, Sonnewald et al., 1993, Cruz et al., 2001). Interestingly, the various metabolic pathways studied and shown to exhibit compartmentalisation were largely of mitochondrial origin or had close metabolic connection to the mitochondria.

Mitochondria are the main source of energy in the cell. In addition to being the main intracellular source of ATP needed for normal function and viability, they also serve as major controllers in many cellular pathways. It is now accepted that mitochondria not only vary between different cell types but that mitochondria localized in specific regions of a single cell can have different functional properties. Advances in various imaging techniques such as confocal microscopy, fluorescence resonance energy transfer (FRET) for imaging protein-protein interactions and conformational changes, 4Pi and STED microscopy have allowed the direct visualization of the intracellular heterogeneity of mitochondria and mitochondrial function (Egner et al., 2002, Plecita-Hlavata et al., 2008, Kuznetsov et al., 2006, Collins et al., 2002). Mitochondria localized in different cell regions have different morphology (Collins and Bootman, 2003), biochemical properties (Zhang et al., 2001), respiratory activity (Kiebish et al., 2008, Kuznetsov et al., 1998), redox states (Kuznetsov et al., 1998), mitochondria ROS (Zorov et al., 2000), different levels of uncoupling proteins (Jimenez et al., 2002) and/or interact with other intracellular structures (Egner et al., 2002), demonstrating clear intrinsic heterogeneity. Cardiomyocytes are a perfect example of well characterised mitochondrial subpopulations present in cells that are involved in different physiological and pathological processes (Collins et al., 2002). They show functionally and structurally distinct mitochondria, which is very important for the specific functions of these cells.

The phenomenon of mitochondrial heterogeneity can also be attributed to the brain. Based on their cellular localization, mitochondria from brain can be divided into two major populations (Lai et al., 1994). These include synaptic mitochondria (SM), which are predominantly neuronal and presynaptic in origin and non-synaptic mitochondria (NSM), which originate from neuronal and glia cell bodies (Brown et al., 2006). Generally, mitochondria in the brain have an apparent half-life of approximately one month (Menzies and Gold, 1971). SM are synthesized in the cell body of neurons and then transported down the axon or dendrite. These highly dynamic organizations of mitochondrial networks are achieved in the neuron by moving along the intricate cytoskeleton and attaching to the cytoskeleton using specific motor and connector proteins (Hollenbeck and Saxton, 2005).

Pronounced differences exist between NSM and SM and have been reported in the literature. Broad variations in mitochondrial shape and morphology can be observed. SM are typically punctuate and isolated, in contrast to the mitochondrial threads and clusters found in the neuronal cell body and astrocytes (Collins et al., 2002, Muller et al., 2005). Recent advances in electron microscopy now suggest the presence of single synaptic mitochondrion in the nerve terminal (Wilhelm et al., 2014).

Mitochondria in the synapse serve a variety of functions. In addition to ATP synthesis and Ca^{2+} buffering, they contribute to neurotransmitter synthesis and catabolism. Therefore, the bioenergetics demands in the presynaptic terminal are very high, with ATP being required for endo- and exocytosis, redox regulation, in addition to maintenance of ion homeostasis, especially calcium homeostasis (Brown et al., 2006). Mitochondria at the synapse are constantly exposed to high Ca^{2+} transients associated with transmitter release (Friel, 2000). The ability of the organelle tolerate exogenously added calcium is a key aspect of mitochondrial integrity. SM and NSM exhibit different sensitivity to Ca^{2+} induced inner mitochondrial membrane permeability transition indicating that mitochondria are heterogenous in their response to calcium (Brown et al., 2006). Consequently, these presynaptic mitochondria are more susceptible to Ca^{2+} overload and when they succumb, the opening of permeability transition pore (PTP) leads to swelling and eventual rupture of the organelle (Brown et al., 2006). The increased possibility of SM to undergo permeability transition may have pathological

consequences, as degeneration of distal axons is one of the initial events observed in several neurodegenerative disorders and in aging (Brown et al., 2006).

One of the first observations that synaptic and nonsynaptic neuronal elements exhibit differences in metabolic characteristics was based on measurements of enzymatic activity that reported differences with regard to activities of key metabolic enzymes (Lai et al., 1977). The individual activities of the ETC have been studied in isolated SM and NSM and complexes I, II, III and II/III were lower in SM than in NSM. On the other hand, Complexes I/III and IV activities were similar in both populations (Kiebish et al., 2008). SM and NSM populations also display different responses to both substrates and inhibitors. Another important difference observed between the brain mitochondria is their energy threshold level. NSM have higher “reserves” of complex activities as the ETC complex activities have to be reduced by 60-70%, before OXPHOS is severely reduced. This figure is much lower at around 20-30% for SM. In particular, threshold of complex I activity is around 72% in NSM as compared to 25% in SM (Rossignol et al., 1999, Davey et al., 1998).

Membrane lipids can influence the activity of electron transport chain enzymes in the inner mitochondrial membrane (Chicco and Sparagna, 2007). Quantitative differences in their lipid composition has been characterized in SM and NSM. Shotgun lipidomics showed that levels of cardiolipin (CL) were lower, whereas levels of ceramide and phosphatidylserine (PS) were higher in SM than in NSM (Kiebish et al., 2008). CL is required for the activities of complex I and III (Fry and Green, 1981). This may account for the lower enzyme activities observed by Kiebish et al. Differences in cardiolipin content might also influence susceptibility to apoptosis, cytochrome c retention or lipid peroxidation (Kagan et al., 2004). Coenzyme Q9 and Q10 were also lower in SM when compared to levels in NSM (Kiebish et al., 2008). SM and NSM lipidomic heterogeneity could influence energy metabolism which may in turn contribute to metabolic compartmentation of the brain (Kiebish et al., 2008).

Quantitative analysis of SM and NSM proteasomes revealed differences in protein composition and functional assessment of the differing genes suggest lower expression of proteins involved in the ETC and OXPHOS, calcium transport and mitochondrial DNA replication and maintenance (Stauch et al., 2014b). Mitochondrial dynamics,

which includes fission/fusion events controls morphology and organisation of these organelles and are therefore important for mitochondrial function and metabolism (Youle and van der Bliek, 2012). Key proteins involved in regulating mitochondrial fission and fusion such as OPA1, Mfn1 and Mfn2 were decreased in SM, suggesting decreased fusion and the increased fragmentation that can ultimately lead to reduced ATP production. The same study also demonstrated that in comparison to NSM, SM exhibited elevated age-associated mitochondria DNA deletions and decreased bioenergetic function. These proteomic alterations suggest that SM may exhibit changes in their morphology that could impact their function and integrity.

The structural and functional organization of the respiratory complexes in the mitochondrial inner membrane has been the subject of great debate for nearly 60 years. For many years, these protein complexes were thought to be randomly dispersed entities within the inner membrane, with the mobile carriers Co-Q and cytochrome c carrying electrons between the complexes also freely diffusible in the lipid membrane. The model proposes casual interactions between the dispersed components (Hackenbrock et al., 1986). However, with the advent of blue native PAGE (BN-PAGE) technology and other biochemical and biophysical techniques, it is now accepted that a higher organisation level of respiratory complexes exists. Individual components of the ETC can actually inhabit the inner mitochondrial membrane as large macromolecular assemblies, or so called 'supercomplexes'. Further support for the existence of supercomplexes arises from their visualization by electron microscopy (Schafer et al., 2007), flux control experiments which show that the complexes function as units (Lenaz and Genova, 2007), and genetic studies which show that the stability of one complex affects the stability of another complex (Lapiente-Brun et al., 2013). In addition, a three-dimensional structure for a supercomplex termed the functional 'respirasome', composed of complexes I, III and IV with a stoichiometry of 1:2:1, respectively has been proposed (Althoff et al., 2011, Dudkina et al., 2011). In fact, respiratory supercomplexes are believed to co-exist in the membrane with single OXPHOS complexes (Acin-Perez et al., 2008).

The most common supercomplex assemblies documented are I+III₂, I+III₂+IV_X and III₂+IV_X (Berry and Trumpower, 1985, Schagger and Pfeiffer, 2001, Stroh et al., 2004, Dudkina et al., 2011). It appears that complex I is bound complex III dimer in

physiological conditions. Approximately 90% of complex I is associated with a complex III dimer and several units of complex IV to form the two major supercomplexes I-III₂ and I-III₂-IV_X. Around 50-60% of complex III and 70-80% complex IV remain as individual entities but can both dynamically interact in an III₂-IV_X intermediate formation. The association of complex II within supercomplex assemblies is still not clear and remains controversial. However, a few studies show it to associate in small proportions with the supercomplex I-III₂-IV_X (Eubel et al., 2003, Acin-Perez et al., 2008). The supercomplex I-III₂-IV_X is one of the most intriguing because it contains all the redox enzymes required for the complete electron transfer from NADH to molecular oxygen. Complex V exists in large proportions as dimers and homo-oligomers in several organisms and has been intensely studied in yeast and beef heart (Krause et al., 2005, Dudkina et al., 2006). Ribbon like structures composed of ATP synthase dimers can play a major role in shaping the IMM because they exert a bending force on the lipid bilayer and induce the formation of tight bends or ridges, which can extend for several hundred nanometers in the cristae membrane.

Respiratory supercomplexes have previously been revealed and characterized in the rat brain (Reifschneider et al., 2006, Dencher et al., 2007, Frenzel et al., 2010). In the rat brain cortex, isolation and structural characterization of OXPHOS supercomplexes, namely I-III₂, I-III₂-IV_X was performed (Dencher et al., 2007) and demonstrated that the abundance of supercomplexes can differ between young and aged cortex. Frenzel et al., quantified changes in the abundance of individual OXPHOS complexes and of the respective supercomplexes I-III₂-IV₀₋₃, V₂-V₄ caused or accompanied by aging (Frenzel et al., 2010).

However, the ramifications of such large protein complexes on electron transport chain function are still poorly understood (Section 1.8.7). The arrangement of ETC complexes into supercomplexes may lead to quick and efficient transport of electrons (Bianchi et al., 2004b, Bianchi et al., 2003, Lenaz et al., 2007). This may result in the sequestration of reactive intermediates to prevent the generation of reactive oxygen species (ROS) (Ghelli et al., 2013, Maranzana et al., 2013b). Further, there is evidence that the complexes are more stable when organized as a supercomplex (Acin-Perez et al., 2004, Diaz et al., 2012, Vempati et al., 2009). The significance of this role still remains to be determined physiologically (Acin-Perez and Enriquez, 2014, Diaz et al., 2012).

Experimental evidence has led to the proposal that the formation of respirasomes may be essential for the assembly/stability of Complex I (Acin-Perez et al., 2004, Acin-Perez et al., 2009a, Diaz et al., 2012, Vempati et al., 2009). Differences in cell types and physiological states give rise to different combinations of respiratory supercomplexes. Different metabolic needs for different tissue types may dictate the existence of supercomplexes at any time allowing the entire respiratory chain to accommodate the ATP demands of the cell (Vartak et al., 2013).

Previous studies have compared isolated NSM and SM with regard to metabolism and lipid composition but the assembly of supercomplexes in these two populations has not been investigated. This study investigates whether there is a difference in quality or quantity of supercomplexes in the IMM of NSM compared to SM. Comparison of the aggregated state of the electron transport chain complexes in NSM and SM may help elucidate the nature of recognised respiratory and enzymatic differences that exist between the two isolated populations of brain mitochondria (Pathak and Davey, 2008).

3.2 Aims of the Chapter

1. **To determine quantitative and qualitative differences between SM and NSM.** The use of high-resolution quantitative based proteomics was used to determine the possibility of quantitative and qualitative differences between these two brain mitochondrial populations. The iTRAQ method is based on the covalent labeling of the N-terminus and side chain amines of peptides from protein digestions with tags of varying mass. The two populations of brain mitochondria were pooled, fractionated by nano liquid chromatography and analyzed by tandem mass spectrometry (MS/MS). This data obtained was then used to relatively quantify the peptides and the proteins from which they originated.
2. **To identify interactors of complex I in SM and NSM.** With the application of the co-immunoprecipitation technique, *in vivo* interactions of the respiratory complexes can be determined. A more detailed insight into complex I interactors can be achieved using a complex I immuncapture resin. In this case, all protein associations to that enzyme could be identified by LC/MS.
3. **To determine the composition and assembly of electron transport chain supercomplexes in mitochondria of SM and NSM.** Using native gel electrophoresis techniques combined with western blotting, the composition of supercomplex assemblies in the brain was quantified. Sucrose density gradient centrifugation was performed to largely enrich the proteins that are either truly localized or tightly associated with mitochondria.
4. **To determine if differences in enzyme activities correlated with differences in assembly of supercomplexes.** Using a combination of in-gel activity assays and spectrophotometric assays, the activities of individual ETC enzymes were assessed.

3.3 Methods

3.3.1 Transmission Electron Microscopy (T.E.M)

NSM and SM were isolated as described in Section 2.7.2 and processed immediately for microscopy. Each population of mitochondria was treated identically using the same techniques and solutions.

The mitochondrial pellet was placed in primary fixative solution (gluteraldehyde (3% v/v) in 0.1 M sodium cacodylate buffer, pH 7.4), transferred to corked vials and allowed to fix for up to 2 hours at room temperature with agitation. The sample was then washed six times for 1 hour with 0.1 M cacodylate buffer to remove any unreacted gluteraldehyde before post fixation. The sample was placed in buffered secondary fixing solution (2% osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.4) for 30°C minutes at room temperature. Following fixation, the sample was dehydrated in solutions of increasing ethanol concentration (50%, 75%, 90% and 100% (v/v)) before being treated with a 100% propylene oxide for 45 minutes at room temperature. Agar 100 resin was prepared according to the manufacturer's instructions. The final 100% propylene oxide solution was replaced with a 50% resin solution for 2-3 hrs. The sample was then placed in 100% resin and baked in an oven under vacuum at 60°C for 24 hrs. Sections were cut using a LKB 8800 Ultra microtome III in the E.M. Unit, Trinity College. The final magnification of the micrographs was 10,000-fold.

3.3.2 Clear Native PAGE

Clear Native Page (CN-PAGE) was performed as described by Shagger and Jagow (Schagger et al., 1994) to isolate supercomplex assemblies. Solubilisation of biological membranes was identical to BN-PAGE (Section 2.9.3). However, in place of Serva Blue G, Ponceau S (0.001%) was added to color the samples faintly. This dye does not bind to proteins and the proteins separate according to their isoelectric points. Sample (80-150 µg per lane) was separated on a 4-16% gradient gel as described in Section 2.9. Electrophoresis was carried out using a cathode buffer of 50 mM Tricine, 15 mM BisTris, pH 7.0 and an anode buffer of 50 mM BisTris-HCl, pH 7.0. Serva Blue G was

omitted from the cathode buffer (Table 3.1). Electrophoresis was started at 40 volts for the first 30 min until the samples had entered the stacking gel following which the voltage was increased to 10 mAmps. Electrophoresis was stopped after 3-4 hrs when the sharp line of the red Ponceau S dye approached the gel front.

3.3.3 High-resolution Clear Native PAGE

High resolution Clear Native PAGE (hrCNE) was developed to preserve the advantages of high resolution obtained using BN-PAGE but separating the proteins in clear gels without the interfering SBG dye (Wittig et al., 2007). hrCNE induces a charge shift just like BNE but in this case, the charge shift is induced by non-colored mixed micelles of neutral and anionic detergents supplemented in the cathode buffer (Wittig et al., 2007). Digitonin was the choice of detergent for membrane solubilisation using a 10:1 (w/w) ratio of detergent to protein. Low amounts of dodecylmaltoside (DDM) (0.01%) and 0.5% deoxycholate (DOC) was added to the cathode buffer (Table 3.1) and the same anode buffer as in CNE and BNE was used. Electrophoresis was started at 40 volts for the first 30 min until samples had entered the stacking gel at which point, the voltage was increased to 10 mAmps. Electrophoresis was stopped after 3-4 hr when the sharp line of the red Ponceau S dye approached the gel front.

	CNE	BNE	hrCNE-3
Tricine (mM)	50	50	50
Bis-Tris (mM)	15	15	15
DDM (%)			0.01
DOC (%)			0.05
Serva Blue G (%)		0.02	
pH	7.0	7.0	7.0

Table 3.1 Composition of the cathode buffers used in CNE, BNE and hrCNE variant 3.

3.3.4 Measurement of Respiratory Chain Complex Activities

After electrophoretic separation by native electrophoresis, the respiratory complexes retain enzymatic activity. In-gel activity assays followed the principles described by Diaz et al., 2009. 150 µg of purified protein was loaded in each lane. All staining solutions were freshly made.

3.3.4.1 Complex I in-gel activity assay

Specific staining of individual complex I (NADH-ubiquinone oxidoreductase) and of respiratory supercomplexes containing complex I in NSM and SM was achieved by incubating the 1D BN- PAGE gel in 0.1 M Tris-HCL pH 7.4 containing 1 mg/ml nitro blue tetrazolium (NBT) and 0.14 mM NADH at room temperature. Depending on the sample, the blue purple color developed as early as in 30 min.

3.3.4.2 Complex III in-gel activity assay

A method for the in gel staining for complex III (cytochrome c reductase) was described by (Wittig et al., 2007). Specific staining of individual complex III and of respiratory supercomplexes containing complex III in NSM and SM was achieved by incubating the hrCNE-3 gel in complex III assay buffer (5 mg iaminobenzidine dissolved in 10 ml 50 mM sodium phosphate, pH 7.4). Depending on the sample, a brown colour developed in 1 hour.

3.3.4.3 Complex IV in-gel activity assay

Specific staining of individual complex IV (Cytochrome c oxidase) and of respiratory supercomplexes containing complex IV in NSM and SM was achieved by incubating the BN-PAGE in 5 mg diaminobenzidine tetrahydrochloride (DAB) dissolved in 9 ml of 50 mM potassium phosphate buffer pH 7.4, 10 mg cytochrome c and 750 mg sucrose. Depending on the sample, a brown colour developed in 1 hour.

3.3.4.4 Complex V in-gel activity assay

Specific staining of monomeric complex V (ATP synthase) and of dimeric and homo oligomeric supercomplexes containing complex V in NSM and SM was achieved by washing the 1D BN-PAGE a twice in water and then incubating in 50 mM phosphate buffer pH 8.6 for 1 hour to equilibrate gel. A reaction mix was prepared in the following order: 35 mM Tris, 270 mM glycine, 14 mM MgSO₄, 5 mM ATP and adjusted to pH

7.8, then add 0.2% $\text{Pb}(\text{NO}_3)_2$. As the reaction is slow the gel was incubated overnight before white bands were observed.

A gel imaging system was used to digitize gel images (see Chapter 2, section 2.11.1) and the densitometry in-gel quantification of band intensity was performed using ImageJ (National Institutes of Health).

3.3.4.5 Complex I Spectrophotometric Assay

The specific activity of Complex I (NADH-ubiquinone oxidoreductase) in NSM and SM was determined by the method of Ragan et al. (1987), with modifications. The spectrophotometric assay follows the oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+ by Complex I (with Decyl Q as an artificial electron acceptor), which is seen as a decrease in absorbance at 340 nm. Samples were thrice cryolysed in liquid nitrogen before 2 μg of pure mitochondria were added to a well containing assay buffer (25 mM potassium phosphate, 10 mM magnesium chloride (MgCl_2), pH 7.4), 250 μM potassium cyanide (KCN), 250 μM NADH and 3mg/ml BSA with a final volume of 200 μl .

The assay was carried out at 37°C in a SpectraMax spectrophotometer. The basal rates of ΔAbs_{340} were monitored in the samples for 2 min before the redox reaction was initiated by the addition of 50 μM Decyl Q. The reaction was monitored for 3 min before 10 μM rotenone was added to each sample to inhibit complex I and obtain rotenone-insensitive rates. The rotenone-insensitive rates of ΔAbs_{340} were subtracted from the rotenone-sensitive rates and specific activities expressed as nmol/min/mg.

3.3.5 Sucrose Gradient Centrifugation

The physiological supramolecular assemblies were further purified by sucrose gradient centrifugation. Isolated NSM and SM were solubilized by digitonin using a 10:1 ratio of detergent to protein. 2.5 mg of mitochondria was suspended in 500 μ l extraction buffer (1M Hepes pH 7.2, 20 μ L KCl, 5 μ l protease inhibitor cocktail) and incubated on ice for three hours. This was centrifuged at 16,000 g for 30 min and the supernatant collected. A discontinuous gradient containing sucrose solutions from 1.5 M to 0.3 M was prepared and the mitochondrial extract applied to the top of the gradient (Figure 3.1). The gradient was centrifuged in a Sorvall Discovery 100 centrifuge, fitted with a Sorval AH650 rotor, at 132,000 g (29,000 rpm) for 16 hr at 4°C. Acceleration and deceleration profiles were set to 0. For fraction collection, the tube was held steady and upright by a clamp stand. Fractions were collected from bottom up, using a peristaltic pump and a fine glass capillary tube. The capillary glass tube was inserted to the bottom of the centrifuge tube carefully without disturbing the sucrose layers. The pump was started at a slow speed of 1ml/min and 0.5 ml fractions collected. These were stored at -80°C till further analysed.

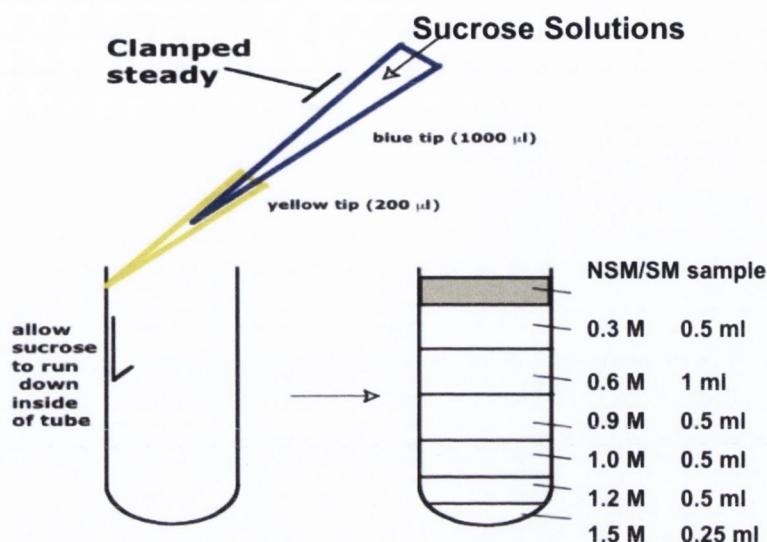


Figure 3.1 Preparation of the discontinuous sucrose gradient. A discontinuous gradient containing sucrose solutions from 1.5 M % to 0.3 M was prepared and the mitochondrial extract applied to the top of the gradient. The gradient was centrifuged in a Sorvall Discovery 100 centrifuge at 132,000 g (29,000 rpm) for 16 hr at 4°C. For fraction collection, the tube was held steady and upright by a clamp stand. Fractions were collected from bottom up, using a peristaltic pump and a fine glass capillary tube. The capillary glass tube was inserted to the bottom of the centrifuge tube very carefully without disturbing the sucrose layers. The pump was started at a slow speed of 1ml/min and 0.5 ml fractions collected.

3.3.6 Co-immunoprecipitation (Co-IP)

0.5 mg of pure NSM and SM was resuspended in 1 ml of immunoprecipitation (IP) buffer (20 mM Hepes pH 7.4, 100 mM KCl, 0.2 mM EDTA, 10% (w/v) glycerol) and digitonin using a 10:1 ratio of detergent to protein. This was incubated on ice for 30 min followed by centrifugation at 16,000 g for 30 min at 4°C. The supernatant was collected and 1X protease inhibitor added. The NSM and SM supernatant extracts were precleared with washed Protein G agarose by passing the extracts over beads that were not coated with antibody. This removed proteins that non-specifically bound to the beads. The precleared extracts were then mixed with 10 µg monoclonal anti-NDUFA9 (complex I), anti-Core I (complex III), anti-Subunit Va (complex IV) or anti-ATP synthase subunit- α (complex V) respectively at 4°C overnight. Following mixing, the beads were collected by centrifugation at 3,000 g for 1 min. The supernatant was removed and discarded. The complex of interest was now specifically bound to the antibody coating the beads. The beads were washed in 1 X PBS containing digitonin. The beads were collected by gentle centrifugation and supernatant removed and discarded. Beads were resuspended in 2-5 volumes of 1% SDS, incubated for 10 min with frequent agitation and the supernatant containing the eluted complexes collected from above the beads. The immunoprecipitates were resolved by SDS-PAGE and Western blotted with complex I and complex III antibodies (see Chapter 2, sections 2.8.1 and 2.11 respectively).

3.3.7 Immunocapture of complex I

0.5 mg of pure NSM and SM was resuspended in 1 ml of PBS and digitonin using a 10:1 ratio of detergent to protein. This was incubated on ice for 30 min and then centrifuged at 16,000 g for 30 min at 4°C. The supernatant was collected and 1X protease inhibitor added. 20 µl of antibody loaded agarose beads (complex I immunocapture resin from Mitosciences) was added to the supernatant and mixed overnight at 4°C. The resin was washed as per manufacturer's instruction. Following mixing, the beads are collected by centrifugation at a low speed for 1 min and supernatant was removed and discarded. The complex of interest was now specifically bound to the antibody coating the beads. The beads were washed in 1 X PBS containing digitonin and collected by gentle centrifugation. The supernatant was removed and

discarded. The collected beads were resuspended in 2-5 volumes of 1% SDS, incubated for 10 min with frequent agitation and the supernatant containing the eluted complexes collected from above the beads. The captured proteins were resolved on 12% SDS-PAGE and stained with Coomassie blue (see Chapter 2, section 2.10.2). The gel lane was sliced into bands (Figure 3.2), which were processed for tryptic digestion (see Chapter 2, section 2.12) and LC-MS analysis (see below).

3.3.8 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS was performed at the Royal College of Surgeons, Dublin 2. Samples were run on a Finnigan LTQ mass spectrometer connected to a Surveyor chromatography system incorporating an auto sampler. A tryptic Picofrit column (100 mm length, 75 μ m ID) at a flow rate of 30 $\text{nl}\cdot\text{min}^{-1}$ was used. Samples were then eluted from the C18 Picofrit column by an increasing acetonitrile gradient. The mass spectrometer was operated in positive ion mode with a capillary temperature of 200 $^{\circ}\text{C}$, a capillary voltage of 46V, a tube lens voltage of 140 V and with a potential of 1800 V applied to the frit. All data were acquired with the mass spectrometer operating in the automatic data-dependent switching mode. Raw mass spectral data was analysed using Proline, a proteomics analysis platform. ThermoRAW files were converted into mzXML format. X! Tandem release 2005.02.01 was used to analyse these files with a static iodacetamide modification, with Uniprot/SwissProt release 7.6 used as the FASTA search database. The results were stored in a MySQL database and were interrogated using Proline's webbased proteomics toolset.

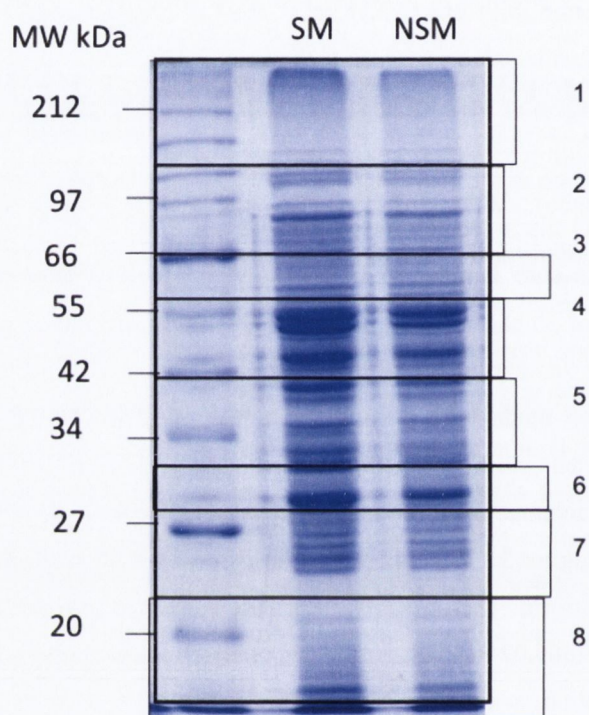


Figure 3.2 Immunocapture of complex I from NSM and SM for LC-MS/MS. Digitonin solubilised extracts from NSM and SM were immunocaptured using complex I immunocapture resin. The captured proteins were resolved on 12% SDS-PAGE and stained with Coomassie blue. Each lane was sliced into eight pieces and processed for trypsin digestion and LC-MS/MS.

3.4 Results

3.4.1 Analysis of mitochondrial purity

Use of a Percoll discontinuous gradient following the Ficoll discontinuous gradient ensured high purity yield of NSM and SM. Western blotting showed that these double gradient mitochondrial preparations were free from cytosolic (GAPDH), plasma membrane (glut 4), golgi apparatus (GP130), endoplasmic reticulum (calnexin) and nuclear (lamin) markers. Reassuringly, the purified mitochondria contained only mitochondrial-enriched markers representing the IMM (complex IV) and the OMM (monoamine oxidase-A) (data not shown). TEM confirmed the isolated SM and NSM were pure and intact (Figure 3.3). The mitochondria were bound by a double membrane; the outermost membrane was smooth and the inner membrane had the characteristic folds of the cristae.

3.4.2 Quantitative analysis of NSM and SM proteomes using iTRAQ quantitative labeling and mass spectrometry.

To examine the differences in the NSM and SM mitochondrial proteomes, a preliminary duplex experiment was performed on isolated mitochondria. This was followed by a 6-plex, which allowed for three separate preparations of NSM and SM to be analyzed in one experiment under identical conditions. This quantitative proteomic study involved differential labeling of NSM and SM proteins or proteolytic peptides by stable isotopes, mixing of samples in a 1:1 ratio followed by combined sample process and subsequent MS analysis. The relative quantification of proteins was then performed by comparing ion signal intensities of peak area of isotope encoded peptide pairs observed in the corresponding mass spectra. To interpret the expression array data, proteins with relative ratio of 1.5 and 2 were chosen because greater disparity in expression may indicate a more significant role for the protein.

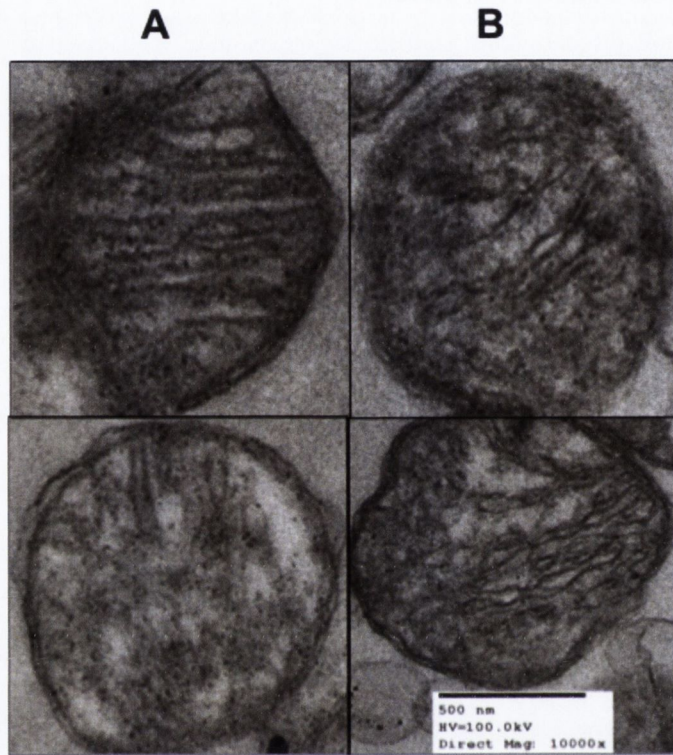


Figure 3.3 NSM and SM isolated following a double gradient procedure are pure and intact. Representative transmission electron micrograph of isolated (A) NSM and (B) SM showing the double outer membranes and numerous internal membranes (cristae) where respiration takes place. Magnification was 10000 x and scale bar 500 nm.

Figure 3.4 illustrates the quantitated and unquantitated proteins in the two experiments. Thirty-seven proteins whose relative levels were increased > 1.5 fold in NSM compared to relative levels in SM was identified. The list of these proteins and their functional categories is shown in Table 3.2. Conversely, twenty-five proteins whose relative levels were increased > 2 fold in SM compared to relative levels in NSM was identified. The list of these proteins and their functional categories is shown in Table 3.3. The differentially expressed proteins were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation tool for enrichment analysis and classified according to the Gene Ontology (GO) terms cellular component, biological process and molecular function. The GO analysis indicated that the list of differentially expressed proteins were associated with functions important to each mitochondrial population. The top five enriched biological processes using DAVID based on NSM/SM > 1.5 proteins included generation of precursor metabolites and energy, glycolysis, glucose catabolic process, monosaccharide catabolic process and hexose catabolic process (Table 3.4). In contrast, the top five enriched biological processes using DAVID based on the 25 SM/NSM > 2 proteins included regulation of action potential in neuron, regulation of action potential, homeostasis, cytoskeleton organization and regulation of membrane potential (Table 3.5). The cellular localisation for the 37 NSM proteins (Table 3.6) and the 25 SM proteins (Table 3.7) were also identified using DAVID and classified according to the GO term cellular component.

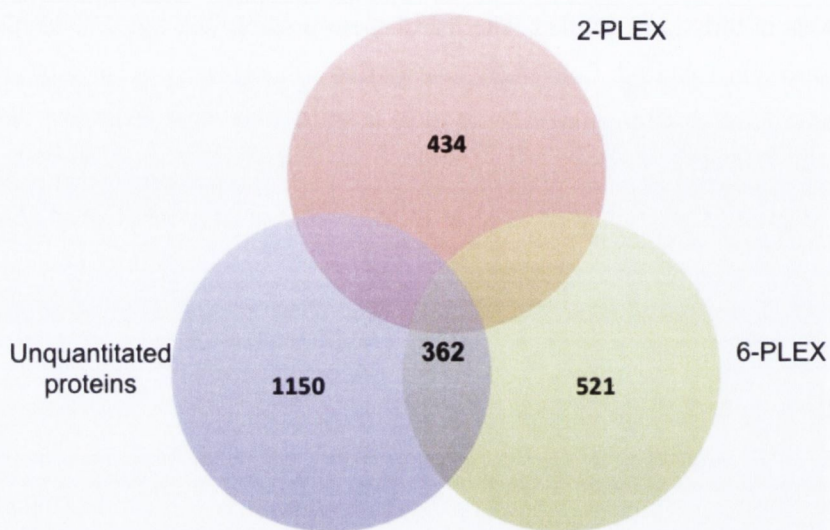


Figure 3.4 Venn diagram of the common and unique proteins that were identified from isolated mitochondria from the preliminary duplex and 6-plex. For the 6-plex three separate isolations were prepared. The 6-plex allows inclusion of a triplicate within the same experiment. Once labelled with the iTRAQ Reagents-6plex, the 6 individual samples are then pooled for further processing and analysis. During subsequent MS/MS of the peptides, each isobaric tag produces a unique reporter ion that identifies which samples the peptide originated and its relative abundance. A list of proteins whose relative levels were increased in each of the two populations of mitochondria is identified and shown in Table 3.2 and Table 3.3.

Table 3.2 Functional categories of the increased relative levels of NSM proteins compared to corresponding protein levels in SM.

Accession Number	Name	UniProt ID	Gene	RGD ID	Ratio NSM/SM
Cellular Component Organization (binding, organelle organization, multicellular organization, membrane organization, complex subunit)					
O35179	endophilin A1	SH3G2_RAT	Sh3gl2	620276	4.08
P63025	vesicle associated membrane protein 3	VAMP3_RAT	Vamp3	61880	3.2
Q5XIT1	microtubule-associated protein RP/EB family member 3	MARE3_RAT	Mapre3	1359297	2.53
P60711	actin, cytoplasmic 1	ACTB_RAT	Actb	628837	2.36
P45479	palmitoyl-protein thioesterase 1 precursor	PPT1_RAT	Ppt1	61994	2.19
Transport (transporter activity, binding, cellular process)					
O35179	endophilin A1	SH3G2_RAT	Sh3gl2	620276	4.08
P63081	V-type proton ATPase 16 kDa proteolipid subunit	VATL_RAT	Atp6v0c	621394	3.8
P8049	ADP-ribosylation factor 1	ARF1_RAT	Arf1	621270	3.2
P09951	synapsin-1 isoform a	SYN1_RAT	Syn1	3797	2.56
Q63537	synapsin-2 isoform 2	SYN2_RAT	Syn2	3798	2.32
Q794C0	v-type proton ATPase subunit E1	VA0E1_RAT	Atp6v0e1	621393	2.27
P45479	palmitoyl-protein thioesterase 1 precursor	PPT1_RAT	Ppt1	61994	2.19
Q2I6B4	v-H ⁺ ATPase subunit a1-iv	Q2I6B4_RAT	Atp6v0a1	68405	2.19
Q5M7T6	V-type proton ATPase subunit d1	Q5M7T6_RAT	Atp6v0d1	1306159	2.15
P62744	AP-2 complex subunit sigma	AP2S1_RAT	Ap2s1	620188	2.04
Q02563	synaptic vesicle glycoprotein 2A	SV2A_RAT	Sv2a	619715	2.01
Biological Regulation					
Q04940	neurogranin	NEUG_RAT	Nrgn	61833	2.8
Q6P7Q4	glyoxalase	LGUL_RAT	Glo1	2702	2.8
P62749	hippocalcin-like protein 1	HPCL1_RAT	Hpcal1	708375	2.45
Q71UE8	NEDD8 precursor	NEDD8_RAT	Nedd8	3158	2.31
G3V7Z4	glia-derived nexin isoform a precursor	G3V7Z4_RAT	Serpine2	3748	2.2
P62762	visinin-like protein 1	VISL1_RAT	Vsnl1	3966	2.12
P82995	heat shock protein HSP 90 alpha	HS90A_RAT	Hsp90aa1	631409	2.03
Metabolic Processes					
<i>Pyrimidine Nucleobase Catabolic process</i>					
P47942	dihydropyrimidinase-related protein 2	DPYL2_RAT	Dpysl2	2517	2.13
P8049	ADP-ribosylation factor 1	ARF1_RAT	Arf1	621270	3.2
P11442	clathrin heavy chain 1	CLH1_RAT	Cltc	2364	2.24

Accession Number	Name	UniProt ID	Gene	RGD ID	Ratio NSM/SM
<i>Generation of Precursor Metabolites and Energy</i>					
P63081	V-type proton ATPase 16 kDa proteolipid subunit	VATL_RAT	Atp6v0c	621394	3.8
Q794C0	v-type proton ATPase subunit E1	VA0E1_RAT	Atp6v0e1	621393	2.27
Q2I6B4	v-H+ ATPase subunit a1-iv	Q2I6B4_RAT	Atp6v0a1	68405	2.19
Q5M7T6	V-type proton ATPase subunit d1	Q5M7T6_RAT	Atp6v0d1	1306159	2.15
P07323	gamma-enolase	ENOG_RAT	Eno2	2554	2.62
P13221	Aspartate aminotransferase, cytoplasmic	AATC_RAT	Got1	2721	2.46
	pyruvate kinase isozymes M1/M2			1592113	2.11
P04797	glyceraldehyde-3 -phosphate dehydrogenase	G3P_RAT	Gapdh	2661	2.19
P48500	triosephosphate isomerase isoform 2	TPIS_RAT	Tpi1	3896	2.24
P62744	AP-2 complex subunit sigma	AP2S1_RAT	Ap2s1	620188	2.04
Q02563	synaptic vesicle glycoprotein 2A	SV2A_RAT	Sv2a	619715	2.01
<i>Nucleotide biosynthetic synthesis</i>					
P63081	V-type proton ATPase 16 kDa proteolipid subunit	VATL_RAT	Atp6v0c	621394	3.8
Q794C0	v-type proton ATPase subunit E1	VA0E1_RAT	Atp6v0e1	621393	2.27
Q2I6B4	v-H+ ATPase subunit a1-iv	Q2I6B4_RAT	Atp6v0a1	68405	2.19
Q5M7T6	V-type proton ATPase subunit d1	Q5M7T6_RAT	Atp6v0d1	1306159	2.15
	pyruvate kinase isozymes M1/M2			1592113	2.11
C7C5T2	6 phosphofructokinase type C	C7C5T2_RAT	Pf1k	61893	2.03
<i>Lipid Catabolic Process</i>					
P45479	palmitoyl-protein thioesterase 1 precursor	PPT1_RAT	Ppt1	61994	2.19
Receptor internalization and recycling					
Q35179	endophilin A1	SH3G2_RAT	Sh3g12	620276	4.08
P63025	vesicle associated membrane protein 3	VAMP3_RAT	Vamp3	61880	3.2
P8049	ADP-ribosylation factor 1	ARF1_RAT	Arf1	621270	3.2
P11442	clathrin heavy chain 1	CLH1_RAT	Cltc	2364	2.24
GTP Binding					
Q9WU34	Neuronal-specific septin-3	SEPT3_RAT	Sep-03	621745	2.18

Accession Number	Name	UniProt ID	Gene	RGD ID	Ratio NSM/SM
Catalytic activity					
Q04940	neurogranin	NEUG_RAT	Nrgn	61833	2.8
P61983	14-3-3 protein gamma, Monooxygenase	1433G_RAT	Ywhag	62002	2.67
Q5XIT1	microtubule-associated protein RP/EB family member 3	MARE3_RAT	Mapre3	1359297	2.53
P13221	Aspartate aminotransferase, cytoplasmic	AATC_RAT	Got1	2721	2.46
P63329	serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	PP2BA_RAT	Ppp3ca	3382	2.42
P24268	Cathepsin D	CATD_RAT	Ctsd	621511	2.15
O88831	Calmodulin-dependent protein kinase II beta M isoform	KKCC2_RAT	Camkk2	620092	2.02

Table 3.3 Functional categories of the increased relative levels SM compared to corresponding protein levels in NSM

Accession Number	Name	UniProt ID	Gene	RGD ID	Ratio SM/NSM
Regulation of Action Potential					
P60203	Myelin proteolipid protein	MYPR_RAT	Plp1	3354	6.35
P02688	myelin basic protein isoform 1	MBP_RAT	Mbp	3054	4.36
P13233	Claudin-11	CLD11_RAT	Cldn11	71081	4.02
G3V7A1	Calcium-activated potassium channel subunit beta-2	G3V7A1_RAT	Kcnmb2	631398	1.5
Cellular Homeostasis					
P60203	Myelin proteolipid protein	MYPR_RAT	Plp1	3354	6.35
P02688	myelin basic protein isoform 1	MBP_RAT	Mbp	3054	4.36
P13233	Claudin-11	CLD11_RAT	Cldn11	71081	4.02
D3ZYW7	Frataxin, mitochondrial	FRDA_RAT	Fxn	1565754	1.53
Q6AXW1	Glutaredoxin-2, mitochondrial	GLRX2_RAT	Glxr2	1307950	1.53
Q6AXW1	Calcium-activated potassium channel subunit beta-2	G3V7A1_RAT	Kcnmb2	631398	1.5
Cytoskeleton Organization					
P13233	2,3, cyclic nucleotide 3 phosphodiesterase, CNP	CN37_RAT	Cnp	2368	2.7
P23565	Alpha-internexin	AINX_RAT	Ina	2911	2.09
Q9JKC6	Cell cycle exit and neuronal differentiation protein 1	CEND_RAT	Cend1	1309401	2
Q61FW6	putative uncharacterized protein Krt10	K1C10_RAT	Krt10	1359092	1.9
P08644	GTPase Kras	RASK_RAT	Kras	2981	1.76
Q63345	myelin oligodendrocyte glycoprotein	MOG_RAT	Mog	3102	1.55
Q5PPN5	tubulin polymerization-promoting protein	TPPP3_RAT	Tppp3	1305061	1.53
Metabolic Processes					
<i>Oxidation and Reduction</i>					
D3ZUY0	Protein Rdh14	D3ZUY0_RAT	Rdh14	1565196	1.8
Q64591	2,4 dienoyl Co A reductase	DECR_RAT	Decr	70999	1.69
Q64057	alpha aminoacidipic semialdehyde dehydrogenase	AL7A1_RAT	Aldh7a1	1308614	1.63
Q6AXW1	Glutaredoxin-2, mitochondrial	GLRX2_RAT	Glxr2	1307950	1.53
<i>GTP Catabolic Process</i>					
Q8CFN2	Cell division control protein 42 homolog	CDC42_RAT	Cdc42	71043	1.84
F1LW77	Protein Rab33b	F1LW77_RAT	Rab33b	1308353	1.8
P08644	GTPase Kras	RASK_RAT	Kras	2981	1.76
<i>Fatty Acid Metabolism</i>					
P60203	myelin proteolipid protein	MYPR_RAT	Plp1	3354	6.35
Q64591	2,4 dienoyl Co A reductase	DECR_RAT	Decr1	70999	1.69
P14604	enoyl Co A hydratase, mitochondrial precursor	ECHM_RAT	Echs1	69330	1.61

Accession Number	Name	UniProt ID	Gene	RGD ID	Ratio SM/NSM
<i>Cyclic Nucleotide Catabolic Process</i>					
Q02563	synaptic vesicle glycoprotein 2A	SV2A_RAT	Sv2a	619715	2.01
<i>Methylation</i>					
Q5I0P2	Glycine cleavage system H protein, mitochondria	GCSH_RAT	Gcsh	619946	1.84
<i>Oxidative Phosphorylation</i>					
C6ZII9	SIRT3L	C6ZII9_RAT	Sirt3	1308374	2.0
D3ZYW7	Fratxin, mitochondrial	FRDA_RAT	Fxn	1565754	1.53
<i>Purine Nucleotide Metabolic Process</i>					
Q8K4G6	O-acetyl-ADP-ribose deacetylase MACROD1	MACD1_RAT	Macrodl	628701	1.84
<i>Cation Transmembrane Transporter Activity</i>					
Q9JHY2	Sideroflexin-3	SFXN3_RAT	Sfxn3	620716	1.53
<i>Syntaxin Binding</i>					
B5DF41	syntaphilin isoform CRA d	SNPH_RAT	Snph	1305867	1.96

Table 3.4 Gene Ontology (GO) Annotation for Biological Process and Molecular Function Obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) for the 37 NSM/SM ratios > 1.5 (Max Probe <0.1 and Min Count >2).

		%	<i>P</i> value
GO: Biological Process			
1	generation of precursor metabolites and energy	27.0	7.2 x 10 ⁻⁹
2	glycolysis	13.5	5.6 x 10 ⁻⁶
3	glucose catabolic process	13.5	1.4 x 10 ⁻⁵
4	monosaccharide catabolic process	13.5	1.7 x 10 ⁻⁵
5	hexose catabolic process	13.5	1.7 x 10 ⁻⁵
6	cellular carbohydrate catabolic process	13.5	3.7 x 10 ⁻⁵
7	alcohol catabolic process	13.5	3.7 x 10 ⁻⁵
8	ATP biosynthetic process	13.5	1.1 x 10 ⁻⁴
9	purine ribonucleotide triphosphate biosynthetic process	13.5	1.7 x 10 ⁻⁴
10	ribonucleoside triphosphate biosynthetic process	13.5	1.8 x 10 ⁻⁴
GO: Molecular Function			
1	hydrogen ion transmembrane transporter activity	10.8	1.5 x 10 ⁻³
2	monovalent inorganic cation transmembrane transporter activity	10.8	1.8 x 10 ⁻³
3	inorganic cation transmembrane transporter activity	10.8	5.2 x 10 ⁻³
4	purine ribonucleotide binding	27.0	1.2 x 10 ⁻²
5	ribonucleotide binding	27.0	1.2 x 10 ⁻²
6	purine nucleotide binding	27.0	1.6 x 10 ⁻²
7	cytoskeleton protein binding	13.5	1.6 x 10 ⁻²
8	nucleotide binding	29.7	1.7 x 10 ⁻²
9	calmodulin binding	8.1	2.8 x 10 ⁻²
10	ATP binding	21.6	3.1 x 10 ⁻²

Table 3.5 Gene Ontology (GO) Annotation for Biological Process and Molecular Function Obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) for the 25 SM/NSM ratios > 2.0 (Max Prob <0.1 and Min Count >2).

		%	<i>P</i> value
GO: Biological Process			
1	regulation of action potential in neuron	16	1.7 x 10 ⁻⁴
2	regulation of action potential	16	3.27 x 10 ⁻⁴
3	cellular homeostasis	16	7.07 x 10 ⁻⁴
4	Cytoskeleton organization	24	1.67 x 10 ⁻³
5	regulation of membrane potential	20	1.9 x 10 ⁻³
6	axon ensheathment	16	2.8 x 10 ⁻³
7	ensheathment of neurons	12	2.8 x 10 ⁻³
8	cellular ion homeostasis	12	2.8 x 10 ⁻³
9	ion homeostasis	20	3.0 x 10 ⁻³
10	homesostatic process	20	3.8 x 10 ⁻³
GO: Molecular Function			
1	structural constituent of myelin sheath	8	8.7 x 10 ⁻³
2	structural molecule activity	20	1.7 x 10 ⁻²
3	2 iron, 2 sulfur cluster binding	8	3.1 x 10 ⁻²
4	Co-enzyme binding	12	5.9 x 10 ⁻²
5	iron-sulfur cluster binding	8	8.9 x 10 ⁻²
6	metal cluster sulfur binding	8	8.9 x 10 ⁻²
7	iron ion binding	12	9.0 x 10 ⁻²
8	Cofactor binding	12	9.7 x 10 ⁻²

Table 3.6 Gene Ontology (GO) Annotation for Cellular Component Obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) for the 37 NSM/SM ratios > 1.5 (Max Prob <0.1 and Min Count >2).

		%	<i>P</i> value
GO: Cellular Component			
1	soluble fraction	29.7	8.5 x 10 ⁻⁹
2	cell fraction	43.2	2.3 x 10 ⁻⁸
3	cytosol	40.5	1.2 x 10 ⁻⁶
4	clathrin-coated vesicle	18.9	1.5 x 10 ⁻⁶
5	coated vesicle	18.9	3.5 x 10 ⁻⁶

Table 3.7 Gene Ontology (GO) Annotation for Cellular Component Obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) for the 25 SM/NSM ratios > 2.0 (Max Prob <0.1 and Min Count >2)

		%	<i>P</i> value
GO: Cellular Component			
1	mitochondria	40.0	4.5 x 10 ⁻⁴
2	compact myelin	8.0	1.0 x 10 ⁻²
3	myelin Sheath	8.0	3.0 x 10 ⁻²
4	internal side of plasma membrane	12.0	4.0 x 10 ⁻²
5	plasma membrane part	28.0	5.0 x 10 ⁻²

3.4.3 Native Gel electrophoresis allows the immediate study of OXPHOS proteins in mitochondria.

GO analysis of differentially expressed NSM proteins showed enrichment of biological processes related to generation of energy, metabolic processes and protein translation (Table 3.4). Differences between energy metabolism in NSM and SM (Davey et al., 1998, Kiebish et al., 2008) have previously been documented but poorly understood. Using the results from the proteomic study encouraged further exploration into the differences observed in biological pathways relevant to energy production, in particular oxidative phosphorylation (OXPHOS).

Native gel electrophoresis is a technique that has been optimized for the study of OXPHOS proteins (Schagger, 2002) as it isolates membrane protein complexes immediately from purified mitochondria. In order to determine an appropriate detergent concentration for the solubilisation of membrane protein complexes from NSM and SM, a dilution series was carried out using two detergents. Dodecylmaltoside (DDM) and digitonin are both non-ionic detergents but differ in their solubilisation properties. A higher concentration of digitonin was necessary to obtain similar solubilisation effects as with maltoside. Mitochondria were treated with varying detergent to protein ratios and then applied to a 1D BN-PAGE. This lane was excised and further analyzed on Tricine SDS-PAGE. In the case of DDM, the ratio of detergent to protein for optimal solubilisation was 1:1 (w/w) (Figure 3.5). In the case of digitonin, the ratio of detergent to protein for optimal solubilisation, while preserving higher molecular weight supramolecular structure was 10:1 (w/w) (data not shown).

Figure 3.6 shows the separation pattern of proteins by CN-PAGE of both digitonin (Figure 3.6 A) and maltoside (DDM) (Figure 3.6 B) solubilized NSM and SM mitochondria. Clear Native PAGE (CN-PAGE) relies on the protein intrinsic charge. Many of the membrane proteins gave rise to smearing bands. On this basis, it was concluded that CN-PAGE served only as a pre-purification step.

The use of CN-PAGE as a preparatory step is evident when performing the characterization of complex I subunits from isolated mitochondrial samples as demonstrated in Figure 3.7 and Figure 3.8 Individual subunits of complex I can be isolated using CN-PAGE in the first dimension (Figure 3.7 A), a BN-PAGE in the

second dimension (Figure 3.7 B) and a final denaturing Tricine-SDS-PAGE in the third and final dimension (Figure 3.7 C). NSM were carried through the same procedure and the individual subunits of complex I resolved on Tricine-SDS-PAGE (Figure 3.8 A-C). There are 45 known subunits in complex I (Carroll et al., 2003). By using three-dimensional PAGE as described, approximately 75% of individual subunits are recovered.

All subsequent experiments on brain mitochondria involved the use of BN-PAGE or a modified version in the first dimension. BN-PAGE involves the use of the anionic dye Serva Blue G. This dye can impose a negative charge shift on proteins upon binding to the protein surface. This charge shift is especially important for hydrophobic membrane proteins. All SBG-binding membrane proteins migrate to the anode in BN-PAGE independent of the protein intrinsic pI.

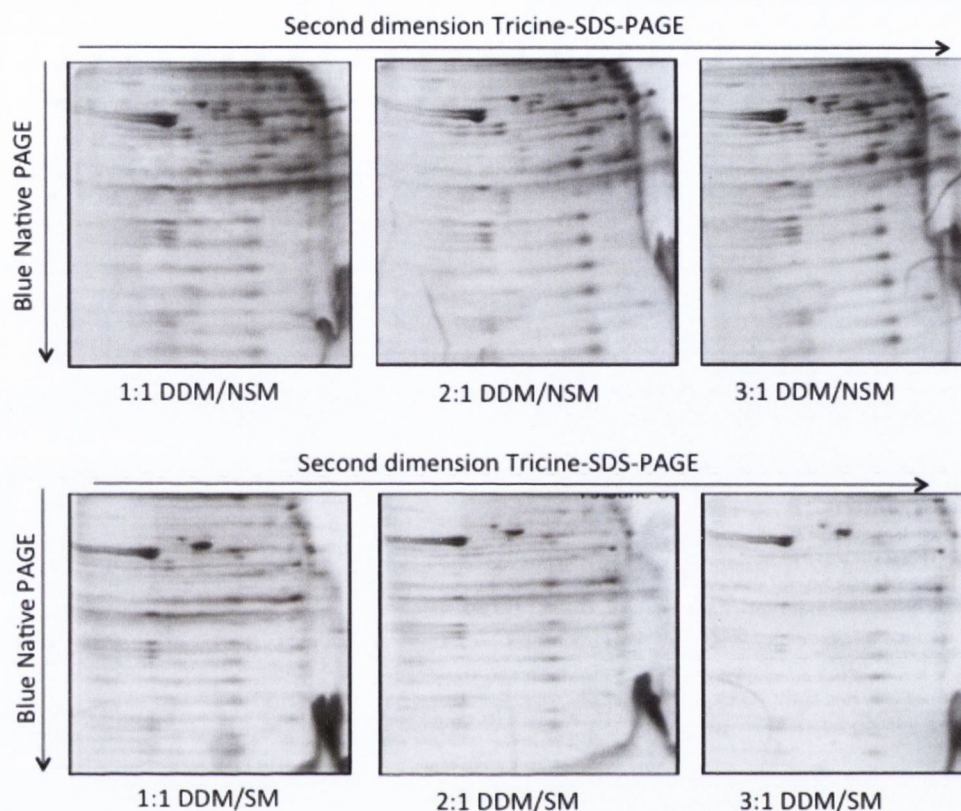


Figure 3.5 Optimal solubilisation of mitochondrial membranes was achieved using 1 g dodecylmaltoside per g of protein for both NSM and SM. (A-E) Representative SBG stained 2D Tricine-SDS-PAGE with varying degrees of detergent used to solubilize membranes in NSM and SM. Three different concentrations of DDM were used in order to evaluate the most suitable for solubilisation of the mitochondrial membranes. Freshly prepared NSM (A-C) and SM (D-E) were treated with detergent and resolved on 1D BN-PAGE (data not shown). Following electrophoresis, in-gel native proteins in each lane were denatured using 20 mM DDT/ 1% SDS and resolved on a uniform 10% Tricine-SDS gel in the second dimension. At a ratio of 1 g detergent to 1 g protein, the staining intensity is highest and the highest number of distinct bands could be identified. At lower ratios of detergent to protein, there was disappearance of bands and staining intensity decreased.

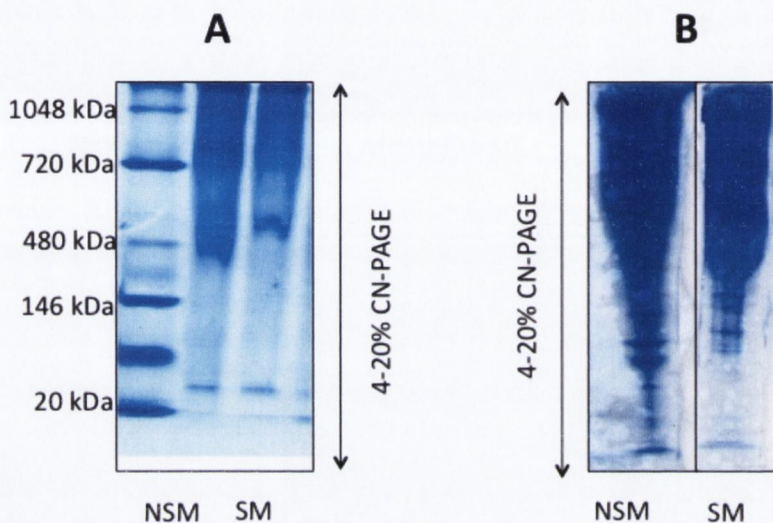


Figure 3.6 Supramolecular assemblies of rat brain mitochondria are poorly resolved by 1D CN-PAGE. (A) NSM and SM were solubilized with a digitonin-to-protein ratio of 10:1 (w/w) and separated on a 4-20% acrylamide gel with an extended 3% sample gel. (B) NSM and SM were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and separated on a 4-20% acrylamide gel with an extended 3% sample gel. One-dimensional CN-PAGE results in poor resolution and smearing of OXPHOS complexes.

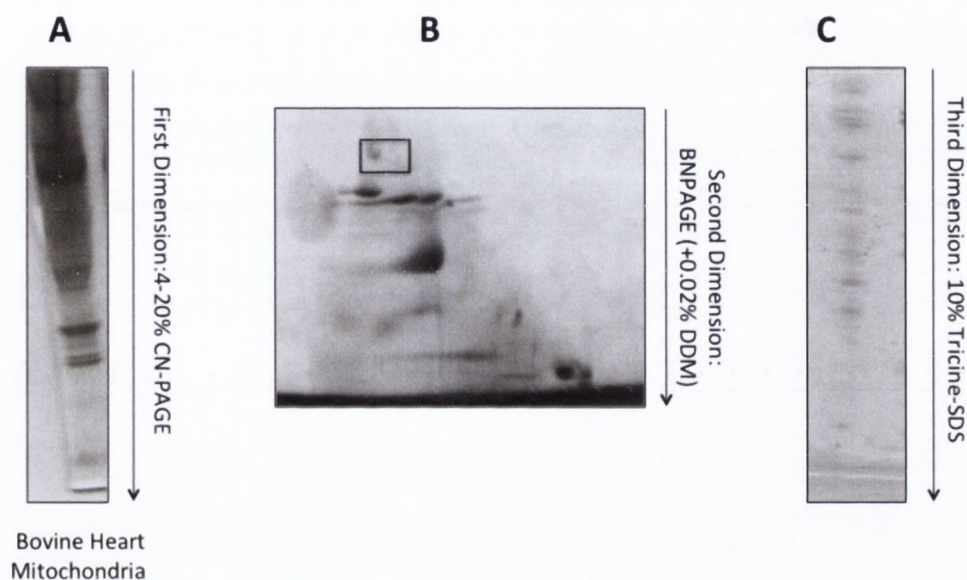


Figure 3.7 Individual subunits of complex I in bovine heart mitochondria can be isolated using three dimensional gel electrophoresis separation. (A) Bovine heart mitochondria were solubilized with a DDM-to-protein ratio of 3:1 (w/w) and separated on a 4-20% CN-PAGE with an extended 3% sample gel. (B) Following electrophoresis, a complete lane from the first dimension CN-PAGE was excised and further resolved by a modified 2D-BN PAGE (1 mm gel, 4.8-12% gradient gel). 0.02% DDM was added to the cathode buffer in the second dimension. Individual complexes of the oxidative phosphorylation system are detected as blue spots during this BN-PAGE stage. Assignment of complexes was possible after identification by Tricine-SDS-PAGE in the third dimension. The boxed spot represents complex I. This was excised and the individual subunits resolved by three-dimensional electrophoresis (C).

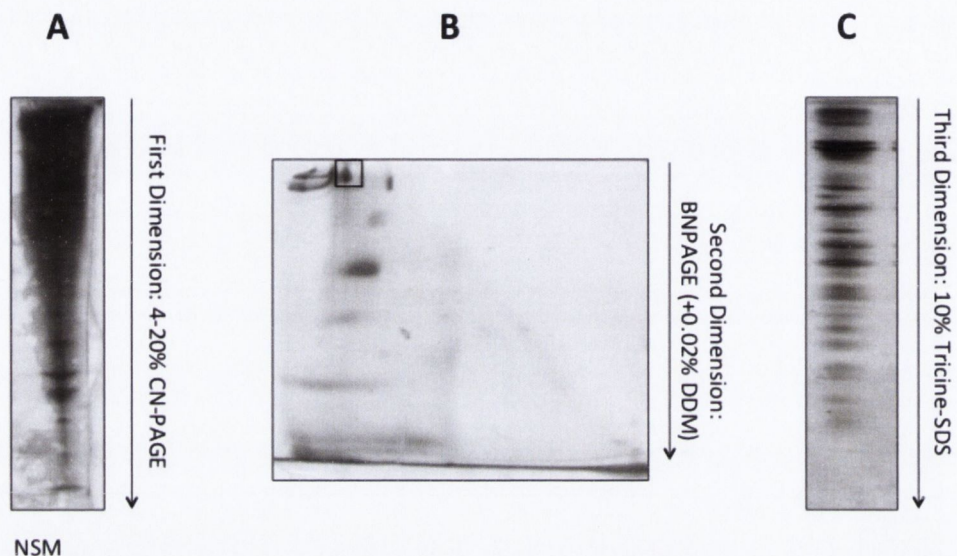


Figure 3.7 Individual subunits of complex I in non-synaptic mitochondria (NSM) can be isolated using three dimensional gel electrophoresis separation. (A) NSM were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and separated on a 4-20% CN-PAGE with an extended 3% sample gel. **(B)** Following electrophoresis, a complete lane from the first dimension CN-PAGE was excised and further resolved by a modified 2D-BN PAGE (1 mm gel, 4.8-12% gradient gel). 0.02% DDM was added to the cathode buffer in the second dimension. Individual complexes of the oxidative phosphorylation system are detected as blue spots during this BN-PAGE stage. Assignment of complexes was possible after identification by Tricine-SDS-PAGE in the third dimension. The boxed spot represents complex I. This was excised and the individual subunits resolved by three-dimensional electrophoresis **(C)**.

3.4.4 Supramolecular assemblies of NSM AND SM are preserved following solubilisation with DDM.

DDM when used to solubilize mitochondrial membranes, results in separation of individual complexes rather than retaining supercomplexes. However, when used in lower amounts it is able to preserve some supercomplex organization (Schagger, 2006, McKenzie et al., 2006). In this study, use of low DDM:protein ratio (1:1(w/w)) resulted in efficient isolation of I+III₂+IV₁ and I+III₂+IV₂ supramolecular assemblies (Figure 3.9). The supramolecular assemblies that were retained during 1D BN-PAGE are dissociated into the individual complexes by applying a modified BN-PAGE for the second native dimension (Figure 3.10). To dissociate the supercomplexes into their constituent complexes, the cathode for the second dimension BN-PAGE was supplemented with 0.2% DDM. This modified 2D BN-PAGE indicated the existence of homodimeric supercomplexes like dimeric complex III and complex V. The 2D modified BN-PAGE released monomeric IV and dimeric III and complex I from bands identified as supercomplexes in the first dimension. This confirmed the identity of the interacting partners and subcomponents within the supramolecular assemblies. Knowing the apparent molecular masses of these supercomplexes, it was determined that these could be assigned I+III₂+IV₁ and I+III₂+IV₂.

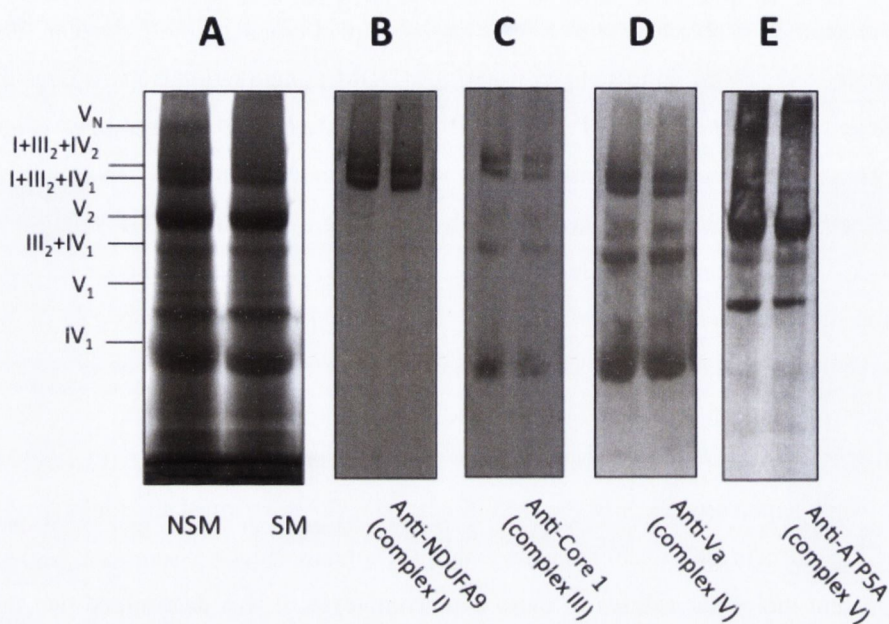


Figure 3.9 Supramolecular assemblies of NSM and SM are preserved following solubilisation with DDM. (A) Representative Serva Blue G stained 1 D BN-PAGE. NSM and SM were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and separated on a 1D 4-8% BN-PAGE. (B) An identical gel was transferred onto PVDF and submitted to immunoblotting. Mitochondrial complexes were detected using mouse monoclonal antibodies against the following subunits: NDUF9 of complex I, core 1 of complex III, Cox Va of complex IV and the α subunit of complex V. The Serva Blue G-stained bands in the ID were then identified on the basis of their protein subunit composition as assessed by the corresponding spots in the immunoblot. Two supercomplex assemblies were identified and were designated S₁ (I+III₂+IV₁) and S₂ (I+III₂+IV₂). A smaller supercomplex assembly of III₂+IV₁ was also identified.

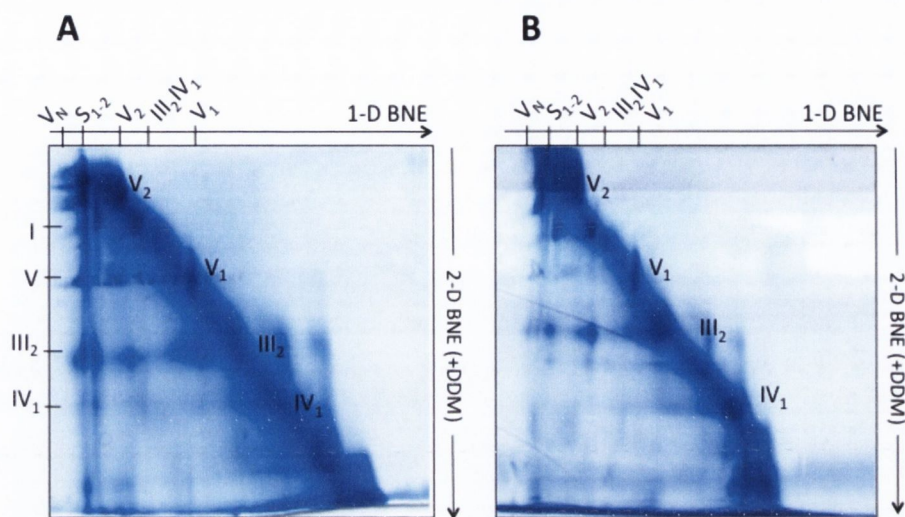


Figure 3.10 Supramolecular assemblies of oxidative phosphorylation complexes are separated into their constituent individual complexes by 2D BN/BN PAGE. Representative Coomassie Stained 2D BN-PAGE obtained with (A) NSM and (B) SM. NSM and SM were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and resolved on 1D BN-PAGE. Homomeric and heteromeric assemblies were further dissociated into their individual complexes by applying the 1D BN-PAGE lane to a second native modified BN-PAGE (0.02% DDM added to cathode buffer). It can be concluded that there is direct interaction of complexes I, III and IV yielding the respiratory supercomplexes denoted S₁ and S₂. The SBG stain was not sensitive enough to stain all proteins and silver staining of the gels was required to stain Complex IV spots.

3.4.5 NSM and SM show no difference in their subunit composition following treatment with DDM.

Protein complexes isolated by 1D BN-PAGE can be separated into their individual subunits using second dimension Tricine SDS-PAGE (Figure 3.11). All protein spots situated vertically below each other are potential subunits of one protein complex. Spots located side by side in a horizontal row potentially represent the same protein localized in a protein complex of different molecular mass. Monomeric complex IV with an apparent mass of 200 kDa was identified by its Cox I and Cox 2 subunits. Complex III dimer with an apparent mass of 500 kDa is known as a structural and functional dimer and was identified by representative Core 1 and Core 2 subunits. The identity of Core 1 was confirmed by transferring the second dimensional gel onto PVDF membrane and probed using an antibody specific for that subunit (results not shown). Due to the apparent molecular mass and representative α and β subunits, Complex V dimer was identified. The characteristic 75 kDa subunit of complex I can be observed faintly under SBG coomassie stain. Supercomplexes built from complexes I, III and IV are identified to the left of dimeric Complex V and are assigned S_{1-2} . The staining intensity of complex III and IV subunits is poor and difficult to identify but their presence has been indicated by 1D BN-PAGE. There is no difference in the subunit composition in NSM and SM. This would suggest that it is their assembly into supercomplexes that differ.

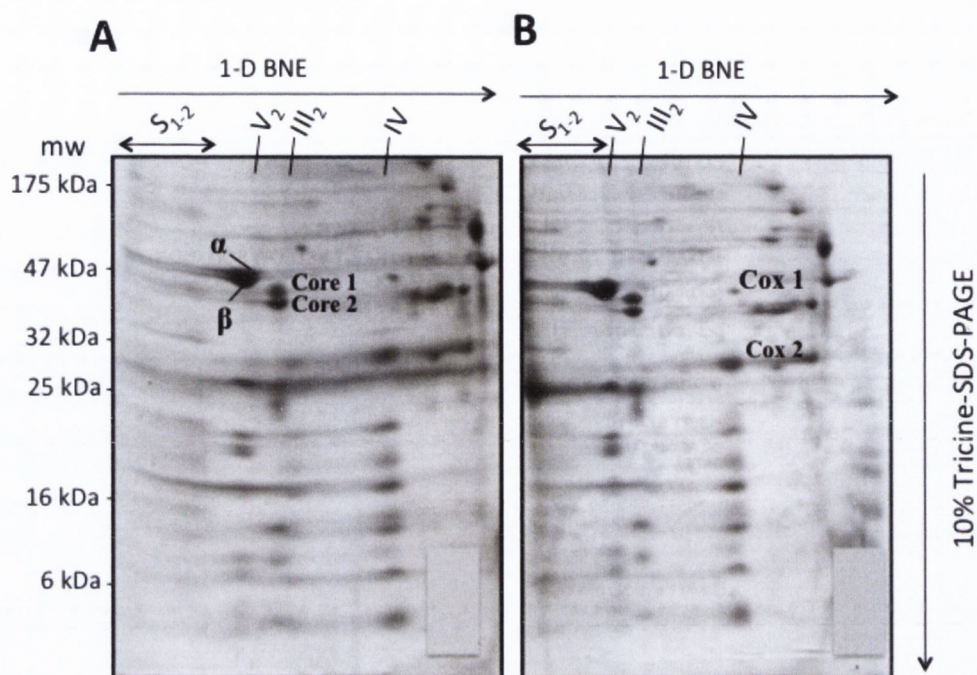


Figure 3.11 NSM and SM show no difference in their subunit composition following treatment with DDM. Representative second dimension Tricine-SDS-PAGE and the polypeptide pattern from (A) NSM and (B) SM. Mitochondria isolated from rat brain were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and individual and supercomplexes resolved on 1D BN-PAGE. The in-gel native proteins were denatured by incubating the first dimension lane in 20 mM dithiothreitol /1% SDS and then resolved using 10% Tricine SDS gel in the second dimension. The positions of Complexes I, V, III, IV and II are indicated at the top of the gels. The characteristic α and β bands of complex V are easily recognizable. Western blotting of an identical gel and immunodetection with Core 1 antibody allowed the identification of the complex III subunit and hence the row of polypeptides representing complex III. Knowing the location of these subunits allowed the identification of complex IV subunits, Cox 1 and Cox 2. Supercomplexes I+III₂+IV₁ and I+III₂+IV₂ are denoted S₁ and S₂ respectively.

3.4.6 Supercomplex assemblies of NSM and SM are preserved following solubilisation with the detergent digitonin.

Digitonin has been found to be very effective in preserving the supra-molecular associations intact and has been used successfully to extract a variety of stable supercomplexes namely the I+III₂, I+III₂+IV₁₋₄, V₂, V₃₋₄ (Schagger et al., 2004). Figure 3.12 shows the separation pattern of proteins by BN-PAGE of digitonin-solubilized NSM and SM. Western blotting using antibodies specific for each complex, revealed the presence of individual complexes and supramolecular assemblies. Three supercomplex assemblies containing complex I were identified. As with DDM, I+III₂+IV₁ and I+III₂+IV₂ were observed but digitonin preserved an I+III₂+IV₃ and I+III₂+IV₄ assembly. These complex I-containing supercomplexes appear to be in greater abundance in NSM when compared to SM.

The supramolecular assemblies retained during 1D BN-PAGE were dissociated into the individual complexes by applying a modified BN-PAGE for the second native dimension as described above. This confirmed the identity of the interacting partners and subcomponents within the supramolecular assemblies (Figure 3.13). The 2D modified BNE-PAGE released monomeric IV, dimeric III and complex I from bands identified as supercomplexes in the first dimension. Knowing the apparent molecular masses of these supercomplexes, it was determined that these could be assigned I+III₂+IV₁, I+III₂+IV₂, I+III₂+IV₃ and I+III₂+IV₄ (S₁₋₄).

Protein complexes isolated by 1D BN-PAGE can be separated into their individual subunits using second dimension Tricine SDS-PAGE (Figure 3.14). Special representative subunits were used to identify complex V (identified by α and β subunits), Complex III (Core I and 2) and Complex IV (identified by Cox I and Cox 2). Supercomplexes built from complexes I, III and IV were identified to the left of dimeric Complex V and are assigned S₁₋₄. The staining intensity of complex III and IV subunits is poor and difficult to identify but their presence has been indicated by 1D-BN-PAGE (Figure 3.10). There is no difference in the subunit composition in NSM and SM again suggesting that it is their assembly into supercomplexes that differ.

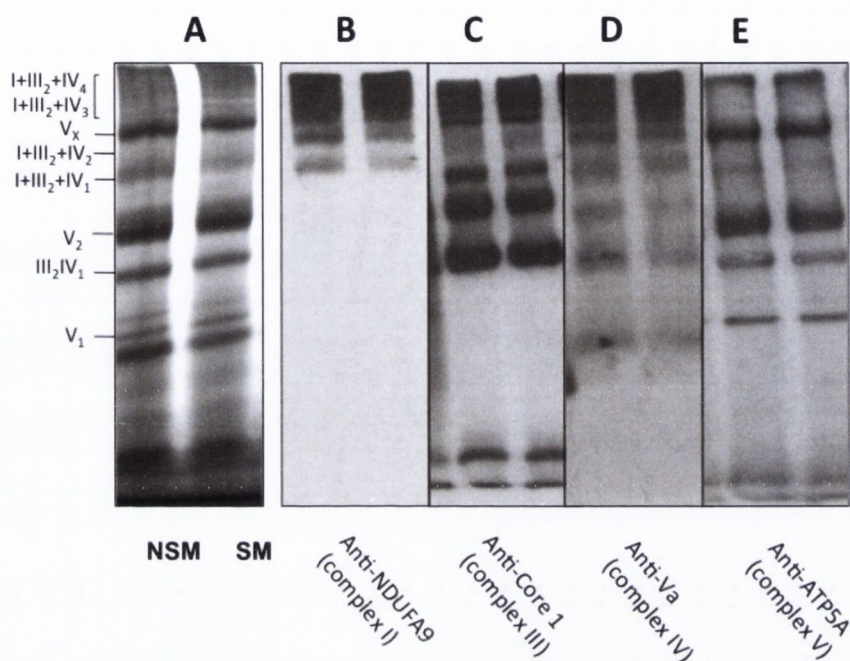


Figure 3.12 Complex I-containing supercomplexes are present in greater abundance in NSM compared to SM. (A) Representative Serva Blue G stained 1 D BN-PAGE. Greater preservation of supramolecular assemblies of NSM and SM was achieved following solubilisation with digitonin. NSM and SM were solubilized with a digitonin-to-protein ratio of 1:1 (w/w) and separated on a 1D BN-PAGE. **(B)** An identical gel was transferred onto PVDF and submitted to immunoblotting. Mitochondrial complexes were detected using mouse monoclonal antibodies against the following subunits: NDUFA9 of complex I, core 1 of complex III, Cox Va of complex IV and the alpha subunit of complex V. The Serva Blue G-stained bands in the ID were then identified on the basis of their protein subunit composition as assessed by the corresponding spots in the immunoblot. S₁ to S₄ indicate different mitochondrial supercomplex assemblies comprised of complex I, dimeric complex III and varying units of complex IV. A smaller supercomplex assembly of III₂+IV₁ was also identified.

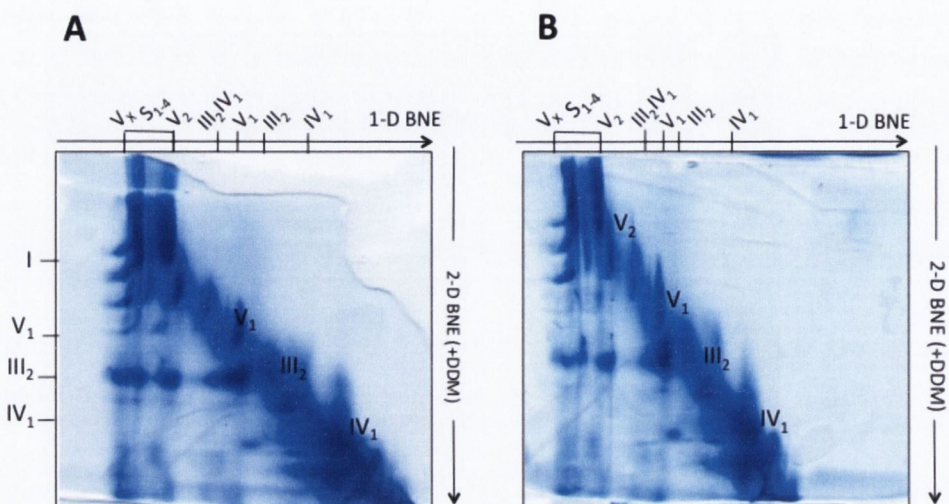


Figure 3.13 Supramolecular assemblies of oxidative phosphorylation complexes are separated into their constituent individual complexes by 2D BN/BN PAGE. (A) Representative Coomassie Stained 2D BN-PAGE obtained with NSM. (B) Representative Coomassie Stained 2D BN-PAGE obtained with SM. NSM and SM were solubilized with a digitonin-to-protein ratio of 10:1 (w/w) and resolved on 1D BN-PAGE. Homomeric and heteromeric assemblies were further dissociated into their individual complexes by applying the 1D BN-PAGE lane to a second native modified BN-PAGE (0.02% DDM added to cathode buffer). It can be concluded that there is direct interaction of complexes I, III and IV yielding the respiratory supercomplexes denoted S₁₋₄. The SBG stain was not sensitive enough to stain all proteins and silver staining of the gels was required to stain complex IV spot.

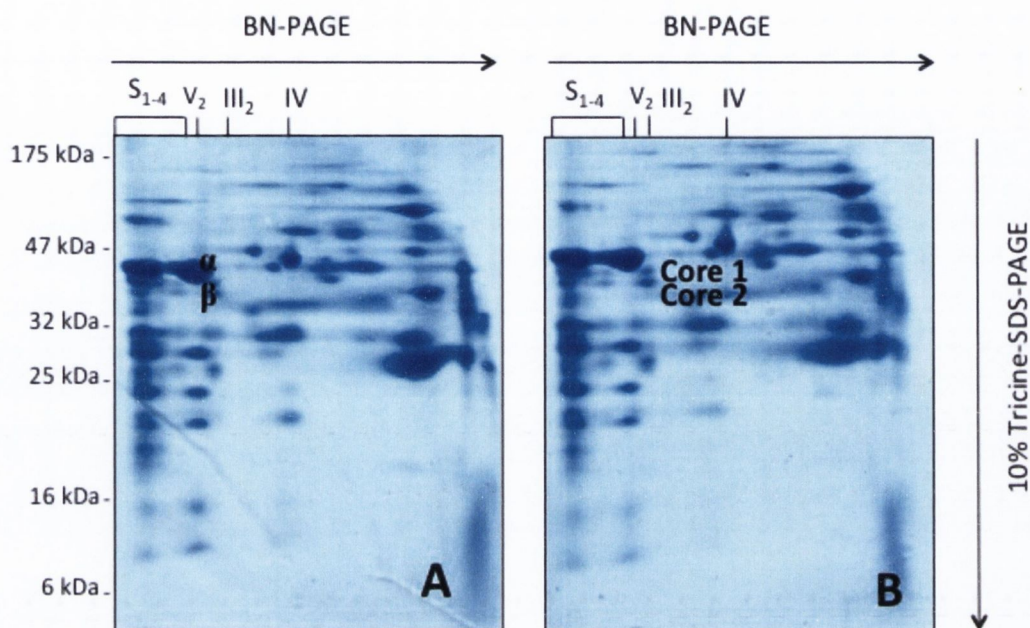


Figure 3.14 NSM and SM show no difference in their subunit composition following treatment with digitonin. (A) Representative second dimension Tricine SDS PAGE and constituent polypeptide pattern from NSM. **(B)** Representative second dimension Tricine-SDS-PAGE and constituent polypeptide pattern from SM. NSM and SM mitochondria isolated from rat brain were solubilized with a digitonin-to-protein ratio of 1:1 (w/w) and the individual and supercomplexes resolved on 1D BN-PAGE. The in-gel native proteins were denatured by incubating the first dimension lane in 20 mM DDT/1% SDS and then resolved 10% Tricine-SDS-PAGE in the second dimension. The positions of complexes I, V, III, IV and II are indicated at the top of the gels. The characteristic α and β bands of complex V are easily recognizable. Western blotting of an identical gel and immunodetection with Core 1 antibody allowed the identification of the complex III subunit and hence the row of polypeptides representing complex III. Knowing the location of these subunits allowed the identification of complex IV subunits, Cox 1 and Cox 2. S_1 to S_4 indicate different mitochondrial supercomplex assemblies comprised of complex I, dimeric complex III and varying units of complex IV.

3.4.7 High resolution Clear Native Page preserves supercomplex assemblies

To further consolidate evidence that Complex-I containing supercomplexes are present in NSM and SM, high resolution CN-PAGE (hrCNE) was performed on the purified samples. hrCNE was developed to preserve the advantages of high resolution of BN-PAGE but the proteins separated in clear gels. hrCNE is a charge shift similar to BN-PAGE. Using the non-coloured anionic micelles instead of the SBG dye preserves the advantages of CN-PAGE for in-gel enzyme activity assays. There are three variants, (hrCNE 1,2 and 3) differing in the composition of the mixed anionic detergent micelles, which influence and determine the mildness of the electrophoretic system (Wittig et al., 2007). hrCNE-3 is the mildest of the three and so was chosen to study the assembly of supermolecular assemblies in NSM and SM. The anode buffer remained the same as in BN-PAGE and the cathode buffer contained no SBG but 0.01% neutral DDM and 0.05% anionic deoxycholate (DOC). Figure 3.15 A shows the separation pattern of proteins by hrCNE-3 of digitonin solubilized NSM and SM. As observed in BN-PAGE, western blotting for complex I and III subunits, confirmed the presence of complex I-containing supercomplexes in brain mitochondria (Figure 3.15 B). In-gel activity staining was used to confirm the identity of complex I containing bands (Figure 3.15 C). An in-gel complex III assay has been developed which allows the observation of complex III activity in the high-resolution clear native gel that is not possible in BN-PAGE (Wittig et al., 2007). No apparent difference in the intensity of complex III activity staining between the two mitochondria populations (Figure 3.15 D).

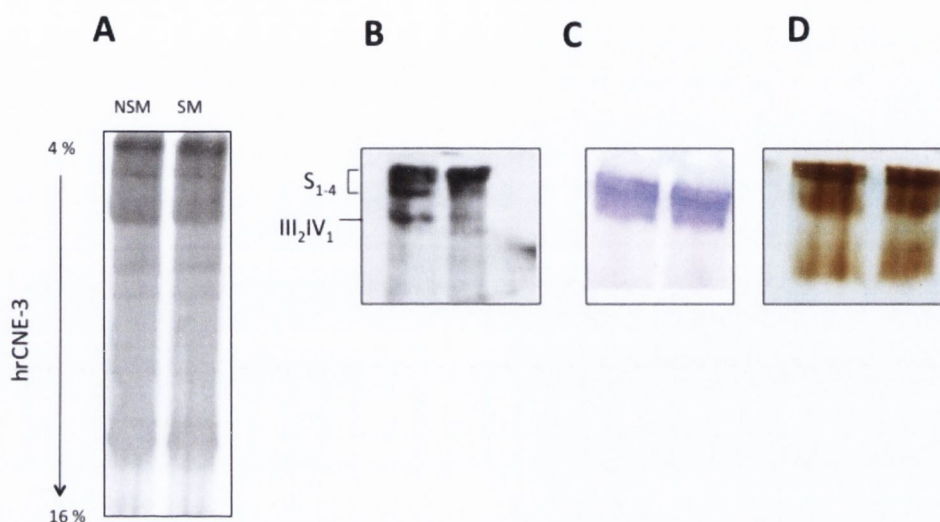


Figure 3.15 Complex I-containing supercomplexes are identified in NSM and SM using hrCNE-3. (A) Representative Serva Blue G stained 1D hrCNE-3 PAGE. NSM and SM were solubilized with a digitonin-to-protein ratio of 1:1 (w/w) and separated on a 1D 4-8% CN-PAGE. Mixed anionic micelles from neutral detergent DDM (0.01%) and anionic detergent DOC (0.05%) was added to the CNE cathode buffer to induce a negative charge shift on proteins. (B) An identical gel was transferred onto PVDF and submitted to immunoblotting with monoclonal mouse antibodies specific for single subunits of complex I and complex III. (C) In-gel catalytic and histochemical assay for complex I. (D) In-gel catalytic staining and histochemical assay for complex III.

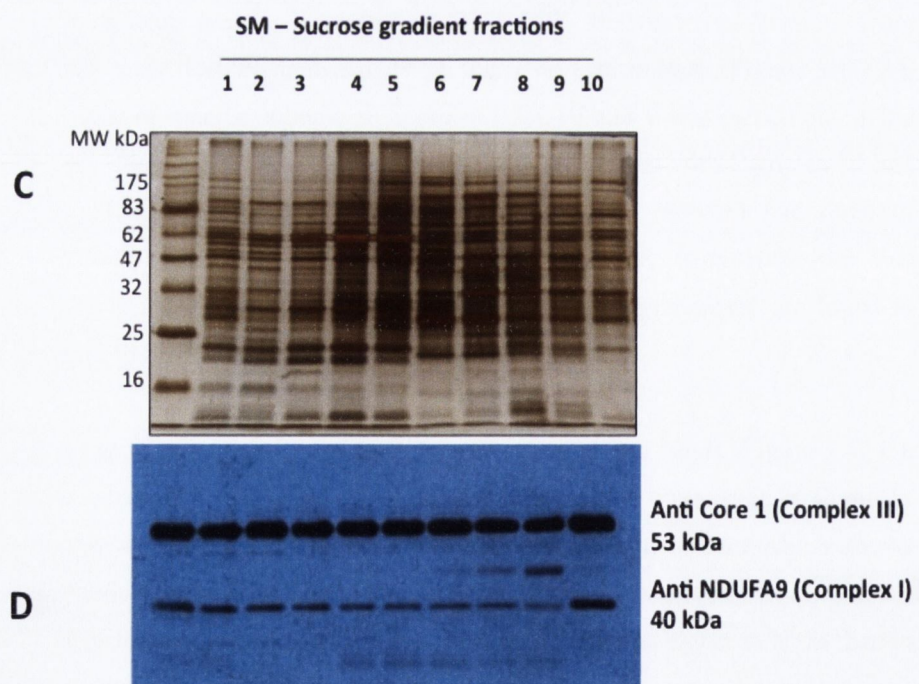
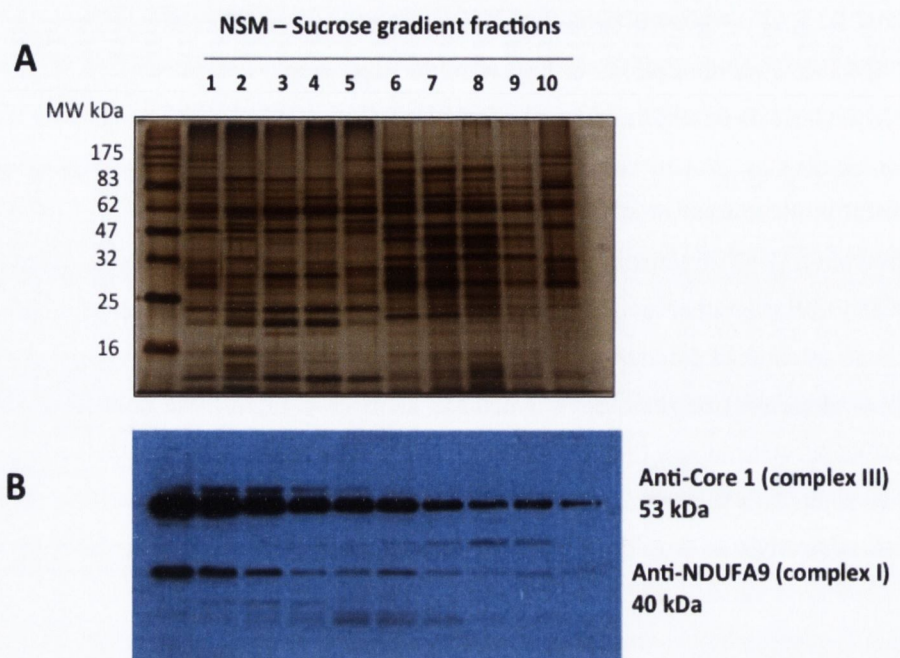
3.4.8 Mitochondrial supercomplexes are not electrophoretic artifacts.

To confirm these supercomplexes are not electrophoretic artifacts, and are in fact pre existing structures, isolated NSM and SM were solubilized by digitonin and the protein complexes and supercomplexes resolved by sucrose gradient ultracentrifugation. Subsequently, fractions were removed from the gradient from bottom to top. Ten fractions were obtained from high molecular weight (fraction 1) to low molecular weight (fraction 10) and the components of each fraction resolved on a 10% SDS-PAGE (Figure 3.16 A, C). An identical SDS-PAGE was transferred to PVDF membrane and probed with anti-NDUFA9 (complex I) and anti-Core 1 (complex III). Figure 3.16 B shows that in NSM, most of complex I and III was present in the first few fractions. These bottom fractions represent proteins of higher molecular weight which would suggests that complex I and III are organized into supercomplexes of high molecular weight and hence settle at the bottom of the sucrose gradient. This was not observed in SM as see in Figure 3.14 D. Here, there was no obvious enrichment of complex I and complex III in the first few fractions. Instead there was equal distribution throughout all the ten fractions.

Since supercomplexes are best resolved on BN-PAGE, the first five fractions from NSM and SM were further analyzed using this native technique (Figure 3.17 A). Immunodetection of specific subunits of complex I, III, IV and V was carried out on the first dimension. Figure 3.17 B, C shows that complex I migrated with Complex III and Complex IV. This is agreement with other studies which reported that most of mammalian complex I exist in a supercomplex assembly. Comparing the two mitochondria populations, greater complex I-containing supercomplexes are found in NSM compared to SM.

Western blotting for complex III and Complex IV revealed five bands (Figure 3.17 C). These bands correspond to III_2+IV_{1-4} . Again there was greater abundance of Complex III and IV containing supercomplexes in NSM. Western blotting with complex V shows that it mostly exists in the dimeric and oligomeric state. It was observed that the amount of complex V present in dimeric and oligomeric associations was higher in NSM than in SM.

OVERLEAF: Figure 3.16 Greater organisation of complex I and complex III in NSM into supercomplexes of higher molecular weight compared to SM. NSM and SM isolated from rat brain were solubilized with a digitonin-to-protein ratio of 1:1 (w/w) and the protein complexes resolved by sucrose gradient centrifugation. SDS-PAGE with silver staining was carried out to analyze the protein content of the NSM (A) and SM (C) fractions. Identical NSM (B) and SM (D) gels were transferred onto PVDF and submitted to immunoblotting with monoclonal mouse antibodies specific for single subunits of the individual electron transport complexes. Mitochondrial complexes were detected using mouse monoclonal antibodies against the following subunits: NDUFA9 of complex I, core 1 of complex III. This shows the relative quantity of the two complexes present together in the different fractions isolated from NSM and SM.



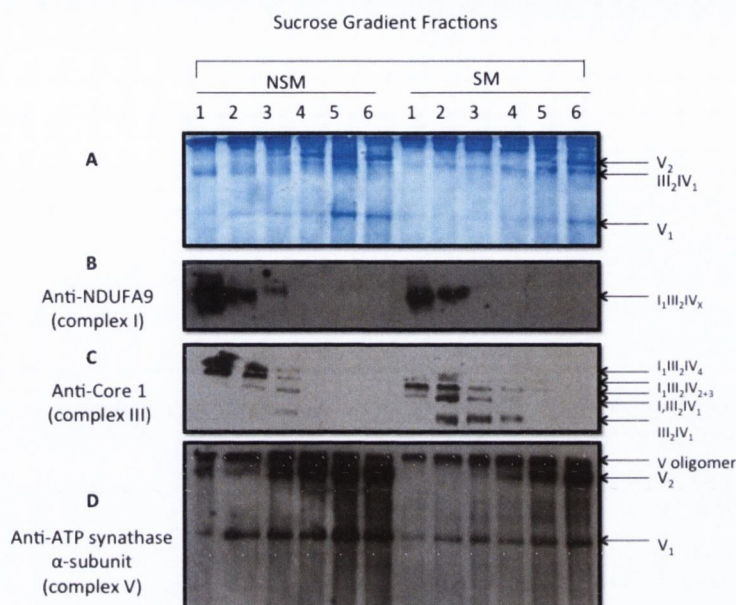


Figure 3.17 Blue Native gel electrophoresis of sucrose gradient fractions confirms higher abundance of supercomplexes in NSM compared to SM. (A) SBG staining of the sucrose gradient fractions (1-6) obtained from digitonin-treated rat brain mitochondria of non-synaptic and synaptic origin on a 1D BN-PAGE. (B) Western blot analysis using anti-NDUFA9 antibody reveals supercomplexes containing complex I. (C) Western blot analysis using anti-core 1 reveals supercomplexes containing complex III. (D) Western blot analysis using anti-subunit- α reveal monomer, dimer and oligomer assemblies of complex V. NSM and SM isolated from rat brain were solubilized by digitonin (10:1 ratio) and the protein complexes were resolved by sucrose gradient centrifugation. 1D BN-PAGE was carried out to analyze the supercomplexes in the different fractions. Identities of the complexes on the 1D gel were elucidated by western blotting with antibodies as indicated. This shows the relative quantity of the complexes present together in supercomplex assemblies in the different fractions isolated from NSM and SM.

3.4.9 Identification of proteins that interact with physiological supramolecular assemblies proteins in NSM and SM.

To further confirm the *in vivo* interactions of the respiratory complexes, individual enzymes of the electron transport chain were immunocaptured from extract derived from NSM and SM. An antibody to one of the component subunits of each electron transport chain complex, with the exception of complex II, was used as the bait. As Complex II had not been shown to associate in supermolecular assemblies, this enzyme was omitted from immunocapture studies. The complex and its associated *in vivo* partners were then pulled-down. The immuno-precipitates were resolved on a SDS-PAGE and transferred onto PVDF and probed for complex I and complex III. The top left panel in Figure 3.18 shows immunocapture with Complex I and immune detection with anti-NDUFA9, a Complex I subunit. The 2nd bottom panel shows immunocapture with complex III and immunodetection with Core 1, a complex III antibody. Staining intensities in the 2nd upper and 1st lower panels represent complex I and complex III interactions within NSM and SM. Complexes IV and V were immunocaptured and the level of complex I and complex III that pulls down along with them assayed by immune detection with an anti NDUFA9 and core 1, respectively (3rd, 4th upper and lower panels in Figure 3.16).

To elucidate *in vivo* interactors of complex I in detail, the protein was immunocaptured using an immunocapture resin available from Mitosciences. The resin has polyclonal antibodies against the complex I holoenzyme (~45 subunits) adsorbed on its surface. It was expected that the whole enzyme along with its supercomplex partners and other unknown partners would immunoprecipitate. Many subunits of all the five ETC complexes were brought down by the immunocapture (Table 3.8). The table shows that the score and the percentage of sequence covered of the tryptic peptides detected by LC-MS/MS were comparable amongst NSM and SM. This implies that, following the immunocapture procedure, the supercomplex assemblies were similar in composition in the two type of mitochondria. The only difference between the ETC interactors of complex I in NSM and SM is in the complex IV subunit V (Cox-V), GBAS and Piccolo. Cox-V is a nuclear coded subunit and has two isoforms Va and Vb. In this study, Cox-Va isoform is present in NSM as well as SM, but Cox-Vb isoform was present only in NSM. In addition to a NSM specific interactor, the study identified two unique interactors of complex I in SM: GBAS and Piccolo.

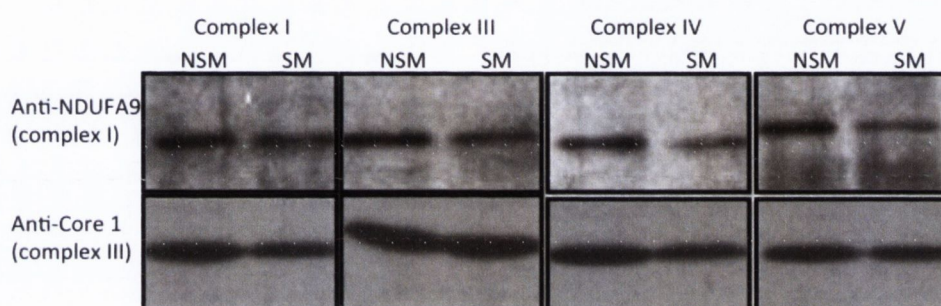


Figure 3.18 Co-immunoprecipitation reveal in vivo interactions of the respiratory complexes and show that complex I, complex III, complex IV and complex V exist in conjunction with each other. Precleared extract obtained by digitonin treatment of NSM and SM were immunocaptured with 10 μ g monoclonal anti-NDUFA9 (complex I), anti-Core I (complex III), anti-Subunit Va (complex IV) or anti-ATP synthase subunit- ∞ (complex V) respectively. The immunocaptured protein complexes were resolved on a 10% SDS-PAGE and silver stained. An identical gel was transferred onto PVDF and submitted to immunoblotting with monoclonal mouse antibodies specific for single subunits of the individual electron transport complexes; NDUFA9 of complex I and core 1 of complex III. The upper panels show immunocapture with complex I, III, IV and V and immune detected with an antibody against NDUFA9, a complex I subunit. The lower panels show immunocapture with complex I, III, IV and V and immune detected with an antibody against Core 1, a complex III subunit. The complexes immunocaptured together were then identified. This pattern is representative expression found from three separate isolations.

The protein extraction protocol is mild enough to preserve relatively weak protein-protein interactions and allow the co-purification of several non-ETC mitochondrial protein along with complex I. Most prominent amongst them are the components of the mitochondrial permeability transition pore (MPTP) complex. All the three mammalian voltage-dependent anion-selective channel proteins (VDAC1, VDAC2, VDAC3), the two adenine nucleotide translocator proteins (ADT1 and ADT2) and the phosphate carrier protein (PiC) were found to be present as interactors of complex I (Table 3.8).

In the mitochondria the TCA cycle is always followed by oxidative phosphorylation. So it was no surprise that several enzymes for pyruvate oxidation (PDH complex) and TCA cycle (Aconitase, Isocitrate dehydrogenase, Malate dehydrogenase, 2-oxoglutarate dehydrogenase and Succinyl CoA ligase) physically interact with the complex I. This is an important observation as it would lead to easy substrate availability for subsequent processes and can even control the two processes by feedback mechanism. Physical proximity of these enzymes to ETC-supercomplexes translates into easy availability of NAD^+ , NADP and ATP for regulation of this very tightly regulated step of TCA cycle.

Other proteins identified that co-immunoprecipitate with complex I include Prohibitins, Hsp60, Stress-70, TOM70, MPPB and Tufm (Table 3.8). Prohibitins, Hsp60 and Stress-70 act as chaperones for complex I assembly. TOM70, MPPB and Tufm as identified here as interactors of complex I by the virtue of their role in either import and processing of nuclear encoded complex I polypeptides or translation of the mtDNA encoded complex I polypeptides. TOM70 is a part of the mitochondrial import machinery. It forms part of the preprotein translocase complex of the OMM. MPPB is the mitochondrial peptidase that cleaves presequences from mitochondrial protein precursor. Mitochondrial elongation factor Tufm, is the part of a dedicated protein-translation apparatus in mitochondrial matrix that synthesizes the 13 polypeptides encoded in mammalian mitochondrial DNA.

Another class of proteins that are found to interact with complex I are the ones involved in mitochondrial tubular network formation and cristae morphology. Mitofilin is an IMM known to control mitochondrial cristae morphology. These interactions are common to NSM as well as SM, probably to derive energy to maintain the mitochondrial shape and cristae morphology.

Mitochondrial matrix enzymes involved in amino acid metabolism and lipid metabolism (aspartate aminotransferase and trifunctional enzyme), Nipsnap1, a strictly neuronal protein (involved in branched-chain amino acid metabolism), were found to be associated to complex I. The multi-pass inner mitochondrial carrier proteins (oxoglutarate/malate carrier and glutamate carrier) too were found to associate with the complex I-supercomplexes. Sideroflexins on the IMM are a family of tricarboxylate carrier protein too interact with complex I. These interactions of unknown significance were found to similar in the NSM and SM.

The other interactors are cysteine desulfurase, mitoNEET, and Cend1 (Table 3.8). Cysteine desulfurase provides sulphur that becomes incorporated in Fe-S cluster assembly. Fe-S clusters are important for *de novo* synthesis of many of the complex I subunits. The CDGSH iron-sulphur domain containing protein, mitoNEET is also an OMM protein. It has been shown to regulate mitochondrial oxidative capacity by an unknown mechanism. Its interaction with complex I supports its proposed role in mitochondrial respiration. Cend1 is a neuronal membrane integrated protein. It is anchored to the OMM, in a manner that the bulk of the protein faces the cytoplasm (Politis et al., 2007). It is associated with the molecular machinery that coordinates cell cycle exit and differentiation of neuronal precursors. It has also been implicated in inherited human ataxias (Humbert and Saudou, 2006). Association of Cend1 with complex I, is reported here for the first time. What such an interaction means is yet to be elucidated.

Table 3.8. Associated proteins on immunocaptured complex I from NSM and SM preparation.

Accession No.	Protein Name	Description	NSM			SM		
			Score	Unique peptides	% Cover	Score	Unique peptides	% Cover
Complex I subunits (EC 1.6.5.3)								
P11661	NU5M_RAT	NADH-ubiquinone oxidoreductase chain 5	0.997	2	4.6	0.999	4	8.7
B2RZD6	NDUA4_RAT	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 4	0.963	2	22	1	4	32.9
Q63362	NDUA5_RAT	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 5	1	2	17.2	1	2	17.2
A9UMV9	A9UMV9_RAT	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 7	0.999	3	25	1	4	23.2
Q5BK63	NDUA9_RAT	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 9	1	12	39.7	1	15	43.2
Q561S0	NDUAA_RAT	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 10	1	12	34.6	1	13	41.1
Q2XTA8	Q2XTA8_RAT	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4	1	4	45.7	1	3	39.8
B2RYS8	B2RYS8_RAT	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	1	2	14.5	0.909	1	5.9
B2RYW3	B2RYW3_RAT	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9	0.999	3	23.3	0.999	3	23.5
Q5PQZ9	Q5PQZ9_RAT	Putative uncharacterized protein Ndurf2	1	3	25.0	1	3	27.5
Q66HF1	NDUS1_RAT	NADH-ubiquinone oxidoreductase 75 kda subunit	1	19	35.2	1	19	34.1
Q64IY2	NDUS2_RAT	NADH dehydrogenase (Ubiquinone) Fe-S protein 2	1	12	35.9	1	10	26.3

Accession No.	Protein Name	Description	NSM			SM		
			Score	Unique peptides	% Cover	Score	Unique peptides	% Cover
Q5XIF3	NDUS4_RAT	NADH dehydrogenase [ubiquinone] Fe-S protein 4	0.999	2	19.4	0.888	2	11.4
B5DEL8	B5DEL8_RAT	NADH dehydrogenase (Ubiquinone) Fe-S protein 5	1	4	35.8	1	3	32.1
Q5RJN0	Q5RJN0_RAT	NADH dehydrogenase (Ubiquinone) Fe-S protein 7	1	6	30.7	1	7	24.3
B0BNE6	B0BNE6_RAT	NADH dehydrogenase (ubiquinone) Fe-S protein 8	1	5	17.0	1	7	24.1
Q5XIH3	Q5XIH3_RAT	NADH dehydrogenase (Ubiquinone) flavoprotein 1	1	11	21.3	1	8	16.6
P19234	NDUV2_RAT	NADH dehydrogenase [ubiquinone] flavoprotein 2	1	4	18.1	1	6	31.5
<i>Complex II subunits (EC 1.3.5.1)</i>								
Q920L2	DHSA_RAT	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	1	8	16.3	1	6	15.4
P21913	DHSB_RAT	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	1	5	20.9	1	4	10.6
<i>Complex III subunits (EC 1.10.2.2)</i>								
Q68FY0	QCR1_RAT	Cytochrome b-c1 complex subunit 1	1	10	29.8	1	15	29.4
P32551	QCR2_RAT	Cytochrome b-c1 complex subunit 2	1	14	42.3	1	16	42.7
P20788	UCR1_RAT	Cytochrome b-c1 complex subunit rieske	1	9	37.6	1	12	37.6
Q7TQ16	QCR8_RAT	Cytochrome b-c1 complex subunit 8	0.999	2	15.9	0.985	1	15.9
B2RYS2	B2RYS2_RAT	Ubiquinol-cytochrome c reductase binding protein	1	7	53.2	1	6	39.6
<i>Complex IV subunits (EC 1.9.3.1)</i>								
Q37652	COX2_RAT	Cytochrome c oxidase subunit 2	1	4	19.4	1	5	19.4
P10888	COX4I_RAT	Cytochrome c oxidase subunit 4 isoform 1	1	7	52.7	1	8	36.7
P11240	COX5A_RAT	Cytochrome c oxidase subunit 5a	1	5	71.2	1	7	47.3
P12075	COX5B_RAT	Cytochrome c oxidase subunit 5b	1	4	47.3	-	-	-

Accession No.	Protein Name	Description	NSM			SM		
			Score	Unique peptides	% Cover	Score	Unique peptides	% Cover
Complex V subunits (EC 3.6.3.14)								
P11951	CX6C2_RAT	Cytochrome c oxidase polypeptide vic-2	0.995	2	18.4	0.995	2	18.4
Complex V subunits (EC 3.6.3.14)								
P15999	ATPA_RAT	ATP synthase subunit alpha	1	35	61.7	1	32	57.3
P10719	ATPB_RAT	ATP synthase subunit beta	1	25	64.1	1	25	52.2
P35435	ATPG_RAT	ATP synthase gamma chain	1	9	34.8	1	7	24.5
P35434	ATPD_RAT	ATP synthase delta chain	1	2	13.7	0.999	3	25.0
P19511	AT5F1_RAT	ATP synthase b chain	1	6	25.0	1	6	27.7
P31399	ATP5H_RAT	ATP synthase d chain	1	4	24.2	1	6	41.0
P29419	ATP5I_RAT	ATP synthase e chain	1	3	36.6	1	4	46.5
Q06647	ATPO_RAT	ATP synthase subunit o	1	13	60.1	1	6	36.6
PDH Complex subunits (EC 1.2.4.1)								
P08461	ODP2_RAT	Dihydrolipoyllysine-residue acetyltransferase (PDH complex)	1	3	15.8	1	5	16.1
P26284	ODPA_RAT	Pyruvate dehydrogenase E1 component subunit alpha	1	5	22.3	1	5	24.4
P49432	ODPB_RAT	Pyruvate dehydrogenase E1 component subunit beta	0.999	2	10.9	1	3	7.8
Mitochondrial outer membrane proteins								
P05708	HXK1_RAT	Hexokinase-1 (EC 2.7.1.1)	1	24	26.9	1	28	26.8
B0K020	CISD1_RAT	CDGSH iron-sulphur domain containing protein mitoneet	1	3	50.9	0.988	1	13.9
Q75Q39	TOM70_RAT	Translocase of outer membrane TOM70	1	3	7.2	1	3	7.2
Q9Z2L0	VDAC1_RAT	Voltage-dependent anion-selective channel protein 1	1	14	51.9	1	14	47.7

Accession No.	Protein Name	Description	NSM			SM		
			Score	Unique peptides	% Cover	Score	Unique peptides	% Cover
Mitochondrial outer membrane proteins								
P81155	VDAC2_RAT	Voltage-dependent anion-selective channel protein 2	1	9	31.9	1	9	36.6
Q9R1Z0	VDAC3_RAT	Voltage-dependent anion-selective channel protein 3	1	5	17.7	1	5	23.7
Mitochondrial inner membrane proteins								
Q6P9Y4	ADT1_RAT	ADP/ATP translocase 1 (Adenine nucleotide translocator 1)	1	18	40.9	1	11	39.9
Q09073	ADT2_RAT	ADP/ATP translocase 2 (Adenine nucleotide translocator 2)	1	14	38.6	1	12	36.2
Q505J6	GHC2_RAT	Glutamate carrier	0.999	2	9.2	1	4	23.1
Q3KR86	Q3KR86_RAT	Inner membrane protein mitochondrial, Mitofilin	1	16	31.2	1	13	20.7
Q5XIN6	LETM1_RAT	Leucine zipper-EF-hand-containing transmembrane protein 1	1	3	7.4	1	4	9.9
Q5EBA4	Q5EBA4_RAT	Nipsnap 1	1	4	20.1	0.995	2	14.1
Q2TA68	OPA1_RAT	Optic atrophy protein 1	0.997	4	6.8	1	2	4.0
P97700	M2OM_RAT	2-oxoglutarate/malate carrier protein (OGCP)	1	4	15.9	1	4	14.3
P16036	MPCP_RAT	Phosphate carrier protein (PTP)	1	7	19.4	1	7	16.6
P67779	PHB_RAT	Prohibitin (B-cell receptor-associated protein 32)	1	8	36.0	0.999	4	16.5
Q5XIH7	PHB2_RAT	Prohibitin-2 (B-cell receptor-associated protein 37)	1	9	31.8	1	10	42.1
Q63965	SFXN1_RAT	Sideroflexin-1	0.992	3	13.0	0.999	5	19.6
Q9JHY2	SFXN3_RAT	Sideroflexin-3	1	5	19.9	1	10	36.8
Q8CFD0	SFXN5_RAT	Sideroflexin-5	1	2	9.4	1	5	17.3

Accession No.	Protein Name	Description	NSM			SM		
			Score	Unique peptides	% Cover	Score	Unique peptides	% Cover
Mitochondrial matrix proteins								
Q9ER34	ACON_RAT	Aconitase hydratase (EC 4.2.1.3)	1	4	5.4	1	5	7.9
P00507	AATM_RAT	Aspartate aminotransferase (EC 2.6.1.1)	1	2	5.3	0.999	3	6.7
Q3MHT2	NFS1_RAT	Cysteine desulfurase (EC 2.8.1.7)	1	17	39.9	1	14	35.1
Q99NA5	IDH3A_RAT	Isocitrate dehydrogenase subunit alpha (EC 1.1.1.41)	0.990	1	3.0	1	5	16.4
P04636	MDHM_RAT	Malate dehydrogenase (EC 1.1.1.37)	1	4	16.3	1	4	18.6
Q5XI78	ODO1_RAT	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	1	3	2.6	0.996	2	1.9
B2RZ24	B2RZ24_RAT	Succinate/Malate CoA ligase, beta subunit (EC 6.2.1.5)	1	6	21.5	1	5	12.3
Q60587	ECHB_RAT	Trifunctional enzyme subunit beta (EC 2.3.1.16)	1	2	6.9	1	2	4.2
Other proteins								
Q5FVI4	CEND_RAT	Cell cycle exit and neuronal differentiation protein	1	5	56.4	1	7	63.8
P85834	EFTU_RAT	Elongation factor Tu, mitochondrial	1	8	23.7	1	9	26.1
P63039	CH60_RAT	60 kD heat shock protein (Hsp60)	1	11	27.1	0.996	4	13.1
Q03346	MPPB_RAT	Mitochondrial-processing peptidase subunit beta	0.999	2	8.2	0.976	2	4.9
Q62046	RUNX1_RAT	Runt related transcription factor 1	1	3	12.9	1	2	10.7
P48721	GRP75_RAT	Stress-70 protein	1	8	17.4	1	3	6.2
Q9Z0V6	PRDX3_RAT	Thioredoxin-dependent peroxide reductase 3 (EC 1.11.1.15)	0.986	1	4.3	0.986	1	4.3
Q64649	KPB1_RAT	Phosphorylase B kinase regulatory subunit alpha	0.999	3	2.0	-	-	-
Q5RK08	Q5RK08_RAT	Glioblastoma amplified sequence	-	-	-	1	3	9.6
Q9JKS6	PCLO_RAT	Piccolo, multidomain presynaptic cytomatrix protein	-	-	-	1	8	3

3.4.10 Enzyme activities of complex I and complex V in their supercomplex assemblies.

Previous studies and a study here (Figure 3.19) suggest increased complex I activity in NSM compared to SM (Davey et al., 1998, Kiebish et al., 2008). Two questions were considered: (i) Is complex I activity greater when assembled into supramolecular formations? (ii) Does the increase in supercomplex formation explain the increase in complex I activity found in NSM? The NADH:NTP reductase assay for complex I and its hydrophilic catalytic parts is a highly robust in-gel histochemical assay. It is not disturbed by the SBG dye used for 1D BN-PAGE. This allows the direct visualization of complex I activity when they are present in supramolecular associations (Figure 3.20 A). Interestingly, complex I present in the assembly $I+III_2+IV_1$ isolated by 1D BN-PAGE is greater in NSM as confirmed by densitometry analysis (Figure 3.20 B).

Blue Native PAGE with the sucrose gradient fractions suggests greater complex V dimer and oligomeric assemblies in NSM when compared to SM. Correlation of these assemblies with increased enzyme activity was tested.

Complex V in gel activity staining in 1-D BN-PAGE revealed several forms of complex V activity assigned to monomeric, dimeric and oligomeric states (Figure 21 A). Stained bands were quantified densitometrically and shows that greater enzyme activity present in CV dimers and oligomers identified in NSM when compared to corresponding supercomplexes in SM (Fig 3.21 B).

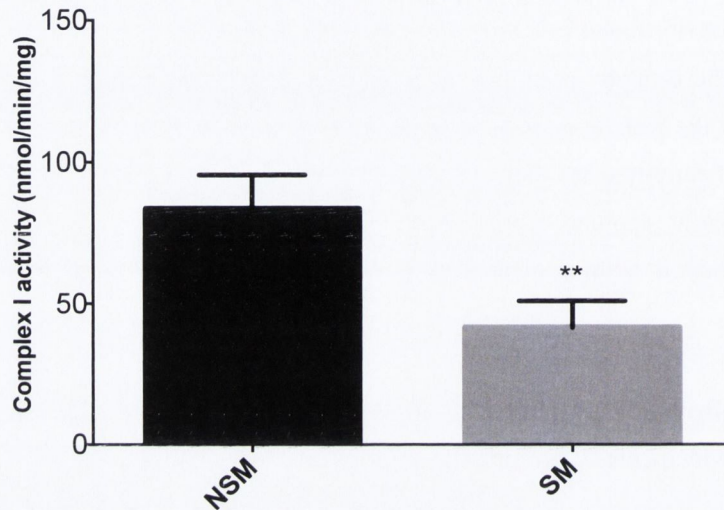


Figure 3.19 Complex I activity is greater in NSM compared to SM. The samples were frozen at - 80 °C and freeze fractured three times and examined for complex I activity in a spectrophotometer at 340 nm (Section 3.3.4.5). Experiments were performed on three individual preparations and results are expressed as mean $\pm$ SEM (error bars). Enzyme activities are expressed as nmol/min/mg protein. Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism. Samples significantly different to the control are shown with ** for $p < 0.01$.

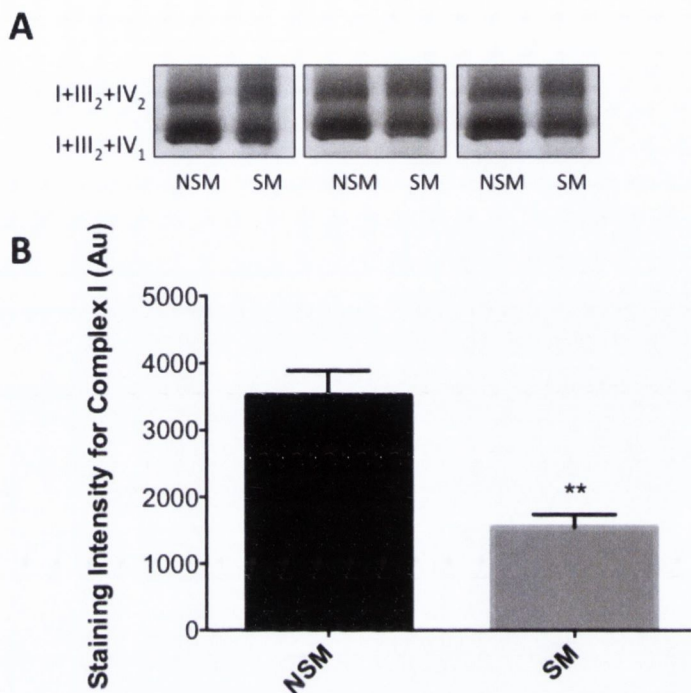


Figure 3.20 Greater complex I activity is observed in supercomplexes of non-synaptic origin compared to synaptic mitochondria. (A) Non-synaptic (NSM) and synaptic mitochondria (SM) were solubilized with a digitonin-to-protein ratio of 10:1 (w/w) and separated on a 1D 4-8% BN-PAGE. Specific staining of respiratory supercomplexes containing complex I in NSM and SM was achieved by incubating the Blue Native PAGE gel in 0.1 M Tris-HCL pH 7.4 containing 1 mg/ml nitro blue tetrazolium and 0.14 mM NADH at room temperature. (B) Densitometry in-gel quantification of complex I band intensity activity was performed using Image J (National Institutes of Health) after identification of the proteins by western blot. Experiments were performed on three individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism. Samples significantly different to the control are shown with ** for $p < 0.01$.

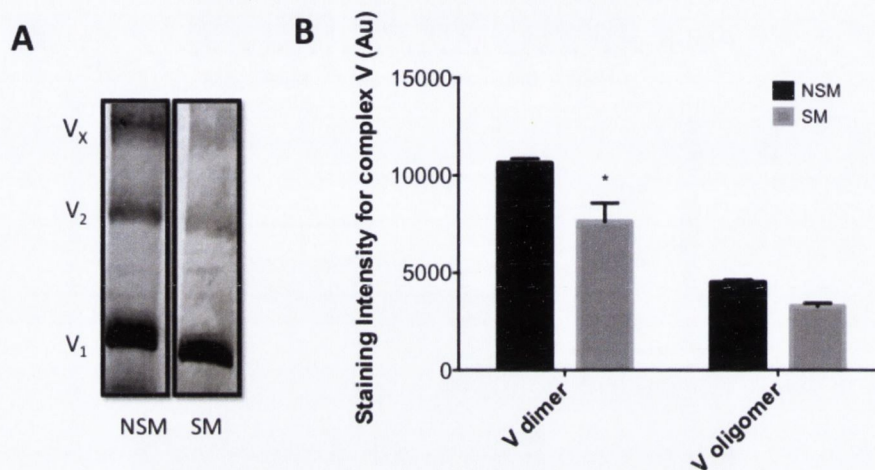


Figure 3.21 Greater complex V dimer and oligomer activity is observed in supercomplexes of non-synaptic origin compared to synaptic mitochondria. (A) NSM and SM were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and separated on a 1D 4-8% BN-PAGE. Specific staining of monomeric complex V (ATP synthase) and of dimeric and homo oligomeric supercomplexes containing complex V in NSM and SM was achieved by washing the Blue Native PAGE a couple of times in water and then incubating in reaction mix overnight before stained bands were observed. (B) Densitometry in-gel quantification of complex V band intensity activity was performed using Image J (National Institutes of Health) after identification of the proteins by western blot. Experiments were performed on three individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism. Samples significantly different to the control are shown with ** for $p < 0.01$ and * for $p < 0.05$.

3. 5 Discussion

Based on their cellular localization, mitochondria from the brain can be divided into two major populations (Lai and Clark, 1989). These include non-synaptic mitochondria (NSM), which originate from neuronal and glial cell bodies and synaptic mitochondria (SM), which originate from the synaptic boutons or terminals of neurons. These mitochondrial populations express differences in ETC activities (Kilbride et al., 2008) energy thresholds, in the lipid composition of their membranes (Kiebish et al., 2008) and also differ in their ability to handle added Ca^{2+} . SM are more susceptible to Ca^{2+} overload and when they succumb, the opening of permeability transition pore leads to swelling and eventual rupture of the organelle (Brown et al., 2006). The increased possibility of SM to undergo permeability transition may have pathological consequences, as degeneration of distal axons is one of the initial events observed in several neurodegenerative disorders. This study attempts to understand why synaptic mitochondria exhibit lower complex I energy thresholds, increased oxidative damage and are more vulnerable to calcium insult than NSM. We propose that differences in supercomplex assemblies may hold the key to the differences observed and may explain synaptic mitochondrial susceptibility to damage.

Using BN-PAGE, this present study is the first to describe the existence of macromolecular assemblies of individual ETC complexes, otherwise known as supercomplexes, in NSM and SM (Figure 3.12). No individual complex I was observed, which allows us to assume that all complex I is associated in the form of a supercomplex. This is in agreement with studies that complex I is bound to complex III dimers in physiological studies (Moreno-Lastres et al., 2012). Supercomplex assemblies of $\text{I+III}_2\text{+IV}_{1-4}$ were observed in both populations of mitochondria. However, NSM appear to contain higher amounts of complex-I containing supercomplexes, in particular I+III+IV_1 and I+III+IV_2 . These supercomplexes also had greater complex I activity compared to corresponding supercomplexes in NSM (Figure 3.20). The profile of mitochondrial proteins separated by BN-PAGE was not different between the two populations of mitochondria, so lower abundance of these supercomplex assemblies identified in SM is not caused by any deficit of constituent subunits of ETC complexes. These results suggest that the complex I-containing supercomplex assemblies are dynamic and heterogeneous in different type of brain mitochondria.

The co-immunoprecipitation study showed that the only difference between the ETC interactors of complex I in NSM and SM was in the complex IV subunit V (Cox-V). Cox-V is a nuclear coded subunit and has two isoforms Va and Vb. In this study, Cox-Va isoform is present in NSM as well as SM, but Cox-Vb isoform is present only in NSM (Table 3.8). Previous reports have shown that the isozyme with Vb has a higher turnover rate and a higher intramolecular transfer rate than the isozyme with Va (Allen et al., 1995). Subunit Vb content varies with tissue type and differs even within different cardiac compartments of the heart (Galati et al., 2009). The subunit is phosphorylated in cAMP dependent manner and highest levels of Vb is seen in tissues with higher metabolic demands (Galati et al., 2009). Hypoxia has been shown to lower the Vb protein level as well as enzymatic activity level (Fang et al., 2007). Further, genetic studies in yeast have suggested a role for the subunit in stability of complex IV (Coyne et al., 2007). A recent study by Galati et al. 2009 reports that in murine macrophages, a knockdown of Cox-Vb results in reduced association of complex IV with supercomplexes. The present study, shows for the first time that Cox-Vb subunit level is reduced in SM in parallel to reduced levels of ETC supercomplexes containing I+IV, which may account for a reduction in energy metabolism in SM.

The co-immunoprecipitation study identified only one protein, phosphorylase b kinase (KPBI) as a unique interactor of complex I in NSM (Table 3.8). KPBI is known to catalyse the phosphorylation of serine/threonine residues in certain substrates. The most prominent substrate of KPBI is glycogen phosphorylase in glycogen metabolism. The alpha chain binds calmodulin and the enzyme may be regulated by calcium as well as phosphorylation of serine residues. It is a large protein of ~140 kDa molecular weight and in the LC-MS/MS only 2% of the protein sequence is covered by the peptides sequenced. However two different peptides, with around 85% ions matched, identify the protein with confidence. Its association with NSM is interesting, where it may be involved in phosphorylation of substrates not known till now, leading to differences in energy metabolism.

In addition to a NSM specific interactor, the study identified two unique interactors of complex I in SM (Table 3.8). The glioblastoma-amplified sequence (GBAS) is a mitochondrial protein with unknown function. Although it is proposed to play a role in oxidative phosphorylation, the exact mechanism is unknown. The second protein,

Piccolo, is a large scaffold protein and a core component of cytomatrix at the active zone of synapse. It accumulates at nascent synaptic junctions and organises presynaptic neurotransmitter release. Mitochondria too are known to reside at the synapse to provide energy for exocytosis. The complex I co-immunoprecipitation in this present study indicates a direct interaction between ETC supercomplex and this cytomatrix protein with unknown consequences.

Dimeric and higher oligomeric structures of complex V have previously been identified in rat brain (Krause et al., 2005). This study is the first to provide evidence for the existence of F_0F_1 -ATP synthases as homooligomeric structures that are enzymatically active in NSM and SM. A greater abundance of complex V dimers and oligomers with correlating enzymatic activity was observed in NSM compared to SM (Figure 3.21). It appears that ATP synthase molecules are organised as extended functional homooligomeric structures in the mitochondrial inner membrane. Specific associations of rat liver ATP synthase with other OXPHOS components to built up 'ATP-synthasomes' has been described (Chen et al., 2004, Ardehali et al., 2004).

We confirmed that in brain mitochondria, complex V co-immunoprecipitates with all enzymes of the respiratory chain as well as two adenine nucleotide translocator proteins (ANT1 and ANT2) and the phosphate carrier protein (PiC) (Table 3.8). ATP synthase has an absolute dependence on the PiC and the ANTs as they supply the enzyme with its essential substrates to make ATP. ANTs also transport ATP out of the mitochondria. ATP synthase, ANTs and PiC have been shown to form the ATP synthasome – a complex that facilitate substrate usage during the metabolic generation of ATP (Ko et al., 2003). These results strongly suggest that the complex I-containing supercomplexes and the ATP synthasome are in physical proximity to each other. Such an interaction would further increase the efficiency of ATP generation by oxidative phosphorylation.

A possible role for these homooligomeric structures is in the architecture of the mitochondrial membrane (Velours et al., 2009). Studies of the mitochondrial morphology of yeast mutants with destabilised ATP synthase dimers confirmed this idea that dimeric and oligomeric ATP synthase is involved in determining mitochondrial cristae morphology (Paumard et al., 2002). Another functional role proposed is increased stability (Arnold et al., 1998). This study proposes that

supramolecular structures of ATP synthase and respiratory chain complexes and arrangement relative to each other in NSM may favor faster substrate channeling. In this way, the homooligomeric assembly of ATP synthase would indirectly affect the overall flux through the electron transport chain. The association of monomeric ATP synthase to oligomeric structures may be necessary to maintain bioenergetic competency in mitochondria (Bornhovd et al., 2006), which has been observed in the NSM population. Interestingly, the reduced oligomers of ATP synthase are suggested to be responsible for lowering the membrane potential and thus the bioenergetic competency of mitochondria (Bornhovd et al., 2006).

Why there is a greater abundance of complex I containing supercomplex structures found in NSM when compared to SM is currently unknown. Organisation into respiratory chains depends on phospholipid and composition of membrane. Cardiolipin is one of the earliest assembly factors thought to be involved in the supercomplex assembly (McKenzie et al., 2006). It is primarily found in mitochondrial membranes (Fleischer and Fleischer, 1967) and a decrease in cardiolipin is associated with decreased membrane potential, ATP synthesis and overall mitochondrial functions (Santiago et al., 1973, Jiang et al., 2000, Gohil et al., 2004). It physically binds to all respiratory chain complexes in the inner mitochondrial membrane (Fry and Green, 1981; Armarez et al., 2013). In a comparison of the brain mitochondrial lipidome, Keibish et al. 2008 show that lipid cardiolipin levels were lower in SM than in NSM. Independent studies from two groups (Zhang et al., 2002, Kiebish et al., 2008) had earlier shown that cardiolipin stabilizes respiratory chain supercomplexes and it is required for the ETC complexes to be held together. These studies taken together with our results, indicate that the differences in mitochondrial lipids may be responsible for lower level of ETC supercomplexes in SM. Thus it is possible that greater cardiolipin present in the inner membrane of NSM conserves the stability of supercomplexes and maintains efficient electron transfer between the different complexes.

A few other proteins and lipids have been hypothesised to participate in assembly and stability of supercomplexes (Table 3.8). Prohibitins (PHB1/PHB2) are ubiquitous, abundant and evolutionarily conserved proteins that form a large multimeric complexes in the mitochondrial inner mitochondrial membrane (Berger and Yaffe, 1998). They provide protection to mitochondrial encoded peptides against proteolysis and prevent

misfolding of the newly synthesized peptides. They are thus classified as membrane associated protein chaperones (Nijtmans et al., 2000). They are scaffold-like proteins that are involved in mitochondrial function, fission and fusion and mitochondrial biogenesis (Schleicher et al., 2008). Prohibitin has previously been shown to interact with complex IV (Strub et al., 2011) and co-migrate with supercomplex III₂+IV_x (Marques et al., 2007). This study reports the co-immunoprecipitation of the prohibitins with complex I subunits (Table 3.8). Quantitative 2-DE performed in Chapter 4 describes greater amounts of these prohibitins in NSM. As prohibitins regulate the assembly of the ETC (Nijtmans et al., 2000), loss of supercomplex assembly in synaptic mitochondria due to alterations of prohibitin abundance is a possibility.

The differences observed in supercomplex assembly may explain the previously reported functional differences between the two types of brain mitochondria. An important difference observed between these two types of mitochondria is their energy threshold level. NSM have higher “reserves” of complex activities as the ETC complex activities have to be reduced upto 60-70%, before oxidative phosphorylation is severely reduced. This figure is much lower at around 20-30% for SM. In particular, threshold of complex I activity is around 72% in NSM as compared to 25% in SM (Davey et al., 1998). Only 14-16% of total Complex I is found in the free form in the presence of digitonin (Schagger and Pfeiffer, 2001) which would indicate that under physiological conditions, Complex I is bound to Complex III. Evidence suggests that the formation of supercomplexes is essential for stability of complex I (Acin-Perez et al., 2004, Diaz et al., 2006, Vempati et al., 2009) (see Chapter 1, section 1.8.3 for further detail). The physiological significance of these findings still remains to be determined (Acin-Perez and Enriquez, 2014, Diaz et al., 2012). Therefore it is possible that a decline in ETC enzyme activities in nerve terminal mitochondria correlates to the altered level of complex I-supercomplex organization.

Indirect circumstantial evidence suggests that supercomplex assembly may limit the extent of superoxide generation by the respiratory chain (see Chapter 2, section 1.8.2 for further detail). The supercomplex assembly helps to maintain the redox components of the complexes in the oxidised state through the facilitation of electron flow by channeling, thus limiting ROS formation (Panov et al., 2007, Seelert et al., 2009). NSM and SM show differences in their susceptibility to ROS damage. This may be a

consequence of the extent and tightness of supercomplex organization of their respiratory chains. NSM have been shown to produce less ROS than SM (Davey Lab, unpublished results) and this may be due to the greater presence of stabilising supercomplex assemblies. Peroxiredoxin 3 (PRDX3) is the mitochondrial antioxidant enzyme that controls ROS levels that are a by-product of aerobic respiration and was identified in this study to interact with complex I in NSM and SM (Table 3.8). Its association with complex I would be to reduce the damaging effect of the ROS generated during oxidative phosphorylation. This study proposes that the lower presence of supercomplexes in synaptic mitochondria would render them vulnerable to ROS production and subsequently susceptible to damage.

Their cellular environment may drive differences in supercomplex assembly between NSM and SM. This study characterises the differences between NSM and SM mitochondria using a quantitative approach and indicates that global proteomic differences exist between both mitochondrial populations. Analysis of the data revealed that proteins enriched in several pathways were differentially regulated between NSM and SM (Table 3.4 and Table 3.5). NSM had significantly greater expression of proteins involved in the generation of energy. The results in this study are validated by similar findings published recently (Stauch et al., 2014b). Here Stauch et al., propose that a decline in the expression of energy generating proteins may be compensated for by trafficking of mitochondria resulting in a high density of mitochondria at the synapse (Chang et al., 2006). Mitochondrial biogenesis depends on nuclear encoded genes, and degradation machineries are primarily located at the cell body of the neuron (Chang et al., 2006). Therefore, SM must be transported back and forth between the soma and synapses via mitochondrial transport systems. The synapse relies on high ATP production for vesicle mobilisation and maintenance of membrane potential. A number of proteins involved in regulation of membrane potential were found to be elevated in SM. A number of proteins whose relative levels were greater in NSM were identified as having a role in transport. In addition to producing ATP, mitochondria are necessary for maintaining proper calcium homeostasis at the synapse (Sheng and Cai, 2012). Again, relative levels of proteins whose function is to regulate cellular homeostasis were greatly elevated in SM, possibly indicating an attempt to regulate the cellular environment at the synapse (Table 3.3).

In conclusion, supercomplex assemblies are more numerous in the NSM and reduces as the mitochondria travels down to the synapse. This heterogeneity could influence energy metabolism. This would co-relate to the respiratory and enzymatic differences that exist between the two isolated populations of brain mitochondria. Although, the differences observed between NSM and SM may appear to be relatively subtle, they may trigger the 'vicious cycle' of mitochondrial deterioration at the nerve terminus. Small deficits in mitochondrial supercomplex levels may significantly impact the ETC functions. Despite severe mitochondrial ultra structural and respiratory chain defects observed in Barth Syndrome (McKenzie et al., 2006) and Leigh Syndrome (Schagger et al., 2004) only subtle declines in supercomplex levels were noted. SM display a longer lifespan and increased vulnerability to cumulative damage than NSM and this study proposes that a potential factor in this is due to loss of supercomplexes.

CHAPTER 4

Quantitative Proteomics and Supercomplex Assembly of Synaptic and Non-Synaptic Mitochondria in the Aging Rat

4.1 Introduction

Mitochondrial dysfunction has been implicated as a major player in aging theories for several decades and is among the nine recently characterized hallmarks of aging (Newgard and Pessin, 2014, Lopez-Otin et al., 2013). Impaired mitochondrial function causes an accelerated aging phenotype (Peterson et al., 2012), which is particularly evident in high energy demanding tissues such as brain (Gonzalez-Freire et al., 2015). However, to date, the relationship between aging and mitochondrial function in the neuron is still unclear. Since neurons require large amounts of energy for the firing of action potential, neurotransmission, and other processes, age related decline in mitochondrial function contributes to the cognitive declines associated with aging (Biessels and Kappelle, 2005). Aging is also a risk factor for age-associated diseases such as neurodegenerative disorders such as PD and AD (see Chapter 1, Section 1.10.3). These diseases may occur when neurons fail to respond adaptively to an age-related decline in basal metabolic rates and in energy-driven tasks, such as neuromuscular coordination, cognitive performance, and environmental awareness (Swerdlow, 2007). It should be noted that the brain consists mainly of terminally differentiated neurons and its regenerative capacity is notably lower as compared with other organs (Lazarov et al., 2010). As a result, the brain is highly susceptible to neuronal loss due to these hypometabolic states.

Substantial evidence has emerged from morphological, bioenergetic, biochemical and genetic exists that suggest mitochondrial decay is a major contributor to brain tissue alterations associated with aging (Navarro and Boveris, 2004, Navarro and Boveris, 2007, Lin and Beal, 2006, Floyd and Hensley, 2002). Both significant and subtle alterations in the mitochondrial ultrastructure have been observed (Shigenaga et al., 1994). With increasing age, mitochondria become larger, abnormally rounded and accumulate vacuoles, cristae abnormalities and reduced integrity in the inner membrane (Frenzel and Feimann, 1984, Daum et al., 2013, Cheng et al., 2013). The number of mitochondria also becomes less numerous with age as shown in liver cells of mice (Herbener, 1976), rats (Stocco and Hutson, 1978, Yen et al., 1989) and humans (Tauchi and Sato, 1968).

Decreased basal metabolic rate is of the most recognised traits of old mammals (Boveris and Navarro, 2008, Drew and Leeuwenburgh, 2004, Navarro and Boveris, 2007, Zhou et al., 2009). These observed age-related effects on mitochondrial energy metabolism are tissue specific and again are notably more prominent in tissues whose parenchyma contains mostly postmitotic cells such as brain, heart, and skeletal muscle (Yin et al., 2014).

Parts of the neuron, such as at the nerve terminal, have a higher energy demand and accordingly, have higher density of mitochondria (Shepherd and Harris, 1998). High levels of ATP are required at the synapse for maintaining Ca^{2+} homeostasis (Sheng and Cai, 2012), maintenance of membrane potential and vesicle mobilization (Murthy and De Camilli, 2003). The main source of ATP for these processes is generated from the ETC and OXPHOS. Defects in the ETC and decline in complex activity have been documented as part of the age-associated loss of mitochondria function and was discussed in greater detail in Chapter 1, section 1.9.2. The activities of the complexes I, III and IV all display a decline with increasing age (Lesnefsky et al., 2001, Paradies et al., 2000, Suh et al., 2003). Impairment of the OXPHOS process and inefficient electron transport results in the leakage of electrons that go on to generate ROS (see Chapter 1, Section 1.9.1). These ROS target DNA and, lipids (Chance et al., 1979, Yakes and Van Houten, 1997, Yan and Sohal, 1998), damage mitochondrial macromolecules and trigger cell death (Stadtman, 1992, Kujoth et al., 2005) which ultimately impacts on mitochondria physiology, and may lead to premature senescence and aging. Accordingly, elevated levels of oxidative stress and ROS have been observed in the aging brain and in many age related neuronal pathologies (Floyd and Hensley, 2002, Parihar and Brewer, 2007, Lin and Beal, 2006, Yasuda et al., 2006).

Oxidative damage culminates in an accumulation of dysfunctional mitochondria with age. Accumulation of such damage may contribute to aging and age associated degenerative diseases (Balaban et al., 2005). The cell has developed many mechanisms to prevent accumulation of dysfunctional mitochondria including fission and fusion cycles, biogenesis and elimination of damaged mitochondria by mitophagy. Disruptions in these processes have been implicated in the aging process. A number of studies found that mitochondrial biogenesis is impaired with aging and is associated with a loss of mitochondrial content and function (Short et al., 2005). It has been shown that aging is

associated with defective autophagy that leads to the accumulation of dysfunctional mitochondria, oxidative stress, and inflammation (Sgarbi et al., 2014).

In order to carry out its multiple functions, mitochondria need a large number of different proteins, most of which are nuclear encoded and subsequently post-translationally transported into the organelle (Lithgow, 2000). Protein profiling is an effective tool to monitor changes in protein abundance in response to events like aging. Mitochondria display diversity in their proteome, which allows each tissue to meet their specialized functions (Johnson et al., 2007, Mootha et al., 2003). Proteome databases have been constructed for whole rat brain (Fountoulakis et al., 1999), whole mouse brain, (Gauss et al., 1999) mouse cerebellum (Beranova-Giorgianni et al., 2002) and hippocampus (Edgar et al., 1999). Due to the complexity of higher organisms and the presence of different cell types, global 2-DE protein maps are difficult to establish. However, cell compartmentalization can allow for the establishment of organelle proteomes. This reduces the complexity of the samples, increases the dynamic range and specificity for proteome detection, giving insight into subcellular localization of the proteins and physiology of the organelle in question, in this case, the mitochondrion (Grant, 2003). Organelle proteomes have been constructed for rat neurons and astrocytes (Yang et al., 2005). These proteome maps highlight specific neuronal and astrocyte expressional patterns of several protein classes. A proteome map of rat cerebral cortex synaptosome fractions, which identified over 900 proteins, has also been established (Witzmann et al., 2005). It is over a decade now since one of the most recognized brain mitochondrial proteomes was published (Mootha et al., 2003).

Age related changes in the mitochondrial proteome are also tissue specific (Chang et al., 2007). Several studies have compared the mitochondrial profile of aging skeletal, heart and liver mitochondria (O'Connell and Ohlendieck, 2009, Chakravarti et al., 2008). However, studies on the mitochondrial proteome of aging neurons are limited. A partial mitochondrial proteome map of rat brain was established a couple of years later and revealed age dependent changes in abundance, in protein-protein interactions, and in the supramolecular architecture of protein complexes (Dencher et al., 2007). A recent publication investigated the synaptic mitochondrial proteomic and bioenergetic alterations that occur with age and the results indicate that dynamic proteomic changes occur with aging to preserve synaptic mitochondrial function (Stauch et al., 2014a). Up

to now, there was no age related proteomic comparison available for different populations of brain mitochondria, especially mitochondria present in the nerve terminal that are known to differ bioenergetically to NSM (Davey et al 1997).

To unravel the role of mitochondria in aging and to identify key proteins involved, this study analyses the age related alterations of the mitochondrial proteome profile in the hope to understand the cellular and molecular processes that ultimately lead to ETC defects from aging synaptic (SM) and non-synaptic (NSM). Protein expression levels in SM and NSM from 6 and 22 months old rats were monitored in this study. 2-DE, followed by mass spectrometry identified a total of 34 proteins with differential expression in the aging proteome. Apart from quantitative changes, eight proteins show variations in pI at different ages, which means that they were differentially post-translationally modified according to the age of the rat. In addition, a quantitative iTRAQ method was used to monitor changes in protein abundance in response to the aging process and revealed that dynamic protein changes occur with aging in SM.

As described in Chapter 1, section 1.7, a feature of the respiratory chain complexes is their existence in stoichiometrically defined functional units. Assembly of these supercomplexes has many advantages for the mitochondria, including substrate channeling, stabilization of individual complexes, and higher efficiency of electron transport and minimizing proton leak. It has been suggested that through substrate channeling, supercomplex assembly may limit the extent of ROS generation by the respiratory chain (Panov et al., 2007, Seelert et al., 2009). This is achieved by maintaining the redox components of the complexes in the oxidized state through electron flow channeling. The role of mitochondrial supercomplexes during the aging process cannot be underestimated and requires further exploration. To date, age-related loss of mitochondrial supercomplex assembly has been identified in the aging rat heart (Gomez et al., 2009) and the rat brain cortex (Frenzel et al., 2010). Beyond the expression/abundance changes in proteins, insight into age dependent changes in supercomplex levels and composition can provide structural and functional insight into the aging proteome. To provide a structural insight, supercomplex assembly was also monitored in the two age groups. The results show that the I+III₂+IV₁ supercomplex in both SM and NSM was shown to decline with age. All observations combined provide further insight into a phenomenon that is still poorly understood.

4.2 Aims of the Chapter

1. **To investigate the SM and NSM mitochondrial proteomic alterations that occurs with age.** The utilization of a state of the art quantitative proteomics approach (iTRAQ) allowed for the comparison of protein expression levels in SM and NSM isolated from 6 (young adult), 10 (adult) and 22 (old) month old rats.
2. **To define the age-dependent changes in the protein expression profiles of rat SM and NSM.** With the aim of further investigation into the molecular and cellular events characterizing the changes that occur in the brain mitochondria during aging in normal rats, a 2-DE proteomic approach, combined with mass spectrometry was used. Two groups, 6 (young adult) and 22 (old) month old rats were used throughout.
3. **To investigate if age-associated alterations in supercomplexes take place in SM and NSM.** Using BN-PAGE separation of membrane proteins and western blotting procedures, the composition of supercomplexes in young adult and old rat brain SM and NSM could be determined and quantified.

4.3 Methods

4.3.1 Isolation of mitochondria

SM and NSM from 6 (young adult), 10 (adult) and 22 (old) month old male Hanover rats were isolated according to the protocol described in Section 2.7.2

4.3.2 Native Gel Electrophoresis

Blue Native Page was performed as described in Section 2.9.3.

4.3.3 Proteomic Analysis

Two-dimensional gel electrophoresis and iTRAQ quantitative proteomics was performed as described in Section 2.8.2 and 2.13, respectively.

4.4 Results

4.4.1 Mitochondrial supercomplexes are diminished in the aging brain.

BN-PAGE is a powerful tool for the analysis of the mitochondrial proteome, since it separates many of the membrane proteins that cannot be resolved using conventional 2-DE. It provides information of mitochondrial protein-protein interactions and any contacts with other cell compartments (Reifschneider et al., 2006). As a result, BN-PAGE can represent a technique of true functional proteomics. In the previous chapter, BN-PAGE was used to investigate mitochondrial protein-protein interactions and the assembly of supercomplexes in SM and NSM. Having identified supercomplex assemblies in brain mitochondria, the next step was to explore their fate in the aged animal, as part of the study into the aging mitochondrial proteome.

SM and NSM were isolated as described in Section 2.7.2 from two age groups: 6 (young adult) and 22 (old) month old rats. Dodecylmaltoside (DDM) was used to solubilize mitochondrial membranes and separate the individual complexes and supercomplexes. Here, use of low DDM:protein ratio (1:1(w/w)) resulted in the same overall separation of proteins in SM and NSM mitochondria isolated from young adult (Y) and old rats (O). Efficient isolation of I+III₂+IV₁ (S₁) and I+III₂+IV₂ (S₂) supramolecular assemblies (Figure 4.1) was observed in both ages. The supercomplex identified as I+III₂+IV₁ was enriched in young adult NSM compared to SM. This coincides with our observations described in the previous chapter. Interestingly, the I+III₂+IV₁ supercomplex in both SM and NSM was shown to decline with age.

Digitonin has been found to be very effective in preserving the supra-molecular associations intact and has been used successfully to extract a variety of stable supercomplexes namely the I+III₂, I+III₂+IV₁₋₄, V₂, V₃₋₄ (Schagger and Pfeiffer, 2000). Solubilisation of mitochondrial membranes with digitonin (10:1(w/w)) versus DDM resulted in a more complex separation profile of proteins by BN-PAGE. Protein bands of very high mass were observed in both age groups (Figure 4.2). Western blotting using antibodies specific for each complex, revealed the presence of individual complexes and supramolecular assemblies in young adult (Y) and old (O) SM and NSM. Four supercomplex assemblies containing complex I were identified (S₁₋₄). As with DDM, I+III₂+IV₁ and I+III₂+IV₂ were observed but digitonin preserved an

I+III₂+IV₃ and an I+III₂+IV₄ assembly. The supercomplexes identified as I+III₂+IV₁ and I+III₂+IV₂ were enriched in young NSM compared to SM in the young adult animal. Again, this coincides with our observations described in the previous chapter. Interesting, not all supercomplexes identified were shown to decline with age to the same degree. As observed when DDM was used as the detergent (Figure 4.1), the supercomplex identified as I+III₂+IV₁ was shown to decline with age in both SM and NSM.

Protein complexes isolated by solubilisation with digitonin (10:1(w/w)) and resolved by 1D BN-PAGE can be separated into their individual subunits using second dimension Tricine SDS-PAGE (Figure 4.3). Special representative subunits were used to identify complex V (identified by α and β subunits), Complex III (Core I and 2) and Complex IV (identified by Cox I and Cox 2). Supercomplexes built from complexes I, III and IV were identified to the left of dimeric Complex V and were assigned S₁₋₄. The staining intensity of complex III and IV subunits was poor and difficult to identify but their presence has been indicated by 1D-BN-PAGE (Figure 4.2). The same overall separation of proteins was observed in SM and NSM isolated from young adult and old rats. No obvious difference in subunit composition was observed between the two populations of mitochondria and the two age groups. As in the previous chapter, this would suggest that it is the assembly into supercomplexes that differ.

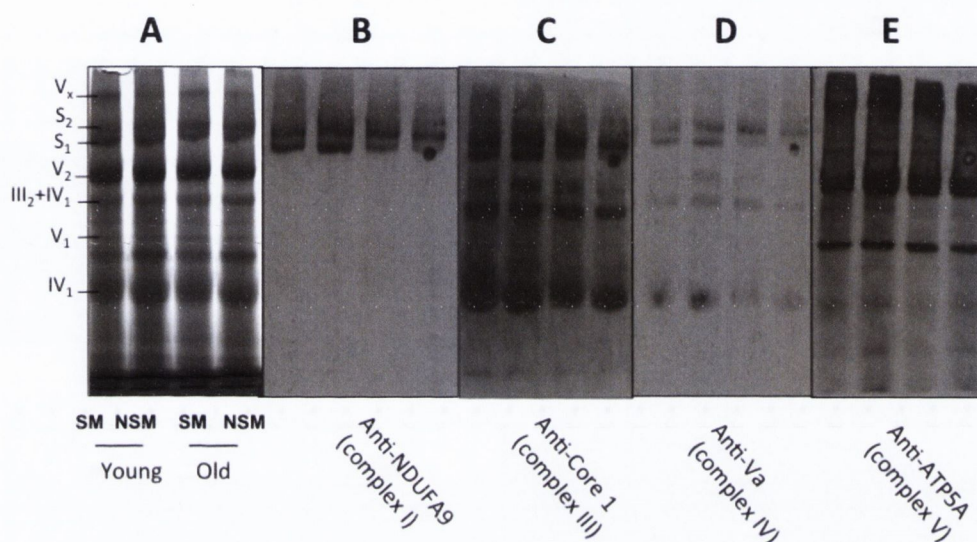


Figure 4.1 Mitochondrial supercomplexes isolated by dodecylmaltoside (DDM) extraction diminish in the aged brain. (A) Representative Serva Blue G stained 1 D BN-PAGE. NSM and SM from three age groups were solubilized with a DDM-to-protein ratio of 1:1 (w/w) and separated on a 1D 4-8% BN-PAGE. An identical gel was transferred onto PVDF and submitted to immunoblotting. Mitochondrial complexes were detected using mouse monoclonal antibodies against the following subunits: (B) NDUFA9 of complex I, (C) core 1 of complex III, (D) Cox Va of complex IV and (E) the α subunit of complex V. The Serva Blue G-stained bands in the ID were then identified on the basis of their protein subunit composition as assessed by the corresponding spots in the immunoblot. The same overall separation of proteins was observed in NSM and SM isolated from young, adult and old rats. Two supercomplex assemblies were identified as I+III₂+IV₁ and I+III₂+IV₂. A smaller supercomplex assembly of III₂+IV₁ was also identified. Levels of supercomplex, I+III₂+IV₁ appear to diminishes in SM and NSM from old rats.

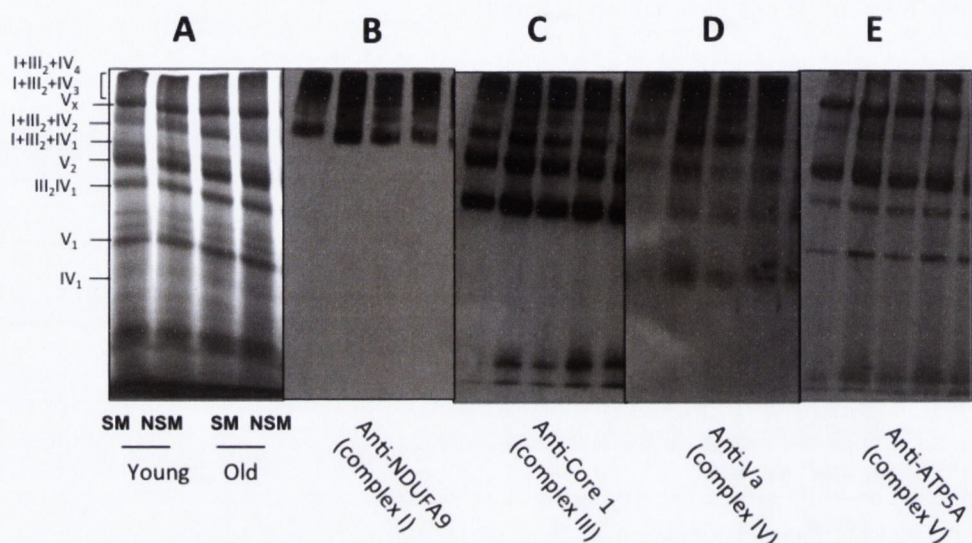


Figure 4.2 Mitochondrial supercomplexes isolated by digitonin extraction diminish in the aged brain. (A) Representative Serva Blue G stained 1D BN-PAGE. SM and NSM from three age groups were solubilized with a digitonin-to-protein ratio of 10:1 (w/w) and separated on a 1D 4-8% BN-PAGE. An identical gel was transferred onto PVDF and submitted to immunoblotting. Mitochondrial complexes were detected using mouse monoclonal antibodies against the following subunits: (B) NDUFA9 of complex I, (C) core 1 of complex III, (D) Cox Va of complex IV and (E) the α subunit of complex V. The Serva Blue G-stained bands in the 1D were then identified on the basis of their protein subunit composition as assessed by the corresponding spots in the immunoblot. The same overall separation of proteins was observed in SM and NSM isolated from young, adult and old rats. Four supercomplex assemblies containing complex I, complex III and complex IV were identified and assigned S1-4 representing I+III₂+IV₁, I+III₂+IV₂, I+III₂+IV₃ and I+III₂+IV₄ respectively. A smaller supercomplex assembly of III₂+IV₁ was also identified. Levels of supercomplex, I+III₂+IV₁ diminishes in aged SM and NSM.

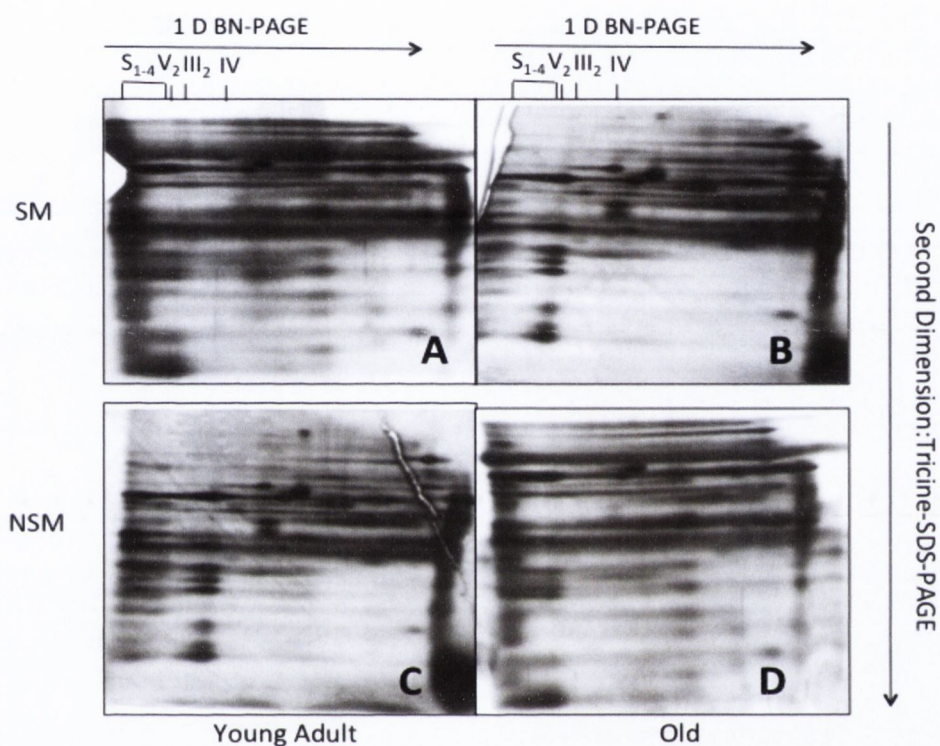


Figure 4.3 Subunit composition in SM and NSM from young, adult and old rat brain does not vary. Representative second dimension Tricine SDS PAGE of SM and NSM from young, adult and old rat brain. Mitochondria were solubilized with a digitonin-to-protein ratio of 10:1 (w/w) and the individual and supercomplexes resolved on 1D BN-PAGE. The in-gel native proteins were denatured by incubating the first dimension lane in 20 mM DDT/1% SDS and then resolved 10% Tricine-SDS-PAGE in the second dimension (**A**) young adult SM, (**B**) old SM, (**C**) young adult NSM, (**D**) old NSM. The positions of Complexes I, V, III, IV are indicated at the top of the gels. S1 to S4 indicate different mitochondrial supercomplex assemblies comprised of complex I, dimeric complex III and varying units of complex IV.

4.4.2 Differentially expressed proteins during aging identified by 2-DE.

2-DE has been the most employed method to define and quantitate the components of the mitochondrial proteome (Chang et al., 2003, Hanson et al., 2001, Lopez and Melov, 2002, Sickmann et al., 2003, McDonald and Van Eyk, 2003, Reinders et al., 2006, McDonald et al., 2006). High resolution of protein spots facilitates the study of protein isoforms generated at transcriptional level or by post-translational modifications (Hunzinger et al., 2006). The first dimension separates proteins on the basis of differences in the net charge by isoelectric focusing (IEF). The second dimension separates the focused proteins on the basis of differences in their molecular weight using sodium dodecyl (SDS) polyacrylamide gels (O'Farrell, 1975). Extracts of SM and NSM from the young adult and old rat were separated by 2-DE and several hundred spots were visualized by colloidal Coomassie blue staining (Figure 4.4). Images were analyzed by PDQuest software (Bio-Rad Laboratories) and spot intensities quantified. A total of 34 proteins showed differential expression by a magnitude > 2 . The gel position of each differentially expressed protein spot is identified numerically in Figure 4.5. These are numbered 1-28. The staining intensity differences for each numbered protein is further demonstrated in the partial 2D images in Figure 6A&B. After in-gel digestion, these spots were identified using MALDI-TOF with Mascot database searching. Data obtained from mass spectrometry analysis are presented in Table 4.1, including protein name, theoretical pI, molecular weight values, sequence covered, Mowse score, staining intensity and calculated ratios.

All of the proteins identified showed a decrease in expression with age, except for Annexin A3 (ANAXA3), which showed increased expression in the aged brain. The down-regulated proteins were categorized into several functional groups related to mitochondrial biogenesis, detoxification, signaling, TCA cycle and oxidative phosphorylation, fatty acid and amino acid metabolism and gluconeogenesis (Table 4.1).

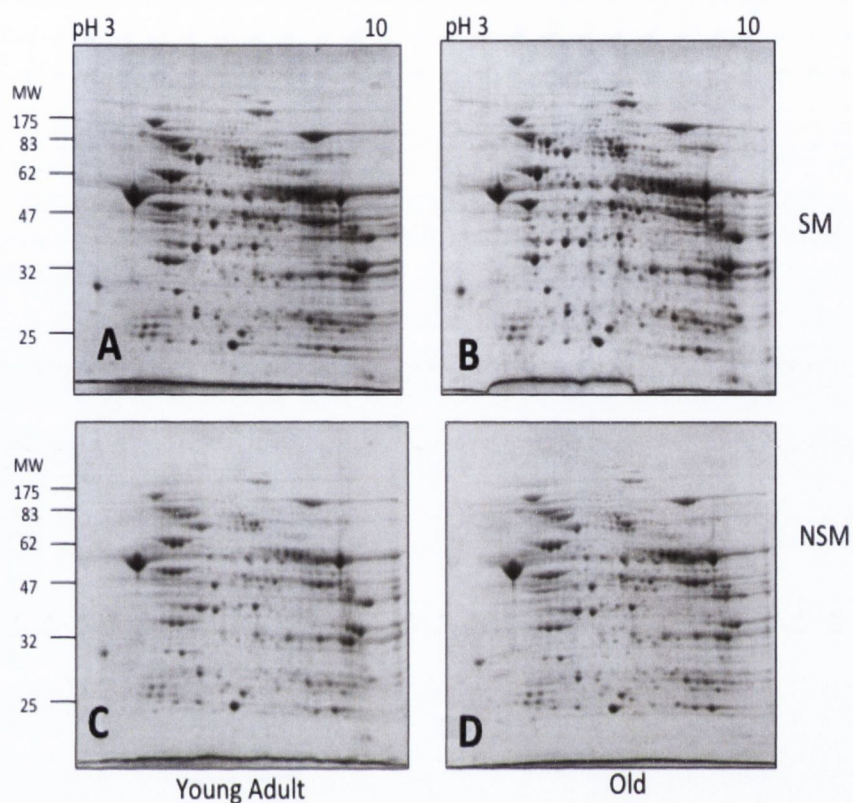


Figure 4.4 Age related changes in mitochondrial associated proteins. Representative 2D-PAGE profiles of synaptic (SM) and non-synaptic (NSM) mitochondrial proteins from brain of young adult and old rats. The first dimension run was performed using a nonlinear pH range of 3-10 and the second dimension SDS-PAGE was performed on a 8% gel. **(A)** young adult SM, **(B)** old SM, **(C)** young adult NSM, **(D)** old NSM.

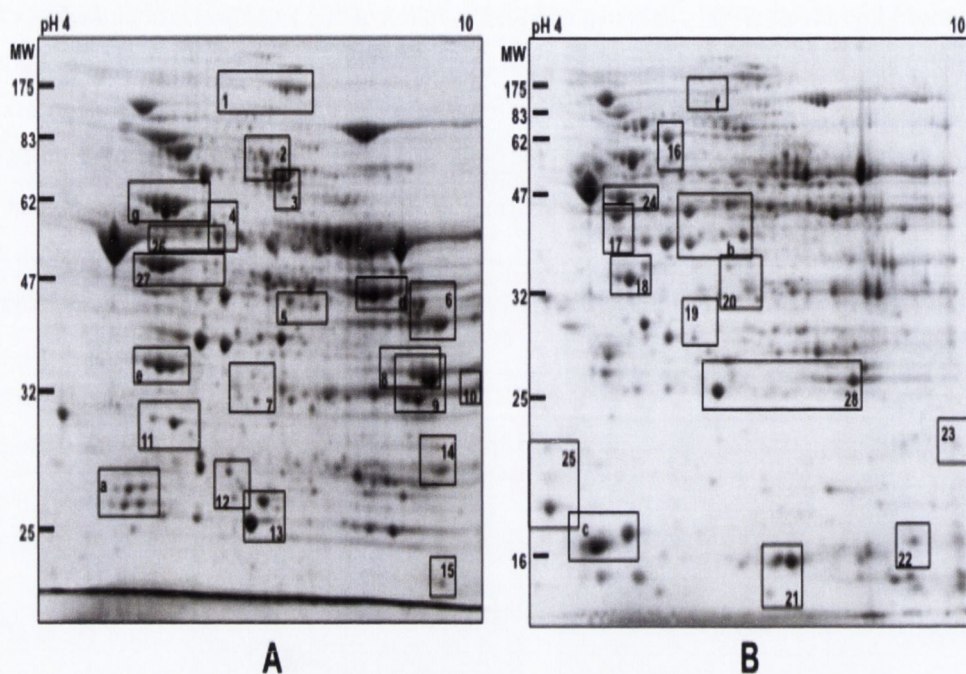


Figure 4.5 Age related changes in mitochondrial associated proteins. Representative 2D-PAGE profile of brain mitochondria with the position of the differentially expressed proteins numbered 1-28. Identified post-translationally modified proteins are labelled a-g.

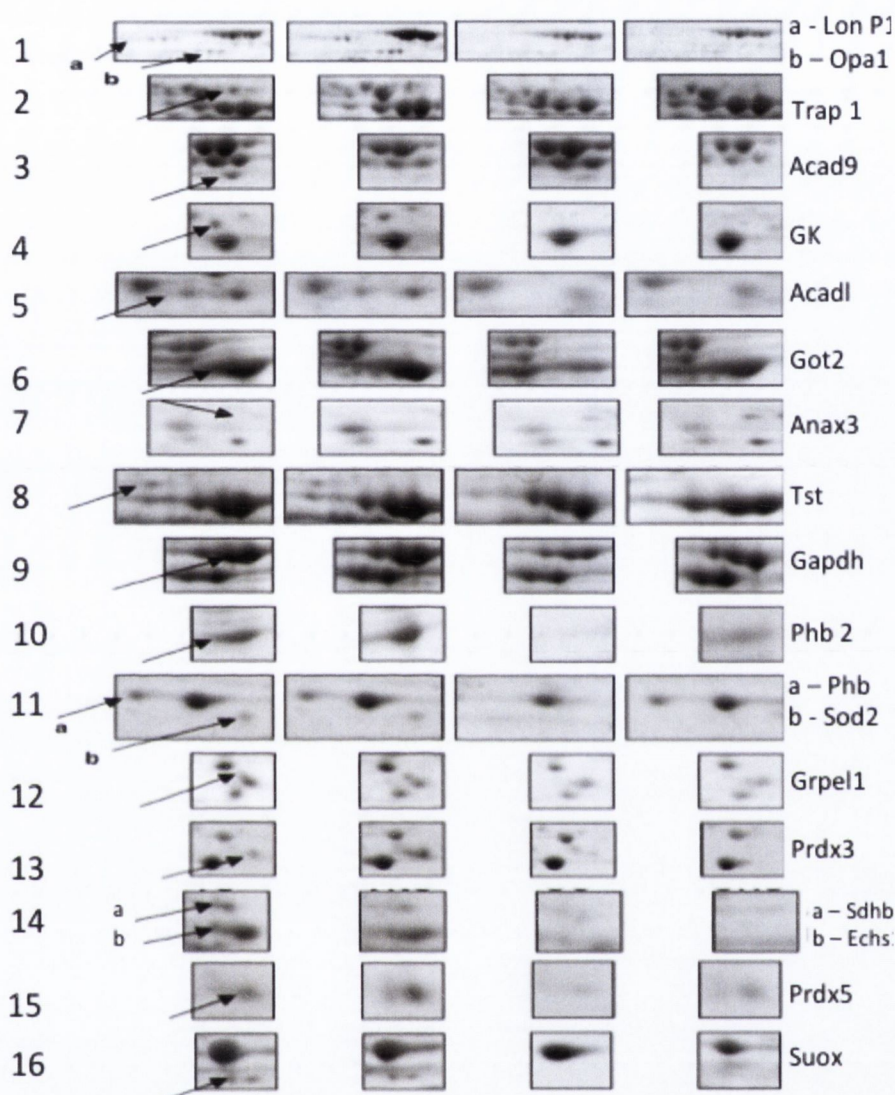


Figure 4.6A Partial 2D gel images showing differentially expressed proteins in young and old SM and NSM. The proteins were identified by MALDI-TOF following in gel digestion with trypsin.

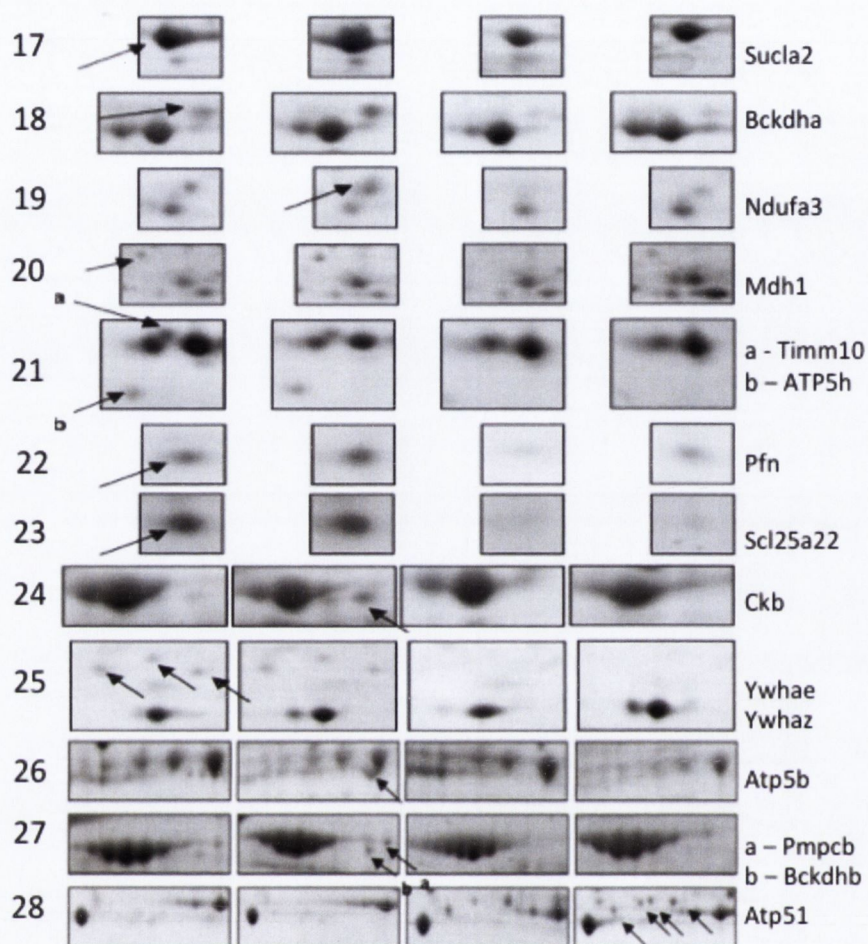


Figure 4.6B Partial 2D gel images showing differentially expressed proteins in young and old SM and NSM. The proteins were identified by MALDI-TOF following in gel digestion with trypsin.

Tale 4.1 Differentially expressed proteins from SM and NSM of young and old rats identified by MALDI-TOF MS

Protein/ Gene Name	Mr/PI	Accession Number	Seq covered	Staining intensity			Ratio			
				Mowse Score	YSM	YNSM	OSM	ONSM	O/Y SM	O/Y NSM
Mitochondrial Biogenesis, Cristae Morphology, Protein Homeostasis										
Optic atrophy protein 1 Opa1	111.6/7.01	OPA1_RAT Q2TA68	30%	99	347 ±22	863 ±67	56 ±12	175 ±10	0.16	0.20
Prohibitin Phb	29.8/5.57	PHB_RAT P67779	53%	143	433 ±33	745 ±56	56 ±12	175 ±10	-	0.92
Prohibitin 2 Phb2	32.2/9.77	PHB2_RAT Q5XIH7	66%	177	1127 ± 29	1531 ± 98	- ±75	691 ±75	-	0.81
GrpE protein homolog 1 Grpel1	24.5/8.57	GRPE_RAT P97576	47%	107	219 ±15	415 ±27	- ±63	1245 ±63	-	-
TNF receptor associated protein 1 Trap1	80.6/6.56	TRAP1_RAT Q5XHZ0	40%	176	614 ±43	710 ±50	- ±50	- ±50	0.07	0.21
Lon Peptidase 1 Lon P1	106.2/6.17	LONM_RAT Q924S5	34%	122	306 ±26	550 ±34	45 ±6	151 ±26	-	0.05
Mitochondrial processing peptidase beta Pmpcb	55.0/6.38	MPPB_RAT Q03346	32%	98	463 ±91	679 ±140	- ±5	28 ±5	-	-
Translocase of inner Timm10	10.5/5.89	TIM10_RAT P62074	64%	70	350 ±66	483 ±98	- ±98	- ±98	-	-

Protein/ Gene Name	Mr/PI	Accession Number	Seq covered	Staining intensity			Ratio			
				Mowse Score	YSM	YNSM	OSM	ONSM	O/Y SM	O/Y NSM
Cytosolic Enzymes										
Glyceraldehyde 3 phosphate dehydrogenase	36.0/8.14	G3P_RAT P04797	19%	68	16685 ±875	23387 ±1023	6087 ±345	12080 ±764	0.36	0.52
Glycerol kinase, Gk	58.2/5.49	GLPK_RAT Q63060	29%	98	402 ±27	408 ±29	-	-	-	-
Creatine kinase B Ckb	42.9/5.33	KCRB_RAT P07335	39%	131	110 ±25	379 ±67	-	-	-	-
TCA cycle and Oxidative Phosphorylation										
Succinate CoA ligase Sucla2	47.7/6.06	B2RZ24_RAT B2RZ24	25%	107	1000 ±154	1715 ±370	817 ±43	1077 ±70	0.81	0.63
NADH dehydrogenase Fe-S protein 3 Ndufs3	34.7/8.67	D3ZG43_RAT D3ZG43	47%	86	514 ±23	833 ±236	-	472 ±86	-	0.56
Succinate dehydrogenase subunit B Sdhb	27.3/8.33	DHSB_RAT P21913	43%	108	1182 ±239	1223 ±108	-	-	-	-
ATP synthase subunit alpha Atp5a1	54.6/8.24	ATPA_RAT P15999	32%	132	-	-	1505 ±324	1971 ±456	-	-

Protein/ Gene Name	Mr/PI	Accession Number	Seq covered	Staining intensity			Ratio			
				Mowse Score	YSM	YNSM	OSM	ONSM	O/Y SM	O/Y NSM
ATP synthase subunit beta Atp5b	51.1/4.92	ATPB_RAT P10719	32%	118	1239 ±255	1500 ±292	-	-	-	-
ATP synthase subunit d Atp5h	18.8/6.17	ATP5H_RAT P31399	52%	87	656 ±110	897 ±184	-	-	-	-
Amino-acid Metabolism										
2-oxoisovalerate dehydrogenase Bckdha	37.5/6.4	ODBA_RAT P11960	30%	72	617 ±42	1302 ±221	-	551 ±24	-	0.42
2-oxoisovalerate dehydrogenase Bckdhb	43.5/6.41	ODBB_RAT P35738	21%	77	559 ±105	825 ±171	-	-	-	-
Fatty-acid metabolism										
Acyl CoA dehydrogenase member 9 Acad9	42.5/8.41	B1WC61_RAT B1WC61	29%	73	831 ±85	-	234 ±27	-	0.28	-
Acyl CoA dehydrogenase long chain Acadl	48.2/7.63	ACADL_RAT P15650	34%	127	618 ±40	354 ±26	-	-	-	-
Enoyl CoA hydratase Echs1	33.6/9.49	ECHM_RAT P14604	34%	131	4423 ±344	3182 ±126	-	-	-	-

Protein/ Gene Name	Mr/PI	Accession Number	Seq covered	Mowse Score	Staining intensity			Ratio		
					YSM	YNSM	OSM	ONSM	O/Y SM	O/Y NSM
Malate-Aspartate shuttle										
Glutamate oxaloacetate transaminase 2 Got2	47.6/9.13	AATM_RAT P00507	35%	112	4166 ±178	7639 ±325	1711 ±81	3838 ±141	0.41	0.50
Malate dehydrogenase cytoplasmic Mdh1	36.6/6.16	MDHC_RAT O88989	23%	97	309 ±30	473 ±52	-	255 ±27	-	0.53
Mitochondrial glutamate carrier1 Slc25a22	24.6/8.91	Q5FVG4_R Q5FVG4	24%	64	1236 ±213	2285 ±451	-	-	-	-
Detoxification and signaling										
Peroxiredoxin 3 Prdx3	28.5/7.14	PRDX3_RA Q9Z0V6	17%	64	255 ±14	516 ±43	-	-	-	-
Peroxiredoxin 5 Prdx5	22.5/8.94	PRDX5_RAT Q9R063	68%	100	1127 ±272	1531 ±196	429 ±51	763 ±125	0.38	0.49
Sulfite oxidase Suox	54.6/5.79	SUOX_RAT Q07116	29%	86	515 ±75	795 ±289	-	-	-	-
Superoxide dismutase (Mn) Sod2	24.6/5.89	SODM_RAT P07895	43%	104	278 ±54	370 ±42	-	-	-	-
Thiosulfate sulfurtransferase Tst	33.6/7.71	THTR_RAT P24329	54%	157	728 ±72	503 ±47	-	-	-	-

Protein/ Gene Name	Mr/PI	Accession Number	Seq covered	Staining intensity			Ratio			
				Mowse Score	YSM	YNSM	OSM	ONSM	O/Y SM	O/Y NSM
14-3-3 Epsilon Ywhae	21.7/4.46	1433E_RAT P62260	53%	96	340 ±67	379 ±80	-	-	-	-
14-3-3 Zeta/Delta Ywhaz	27.9/4.73	1433Z_RAT P63102	67%	84	312 ±55	409 ±76	-	-	-	-
Miscellaneous										
Annexin A3 Anax3	36.5/6.04	ANXA3_RAT P14669	48%	158	-	-	365 ±28	411 ±36	-	-
Profilin 2 Pfn2	16.4/8.18	PROF2_RAT Q9EPC6	52%	66	1005 ±199	1466 ±282	259 ±55	590 ±139	0.25	0.40

4.4.3 Differentially modified proteins during aging identified by 2-DE

Apart from quantitative changes in the aging proteomes, several proteins were found to be post translationally modified (PMF) during aging. Eight proteins showed variations in pI at different ages, which means that they were differentially post-translationally modified according to the age of the rat. These are labeled a-g in Figure 4.5. Variations in pI with significant change in molecular weight could be due to phosphorylation, carbonylation or malondialdehyde addition to proteins. The list of proteins that show variation in pI with age is provided in Table 2. Figure 4.7 shows the variations in pI on the 2D gel. Proteins Pdha1 (pyruvate dehydrogenase E1 α), Pdha2 (pyruvate dehydrogenase E1 β) and Cox5A show an acidic shift in pI with age. Ndufv2, Ndufa8, Ndufa10, IMMT (mitofilin) and Hsp60 show a basic shift in pI with age. A basic shift in pI is likely to be caused by a dephosphorylating event. Of these proteins, phosphorylation has been reported for Pdha1 and Pdhb, Ndufa10 and Hsp60 (Denton et al., 1975, Schilling et al., 2005, Helling et al., 2008). Thus age related shift in pI that we observe for these proteins in the present study might be due to phosphorylation events.

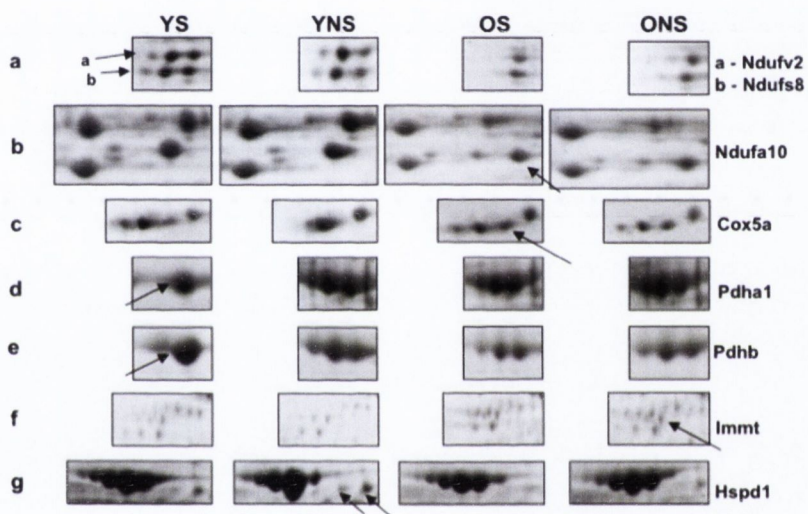


Figure 4.7 Partial 2D gel images showing post-translationally modified (PMF) proteins in young and old SM and NSM. The proteins were identified by MALDI-TOF following in gel digestion with trypsin.

Table 4.2 Differentially modified proteins from SM/NSM of young and old rats identified by MALDI-TOF MS

Protein Name	Mr/PI	Acc Number	Seq covered	Mowse Score	Differences observed
Pyruvate dehydrogenase E1 alpha Pdha1	44.8/8.49	ODPA_RAT P26284	32%	106	Three isoforms observed in young NSM and old SM/NSM Only the most basic isoform observed in young SM
Pyruvate dehydrogenase E1 beta Pdhb	39.2/6.2	ODPB_RAT P49432	38%	75	Three isoforms observed in young NSM and old SM/NSM Only the most basic isoform observed in young SM
NADH dehydrogenase Flavoprotein 2 Ndufv2	26.5/6.0	NDUV2_RAT P19234	49%	120	Three isoforms observed in young SM/NSM Only the most basic isoform observed in old SM/NSM
NADH dehydrogenase Fe-S protein 8 Ndufs8	24.4/5.87	B0BNE6_RAT B0BNE6	41%	74	Three isoforms observed in young SM/NSM Only the most basic isoform observed in old SM/NSM
NADH dehydrogenase alpha subunit 10 Ndufa10	40.7/7.64	NDUAA_RAT Q561S0	52%	171	Two isoforms observed in young SM/NSM An additional basic isoform observed in old SM/NSM
Cytochrome C Oxidase Va Cox5a	16.3/6.08	COX5A_RAT P11240	54%	84	Three isoforms observed in young SM/NSM Loss of basic isoform observed in old SM/NSM
Inner membrane protein Immt	67.4/5.57	IMMT_RAT Q3KR86	31%	146	Three isoforms observed in young SM/NSM Additional basic isoform observed in old SM/NSM
60 kD heat shock protein Hspd1	58.0/5.35	CH60_RAT P63039	32%	129	Three isoforms observed in young SM/NSM Only the most basic isoform observed in old NSM

4.4.4 Differentially expressed proteins identified by iTRAQ quantitative analysis.

To bypass the limitations of 2-DE such as the under representation of membrane and membrane-associated proteins, the isobaric tags for relative and absolute quantitation (iTRAQ) technique was used for relative quantitative analysis of SM and NSM populations and to investigate altered protein expression during the aging process. The iTRAQ technique, in association with liquid chromatography, is a more advanced method for proteomic research and is capable of detecting a much larger number of proteins, even those with low abundance, in addition to identifying and quantifying the proteins simultaneously (Wu et al., 2006).

The iTRAQ method enables multiplexing (comparing) of up to eight different samples in one mass spectrometry-based experiment. This allowed three independent preparations of SM and NSM from three different age groups to be analyzed in the one experiment under identical conditions. This quantitative proteomic study involved differential labeling of SM and NSM proteins or proteolytic peptides from three age groups (6 month, 10 month and 22 month) by stable isotopes, mixing of samples in a 1:1 ratio followed by combined sample process and subsequent MS analysis. The relative quantification of proteins was then performed by comparing ion signal intensities of peak area of isotope encoded peptide pairs observed in the corresponding mass spectra. Data generated by the iTRAQ analysis produced a robust trend.

The identification list included proteins known or presumed to localise to non-mitochondrial compartments (Table 4.3 and Table 4.4). Mitochondria communicate with other cell compartments, such as the nucleus, endoplasmic reticulum, golgi, lysosome, cytosolic proteins, and the cytoskeleton by close physical contacts, which are tightly regulated. This provides insight into mitochondrial biogenesis, cross-talk to different other cellular structures and mitochondrial specific protein interaction networks.

A greater fold difference between mitochondria in the adult group of rats (compared to young and old) was observed (Figure 4.7 A). Individual analysis of SM and NSM consolidated the trend. It was mainly SM that had an increase in protein expression from young to adult with a decrease in expression from adult to young (Figure. 4.7 B).

Considering O-Y and A-Y ratios, it was clear that significant alterations in expression levels between the ages was present in SM (Figure 4.7 C).

These results indicate that the synaptic mitochondrial proteomic profile changes in response to aging. The fits the theory that synapses are immature when young, reach maturity when adult and then decline again with age. The individual synaptic mitochondrial proteins that fluctuate Y-A and A-O were identified. Proteins with relative ratio of 2 and 3 respectively were chosen for further exploration because greater disparity in expression may indicate a more significant role for the protein. Also, more than a twofold difference in expression is a common starting point in interpreting expression array data.

A list of identified SM proteins whose relative levels were increased two fold in old compared to relative levels in young was categorized into groups based on their representative biological roles (Table 4.3). A list of identified proteins whose relative levels were increased threefold in the adult rat compared to relative levels in the young rat was categorized into groups based on their representative biological roles (Table 4.4).

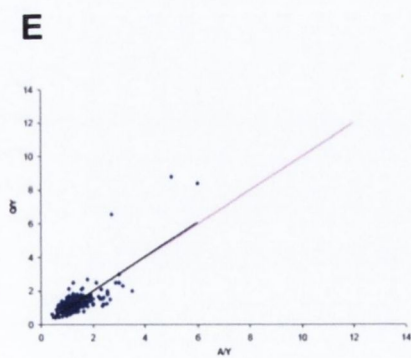
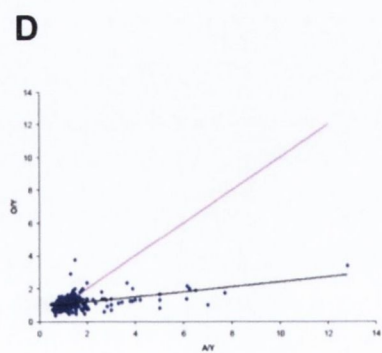
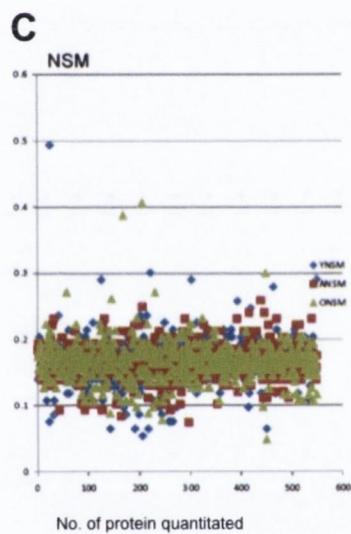
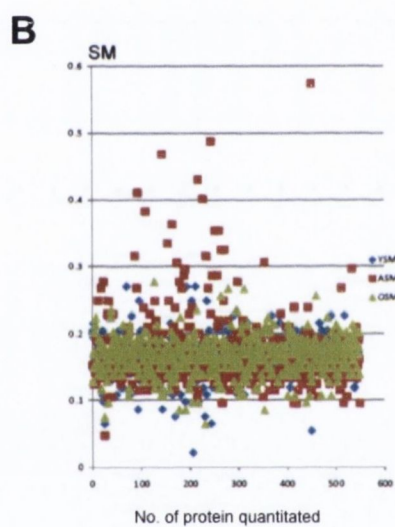
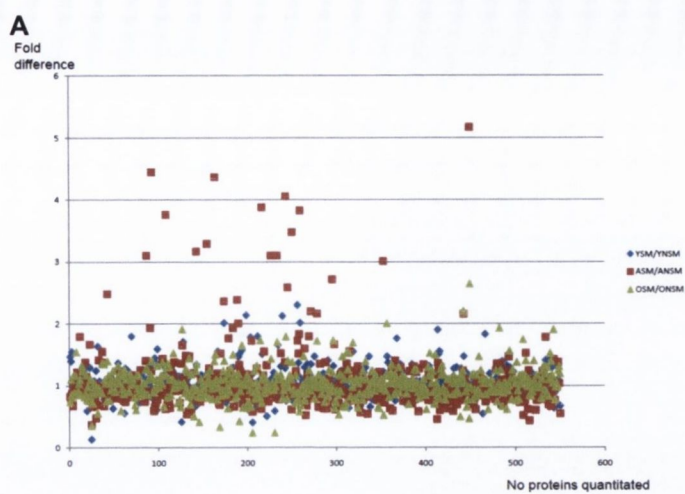
The proteins identified as up-regulated during the aging process have roles in nervous system development and metabolic activity. Nervous system development includes processes such as axon ensheathment, regulation of action potential, regulation of membrane potential and cellular ion homeostasis. Optimal levels of these processes in synaptic mitochondria are necessary for the regulation of neurotransmission. Proteins involved in metabolic processes and mitochondrial bioenergetics also undergo alterations during the aging process. An increase in expression may suggest a change in the synaptic mitochondrial proteome in response to aging. Because energy production at the synapse is necessary for cell function, any age related decline of the electron transport chain and ATP production might result in a protective mechanism to compensate for this decline.

The differentially expressed proteins were also analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation tool for enrichment analysis and classified according to the Gene Ontology (GO) term

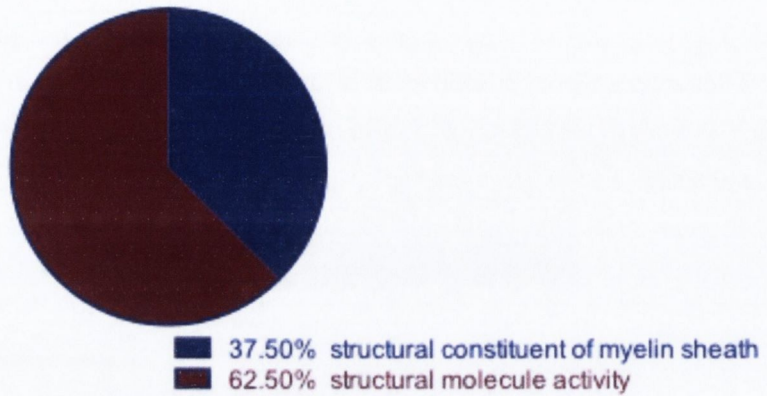
molecular function (Figure 4.8). The GO analysis indicated that the list of differentially expressed proteins were associated with functions involved in structural molecule activity, structural constituent of the myelin sheath, purine ribonucleotide binding and GTP binding. Mechanisms therefore involved in the integrity of the synapse undergo changes during the aging process.

Five proteins from SM came to attention in this study. Alterations in their expression levels were identified in both categories (i.e. Y/A and Y/O). Their relative levels appeared to increase in adult and decrease again during old age (Figure 4.9). Claudin 11, cathepsin D, myelin basic protein are involved in ensheathment of neurons and structural constituent of myelin sheath. Calcium calmodulin dependent protein kinase plays a role in synaptic transmission, while cathepsin D plays a role in mitophagy. This is also the first study to report the presence of Claudin 11 in brain mitochondria. Fascinatingly, similar quantitative analysis performed in Chapter 3 identified higher levels of Claudin 11 in SM compared NSM (Chapter 3) and this current chapter indicates for the first time that it may have a possible role to play in the aging process. This study also identifies the protein 2,4 CNPase as being up-regulated in the aged SM which is also emerging as an important player in the aging process.

OVERLEAF: Figure 4. 8 (A) A greater fold difference for a cohort of proteins present in SM in the adult rat compared to NSM was observed. These ratios were not observed in mitochondria from young or old rats. (B) quantitative comparison of proteins in SM from young, adult and old rats (C) quantitative comparison of proteins in NSM from young, adult and old rats. Here, mainly SM have an increase in protein expression from young to adult with a decrease in expression from adult to old. (D) & (E) show O-Y and A-Y ratios for SM and NSM, respectively. The most significant alterations in expression levels between the ages is present when comparing adult SM to young SM.



A



B

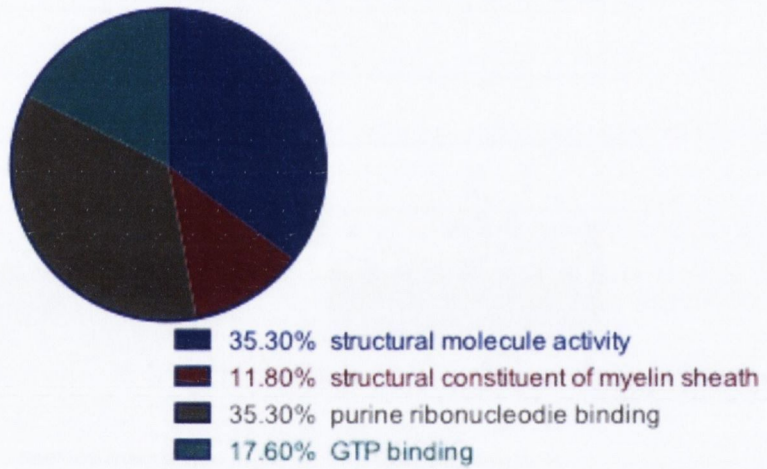


Figure 4.9 Ontology of the cumulative identified SM proteins whose relative levels were altered during aging. Gene Ontology (GO) Annotation for Molecular Function Obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) for the SM proteins whose relative levels were altered during aging. **(A)** Molecular function of proteins identified in old SM whose relative levels were increased two fold compared to levels in the young animal. **(B)** Molecular function of proteins identified in the adult whose relative levels were increased three fold compared to levels in the young animal.

Table 4.3 Biological Process categories of the proteins identified by iTRAQ whose relative levels increased two fold in SM fractions from old rat compared to young adult rat.

Accession No.	Name	Uniprot ID	Gene	RGD ID	Ratio A/Y
System Development (axon ensheathment, regulation of action potential and membrane potential)					
Q99P82	claudin-11	CLD11_RAT	Cldn11	71081	3.4
P02688	Myelin basic protein isoform 1	MBP_RAT	Mbp	3054	2.14
P60203	myelin proteolipid protein	MYPR_RAT	Plp1	3354	2
Q9Z2F1	Calmodulin dependent protein kinase II beta M isoform	Q9Z2F1_RAT	Camk2b	2262	2
Metabolic Processes (oxidative phosphorylation, autophaghy)					
P05508	NADH-ubiquinone oxidoreductase chain 4	NU4M_RAT	Mtnd4	620240	2.9
Q6PCU8	NADH dehydrogenase flavoprotein 3, mitochondrial isoform 1	NDUV3_RAT	Ndufv3	1583292	2
P24268	Cathepsin D	CATD_RAT	Ctsd	621511	2.33
Miscellaneous Proteins					
	Proapopin precursor				3.75
	G-rich RNA sequence binding factor 1				2.33
	ATPase inhibitor, mitochondrial precursor				2

Table 4.4 Biological Process categories of the proteins identified by iTRAQ whose relative levels increased three fold in SM fractions from young adult rat compared to young rat.

Accession No.	Name	Uniprot ID	Gene	RGD ID	Ratio A/Y
Nervous System Development					
Q99P82	claudin-11	CLD11_RAT	Cldn11	71081	12.8
P60203	myelin proteolipid protein	MYPR_RAT	Plp1	3354	6.35
P02688	Myelin basic protein isoform 1	MBP_RAT	Mbp	3054	6.14
Q9QUL6	Vesicle fusing ATPase	NSF_RAT	Nsf	621594	3
Q9JJK1	Glycoprotein m6b	GPM6B_RAT	Gpm6b	620580	5
P31596	Excitatory amino acid transporter 2	EAA2_RAT	Slc1a2	3697	7
Q9Z2F1	Calmodulin dependent protein kinase II beta M isoform	Q9Z2F1_RAT	Camk2b	2262	3.89
Metabolic Processes					
P62982	Ribosomal protein S27a	RS27A_RAT	Rps27a	620240	3.3
Q9QUL6	Vesicle fusing ATPase	NSF_RAT	Nsf	621594	3
P09606	Glutamine synthetase	GLNA_RAT	Glul	2710	3.45
P24268	Cathepsin D	CATD_RAT	Ctsd	621511	3.67
P27139	Carbonic anhydrase	CAH2_RAT	Ca2	2240	4.2
P13233	2,3, cyclin nucleotide 3 phosphodiesterase CNP	CN37_RAT	Cnp	2368	5
Response to Stress					
Q63345	Myelin oligodendrocyte glycoprotein	MOG_RAT	Mog	3102	7
Cellular component organisation					
Q6XUZ7	EG3RVC	Q6XUZ7_RAT	Sep-04	1308781	7.71
P59215	Isoform α 2 of guanine nucleotide binding protein G	GNAO_RAT	Gnao1	628732	6.13
Q9JMB2	Type II brain 4.1 minor isoform	Q9JMB2_RAT			5
Q6P9T8	Tubulin, beta 4	TBB4B_RAT	Tubb4b	735101	4
Q9QUL6	Vesicle fusing ATPase	NSF_RAT	Nsf	621594	3
P09606	Glutamine synthetase	GLNA_RAT	Glul	2710	3.45

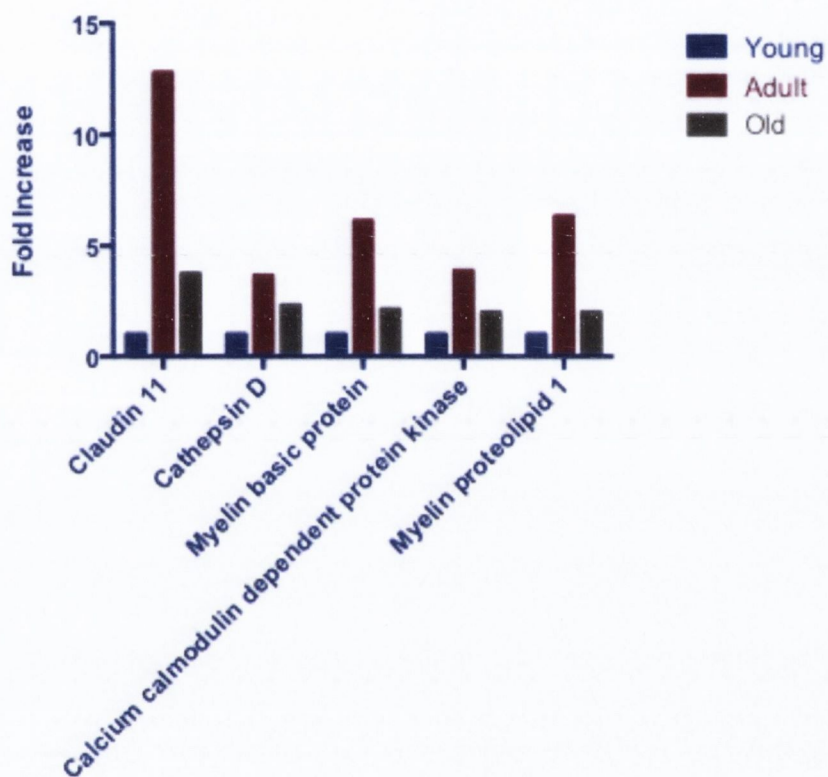


Figure 4.10 Relative levels of synaptic proteins increase on entering adulthood but decrease with age. Five proteins from SM were identified whose relative levels altered during the aging process compared to the young adult. These results indicate that the synaptic mitochondrial proteomic profile changes in response to aging.

4. 5 Discussion

The energetic demands of neurons are heavily dependent on optimal mitochondrial function. Beyond doubt, mitochondria are affected during aging, and studies in several species reveal a wide spectrum of alterations in mitochondria and mitochondrial DNA (mtDNA) associated with aging, including (1) increased disorganization of mitochondrial structure, (2) decline in mitochondrial oxidative phosphorylation (OXPHOS) function, (3) accumulation of mtDNA mutation, (4) increased mitochondrial production of reactive oxygen species (ROS) and (5) increased extent of oxidative damage to DNA, proteins, and lipids (Lee and Wei, 2012). The present study investigates the proteomic and functional changes corresponding to aging in rat SM and NSM mitochondria and identifies alterations in protein abundance, expression levels, post-translational modifications, protein-protein interactions and supercomplex assembly in the aging brain.

4.5.1 Metabolic Processes and Aging

The function of mitochondria is balanced by synchronization of numerous intersecting key metabolic pathways such as the tricarboxylic acid (TCA) cycle, fatty acid oxidation and the urea cycle, which fuel oxidative phosphorylation, the ultimate end point of cellular metabolism and major source of cellular ATP (Koopman et al., 2013, Mazat et al., 2013). Aging of the mammalian brain is associated with a continuous decrease in the capacity to produce ATP via oxidative phosphorylation. In agreement with this, the present study has found that the majority of the proteins identified to be altered in an age-related manner are involved in these different metabolic pathways.

Neural cells have very high, but fluctuating ATP requirements. Despite the fact that fatty acids are substrates that supply redox energy to the mitochondrial ETC, the brain does not produce significant energy from fatty acids. This low generation of brain energy metabolism from energy-rich long-chain fatty acids is not well understood and the high susceptibility of brain tissue to oxidative stress is believed to be a factor. On the other hand, other organs with high-energy turnover, such as the heart and kidney, largely oxidize fatty acids (Hagen et al., 2002, Grynberg and Demaison, 1996). The release of energy from fatty acids is accomplished through the cleavage of two-carbon units of Acetyl-CoA, which enters the TCA cycle. This present study identifies three

enzymes related to β -oxidation of fatty acids, namely enoyl CoA hydratase (ECHM), acyl CoA dehydrogenase long chain (ACADL) and acyl CoA dehydrogenase member 9 (ACAD9), that are down-regulated in both SM and NSM during aging. Down-regulation of enzymes involved in β -oxidation of fatty acids have been reported in aging heart mitochondria (Chakravarti et al., 2008). Consequently, mitochondria may not be able to generate the energy requirement for proper heart function. Interestingly, when levels are compared in young animals, higher quantities are present in SM than in NSM. It should also be noted here that during the quantitative study carried out between SM and NSM proteins in Chapter 3, relative levels of 2,4 dioenyl CoA reductase, enoyl coA hydratase and myelin proteolipid protein (all associated with fatty acid metabolism) were elevated in synaptic mitochondria.

Of these enzymes, ACAD9 (Figure 4.6A, spot no. 3) is most interesting as it is found to be totally absent in NSM of both 6 month and 22 month old rats. Recently it has been shown that *in vivo* ACAD9 does not have β -oxidation activity and instead plays an essential role in the functional assembly of functional complex I, and mutations in the gene are a cause of complex I deficiency (Haack et al., 2010). A non-functional complex I would lead to severely impaired oxidative phosphorylation and its pathological consequences. ACAD9 appears to be a SM specific enzyme down-regulated during aging. The higher levels of these enzymes in SM may be indicative of mechanisms in neuronal cells that replenish the oxidised lipids in mitochondria at neuronal synapses. Of great interest to this study is the fact that multifunctional fatty acid oxidation (FAO) complex has been found to be physically and functionally associated with ETC supercomplexes (Wang et al., 2010). Existence of a multifunctional FAO complex within mitochondria that is physically associated with OXPHOS supercomplexes optimizes the efficiency of energy metabolism. Again, this highlights just some of the possible advantages of supercomplex formation and an ability to associate with many other complexes in the mitochondria.

This study identifies reduced expression of the glycerol metabolising enzyme, glycerol kinase (GK) and the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the SM and NSM fractions prepared from aged rats (Figure 4.6A, spot no. 4 and no. 9). Although GK and GAPDH are cytosolic enzymes, they are known to localise to the OMM (Graham et al., 2007). This provides a mechanism for generating

substrates close to its site of uptake into the mitochondrion where they enter the TCA cycle. GK facilitates the entry of glycerol into the glycolytic pathway. Thus, this is an important gluconeogenic substrate and links several metabolic pathways. The observed decline in expression of GK and GAPDH (Table 4.1) suggest that the level of the enzymes GK and GAPDH (or their association with the mitochondrial surface) decreases with age. In fact, it has been reported that GK activity increases after calorie restriction, correlating with a delay in aging (Hagopian et al., 2008).

A study by Petrak, *et al.* found that the frequency with which GAPDH was shown differentially expressed in 2D-gel electrophoresis (2-DE)-based experiments in human and rodent tissues was ~18%, securing it a spot on the top 15 list of most frequently reported differentially expressed proteins (Petrak et al., 2008). GAPDH is no longer considered a simple glycolytic enzyme that produces glycerate 1,3-bisphosphate and NADH, as recent studies underline its role as (i) a metabolic switch for the Pentose Phosphate Pathway, (ii) a transcription activator, (iii) an activator of apoptosis and (iv) a regulator of vesicle dynamics between the ER and Golgi (Colell et al., 2009). Taking into consideration the multitude of functions GAPDH can carry out under normal conditions, in addition to a variety of subcellular locations, it is not surprising that this enzyme was detected in this aging study. In fact, in age-related diseases, decreased GAPDH glycolytic activity, in addition to oxidative and post-translational modifications, like S-glutathionylation and S-nitrosylation, are distinct markers of cellular stress (Butterfield et al., 2010). It should be noted that initial confirmation into the purity of mitochondrial preparations, cytosolic enzymes such as GAPDH were found to be absent using the immunoblotting technique (Chapter 3, section 3.4.1). However, here both 2-DE and iTRAQ identified the presence of GAPDH in mitochondrial samples. Therefore, it can be concluded that mass spectrometry is extremely sensitive technique able to identify even small quantities of proteins that were not detected using western blotting.

Another cytosolic enzyme involved in energy transduction, soluble creatine kinase (CK-B) (Figure 4.6B, spot no. 24) was also found to be down-regulated during aging. Creatine kinases buffer ADP and ATP levels by reversible transfer of phosphate to creatine and are mainly expressed in tissues with sudden high energy demands like muscle and brain (Castegna et al., 2002) CK-B is the brain isoform distributed across

neuronal and glial cell cytosols. CK-B addresses the sudden transient demand of energy and its ablation leads to diminished learning performances and changes in behaviour (Jost et al., 2002). Other reports have also observed down-regulation of cytosolic CK in aging tissues, for example in the muscle (Capitanio et al., 2009). Increased protein oxidation in the brain, which is a recognized phenomenon in the aged model, can lead to a decrease in the activity of this enzyme in aging (Smith et al., 1991, Castegna et al., 2002). Its up-regulation in brain tissue from aged animals has also been reported (Yang et al., 2008) though it is important to note that these age-related proteomic studies were not completed on purified mitochondria but on whole tissue. We find a consistent lowering of cytosolic CK-B associated with purified brain mitochondrial fractions. The decrease in creatine kinase may be indicative of a perturbed transphosphorylation between phosphocreatine and ADP in the older brain. Lowering of the levels of this protein during aging may ultimately lower the ability of aged brain to handle sudden energy demands.

It is therefore tempting to say that lower levels of these cytosolic enzymes or their lowered association with mitochondria in old SM and NSM may have a detrimental effect, as it may lead to impairment of neuronal functions driven by glycolytic energy as well as cause inefficient channeling of pyruvate to the TCA cycle.

This present study finds that one TCA cycle enzyme (succinate CoA ligase), two subunits of OXPHOS complexes (NADH dehydrogenase Fe-S protein 3, succinate dehydrogenase subunit B) and three subunits of the ATP synthase (subunit α , subunit β and subunit d) are significantly lower in abundance in brain mitochondria from aged rats (Table 4.1). Age-associated decrease in the amount of intact ATP synthase has been previously reported in the rat cortex (Dencher et al., 2007). Decreased levels of ATP synthase subunits has also been reported in the hearts of aged mice (Nishtala et al., 2013, Chakravarti et al., 2008). In an interesting observation, this present study finds that several spots that migrate at ~25 kDa are identified as ATP synthase subunit alpha (ATPA) in both SM and NSM from aged rats. The actual molecular weight of this subunit is 54 kDa and the spots appear to be proteolytically cleaved products of the intact subunit. Or perhaps the protein is labile since it is isolated from purified mitochondria. Previous studies have reported proteolysis of intact F_0F_1 ATP synthase under acute oxidative stress (Dargelos et al., 2010). These changes may lead to a

decrease in ATP generation, thereby perturbing the energy pool and having a significant role in decline of mitochondrial bioenergetics.

One of the gluconeogenic mechanisms that supports OXPHOS and maximizes the number of ATP produced in glycolysis is the malate-aspartate shuttle. This study finds two enzymes associated with the shuttle, namely malate dehydrogenase 1 (MDH1) and aspartate aminotransferase 2 (GOT2) are reduced with age in NSM and are almost absent in SM from aged rats (Figure 4.6B, spot no. 20 and Figure 4.6A, spot no. 6, respectively). Intriguingly, one of the glutamate carriers (GC1) that are important for shuttle activity is also reduced in mitochondria from aged animals (Figure 4.6B, spot no. 23). The lowering of proteins related to the malate-aspartate shuttle in old NSM and old SM suggests that the net energy produced by glycolysis in the aged brain may be low and even lower at synapses.

A recent quantitative proteomic study showed that all the identified subunits in the ETC changed in protein expression during aging, specifically having decreased expression from 5 to 12 months and increased expression from 12 to 24 months in SM (Stauch et al., 2014). This suggests dynamic changes in the mitochondrial proteome as a feedback or compensatory mechanism to manage any age-related decline of the ETC to ensure proper ATP production, which is especially important at the synapse. In line with these reports, our iTRAQ quantitative study also identified proteins in SM whose relative levels were elevated two-fold in old age (Table 4.3). The relative levels of NADH dehydrogenase flavoprotein 3, mitochondrial isoform 1 and NADH-ubiquinone oxidoreductase chain 4 were elevated in aged SM. Observed elevated levels of proteins associated with ATP production suggest a compensatory response to try and maintain ATP levels in presence of declining mitochondrial membrane potentials that occur following oxidative stress (Atlante et al., 2001).

In summary, these results indicate that there is an imbalance in the expression of several proteins and enzymes throughout aging, which are associated with energy metabolism. Through 2D-E and quantitative proteomics, this aging study identifies proteins whose levels are both decreased and increased in the brain over time. These results indicate that energy metabolism is affected during aging and the capacity for ATP synthesis is

reduced in the aged brain. Increased expression of OXPHOS proteins may indicate a survival mechanism to try and maintain ATP levels in the aging brain.

4.5.2 Supercomplexes and Aging

Aging impairs mitochondrial function by affecting both the capacity and the control of OXPHOS. It is well documented that in tissues of animals and humans there is an age-associated decline in the activities of mitochondrial respiratory enzymes and ATP synthase (Papa, 1996). Therefore, the oxidative phosphorylation machinery consisting of complexes and supercomplexes is of great importance when trying to understand mitochondrial dysfunction in the aging process. To gain deeper insights into both the aging mechanisms and the resulting protein-protein interactions, this study employed a blue native gel based approach to examine the abundance of mitochondrial OXPHOS complexes as well as their supercomplex assembly.

Age-related alterations in the supercomplex assembly of respiratory chain enzymes have previously been described for rat brain (Dencher et al., 2007, Seelert et al., 2009, Frenzel et al., 2010), rat skeletal tissue (Lombardi et al., 2009) and rat heart (Gomez et al., 2009). However, the present study is the first to report age-related alterations in OXPHOS supercomplexes from SM and NSM. In line with previous reports, we observe a pronounced loss of complex I-containing supercomplexes isolated from rat SM and NSM mitochondria during aging. Reassuringly, this corresponds to what appears to be an emerging trend in the aging process. Gomez et al. 2009 shows that supercomplexes composed of various stoichiometry of complexes I, III and IV declined significantly with age in rat heart tissue. In another study, mitochondria from old rat skeletal tissue contained significantly lower levels of complex I and III compared to young rats (Lombardi et al., 2009).

With respect to this current study, the most pronounced decrease occurs for the supercomplex $I+III_2+IV_1$, in agreement with the current trend of complex I-containing supercomplexes being altered during the aging process (Figure 4.1 and Figure 4.2). Evidence exists to suggest that the respiratory chain flux is strongly controlled at the level of complex I (Barrientos and Ugalde, 2013). Flux control analysis in aerobic respiration in coupled liver mitochondria show that complex I becomes more rate controlling over all the examined activities of respiratory chain enzymes during aging

(Ventura et al., 2002), suggesting that the aging process induces a profound alteration of complex I that is reflected by the entire OXPHOS pathway.

A decline in complex I-containing complexes would suggest a decrease in the total amount of complex I in the aged brain. However, this study reports no evidence of increased individual complex I nor increased level of individual complex III and IV in SM and NSM. On this basis, it can be assumed that the supercomplex is not dissociated into its individual complexes during aging. To date, it is unclear why supercomplex levels are diminished with age. A compensatory mechanism has been proposed involving the formation of more active, larger supercomplexes to compensate for the reduced level of ATP synthesis (Lombardi et al., 2009) associated with aging. Loss of I+III₂+IV₁ assembly could result in the assembly of larger and more stable complex IV containing supercomplexes. Larger supercomplexes are more stable and the formation of larger complex IV containing supercomplexes is the most favored structure for the activity of the assembled complexes (Schafer et al., 2006, Vonck and Schafer, 2009). In contrast, Gomez et al. reports an age-dependent decline of the mainly higher supercomplexes, while complex I, III and IV did not change. This was observed in rat heart mitochondria, suggesting tissue specificity of the rat mitochondrial proteome that would explain our observations (Reifschneider et al., 2006).

Dissociation of supercomplexes may result in the disassembly of complex I and III subunits and loss of intra-complex electron transfer (Lenaz and Genova, 2007). This phenomenon is described in greater detail in Section 1.8.1. Disruption in the electron transfer may elicit further generation of ROS. There are many reports to demonstrate that the rate of production of ROS from mitochondria increases with age, and coinciding with those observations is indirect evidence suggesting that supercomplex assembly may limit the extent of superoxide generated by the respiratory chain (Panov et al., 2007, Seelert et al., 2009).

Interestingly, in conjunction with the hypothesis that formation of supercomplexes may reduce oxidative damage, the 2-DE study identified several proteins that are categorised as detoxifying enzymes and protect against cellular stress. These identified proteins were down-regulated in aging SM and NSM mitochondria. Thiosulfate sulfurtransferase (rhodanese - THTR) (Figure 4.6A spot no. 8) is a ubiquitous enzyme responsible for cyanide detoxification. It also participates in formation of iron-sulphur clusters and

electron transport chain. Down-regulation of THTR during aging may affect the detoxification process and has been reported by several previous studies (Yi et al., 2010, Lombardi et al., 2009). Enzymes such as superoxide dismutase (SOD) and peroxiredoxins (PRDXs) (Figure 4.6A spot no.s 11 and 15, respectively) are capable of clearing ROS, and the proteomic screen found a lower level of these enzymes during aging. SOD converts superoxide to hydrogen peroxide (H_2O_2) and the PRDXs convert H_2O_2 to molecular oxygen and water. Overexpression of MnSOD increases lifespan not only by efficient detoxification, but also by a regulatory response that involves retrograde signaling of increased H_2O_2 . In *Drosophila*, it was found to be a single gene that when perturbed, had a profound effect on gene expression and organismal longevity (Parkes et al., 1998). Furthermore, the gene sulphite oxidase (SUOX) (Figure 4.6A, spot no. 16) that codes for the terminal enzyme in the degradation pathway of sulphur containing amino acids was also found to be down-regulated during aging. Deficiency of this enzyme in humans leads to neurological abnormalities and early death (Feng et al., 2007).

Reduced ATP synthase oligomers have been reported in previous studies and may be responsible for lowering the membrane potential and consequently, the bioenergetic competency of the organelle (Bornhovd et al., 2006). However, no significant alteration in ATP synthase oligomers was identified in this study. It should be noted that resolution of high molecular weight assemblies on one-dimensional BN-PAGE is poor and may account for the observed absence of ATP synthase alterations in aging.

The small deficits in mitochondrial supercomplex levels observed in this study may significantly impact the ETC functions. Despite severe mitochondrial ultrastructural and respiratory chain defects observed in Barth Syndrome (McKenzie et al., 2006) and Leigh Syndrome (Schagger et al., 2004), only subtle declines in supercomplex levels were noted. Therefore our observations may account for decline in oxidative capacity observed in the aging mitochondria.

4.5.3 Mitochondrial Biogenesis and Aging

A number of proteins important for biogenesis and maintenance of healthy mitochondria were identified in both 2-DE and iTRAQ, suggesting that fragility of mitochondria is affected during the aging process. Mitochondrial chaperones are also essential for mitochondrial biogenesis as they guide proper protein import and folding. As well as biogenesis, they have a role in protecting the organelle from damage as mitochondrial proteins are constantly challenged by stress conditions that result in their destabilization, unfolding and aggregation. The organelle has an elaborate protein quality control setup to safeguard its proteins. This system includes chaperones and ATP dependent proteases. Accumulation of damaged or misfolded proteins is reported to be age-related and leads to oxidative stress cell death (Mattson & Magnus, 2006). Protein aggregation is also associated with a wide variety of age-related pathological conditions like Alzheimers disease. Our proteomic screen identified a large number of proteins that play a role in guiding mitochondrial biogenesis. In all nine of these proteins, expression levels were down-regulated as a function of age. This coincides with similar reports in the aging mouse brain (Mao et al., 2010).

Prohibitins (PHBs) are a class of protein that are uniquely known as membrane-bound chaperones. They are ubiquitous, evolutionarily conserved, and form a ring like high molecular mass complex at the mitochondrial inner membrane. The two prohibitin proteins, PHB1 and PHB2, have been ascribed various functions, including cell cycle regulation, apoptosis, and assembly of mitochondrial respiratory chain enzymes, and their expression levels are indicative of a role in mitochondrial metabolism (Coates et al., 2001). The two proteins are induced by metabolic stress caused by an imbalance in the synthesis of mitochondrial- and nuclear-encoded mitochondrial proteins. Prohibitins may have a role in regulating mitochondrial respiratory activity and in aging (Merkwirth et al., 2012, Piper et al., 2002). As prohibitins regulate the assembly of the ETC (Nijtmans et al., 2000), loss of supercomplex assembly in the aged animal due to alterations of prohibitin abundance in the aging proteome is a possibility. PHBs also have a role in the processing of OPA1. In absence of PHBs, processing of OPA1 is impaired, the lamellar shape of mitochondrial cristae is lost and apoptosis is induced (Merkwirth and Langer, 2009). This study found significantly lower expression of PHBs in SM and slightly lower levels in NSM from aged brain and this was in parallel with the reduced levels of OPA1 (Figure 4.6A, spot no. 10 & 11). As OPA1 controls

mitochondria as discussed in Section 1.4.1, its lowering would lead to punctate mitochondrial morphology and correlate with mitochondrial dysfunction during aging. Recently, another fission protein, dynamin, has been described in the aging rat (Ottis et al., 2013).

This study finds that levels of two chaperone proteins, GRPE1 homolog and TRAP1 are reduced in aging SM and NSM. Of these, TRAP1 is reported to have a ROS-related antagonist role (Hua et al., 2007). The TIM9-TIM10 complex plays an essential role in the process by chaperoning select hydrophobic precursor proteins across the intermembrane space. TIM10 may act in substrate recognition and TIM9 acts in complex stabilization (Baker et al., 2009). We observe a reduced level of TIM10 in aging mitochondria. A lowering of the TIM10 protein during aging may result in reduced import of hydrophobic precursor proteins into mitochondria and ultimately impaired mitochondrial biogenesis and function.

An age-related basic shift in the chaperone Hsp60 was also identified and its activity could be down-regulated in old brain tissue with negative consequences. Two phosphorylated isoforms are absent in NSM from the aged brain. Age-related alterations in the abundance of Hsp60 has been previously reported in the rat cortex (Dencher 2007). Recently, isoform changes for Hsp60 has been shown after complex I inhibition in neuroblastoma cells (Burte et al., 2011).

In the proteomic survey we also found a decrease in the expression of LONP1 during aging. The AAA-protease, LONP1, decreases the accumulation of aggregated polypeptides and degradation of oxidatively modified proteins. Reduced expression and activity was previously correlated with advancing age (Ngo and Davies, 2007). LonP1 participates in the degradation of oxidized proteins within the mitochondrial matrix induced by acute stress. Studies in mice suggest that Lon protease downregulation reduces the ability of the aging mitochondria to respond to stress (Bota et al., 2002). Another protease, the mitochondrial processing peptidase (MPP) which is a soluble mitochondrial matrix peptidase, cleaves the targeting signals of mitochondrial precursor proteins. Lower expression in old mitochondria may result in inefficient precursor protein processing and defects in mitochondrial biogenesis.

Profilins are regulators of actin polymerization and profilin 2 (PFN2) is the neuron specific isoform (Boyl et al. 2007). Reduced levels of PFN2 reduces the dendrite complexity and number of spines in hippocampal neurons (Michaelsen et al., 2010). The present study shows that PFN2 co-purifies with mitochondria. The study finds that the PFN2 level is reduced in aging NSM and SM mitochondria (Figure 4.6B, spot no. 22). This may lead to reduced spine density in aged neurons. It is possible that observation of decreased proteins involved in mitochondrial biogenesis is partly due to perturbations in apoptosis, cell signalling and the accumulation of misfolded and denatured proteins that are characteristic in aging.

4.5.4 Post Translational Modifications and Aging

As well as age-dependent changes in abundance, protein-protein interactions and the supramolecular architecture of protein complexes; modulation of post-translational modifications (PTMs) was analyzed in this aging study. In the last decade PTMs have emerged as powerful modulators of metabolic pathways and impacts many mitochondrial proteins. Mitochondrial complexes are subjected to reversible phosphorylation and dephosphorylation, and are important in regulating activity of the organelle (Salvi et al., 2005). This study identifies eight proteins with variations in pI as a function of age suggesting that they are differentially post-translationally modified according to the age of the rat (Figure 4.7).

The pyruvate dehydrogenase complex (PDH) is at the center of aerobic carbohydrate metabolism. It is localized in the matrix space of mitochondria where it catalyzes the irreversible oxidative decarboxylation of pyruvate entering the organelle to produce acetyl-CoA, NADH and CO₂. PDH is a large complex (around 9 MDa) consisting of multiple copies of three enzymes: pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3). The E1 enzyme is a tetramer comprised of two alpha and two beta subunits. The activity of PDH is regulated by reversible phosphorylation of three serine residues on the E1 α subunit (Patel and Korotchkina, 2006). It has three potential phosphorylation sites, but phosphorylation of just one site can lead to 97% reduction in enzyme activity. Reversible phosphorylation of the pyruvate dehydrogenase enzyme is a critical regulatory mechanism and an important point for monitoring metabolic activity. Increased phosphorylation of the PDH E1 α subunit lead to decreased PDH activity in

the aged rat brains. Similar findings have been reported in depolarized synaptosomes derived from 24-month-old animals with respect to 3-month-old adults (Bogonez et al., 1992). In the present study, SM from young brain possess the active non-phosphorylated form (the most basic) isoform of the enzyme. Young NSM, which represent a mix of glial, astrocyte and neuronal cell body mitochondria, carry the phosphorylated enzyme that is more than likely inactive. In the aged brain, the active form of the enzyme observed in synaptic mitochondria is phosphorylated and inactivated. These results would indicate that compartmentalization of brain-associated fuels as energy sources may be altered during aging. Also, perhaps under stress and decline in electron transport chain efficiency, the cell turns to alternative and compensatory fuel utilization. The decline in BBB and neuronal glucose transporters coupled with decreased hexokinase activity was accompanied by increased phosphorylation of PDH, consistent with decreased PDH activity.

Stable phosphorylation sites in complex IV have been detected at tyrosine, serine and threonine residues in subunits II and III as well as at specific amino acid residues in subunits IV, Vab, VIabc, VIIabc and VIII (Helling et al., 2008). Cox Va and Vab vary in different tissue types, with Vb being more abundant in tissues with a higher metabolic demand. Our study shows increased phosphorylation of subunit Cox Va in old SM and NSM. Hyper phosphorylation of Cox Vb in response to hypoxia has been reported (Prabu et al., 2006). The assembly state of complex IV may be affected by hyper- or hypophosphorylation of some units in the complex. It is tempting to link this phosphorylation PTM to supercomplex assembly. In heart mitochondria, cAMP and PKA-dependent phosphorylation of complex IV was higher in free complex IV than in the supercomplex assembly (Rosca et al., 2011).

The most well studied complex of the oxidative phosphorylation chain is complex I. This study reports that three of its subunits, NDUFV2, NDUFS8 and NDUFA10 are present as phosphorylated and dephosphorylated isoforms in young and old SM and NSM mitochondria, respectively. These proteins show a basic shift in pI with age, which is likely to be caused by a dephosphorylation event. Complex I has 14 conserved cores subunits that form the minimal structure required for functionality of the enzyme. The remaining subunits are involved in stability and biogenesis of the complex. NDUFV2 and NDUFS8 are core subunits and this study shows that they have

phosphorylated forms in young brain. Phosphorylation of these subunits may have direct impact on enzyme activity. NDUFA10, which is not a core subunit, has previously been reported to undergo dynamic phosphorylation and dephosphorylation (Palmisano et al., 2007). This is the first study to report that dephosphorylation of complex I subunits may be a phenomenon that takes place in the aged brain. Phosphorylation of complex I has been shown to modify the activity of the enzyme and subsequent ROS generating capacity (Papa et al., 2008). Phosphorylation of NDUFS4 contributes to the stability of complex I and stimulation of its activity (De Rasmo et al., 2008). Dephosphorylation may contribute to decline in enzyme activity and destabilisation of the complex. This study reports the presence of complex I solely in the assembly of a supercomplex. Phosphorylation may increase the stability of supercomplexes as it increases complex I activity and decreases ROS production.

Mitofilin is a mitochondrial protein anchored to the inner membrane that has been shown to control cristae morphology (John et al., 2005). Here, the study finds that it shows a basic shift in isoelectric point during aging. A similar shift was reported and was correlated to mitochondrial dysfunction and age-associated neurodegeneration in a mouse model for accelerated senescence (Wang et al., 2008). Interestingly, aggregated states of mitofilin were detected in BN-PAGE of rat brain mitochondria (Dencher et al., 2007)

Here, alterations in expression levels of myelin-associated proteins from young synaptic mitochondria to old SM (Figure 4.9) are reported. Three of the most recognized myelin-associated proteins were identified: myelin basic protein (Mbp), proteolipid protein and oligodendrocyte glycoprotein. The role of these proteins with regards to mitochondria is poorly understood at present. Recently, the presence of myelin basic protein as well as 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) was confirmed in SM and NSM (Baburina et al., 2014). These proteins are key components of the myelin sheath and play a role in maintaining the integrity of myelin multi-lamellar structure. The lipid-rich feature of the myelin sheath makes it vulnerable to environmental changes in the brain, such as aging and is thought to be one of the major targets of brain aging (Bartzokis, 2011). Due to advances in technology, there is increasing evidence that the amount of myelin is reduced in the aged nervous system (Lintl and Braak, 1983, Madden et al., 2012). However, the mechanism behind this has not been fully understood and

expression levels of these myelin-associated proteins are limited. This present investigation shows that expression of myelin-associated proteins are low in the young rat, increase on entering adulthood and then decrease in the aged brain. A recent study reports the down-regulation of these proteins in the astrocytes of rats, which correlated with glial activation (Xie et al., 2013). It is difficult to compare these observations to previous reports. Here, a two-fold increase is reported in aged brain. It should be noted that previous attempts to understand myelin expression in the aging CNS involved imaging and staining techniques. This is the first to quantify the expression of these proteins in isolated brain mitochondria. Different experimental methods, different animals and different regions of interest in CNS could induce confusing and even conflicting results. These differences are further evidence that investigation into the age-related changes in the expression of myelin proteins in the CNS is needed.

2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP) is a component of the molecular machinery that mediates early events in myelinogenesis and serves as a useful analytical marker for myelination-related membranes in the CNS even though it does not contribute to the structural lattice of myelin lamellae (Braun et al., 1988). It is believed to undergo significant age-associated changes (Hinman et al., 2008). Here, we report a two-fold increase in aged brain, which is in agreement with and coincides with previously reports in various aging studies (Sloane et al., 2003, Xie et al., 2013). Hinman et al., 2008 suggest that incomplete degradation of CNP leads to age-related CNP accumulation and proteolysis. Our data is in agreement with these observations. CNP is expressed exclusively by oligodendrocytes in the CNS, and the appearance of CNP seems to be one of the earliest events of oligodendrocyte differentiation (Kasama-Yoshida et al., 1997). Oligodendrocytes proliferate with age (Peters and Sethares, 2004) and overexpression of CNP suggests new formation of myelin by oligodendrocytes (Sloane et al., 2003). A study that has just recently emerged sheds a very interesting light on the role of 2, 3-CNP in the aging brain. They report that reduced CNP expression in aging NSM leads to lowering of the threshold Ca^{2+} concentration and an acceleration of mPTP opening (Krestinina et al., 2014, Azarashvili et al., 2014). It is well understood that SM are more susceptible than NSM to opening of the permeability transition pore. Therefore, the observed increase in its expression in synaptic aging mitochondria could be a compensatory mechanism to override the oxidative stresses.

4.5.5 Conclusion

The global assessment of the synaptic mitochondrial proteomic changes, based on protein expression, revealed that SM from 6 and 22 month old rat are slightly more similar than that of 10 months. This change demonstrates a potential proteotypic shift in 22 month old mice back to a 6 month old mitochondrial protein expression pattern. These specific changes in synaptic protein expression may be necessary for the maintenance of ATP production required specifically at the synapse despite age-associated insult. These results are validated by similar findings published recently (Stauch et al., 2014). This trend has also been observed in the aging mouse proteome (Mao et al., 2010), where the average protein concentration of different protein categories showed an overall accelerated turnover rate with time. However, on reaching adulthood, a clear-cut shift is observed and protein concentration turnover rate begins to decrease.

The proteomic profile of SM and NSM mitochondria is in accordance with previous reports, showing decreases in many metabolic pathways and other cellular processes. Taken together the data suggests that impaired biogenesis of mitochondria, altered morphology, impaired OXPHOS activity leading to elevated ROS levels, impaired amino acid catabolism leading to imbalance in neurotransmitter concentration, lack of gluconeogenic enzymes, inefficient detoxification processes, lower protein homeostasis, dissociation of OXPHOS supercomplexes leading to a further lowering of energy supplies, are a few of the processes that are affected severely in mitochondria and connected metabolic pathways from the aged brain. The proteomic changes observed likely allow the cell to adapt to functional requirements and counteract age-associated cellular stress and damage enabling maintenance of mitochondrial energetics at the synapse and other parts of the cell.

Supercomplex disorganization can lead to possible dysfunction in complex I assembly and this, combined with efficient electron channeling, would contribute to decreases in ATP synthesis observed in aging. The functional consequences of reversible phosphorylation, a largely overlooked means of regulating mitochondrial function with respect to age, needs to be established.

With aging, mitochondrial function at nerve terminals would be more susceptible to suffer alterations by the constant calcium changes occurring as a consequence of synaptic activity. NSM would be more resistant to age-related dysfunction and oxidative damage. The summative effect of all these changes could potentially leads to functional failure of mitochondria, neuronal dysfunction and contribute to the aging brain phenotype.

CHAPTER 5

Reconstitution of Mitochondria into the Lipidic Mesophase and Characterisation of Functional Output

5.1 Introduction

The process of oxidative phosphorylation is central to the production of ATP and on average the 7kg of mitochondria present in the resting human body synthesizes approximately 65 kg of ATP daily (Tornroth-Horsefield and Neutze, 2008, Erecinska and Wilson, 1982). Many of the proteins necessary for this phenomenon are embedded in the mitochondrial inner membrane and are organized into the respiratory complexes of the electron transport chain as described in Chapter 1, section 1.6.2. Each complex consists of several protein components that are associated with a variety of redox-active prosthetic groups with successively increasing reduction potentials. In recent years our knowledge of mitochondrial structure has changed enormously and we now know that the electron transport chain protein complexes are arranged into supercomplexes (Acin-Perez et al., 2008) and that the mitochondrial proteome varies from tissues to tissues (Mootha et al., 2003, Reifschneider et al., 2006). Mitochondria are also dynamic organelles, undergoing rapid fusion/fission events (Chapter 1, section 1.4). Despite these advances there remains much to be discovered in terms of how the electron transport chain proteins are structurally arranged in the mitochondrial inner membrane.

It is clear that the lipid composition of the membrane is of vital importance to overall mitochondrial function. The interaction between phospholipids and proteins is of particular importance in the mitochondrial membrane and the amount of inner mitochondrial membrane associated proteins is high with a protein to lipid ratio estimated at 3:1 (Schenkel and Bakovic, 2014). The mitochondrial phospholipid composition varies little amongst cells, suggesting major changes cannot be tolerated. The major phospholipids in the mitochondrial membrane, also present in the plasma membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA). Exclusive to mitochondria are phosphatidylglycerol (PG) and cardiolipin (CL). Unlike plasma membranes, the mitochondrial membranes contain low levels of cholesterol (Houtkooper et al., 2009). The phospholipid diversity in the membrane is also influenced by length and degree of unsaturation of fatty acid acyl chain present in within each class of lipid (Houtkooper et al., 2009). Activity of the electron transport chain proteins depends greatly on this phospholipid composition of the membrane (Fry and Green, 1981). As mentioned in previous chapters (Chapter 1, section 1.9.1),

changes in the mitochondrial phospholipid composition can affect respiration (Ohtsuka et al., 1993).

Membrane proteins can be crystallized by a number of methods. *In surfo* methods make use of detergent- or surfactant-solubilized preparations in crystallization trials (Wiener, 2004). This technique can result in the protein when dispersed in detergent micelles, finding itself in a destabilizing environment. On the other hand, the bilayer method involves a biomolecular lipid leaflet as the pad from which proteins launch into the crystal (Caffrey, 2003). An example of the bilayer method is the *in meso* method, a technique pioneered by scientists for crystallizing membrane proteins. This method is an advancement on the *in surfo* method as instead of the potentially harmful environment of the detergent micelle, the protein is placed into a more familiar, hospitable, stabilizing and organizing lipid bilayer (Caffrey and Cherezov, 2009).

To date, monoolein (MO) (Figure 5.1) is the lipid most successful and commonly used in the *in meso* method and has been shown to be membrane mimetic. For the purpose of this study, monoolein 9.9 MAG was used. The monoacylglycerol [MAG] chain shorthand notation is based on the N.T system introduced previously (Misquitta and Caffrey, 2001). In this system, “N” refers to the neck of the chain extending from, and including, the carbonyl carbon to the first carbon of the *cis* olefinic bond. “T” refers to the tail of the chain extending from, and including, the second olefinic carbon to the carbon of the methyl terminus. The sum of N + T is the total number of carbon atoms in the chain. Thus, 9.9 MAG is monoolein that has an acyl chain that is 18 carbon atoms long and a *cis* double bond between carbons 9 and 10.

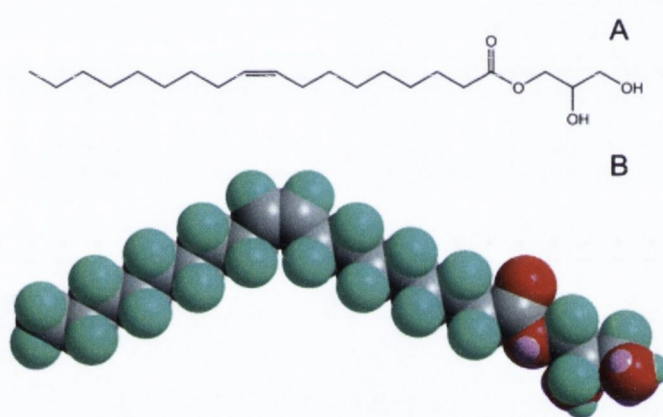


Figure 5.1 Structure and temperature-aqueous composition phase diagram of **monoolein**. (A) Chemical structure of monoolein. (B) Space-filled model of monoolein.

The mechanism behind the *in meso* method is still not fully understood. It is believed that the membrane protein of interest is solubilized in detergent from the parent biomembrane or from inclusion bodies (Ai and Caffrey, 2000). The purified, concentrated protein is combined with lipid that when hydrated forms a cubic mesophase (Figure 5.2). The name cubic phase comes from the fact that when placed in water, lipids spontaneously organize into liquid-crystalline structures, such as, lamellar, hexagonal, or cubic phases (Figure 5.2).

Cubic phase can be characterized as a highly curved, non-intersecting bilayer with two water channels (Figure 5.3). The presence of 5 nm wide bicontinuous water and amphiphilic channels enables water-soluble proteins to be incorporated. At this point, the protein is considered reconstituted into the bilayer of the cubic phase in a native and active conformation and can retain functionality *in meso*. It also has partial or complete mobility within the plane of the cubic phase bilayer (Qiu and Caffrey, 2000). Cubic phases formed in the monoolein/water systems is non-toxic, biocompatible and biodegradable and are commonly used in drug delivery and in biosensing or biofuel cell construction. Incorporated proteins, vitamins and drugs (especially macromolecular drugs) are physically stable in this system (Nazaruk et al., 2012).

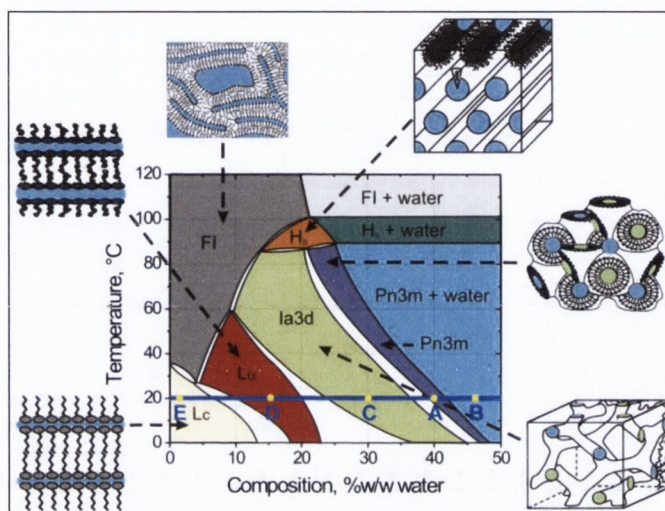


Figure 5.2 Temperature-aqueous composition phase diagram of monoolein. The temperature-aqueous composition phase diagram of monoolein. The phase diagram illustrated in this figure is a summary of the phase tendencies as water content and temperature are changed. The area of interest for this study is on the 20°C isotherm (blue horizontal line). This is where the majority of *in meso* crystallization has taken place. A number of cubic structures can be formed and are classified according to their symmetry that can be deduced from X-ray diffraction. For the requirements of this study, work was carried out in the cubic phase of space group type Pn3m, which is represented by A in the purple region. Here, the monoolein/water system at 20°C has an overall composition of about 40% (w/w) water. Moving in the direction of reduced hydration, the next phase encountered is a second cubic phase, this time of space group type Ia3d. (C in diagram) Increased dehydration triggers formation of the lamellar liquid crystalline or L α phase (D in diagram). A further reduction in water gives rise to the lamellar crystalline or Lc phase (E in diagram), which is a true solid. Image taken from Caffrey et al., 2009, Annu. Rev. Biophys. 38:29-51.

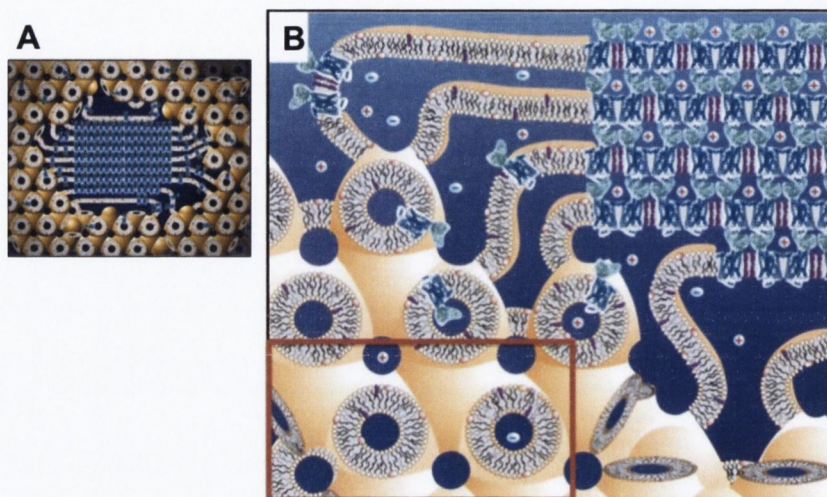


Figure 5.3 Representation of the events proposed to take place during the crystallization of an integral membrane protein from the lipidic cubic mesophase. (A) Cartoon illustration of events during crystallization. (B) Zoomed in representation of events. The process begins with the protein of interest reconstituted into the highly curved bilayers of the 'bicontinuous' cubic phase (red box). The data presented in this current study is based on the assumption that the proteins of rat liver mitochondria are reconstituted in a native and active conformation at this specific stage. As much as possible, the dimensions of the lipid (tan oval with tail), detergent (pink oval with tail), native lipid (purple), protein (blue and green), bilayer and aqueous channels (dark blue) have been drawn to scale. The lipid bilayer is approximately 40 Å thick. To pursue crystallization, which was not the goal in these circumstances, the stability of the cubic phase (red box) is made unstable by overlaying it with an excess of precipitant solution. This leads to phase separation and protein molecules diffuse from the continuous bilayer of the cubic phase of a sheet like portal (upper right quadrant) to lock into the lattice of the advancing crystal face (upper right quadrant). Under conditions leading to crystallization, one of the separated phases is enriched in protein, which nucleates and

develops into a bulk crystal. Figure obtained from Nat Protoc. 2009; 4(5): 706-73 (Caffrey and Cherezov, 2009).

Mitochondria regulate critical cellular processes, from energy production to apoptosis. As a result, the ability to measure their function is an imperative for scientists whose research focuses on different aspects of cellular metabolism. Proper assessment of mitochondrial function has become central to the basic research of mitochondrial physiology and the understanding and diagnosis of many diseases, including the large group of mitochondrial encephalomyopathies (Wallace, 1999), certain neurodegenerative diseases (Lin and Beal, 2006), cancer, oxidative stress, (Balaban et al., 2005) and metabolic adaption. To date, multiple methodological approaches have been developed to access mitochondrial function. These include cytochemical, molecular biology, imaging and biochemical techniques. In an attempt to better understand the basic requirements for OXPHOS function, the present study investigated the functional outcome when mitochondria are reconstituted into the lipidic cubic phase, a technique pioneered by Prof. Martin Caffrey, Trinity College, for crystallizing membrane proteins. Interestingly, it appears that mitochondria were still capable of undergoing OXPHOS and retain enzymatic activity. The system has the potential then to be applied for the study of mitochondrial dysfunction and disease. A potential outcome from this study is the development of a new method for studying mitochondrial function with important applications for the study of mitochondrial dysfunction in human diseases.

5.2 Aims of the chapter:

The lipidic cubic phase provides a native like environment for proteins. On the reconstitution of isolated liver mitochondria into the mesophase, the efficiency of this reconstitution trial must be assessed. A high quality reconstitution of mitochondria into the lipid bilayer could be prerequisite for various biochemical and biophysical studies for mitochondrial membrane proteins. To address this, this study aimed to achieve the following:

1. **To confirm full reconstitution of mitochondria has taken place within the lipid bilayer of the mesophase.** Absorption spectrometry was performed to confirm the incorporation of mitochondria into the mesophase.
2. **To investigate what happens to OXPHOS parameters when isolated mitochondria are reconstituted into the lipidic mesophase.** The oxygen consumption (JO_2) rates of mitochondria, pre and post reconstitution were monitored in a high-resolution respirometer. In each scenario, mitochondria were exposed to individual inhibitors of the electron transport chain and assessed for changes in JO_2 rates.
3. **To quantify complex I enzymatic activity when mitochondria are incorporated into the lipidic mesophase.** A complex I spectrophotometric assay was performed to monitor complex I activity of liver mitochondria present in the lipidic mesophase.

5.3 Methods

5.3.1 Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by a modification of a method by Chappell and Hansford (1972). For each preparation, one Wistar rat was killed by stunning and cervical dislocation and the liver was rapidly dissected and placed in ice-cold isolation buffer (250 mM sucrose, 1 mM EGTA and 5 mM Trizma-HCL, pH 7.4). The rat liver was chopped finely with scissors and the buffer changed at least two times to remove any blood. The chopped liver was homogenised using a hand held Thomas homogeniser by applying 5 passes of a 0.026'' clearance pestle (loose pestle) and 5 passes of a 0.012'' clearance pestle (tight pestle). The rat liver homogenate was centrifuged in a pre-cooled (4°C) Sorvall RC5C centrifuge using a SS34 rotor at 1,050 g for 3 min. The resultant supernatant was centrifuged at 12,000 g for 10 min. The pellet was resuspended in ice-cold and centrifuged at 12,000 g for 10 min. This re-suspension and centrifugation procedure was repeated a further two times retaining the pellet on both occasions. The final pellet was suspended in isolation buffer and the mitochondrial protein content was determined using the method of Coomassie Bradford as described in Chapter 2, section 2.6.1.

5.3.2 Preparation of Crude Submitochondrial particles

Submitochondrial particles (SMP) are for the most part 'inside out'. That is, the mitochondrial inner membrane orientation is inverted relative to intact mitochondria (Figure 5.1). Freshly isolated rat liver mitochondria were subjected to three 30 sec bursts on a Qsonica Sonicator Q50 at 20% output, allowing one full minute before applying each burst to minimize sample degradation.

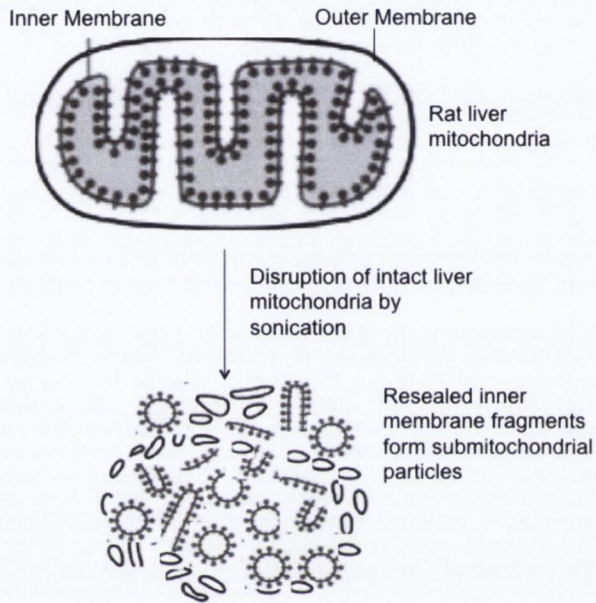


Figure 5.4 Preparation of submitochondrial particles. Intact membranes of mitochondria are disrupted by exposure to ultrasound. This causes the cristae to pinch off forcing the inner mitochondrial membrane inside out, leading to the formation of tiny 'inside out' vesicles derived from the inner membrane. However the electron transport chain complexes remain on the membrane.

5.3.3 Reconstitution of mitochondria into Mesophase

Monoacylglycerol (MAG), monoolein (9.9 MAG) was a kind gift from Prof. Martin Caffrey, TBSI, Trinity College, Dublin. The lipid monoolein (MO) is removed from -80°C storage. Monoolein is hygroscopic so full equilibration to room temperature must be allowed before opening the container.

Two 100 µl gas-tight Hamilton syringes (model no. 1710RN) were used to manually reconstitute mitochondria into the lipidic mesophase. Prior to mitochondrial reconstitution, monoolein was placed in a temperature-controlled block at 42°C to render it molten. Isolated mitochondria were kept on ice throughout the process to minimize degradation.

A lipid to protein ratio of 3:2 was used to form the cubic phase at or close to full hydration. MO was pipetted into a gas-tight Hamilton syringe, slowly pulling the plunger out to force the liquid up the barrel. A Teflon ferrule, which connects the needle to the narrow bore coupler, was slid onto the syringe needle before the coupler was finger-tightened onto the syringe.

In a similar manner to MO, the calculated volume of mitochondria was pipetted into the barrel of the second syringe, sliding a ferrule onto its needle. The MO-containing syringe was primed by pushing the plunger upwards until the MO was injected to the tip of the needle protruding from the coupler. The two syringes were combined by way of a coupler. Torque was applied to both barrels through to the coupler using the fingers and thumb of both hands to ensure that the seal against the ferrules in the coupler remained intact. The two suspensions were injected back and forth through the coupler until it became optically transparent. At this stage, the cubic phase formed and the dispersion appeared optically transparent in the syringe barrel.

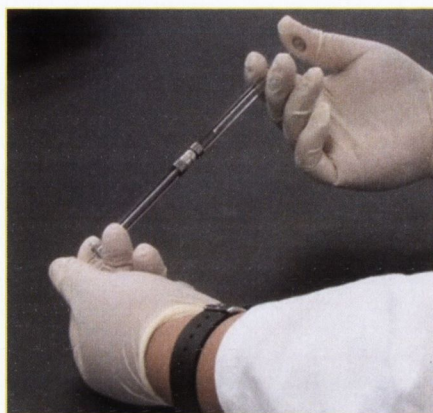


Figure 5.5 Image from a video protocol illustrating the construction of the lipidic mesophase. A gas-tight Hamilton syringe containing MO connected, through a coupler, to a second syringe containing the protein of interest, mitochondria in this case (Caffrey and Porter, 2010). Mixing was achieved by repeatedly moving the contents of the two syringes back and forth through the coupler. This mixing process generated, by spontaneous self-assembly, the cubic mesophase at or close to full hydration. The pure cubic phase was colorless, optically isotropic, transparent and viscous. These characteristics were used to confirm that the proper phase had been accessed.

5.3.4 Reduced and Oxidised Spectrum of Mitochondria

Rat liver mitochondrial sample was solubilized with 1% of sodium deoxycholate in phosphate buffer (KH_2PO_4 100 mM, pH 8) to avoid any problems that may be encountered with light scattering. Cytochromes of the respiratory chain were reduced by addition of several crystals of sodium dithionite to a 1 ml suspension of mitochondria (final concentration 0.25 mg/ml) and oxidized by addition of a few crystals of potassium ferricyanide. The differential spectrum was obtained by scanning from 400 to 600 nm using a dual beam UV-2600 UV-VIS Shimadzu spectrophotometer.

5.3.5 Absorption Spectrum of Reconstituted Mitochondria in the Mesophase

A small volume (5 μl) of cubic phase was placed on a quartz slide that was positioned over one of the wells in the region to be interrogated by the light beam. A sandwich was made of the mesophase by covering it with the second quartz slide. The two slides were secured to the bottom of the 96 well plate placed into SpectraMax M3 spectrophotometer. The absorption spectrum was measured in the appropriate range from 600 nm to 250 nm in 1 nm steps at 100 nm/min with air as the reference.

5.3.6 Measurement of Oxygen Consumption Rates

Oxygen in solution was measured polarographically with a Clark-type oxygen electrode. Clark electrodes have gold or platinum cathodes and silver or silver/silver chloride anodes, which are connected by a salt bridge and covered by an oxygen-permeable membrane. As oxygen diffuses across the membrane, it is reduced by a fixed voltage between the cathode and anode, which generates current in proportion to the concentration of oxygen in solution. By calibrating the voltage with known oxygen concentrations, it was possible to measure the rate of oxygen consumption in a medium containing actively respiring mitochondria (Lanza and Nair, 2009).

High-resolution respiration was performed in a two-chamber respirometer (Oroboros Oxygraph-2k). Two chambers were housed in a temperature-controlled copper block (temperature regulated and insulated). Each chamber was equipped with a polarographic oxygen sensor (POS). The amplified signal was recorded on a computer with parallel

online display of the calibrated oxygen concentration and flux (time derivative of the oxygen signal) at sampling intervals of 1–2 sec. The software DatLab (Oroboros) was used for data acquisition and analysis.

2 ml of respiration medium (135 mM Sucrose, 120 mM KCl, 5 mM KH_2PO_4 , 5mM HEPES, 2.5 mM MgCl_2 , 1 mM EGTA, pH 7.4) was added to the oxygraph chambers and allowed equilibrate with atmospheric oxygen at the required experimental temperature (30°C) with a stirrer speed of 750 rpm for >30 minutes to allow air saturation of the respiration medium. Rat liver mitochondria or sub-mitochondrial particles were prepared as described in Section 5.2.1 and 5.2.2, respectively, and added to the chamber (0.5 mg final concentration). The polyvinylidene fluoride stopper was inserted to generate a closed system with a final volume of 2 ml and baseline respiration was measured in the absence of exogenous substrates. Sequential additions of mitochondrial substrates and inhibitors can allow the study of different segments of the respiratory chain in one experiment.

Using a 25- μl Hamilton microsyringe, 10 mM glutamate and 5 mM malate were added to record resting complex I-supported respiration, without ADP. When using intact mitochondria, ADP was added at a saturating level (0.5 mM) to maximally stimulate respiration in the presence of glutamate and malate. Subsequent addition of 2 μM rotenone inhibited complex I by eliminating electron input to complex I. Addition of 10 mM succinate re-stimulated respiration by providing additional electron flow through complex II. Antimycin A was added at a concentration of 2 μM to inhibit cytochrome bc1 complex (complex III). By inhibiting the reduction of cytochrome c, antimycin A allowed non-mitochondrial respiration to be measured. TMPD is a reducing co-substrate and can act as an artificial electron mediator by passing electrons to complex IV (via cytochrome c). A volume of TMPD/Asc (0.5 mM/2 mM) was added to stimulate respiration. KCN (300 μM) was added to completely inhibit oxidation of all electron donors, thereby inhibiting at complex IV and terminating all oxidation.

Reconstituted mitochondria in mesophase were dispensed into the respiration chamber (2 mg final concentration). With the coupler still attached, the ‘toothpaste-like’ mesophase was injected as a string through the small needle of the coupler into reaction buffer that was already being stirred in the chamber. Surface area was naturally increased when the mesophase is inserted in the form of a string, allowing higher

accessibility of ETC enzymes to substrate/inhibitor additives as performed on intact and sub-mitochondrial particles.

The chambers were washed multiple times with water and with 100% ethanol and sterilized with 70% ethanol for 30 min at 37°C after each experiment and before the next respirometric run. Respiration rates were measured for each condition and were normalized to the amount of mitochondrial protein to allow comparison between pre and post reconstitution without the influence of differences in the amount of mitochondria loaded into the chamber.

5.3.7 Complex I Assay NADH:coenzyme Q oxidoreductase Assay

The specific activity of complex I was determined by the methods of Ragan et al., 1989, with modifications as described in Chapter 3, section 3.3.4.5. Intact rat liver mitochondria were thrice cryolysed in liquid nitrogen before 5 µg was added to a 96 well plate, in a final volume of 200 µl, assay buffer. In the case of reconstituted mitochondria, 10 µl (1 mg) of mesophase was dispensed using a Hamilton repeat dispenser along the inside of the well walls to avoid interference with the light path. Assay performed and specific activity of enzyme calculated as previously described.

5.4 Results

5.4.1 Reconstitution of mitochondria into the lipidic mesophase.

Following the steps outlined in 5.3.3, the assumption is that proteins of the electron transport chain were reconstituted into the bilayer of the cubic phase. The cubic phase was sticky and viscous akin to thick toothpaste and retained slight uniform yellow color of the reconstituted mitochondrial proteins but otherwise it was optically transparent. This characteristic enabled the mesophase to be further investigated spectrophotometrically.

It was important to assess the efficiency and success of the reconstitution process. Further evidence was needed to confirm that mitochondrial proteins were fully incorporated and residing in the lipidic mesophase. Due to the presence of cytochromes, mitochondria exhibit a very characteristic spectrum of oxidized and reduced states of the residential cytochromes.

To show that mitochondria in the mesophase could be oxidized or reduced would provide evidence for a successful reconstitution. Figure 5.6 shows the oxidized and reduced spectrum of intact liver mitochondria prior to reconstitution. The oxidized form has an absorption maximum at 420 nm while the spectra of the reduced state shows absorption maximum at 564 nm. The peaks γ and α are light absorption peaks characteristic to all cytochromes of the respiratory chain – cytochromes a, a_3 , b_H , b_L , c and c_1 . Cytochromes a and a_3 are found in complex IV, cytochromes b and c_1 are found in complex III and c is present in cytochrome c.

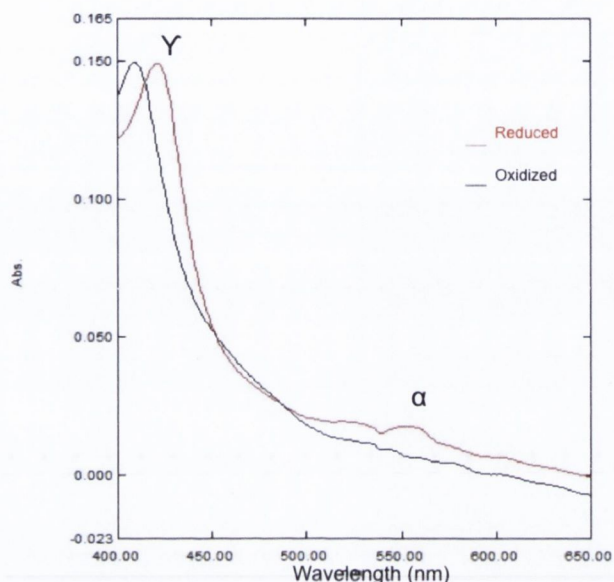


Figure 5.6 Representative oxidized and reduced spectrum of mitochondrial cytochromes in rat liver intact mitochondria. Rat liver mitochondrial sample was solubilized with 1% of sodium deoxycholate (DOC) in phosphate buffer (KH_2PO_4 100mM, pH 8). Cytochromes of the respiratory chain were reduced by addition of several crystals of sodium dithionite to a 1 ml suspension of mitochondria (final concentration 0.25 mg/ml) and oxidized by addition of a few crystals of potassium ferricyanide. The differential spectrum was obtained by scanning from 400 to 600 nm using a Cary 50 Bio spectrophotometer. The oxidized form has an absorption maximum at 420 nm while the spectra of the reduced state shows absorption maximum at 564 nm. The peaks γ and α are light absorption peaks characteristic to all cytochromes of the respiratory chain – cytochromes a, a_3 , b_H , b_L , c and c_1 .

Due to the consistency of the mesophase, it was not possible to monitor changes in the redox state of mitochondrial cytochromes in the reconstituted form using the dual beam UV-2600 UV-VIS Shimadzu spectrophotometer. To overcome this, a spectrum was attempted using a sandwich cuvette as described in Section 5.3.5 in a SpectraMax M3 spectrophotometer. Unfortunately, this instrument was not sensitive enough to detect the various redox states of the cytochromes in native mitochondria or reconstituted mitochondria. However, the optical absorbance of protein can be measured at 280 nm. At this wavelength, the absorbance of protein is *mainly* due to the amino acids tryptophan, tyrosine and cysteine. The absorbance of a solute depends linearly on its concentration, and therefore absorption spectroscopy is ideally suited for quantitative measurements. Protein absorption peaks at 280 nm could be detected and confirmed the present of mitochondrial protein in the mesophase (Figure 5.7).

5.4.2 Electron transfer and oxygen consumption in intact mitochondria reconstituted into the lipidic mesophase.

When placed in an oxygen electrode system isolated intact mitochondria are capable of limited respiration rates as they are depleted of NADH- and FADH₂-linked substrates for oxidation by the ETC. Adding substrates (glutamate, malate, succinate, TMPD/Ascorbate), ADP and phosphate to a suspension of mitochondria in an appropriate medium drives the mitochondria into state 3 respiration where the process of oxidative phosphorylation synthesizes ATP and consumes oxygen. The NADH- and FADH₂-linked substrates channel electrons and protons through these reducing equivalents to the electron transport chain. As protons are pumped through complexes I, III and IV electrons are terminally transferred by cytochrome oxidase complex, oxygen to form H₂O. Experimentally, the decline in total dissolved oxygen as oxygen is reduced to water can be used to measure the rate of electron transport. This process is subject to the limitations imposed upon the mitochondria by removal of intracellular or matrix environment induced by incorporation of the mitochondrial membrane proteins into the cubic mesophase. Therefore the aim of this study was to study the ability of reconstituted mitochondria to carry out electron transfer and oxygen reduction in the cubic mesophase lipid environment.

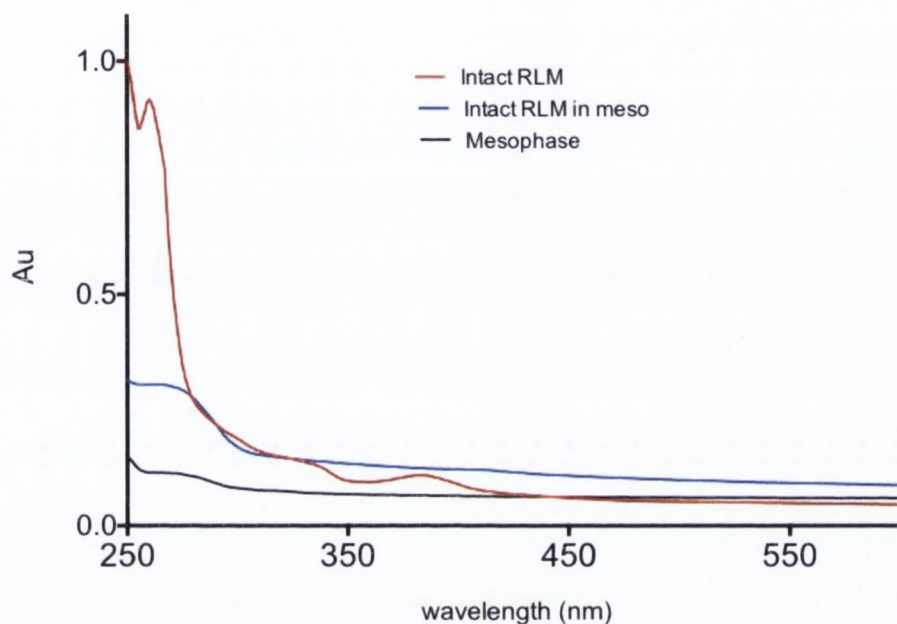


Figure 5.7 Absorption spectra of rat liver mitochondria in the lipidic mesophase.

A small volume (5 μ l) of protein-loaded mesophase was placed on a quartz slide that was positioned over one of the wells in the region to be interrogated by the light beam. A sandwich was made of the mesophase by covering it with the second quartz slide. The two slides were secured to the bottom of the 96 well plate placed into the spectrophotometer. The absorption spectrum was measured in the appropriate range from 600 nm to 250 nm in 1 nm steps at 100 nm/min with air as the reference. The blue line represents the spectrum obtained with protein-loaded mesophase. The red line represents the absorption spectrum obtained from the same volume and concentration of mitochondria, but in the native state (i.e. not reconstituted). To show that the mesophase was not responsible for any absorbance, an absorption spectrum was obtained using 5 μ l of protein-free cubic phase (black line).

Figure 5.8 shows oxygen consumption rates for intact mitochondria in the presence of glutamate/malate, succinate and TMPD/Ascorbate. Mitochondria mediated the NAD^+ linked oxidation of glutamate/malate. As the resulting NADH is oxidized by electron transport chain with O_2 as the terminal acceptor, the O_2 concentration decreased. Addition of succinate, which undergoes FAD-linked oxidation, also increased oxygen consumption rates. The rate of state 3 divided by state 4 is called the respiratory control ratio (R.C.R.) and indicates the tightness of the coupling between respiration and phosphorylation and gives an indication of the integrity and consequently the quality of isolated mitochondria. R.C.R. values between 5 and 9 were obtained for each preparation, confirming that isolated mitochondria were intact and coupled. Although NADH and FADH_2 are main physiological electron donors to the ETC, the non-physiological reagent, TMPD, was used to probe the flow of electrons. Tetramethyl-p-phenylenediamine (TMPD) is an ascorbate-reducible redox carrier that transfers electrons directly to cytochrome c and oxygen consumption was observed.

Intact mitochondria, when reconstituted into mesophase and placed in the chamber of the oxygraph were unresponsive to additions of glutamate and malate and ADP and also succinate. However, on addition of 1 mM NADH, an oxygen consumption rate was observed (Figure 5.9). Because intact mitochondria are impermeable to NADH, this suggests the reconstitution process disrupts the physiology of the inner membrane, dissipates the membrane potential and uncouples this system. Addition of NADH can now directly enter the NADH dehydrogenase component of complex I and promote electron transfer and ultimately oxygen consumption by complex IV.

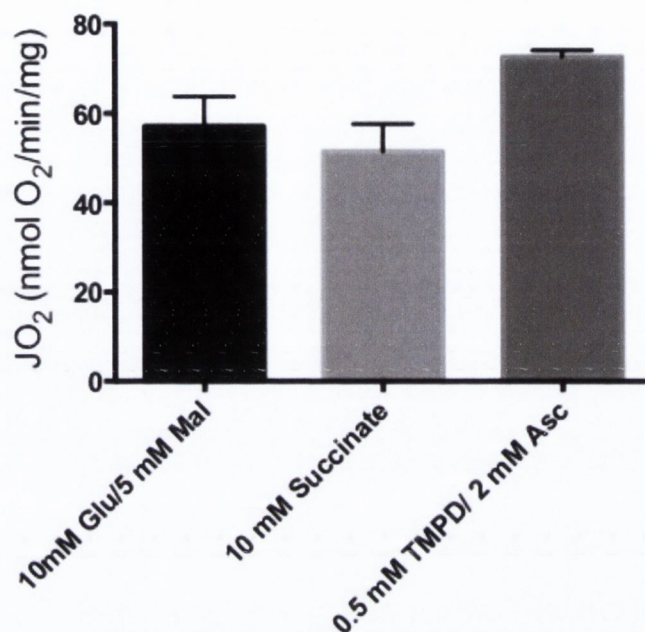


Figure 5.8 Respiration rates in intact rat liver mitochondria with glutamate/malate, succinate and TMPD/ascorbate substrates. Mitochondria (0.25mg/ml) were incubated with respiration medium (135 mM Sucrose, 120 mM KCl, 5 mM KH₂PO₄, 5mM HEPES, 2.5 mM MgCl₂, 1 mM EGTA, pH 7.4) in an Oroboros 2K respirometer (0.25 mg/ml). Baseline respiration was measured in the absence of exogenous substrates. 10 mM glutamate and 5 mM malate were added to record resting NADH-linked respiration. ADP was added at a saturating level (0.5 mM) to maximally stimulate respiration in the presence of glutamate and malate. Subsequent addition of 2 μ M rotenone inhibited complex I by eliminating electron input to complex I. A similar experiment was repeated, this time using 10 mM succinate, which provides electron flow through complex II. Antimycin A was added at a concentration of 2 μ M to inhibit cytochrome bc₁ complex (complex III). Again, this was repeated using TMPD/Asc (0.5 mM/2 mM) to stimulate respiration. Experiments were performed on a minimum of four individual preparations and results were expressed as mean $\pm$ SEM (error bars). Rates were calculated as JO₂ (nmol O₂/min/mg).

For NADH oxidation of intact mitochondria in the mesophase, a rate of around 3 nmol/O₂/min/mg was obtained. Therefore, the rate of oxygen consumption in liver mitochondria decreases fifteen-fold following reconstitution into mesophase. Addition of the complex I inhibitor, rotenone, saw a 15% reduction in oxygen consumption (Figure 5.9). As a control, intact mitochondria were pre-treated with rotenone prior to being reconstituted (Figure 5.9). In this way, complex I supported respiration should be almost irreversibly inhibited by rotenone before reconstitution and remain so inhibited following reconstitution. Oxygen consumption in this case was significantly ($p < 0.001$) reduced by 50% (Figure 5.9). This corresponds to a similar JO₂ when rotenone is added to non-reconstituted mitochondria.

NAD-linked oxidation of mitochondria in the mesophase decreased 50% upon addition of complex III inhibitor, Antimycin A (Figure 5.9). This would suggest that electron transfer has taken place from complex I to complex III via ubiquinone.

A 50% decrease in oxygen consumption was observed on addition of the complex IV inhibitor, KCN, suggesting oxygen consumption is being inhibited to a small degree here (Figure 5.9).

TMPD is a reducing substrate and can act as an artificial electron mediator by passing electrons to complex IV (via cytochrome c). When mitochondria were reconstituted and exposed to TMPD, oxygen consumption rates of 6 nmol/O₂/min/mg were observed (Figure 5.9). This rate was inhibited by KCN, indicating that the decrease in oxygen concentration is not due to autoxidation of TMPD, but in fact due to the transfer of electrons from cytochrome c to complex IV, where oxygen is reduced.

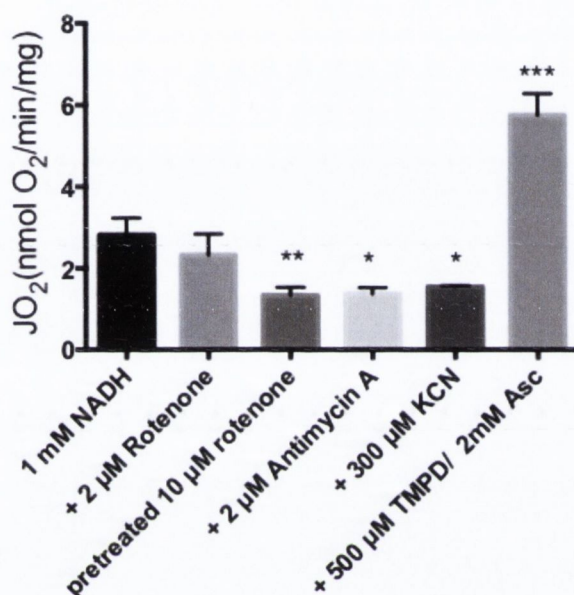


Figure 5.9 Electrons are transferred between the respiratory complexes when intact mitochondria are reconstituted into the lipidic mesophase. Reconstituted intact mitochondria were dispensed into the respiration chamber (2 mg/ml) and oxygen consumption driven by NADH was monitored and investigated using specific inhibitors for individual electron transport chain complexes as described in Section 5.3.6. Experiments were performed on a minimum of four individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using GraphPad Prism. Samples significantly different to the control are shown with * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

5.4.3 Electron transfer and oxygen consumption in sub mitochondrial particles reconstituted into the lipidic mesophase.

Having identified that the mechanical process of reconstitution disrupts the mitochondrial membrane, it was decided that using sub-mitochondrial particles would prove a better vehicle for NADH oxidation. Figure 5.10 shows oxygen consumption rates for sub-mitochondrial particles in the presence of 1 mM NADH, 20 mM Succinate and 0.5 mM TMPD. For NADH oxidation of SMPs in the mesophase, a rate of around 5 nmol O₂/min/mg was obtained (Figure 5.11). The preparation of sub-mitochondrial particles exposed the complex I site to NADH and when reconstituted, the rate of oxygen consumption was increased by 40% when compared to rate achieved from reconstituted intact mitochondria (Figure 5.9). Addition of the complex I inhibitor, rotenone, saw a 61% reduction in oxygen consumption (Figure 11 A, C). This was higher than the rate of inhibition observed in reconstituted intact mitochondria. As a control, SMPs were pre-treated with rotenone prior to being reconstituted into mesophase. In this way, complex I supported respiration should be almost irreversibly inhibited by rotenone before reconstitution and remain so inhibited following reconstitution. Oxygen consumption in this case was significantly ($p < 0.001$) reduced by 64% (Figure 5.11 B, C). Rotenone has the same effect on complex I mediated respiration in SMPs, pre or post reconstitution. When SMP were reconstituted and exposed to TMPD, oxygen consumption rates of 23 nmol/O₂/min/mg were observed (Figure 5.11 C). This rate could was inhibited by KCN, indicating that the decrease in oxygen concentration was not due to autooxidation of TMPD, but in fact due to the transfer of electrons from cytochrome c to complex IV, where oxygen is reduced. NAD-linked oxidation of SMPs in the mesophase decreased 33%, upon addition of complex III inhibitor, Antimycin A (Figure 5.12), slightly less than that observed in reconstituted intact mitochondria. This would suggest that electron transfer has taken place from complex I to complex III via ubiquinone. As with rotenone, SMPs were pretreated with Antimycin A prior to reconstitution and NADH oxidation observed. NAD-linked oxidation of mitochondria in the mesophase decreased by 47% in this case, similar to that observed in reconstituted intact mitochondria. NAD-linked oxidation of SMPs in the mesophase decreased by 80%, upon addition of complex III inhibitor, KCN (Figure 5.12).

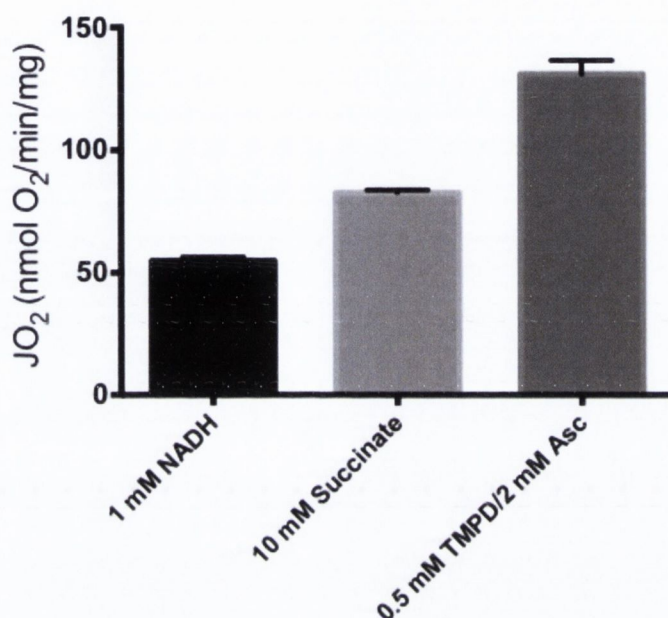
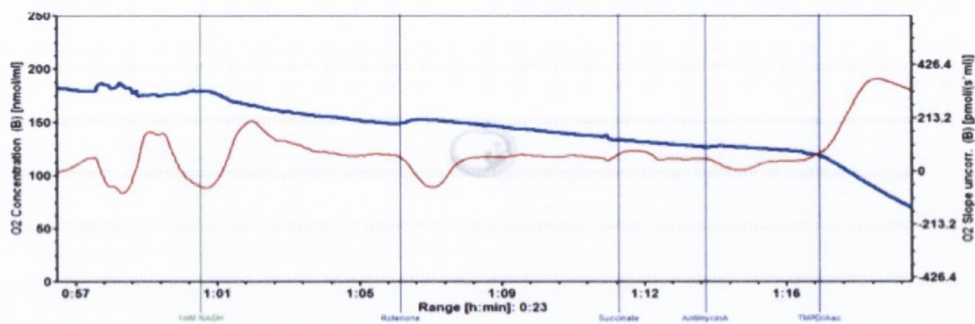


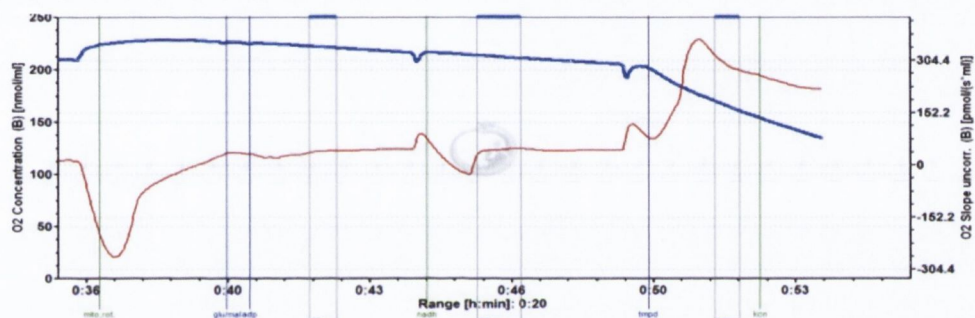
Figure 5.10 Sub-mitochondrial particles (SMP) are oxidized by NADH, Succinate and TMPD/Ascorbate. SMPs (0.25 mg/ml) were incubated with respiration medium (135 mM Sucrose, 120 mM KCl, 5 mM KH₂PO₄, 5mM HEPES, 2.5 mM MgCl₂, 1 mM EGTA, pH 7.4) in an Oroboros 2K respirometer. Baseline respiration was measured in the absence of exogenous substrates. 10 mM glutamate and 5 mM malate were added to record resting complex I-supported respiration. Subsequent addition of 2 μ M rotenone inhibits complex I by eliminating electron input to complex I. A similar experiment was repeated, this time using, 10 mM succinate, which provides electron flow through complex II. Antimycin A was added at a concentration of 2 μ M to inhibit cytochrome bc₁ complex (complex III). Again, this was repeated using TMPD/Asc (0.5 mM/2 mM) to stimulate respiration. Experiments were performed on a minimum of four individual preparations and results are expressed as mean $\pm$ SEM (error bars). Rates were calculated as JO₂ (nmol O₂/min/mg).

OVERLEAF: Figure 5.11 Inhibition of complex I prevents electron transport between the respiratory complexes and molecular oxygen occurs when SMP mitochondria were reconstituted into the lipidic mesophase. (A) Reconstituted SMPs were dispensed into the respiration chamber (1 mg/ml) and oxygen consumption driven by NADH was monitored and investigated using specific inhibitors for individual electron transport chain complexes as described in Section 5.3.6. The blue line indicates the oxygen concentration in the sample. The red line indicates its derivative, the oxygen consumption by the sample in the respiration medium. The responses to the addition of NADH (1 mM), Rotenone (2 μ M), and TMPD (0.5 mM) can be seen. **(B)** As a control, SMPs were pre-treated with rotenone prior to being reconstituted into mesophase. In this way, complex I supported respiration was inhibited. **(C)** Oxygen consumption rates were calculated from the traces in A and B. Experiments were performed on a minimum of four individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using GraphPad Prism. Samples significantly different to the control are show with *** for $p < 0.001$.

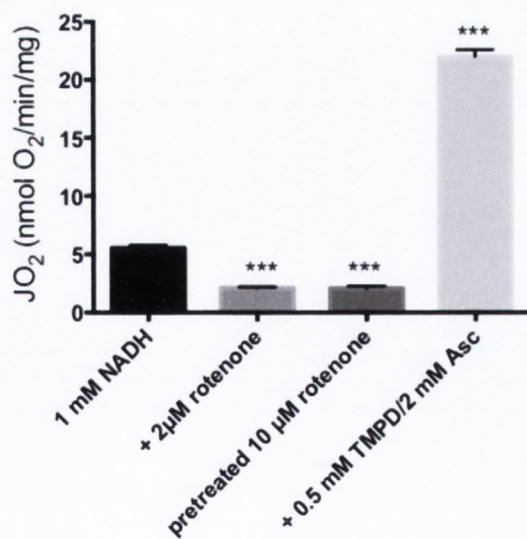
A



B



C



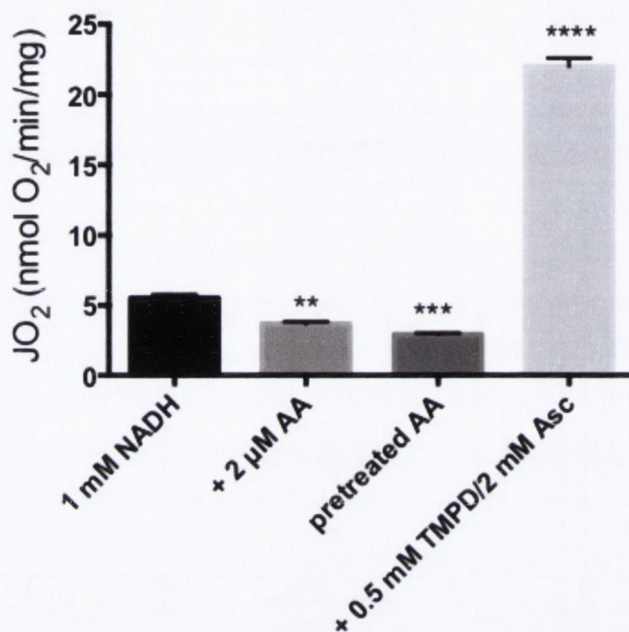


Figure 5.12 Inhibition of complex III prevents electron transport between the respiratory complexes and molecular oxygen when SMPs were reconstituted into the lipidic mesophase. Reconstituted SMPs were dispensed into the respiration chamber (1 mg/ml) and oxygen consumption driven by NADH was monitored and investigated using specific inhibitor of complex III, Antimycin A as described in Section 5.3.6. As a control, SMPs were pre-treated with Antimycin A prior to reconstitution and rates observed. Experiments were performed on a minimum of four individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using GraphPad Prism. Samples significantly different to the control are show with ** for $p < 0.01$; *** for $p < 0.001$.

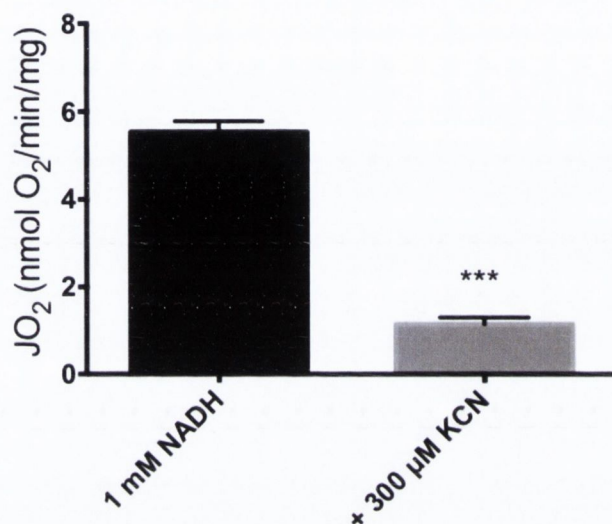


Figure 5.13 Electron transport between the respiratory complexes occurs when SMPs are reconstituted into the lipidic mesophase. Reconstituted SMPs were dispensed into the respiration chamber (1 mg/ml) and oxygen consumption driven by NADH was monitored and investigated using specific inhibitor of complex IV, KCN as described in Section 5.3.6. Experiments were performed on a minimum of four individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using GraphPad Prism. Samples significantly different to the control are show with *** for $p < 0.001$.

5.4.5 Detection of complex I activity in reconstituted mitochondria

Proteins reconstituted in the mesophase are known to retain functionality and activity. The viscous, porous nature of the cubic phase enables continuous spectrophotometric activity assays to be performed in a microplate format. Functional activity assays in meso have been performed in other membrane protein enzymes (Li and Caffrey, 2011). A coupled enzyme assay was used in the case of diacylglycerol kinase (DgkA). With phosphatidylglycerol phosphate synthase (PgsA), activity was quantified by direct assay. Complex I is considered the rate-limiting step for the mitochondrial respiratory chain and having already observed NADH oxidation, it was decided to investigate the properties of complex I in meso further, by enzymatic assay. Complex I activity was first monitored in intact mitochondria and rates observed were similar to previously published reports (Figure 5.14) (Petrosillo et al., 2007). Complex I activity was then monitored when intact mitochondria were reconstituted in mesophase and a rate of 6 nmol/min/mg was detected (Figure 5.14). As a control, intact mitochondria were pretreated with rotenone before reconstitution and then complex I activity assessed. This saw a reduced, almost negligible rate for complex I activity.

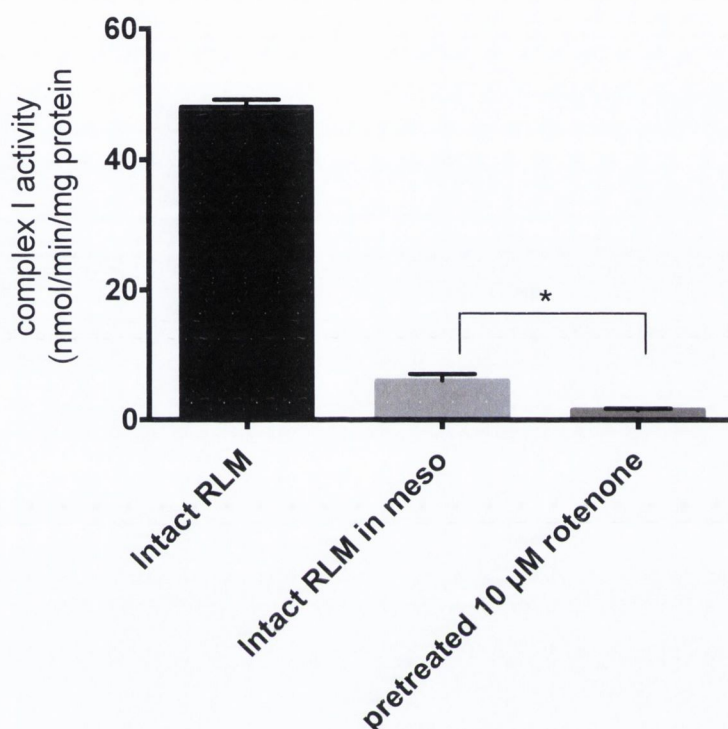


Figure 5.14 Complex I is enzymatically active and functional in reconstituted mitochondria. Intact rat liver mitochondria were thrice cryolysed in liquid nitrogen before 5 μ g was added to a 96 well plate, in a final volume of 200 μ l, assay buffer (25 mM Potassium Phosphate, 10 mM MgCl_2 , pH 7.4), 1 mM potassium cyanide, 150 μ M NADH, 25 mg/ml BSA. In the case of reconstituted mitochondria, 10 μ l (1 mg) of mesophase was added. The basal rates of ΔAbs_{340} were monitored in the samples for two minutes before the redox reaction was initiated by the addition of 50 μ M Decyl Q. The reaction was monitored for ten minutes before 10 μ M rotenone was added to each sample to inhibit complex I and obtain rotenone-insensitive rates. The specific activity of the enzyme was expressed as nmol of NADH oxidized/min/mg of mitochondrial protein. Experiments were performed on a minimum of three individual preparations and results are expressed as mean $\pm$ SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using GraphPad Prism. Samples significantly different to the control are show with * for $p < 0.05$.

5.5 Discussion

To date, the *in meso* method has been very successful in crystallizing purified membrane proteins. Monoolein is the lipid most commonly used in the *in meso* method. It is a membrane mimetic and reconstituted proteins that have been removed from their native membrane and reconstituted into the lipidic mesophase, can adapt to their new hosting membrane environment.

This is the first attempt to reconstitute, not just a purified protein but instead a whole organelle, containing approximately 1000 proteins into the lipidic mesophase. This was an ambitious undertaking and nothing similar has been attempted prior to this. The largest membrane protein so far that has been incorporated into the mesophase is Cytochrome *caa3* oxidase from *Thermus thermophilus*. This is a large 120 kDa heterotrimeric protein with 23 transmembrane helices and a cytochrome *c*-like domain as a C-terminal extension to one of its subunits.

But the default cubic mesophase is remarkably resilient and retains its phase identity in the presence of a vast array of different additives and it was hypothesized that it could hold the membrane proteins of the mitochondria. For this study, the focus was on reconstitution of the four large complexes of the mitochondrial electron transport chain.

Previous small scale X-ray scattering has provided evidence that when a protein of interest is combined with monoolein, the expected cubic-Pn3m phase is produced. This has been done with the vitamin B12 transporter (Cherezov et al., 2006), the invasin/adhesion OpcA (Cherezov et al., 2008) and gramicidin D (Liu and Caffrey, 2005). Quenching of intrinsic tryptophan fluorescence has also been used to demonstrate reconstitution of proteins in the lipidic mesophase (Li and Caffrey, 2011). The data presented in this study tentatively suggests that mitochondrial proteins have been removed from their native membrane and may reside in a new lipid environment (Figure 5.7). It may also be possible that they protein complexes have become trapped within the lipid bilayer.

Spectroscopic measurements have previously been made to examine the conformational state of membrane proteins reconstituted in the cubic phase (Cherezov et al., 2006). Circular dichroism spectra also confirms gross secondary structure of proteins is

insensitive to being in a bilayer environment. It is therefore accepted that cubic phase reconstitution does not dramatically alter the conformation of protein targets. However, proteins that are reconstituted into the bilayer of the cubic phase do so with no preferred orientation. This phenomenon was of particular concern when undertaking this study as it may interfere with electron transport between the individual respiratory chain complexes. However, evidence to the contrary is presented here and shows that reconstituted mitochondria can overcome this and perform a reduced level of electron transfer to molecular oxygen as measured by oxygen consumption rates. Respirometry experiments suggest that transport of electrons take place and are used by complex IV, the cytochrome oxidase complex, to reduce oxygen. The decline in total dissolved oxygen was used to measure the rate of electron transport and compounds that inhibit electron transport, as judged by their effect on oxygen consumption, proved invaluable probes in tracing the paths of the electrons through the transport chain.

This study predicts that the phospholipids and cardiolipin of the native mitochondrial membrane distribute randomly through the monoolein. Individual electron chain enzyme proteins also distribute randomly through the membrane, possibly with the protein and native lipids incorporating as one unit into the monoolein. Cytochrome c is a soluble protein and resides in the water channels. Previous study has shown that cytochrome c resides in the water channels and can be released from the monoolein with an aqueous diffusion coefficient (D_{aq}) of $7.0 \times 10^{-7} \text{ cm}^2/\text{sec}$. This is a similar rate to myoglobin and lysozyme; two well known cytosolic proteins (Clogston and Caffrey, 2005). It may also be possible that cytochrome c may be bound to one of the ETC proteins. Ubiquinone, a hydrophobic carrier is also predicted to now reside in the lipid environment of the mesophase.

Using all the collected data from respiratory experiments, the movement of electrons, that occurs when mitochondria are reconstituted could be traced and the proposed mechanism is illustrated in Figure 5.15. Complex I (NADH-ubiquinone oxidoreductase) receives electrons from NADH and appears to pass them on to ubiquinone. Ubiquinone is reduced to ubiquinol and electrons are then passed to complex III (Figure 5.9 and Figure 5.12). Complex III (Ubiquinol-cytochrome c reductase), an integral protein receives electrons from ubiquinol and passes them on to cytochrome c. Complex IV (Cytochrome c oxidase) receives these electrons and transfers them to molecular oxygen to produce water. The use of the substrate TMPD confirmed the presence of cytochrome

c (Figure 5.11 C). TMPD is a reducing co substrate and can act as an artificial electron mediator by passing electrons to complex IV (via cytochrome c).

The system was unresponsive to the addition of succinate, suggesting that it was unable to participate in electron transfer. This was an unexpected and confusing observation. Succinate Dehydrogenase (SDH) is part of both the citric acid cycle and respiratory electron transfer chain. It is the only oxidative phosphorylation complex to lack subunits encoded by the mitochondrial genome and the only respiratory complex to not pump protons across the IM during its catalytic cycle. Within the citric acid cycle, SDH oxidizes succinate to fumarate. In the respiratory chain, electrons are delivered into the quinone pool (Q) originating from succinate and transferred via FAD to Q. But the latter was not observed when mitochondria were reconstituted into the lipidic mesophase.

SDH The structure of the porcine heart SDH consists of a hydrophilic head that protrudes into the matrix compartment and a hydrophobic tail that is embedded within the IM with a short segment projecting into the soluble intermembrane space (IMS) (Sun et al., 2005). The hydrophobic tail contains two anchor subunits, Sdh C and Sdh D. The hydrophilic head consists of two subunits forming the catalytic core (SdhA, SdhB in mammals). The first hydrophilic subunit is SdhA contains a covalently attached flavin adenine dinucleotide cofactor and the succinate binding site. The catalytic dimer of the hydrophilic subunits transfers electrons from succinate to ubiquinone. However, reduction of ubiquinone requires the presence of the membrane anchoring subunits. The ubiquinone binding site is located in a gap composed of SdhB, SdhC and SdhD. Results in Figure 5.10 indicate that ubiquinone is present and transferring electrons from complex I to complex III. So, the absence of electron transfer in the presence of succinate is not due to absence of ubiquinone. Some other functional or structural flaw within the enzyme that results from the reconstitution process must be interfering in the process. A possible explanation may lie in the fact that succinate dehydrogenase is the only peripheral membrane protein out of the four electron transport enzymes which are all integral membrane proteins. Perhaps during the mechanical reconstitution process, it is somehow unable to integrate within the new lipid environment in a stable manner. The SDH catalytic dimer, which is lipophilic, may become detached from the hydrophobic subunits.

This would then render the enzyme redundant in transferring electrons to ubiquinone and ultimately complex III. It may be possible that the covalently attached FAD is lost in the reconstitution process but the flavin mononucleotide prosthetic group, present in complex I, is identical to FAD in complex I and it appears to be present (Fig 5.9), making this an unlikely explanation. A complex II spectrophotometric assay would need to be performed to confirm the presence of a functional enzyme that has been reconstituted in to the lipidic mesophase.

It is assumed that proteins reconstituted prior to crystallization retain functionality *in meso*. The viscous, porous nature of the cubic phase enables continuous spectrophotometric activity assays to be performed in a microplate format. Functional activity assays *in meso* have been performed in membrane proteins enzymes (Li and Caffrey, 2011). A coupled enzyme assay was used in the case of diacylglycerol kinase (DgkA). With phosphatidylglycerol phosphate synthase (PgsA), activity was quantified by direct assay. In the case of BtuB, functionality was examined by measuring substrate (cyanocobalamin) binding to the protein reconstituted into the cubic phase (Cherezov et al., 2006). Protein-free control samples showed no binding, whereas *in meso* BtuB-containing samples showed evidence of substrate uptake. Similar K_d values have been reported for the native membrane-bound and the micellarised form of the protein.

The results show that complex I is functionally active (Figure 5.14). However, activity was ten fold less than that observed in the native mitochondria. It is difficult to compare to previous enzymatic activities that have been performed on reconstituted proteins. Those reconstituted proteins have been carefully purified before reconstitution. In this case, hundreds of different proteins have been removed from their native membrane and placed into a new lipid environment. This may also explain why rotenone appears unable to completely inhibit complex I driven electron transfer. Perhaps, this new lipid environment means that diffusion rates of substrates and inhibitors have been altered affecting the overall activity of enzymes and electron transfer. These are preliminary results and will require further investigations. But the observation of complex I activity is very promising indeed.

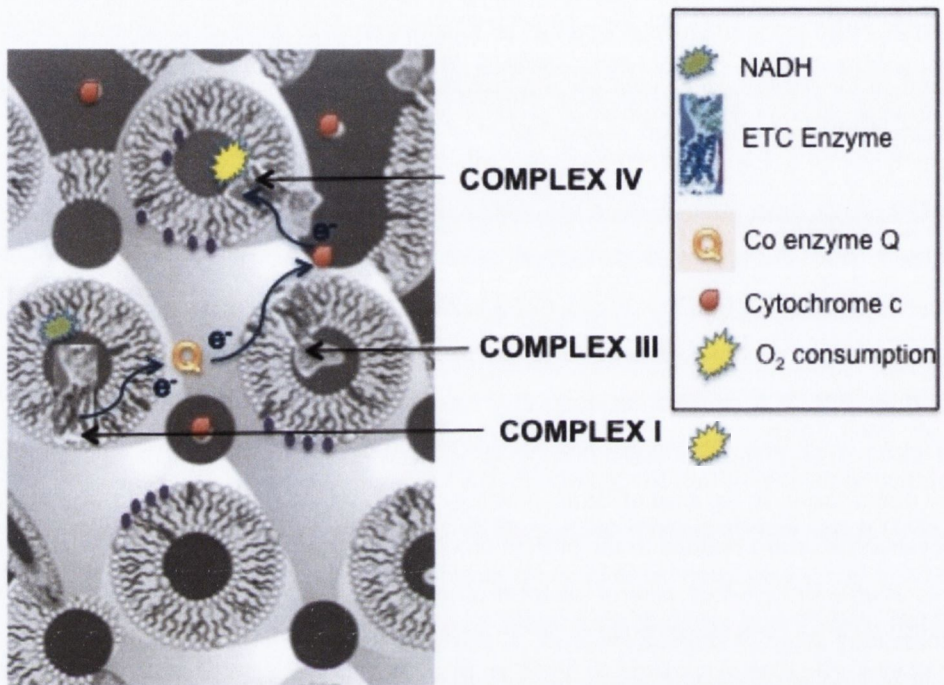


Figure 5.15 Proposed mechanism for the transfer of electrons between respiratory chain complexes in the lipidic mesophase. Phospholipids (purple oval with tail) and cardiolipin (large white oval with tail) of the native mitochondrial membrane distribute randomly through the monoolein (small white oval with tail). Complex I (NADH-ubiquinone oxidoreductase) receive electrons from NADH and appear to pass it on to ubiquinone (Q). Ubiquinone is now reduced to ubiquinol and electrons are then passed to complex III. Complex III receives electrons from ubiquinol and passes them on to cytochrome c (red). Complex IV receives these electrons and transfers them to oxygen to produce water (yellow star).

In conclusion, the data presented here suggests that the proteins of the electron transport chain reconstitute into the bilayer of the cubic phase in an active form. However, observed enzyme activity rates and oxygen consumption rates were disappointingly low. It may be a case that while this technique has been successfully used for the crystallization of purified proteins, the present system may not be suitable for assessing function of the organelle. However, with optimization and modification of this technique, it has the potential to provide insight regarding the mode of action and the physiological function of the protein at the molecular level. It also offers the possibility of studying proteins as they are incorporated within a lipid bilayer, akin to the native environment encountered in a biomembrane. Scientists are always seeking to understand how these membrane proteins function at a molecular level. Mitochondrial dysfunction is a major factor in many different pathologies and scientist are constantly endeavoring to find new ways to understand why it happens. There are then many practical uses to accumulating this information; drug design is an obvious outcome. An example of this, using the lipidic mesophase, is the viral membrane protein neuraminidase. Having determined its crystal structure, successful anti flu drugs such as Tamiflu was developed (Laver et al., 1999). This study is the first of its kind and creates the possibility of finding a new method, which can be used to gain more knowledge about a very intricate and fascinating organelle, the mitochondrion.

CHAPTER 6

GENERAL DISCUSSION

6.1 General Discussion

Mitochondria serve as central hubs of energy metabolism in the cell, transforming the energy deriving from oxidation of substrates into the chemical energy required to perform work, establish ion gradients, and carry out biosynthetic reactions. However, their role extends well beyond that of energy generation and encompasses signaling pathways involved in cell life and death, disease progression, gene expression patterns, protein posttranslational modifications and cellular redox balance. Despite being one of the most intensely studied of the organelles, mysteries still linger with respect to the molecular components involved in the regulation of mitochondrial function, how mitochondrial biogenesis is tuned to the metabolic state of the organ and organism, the dynamics of mitochondria within the cell, and the mechanisms of cross talk between mitochondria, other cellular organelles, the cytoskeleton, and the plasma membrane.

This research thesis takes on the challenge of deciphering the nature of proteins present in brain mitochondria and the arrangement of the electron transport chain complexes within the inner mitochondrial membrane. It attempts to extend knowledge on mitochondrial behavior in the brain, the most complex and heterogeneous organ of the body. In the human brain, there are approximately 10-100 billion neurons and about 1000-10,000 synapses per neuron (Pelvig et al., 2008). So for synaptic functions, proper mitochondrial structure and function is necessary. The location of the mitochondria within the neuron is also of great importance. This study focuses primarily on two populations of brain mitochondria: SM and NSM. SM are predominately neuronal and presynaptic in origin whereas NSM originate from other parts of the neuron and other cell types including glia and astrocytes.

To date, the rat is an accepted model for studying human neurological diseases and so was the chosen model for this research thesis. Classic electron microscopy techniques have revealed morphological differences in mitochondria from different cells. However, despite the apparent physiological diversity, little is known about the molecular basis for the differences and the extent to which mitochondrial composition varies across different tissues. Emerging proteomic technologies can now investigate these properties that may give insight to and further the understanding of mitochondrial mechanics. Mitochondria are ideal targets for proteomic analysis because they have a manageable

level of complexity, a consequence of their apparent prokaryotic ancestry. Their endosymbiotic origins have been preserved in their double membrane structure and they possess their own circular genome with mitochondria specific transcription, translation and protein assembly systems. Chapter 3 characterized the differences between SM and NSM using a quantitative proteomic approach. The data presented indicates that global proteomic differences exist between the two populations of brain mitochondria. The different protein expression levels identified for each population further confirm the heterogeneity that exists in the biological processes and molecular functions in SM and NSM. The synapse relies on high ATP production for vesicle mobilization and maintenance of membrane potential. This was reflected in the high expression of proteins involved in regulation of action potential, membrane potential, ion homeostasis and overall cellular homeostasis. These proteomic alterations observed in synaptic mitochondria allow the organelle to meet the persistent high-energy demands of the synapse. Ironically, these same proteins may also lead to dysfunction in synaptic mitochondria. The results also give us insight into the cross talk between mitochondria, other cellular organelles, the cytoskeleton, and the plasma membrane, an area that is still poorly understood.

Scientists are now taking a holistic approach when endeavoring to understand protein-protein interactions. Presently, the cell is viewed as an assembly of molecular machines made up of proteins interacting harmonically with one another to exert a function. Within mitochondria, the association of more than two binding partners introduces a new level of complexity in their assembly properties. A model of supramolecular organization based on specific interactions between individual respiratory enzymes is now favored (Acin-Perez et al., 2008, Krause et al., 2005, Reifschneider et al., 2006, Wittig and Schagger, 2009). An immunoprecipitation experiment carried out in Chapter 3 reaffirms that complex I, complex III and complex IV exist in conjunction with each other. But it also brings to attention that complex V physically interacts with either complex I or possibly the I+III+IV supercomplex. To date, it is still unclear whether the observation of a direct association between supercomplexes and the ATP synthase is authentic or just an artifact due to the electrophoresis process. A recent group used cryo-electron tomography to study the molecular arrangement of large respiratory chain complexes in mitochondria from bovine heart, potato, and three types of fungi (Davies et al., 2011). ATP synthase dimers and complex I was found to occupy different regions

of the mitochondrial cristae. In all species, the ATP synthase dimer rows were found exclusively on tightly curved cristae edges and the respiratory chain proton pumps were arranged on flat cristae membranes. The rapid consumption of protons by the ATP synthases at the cristae ridges may establish a proton gradient along the membrane, by which protons may tend to flow from source to sink, instead of diffusing equally in all directions. Therefore, supramolecular organization of respiratory chain complexes as proton sources and ATP synthase rows as proton sinks in the mitochondrial cristae ensures optimal conditions for efficient ATP synthesis.

One of the main highlights of this research thesis is that while the total content of complex I is the same, the amount of complex I-containing supercomplexes is higher in NSM than in SM. Therefore, complex I-containing supercomplex assemblies are dynamic and heterogeneous in different types of mitochondria and it is this heterogeneity that could influence energy metabolism. Thus, while supercomplex assembly is more numerous in NSM it reduces in quantity as the mitochondria travel down the axon to the synapse. These results could assist in the understanding understand why SM exhibit lower complex I energy thresholds, increased oxidative damage and are more vulnerable to calcium insult than NSM.

Chapter 3 also describes in detail the *in vivo* interactors of complex I in NSM and SM. Apart from ETC components, the study identifies many proteins from the mitochondrial inner and outer membranes and the matrix as well as a large number of chaperones and proteins involved in the mitochondrial tubular network formation and cristae morphology. One protein, phosphorylase b kinase was identified as a unique interactor of complex I in NSM. It's association with NSM is intriguing as it may be involved in the phosphorylation of substrates not known to now, leading to differences in energy metabolism. Two unique interactors of complex I in SM were identified, glioblastoma amplified sequence and piccolo. The former is a mitochondrial protein with unknown function. Piccolo, is a large scaffold protein and a core component of cytomatrix at the active zone of synapses (Fejtova et al., 2009).

With respect to aging, it is still unclear whether harm to the mitochondria precedes or results from disease processes. Recent studies suggest that it is the former scenario (Ross et al., 2013, Podlesniy et al., 2013). On this basis, unraveling the mechanisms

behind normal brain mitochondrial functions will help understand what precipitates mitochondria dysfunction and ultimately allow the scientist to intervene and potentially delay or reverse this malfunction.

Continuing on from our observation in Chapter 3, the fate of supercomplex assembly was assessed in aging SM and NSM. The study reports a decrease in the supercomplex assembly I+III₂+IV₁ in both SM and NSM. This is in agreement with an emerging trend of complex I-containing supercomplexes undergoing alteration in the aging process (Dencher et al., 2007, Gomez et al., 2009, Frenzel et al., 2010). Age associated destabilization of respiratory supercomplexes would promote production of oxidants and oxidative damage. This study proposes that the observed destabilization may lead to a possible decrease of complex I assembly. Both the lack of efficient electron channeling and the loss of complex I may decrease NAD-linked respiration and ATP synthesis and lead to increased mitochondrial production of reactive oxygen species.

Another possible contributing factor to the loss of supercomplexes may be an age related alteration of the mitochondrial proteome. Chapter 4 explores alterations in the proteomic profile of SM and NSM from aged rats and yielded many exciting and novel results. Thirty-four proteins were identified with differential expression and eight proteins showed evidence of post-translational modifications in the aging proteome. Within the proteomic screen, a decrease in PHB1 and PHB2 in aging synaptic mitochondria was identified. Prohibitins regulate the assembly of ETC and this ablation in their expression may have a knock on effect on supercomplex assembly and explain the observed decrease in aging mitochondria.

Chapter 4 also highlights the dynamic synaptic mitochondrial proteomic changes that occur with aging in rats and results were consistent with a recently published study on mouse brain mitochondria (Stauch et al., 2014a). The present study can confirm that a potential proteome shift in 22-month-old rats back to 6-month-old mitochondrial expression pattern takes place. This change may be necessary for maintenance of ATP production and protection against increase oxidative stress at the synapse. The proteomic changes observed in old synaptic mitochondria compared to corresponding levels in young synaptic mitochondria may allow the cell to adapt to the functional requirements and counteract age-associated cellular stress at the synapse. This study

concludes that within mitochondria, a sophisticated and tightly regulated proteomic response is stimulated in the aging animal to coordinate a mitochondria homeostatic mechanism. This research thesis raises the challenge to view mitochondria as vital targets for therapeutic treatment of human diseases, including aging and age related disorders.

Chapter 5 presents a novel and intriguing reconstitution trial in which mitochondrial function can be studied. The preliminary results achieved show great promise. Submitochondrial particles, reconstituted into the lipidic mesophase results adapt to their new lipid environment. This is evident as the ability to transfer electrons between individual complexes of the respiratory chain was observed. This is an intriguing observation and the potential to go further using this method is unlimited. The high quality reconstitution of mitochondria into the lipid bilayer could be a prerequisite for various biochemical and biophysical studies of mitochondrial membranes. It offers the possibility of studying proteins as they are incorporated within a lipid bilayer, akin to the native environment encountered in a biomembrane. This trial is the first of its kind and potentially provides a new vehicle in which the scientist can gain more knowledge about what is a very intricate and fascinating organelle: the mitochondrion.

6.2 Future Work

Chapter 3 and Chapter 4 characterized the differences between SM and NSM using a quantitative proteomic approach. Findings of the present study provide the basis for further experiments on the role of brain mitochondria proteins in different cellular environments. The quantitative study yielded a large number of proteins that would require further analysis and a more structural approach taken. A lot of these pathways need to be explored systematically. One way to achieve this would be to perform GO trimming to exclude some of the redundant terms in the lists of enriched GO categories identified in the present study. This method should free up the researcher from analyzing overly large, redundant lists, and instead enable the concise presentation of manageable, informative GO lists. From this cleaner list, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database of direct (physical) and indirect (functional) associations could be used to analyze the protein-protein interaction (PPI) of all the proteins identified in the current study and give a global view of the interactions taking place.

One protein that stands out from the iTRAQ study and warrants further research is Claudin 11. Initial identification in Chapter 3 first brought Claudin 11 to our attention, as it is not a recognized mitochondrial protein. But subsequent identification of it during the aging study confirmed its authenticity and increased interest in its purpose and relationship with synaptic mitochondria and a possible role in aging. This research study reports a 4-fold increase of claudin 11 in SM compared to NSM. Claudins are integral membrane proteins and components of tight junctions. Claudin 11 is a major component of the CNS myelin and plays a role in proliferation and migration of oligodendrocytes. It associates with microtubules and plays a role in membrane/cytoskeleton interactions during the formation of myelin (Dyer et al., 1991). Depolarisation at the synapse leads to an increase in post-synaptic calcium influx. Oligodendrocyte processes that may involve claudin 11 take place at sites of high calcium release and accumulated mitochondria (Simpson et al., 1997). Synaptic mitochondria may modulate these Ca^{2+} release processes. This may explain why claudin 11 was identified in the proteomic screen of synaptic mitochondria. Western blotting for the protein in both populations of mitochondria should validate the proteomic observations. Immunoprecipitation would shed light on any potential *in vivo* interactors. However, this research study may also

have highlighted claudin 11 an important player in the aging process. A 12-fold increase of claudin 11 in adult synaptic compared to young synaptic mitochondria was identified. These observations alone suggest claudin 11 serves an important regulatory role in synaptic mitochondrial function. A role for claudin 11 as an auto antigen in autoimmune demyelinating disease has been reported but our observations suggest further exploration into a possible role in the natural aging process (Bronstein et al., 2000).

The 2-DE proteomic study in Chapter 4 also highlighted a couple of proteins that demand further investigation. ACAD9 and its role as a complex I assembly factor in synaptic mitochondria is poorly understood. This study suggests that it is specific to synaptic mitochondria and why this is the case is unclear. One way to investigate this would be to use live imaging of a neuron and fluorescently tag complex I and ACAD9 with antibodies to see if they co-localise. It would then be interesting to track their movement as the mitochondria travel down the axon to the synaptic terminal. It would also be interesting to knockdown ACAD9 in differentiated and undifferentiated PC12 cells and using BN-PAGE, determine if there is any affect on supercomplex assembly. In addition, by knocking it down in primary neurons and performing fusion experiment using confocal microscopy, alongside membrane potential and complex assay, it may be possible to determine if ACAD9 knockdown reduces fusion rates with alteration on membrane morphology and membrane potential.

The 2-DE study in Chapter 4 also highlighted a great reduction of OPA1 in aging mitochondria. This would have a significant effect on morphology and function of the mitochondria. Therefore, it is essential to consolidate these findings. One way to achieve this would be to perform immunoblotting with an OPA1 primary antibody on these aging samples. The confirmation of decreased OPA1 in aging mitochondria would be an exciting result and explain greater susceptibility of mitochondria to apoptosis in the aged animal.

The importance of complex IV subunit Vb in supercomplex assembly could be further explored. Chapter 3 reports it as a complex I interactor specific to non-synaptic mitochondria. Again, this observation would need to be confirmed by another method. Immunoblotting for the protein in NSM and SM may authenticate these results.

Chapter 5 introduced a new proposed mechanism by which to study mitochondrial function. The results presented were preliminary observations and will require further investigation. A sensible starting point would be to perform additional inhibitor controls. It would be suitable to treat submitochondrial particles with KCN prior to reconstitution. The absence of JO_2 rates on addition of NADH would authenticate rates previously observed within the mesophase. The same applies when exploring complex I activity spectrophotometrically. The absence of mesophase rates in both systems would authenticate the preliminary results and allow the confident conclusion that electron transfer takes place between the enzymes of the respiratory chain that have been reconstituted into the lipidic mesophase.

In all the preliminary experiments, crude mitochondria were used as the source of electron transport chain. Mitochondria can contain up to 1000 proteins, so reconstituting the whole organelle is quite ambitious and may deflect from the mechanism that is being explored, i.e. electron transport. Throughout this research study, native gel electrophoresis plays an important role in understanding oxidative phosphorylation. Native Page Gel Electrophoresis enables isolation of individual electron transport chain complexes and supercomplexes. It would be very interesting to combine this technique with this the lipidic mesophase. The reconstitution of pure, individual enzymes of the electron transport chain into the lipidic mesophase should enhance already reported observations. Therefore, it would be suggested to perform blue native page on submitochondrial particles. The resolved bands corresponding to native respiratory enzymes could be excised. The next step involves combining the excised bands and eluting the protein. This elute contains a mixture of all proteins necessary to perform electron transport and can be reconstituted into the mesophase. Oxygen consumption on addition of NAD-linked substrates can then be investigated. If a positive result emerged, the limitations to this novel technique are endless. Mitochondria from any tissue could be studied and explored in such a manner.

The understanding behind the importance of supercomplex assemblies is still in its early stages of research. I predict this area to become a key player in understanding many metabolic disorders and neurodegenerative diseases. It would be very interesting to explore the fate of supercomplexes in HeLa cells. Cancer cells are characterized by their

increased glycolysis and reduced OXPHOS. Therefore, it would be intriguing to investigate if these characteristics correlate with loss of supercomplex assemblies.

To conclude, this research thesis presents some important results relating to oxidative phosphorylation in SM and NSM, thus contributing to our understanding of mechanism behind energy production in brain mitochondria. It is anticipated that further studies will address issues that have emerged in this thesis and hopefully contribute to mitochondrial research in the context of cell biology, aging and neurodegeneration.

6.3 Conclusions of the Thesis

These studies have investigated in detail the mechanisms behind the observed heterogeneity of brain mitochondria. The most significant conclusions that can be drawn from this work are:

1. The quantities of respiratory chain supercomplexes differ in synaptic and non-synaptic mitochondrial inner membranes.
2. As brain mitochondria age, they show dissociation of these assembled supercomplexes.
3. Proteomic profiles of synaptic and non-synaptic mitochondria reflect the ability of these organelles to adapt to their cellular environment.

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