Uncovering the Cause of A Novel Neurological Syndrome: Autosomal Recessive Non-Progressive Dystonia-Myoclonus with Sensorineural Hearing Impairment

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Institute of Medical Sciences IMS
University of Toronto

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Uncovering the Cause of A Novel Neurological Syndrome: 
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2014

Abstract

Mitochondrial disorders manifest with a spectrum of presentations, most of which are progressive. Mitochondrial and nuclear DNA encode proteins that regulate mitochondrial functions; genetic defects in these proteins cause human diseases. A combination of dystonia, sensorineural hearing impairment, and progressive encephalopathy is described in mitochondrial depletion syndrome V, where a nuclear DNA encoded SUCLA2 gene mutation leads to a mitochondrial depletion phenotype. We describe a family manifesting autosomal recessive congenital sensorineural hearing impairment, with a non-progressive childhood-onset movement disorder.

In this thesis, I identify a novel mutation in SUCLA2 causing a unique phenotype. The disease is allelic to mitochondrial depletion syndrome type-V but is associated with normal mitochondria as evidenced by muscle histology and biochemical testing. I describe the clinical features and elucidate the pathological basis of this genetic disorder. Then, I explore and implement a rational therapy. The knowledge gained from this study affords significant insights into mitochondrial function.
Acknowledgments

Firstly, I would like to thank my supervisor and mentor, Dr. Berge Minassian.

His guidance bolstered my confidence and his teaching and questioning style fostered my passion for science and a thirst for knowledge. His ongoing support has changed my career path from clinician to clinician-researcher.

I would also like to thank Dr. Carter Snead, my mentor and committee member. His advice has given me motivation and support. His respect and trust in my decision-making gave me reassurance that I can do what initially seemed impossible and succeed.

I would like to especially acknowledge Dr. Lucy Osbourne, my committee member. Her honest opinion is what helped me reach my goal and change the way I look at science from a clinician’s point of view to a scientist’s point of view. I appreciate this guidance.

I would like to thank the group at The Center for Applied Genomics, The Hospital for Sick Children. Their professional and friendly attitude helped ease the learning process and their discussion style built in me the interest to acquire more knowledge in that field.

Going through tough periods in life make you build true friendships with strong bonds that last a lifetime. The friends I met in the Minassian laboratory have only enriched my experience and I couldn’t have completed this work without them and their support.

I would also like to thank the patients and their families for their trust in me. This gave me the push to go on with this work and the will to explore facts in depth.

A very special thanks to my family and the sacrifices they made to help me succeed in this endeavor.
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AUB</td>
<td>American University of Beirut</td>
</tr>
<tr>
<td>A(_{2a}) R</td>
<td>Adenosine 2a receptors</td>
</tr>
<tr>
<td>aCGH</td>
<td>array Comparative Genomic Hybridization</td>
</tr>
<tr>
<td>BG</td>
<td>Basal Ganglia</td>
</tr>
<tr>
<td>BAEP</td>
<td>Brainstem Auditory Evoked Potentials</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CoQ10</td>
<td>Coenzyme Q10</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>POLG</td>
<td>DNA polymerase Gamma</td>
</tr>
<tr>
<td>D(_2) R</td>
<td>Dopamine 2 receptors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ENK</td>
<td>Enkephalin</td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>FADH2</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma Amino Butyric Acid</td>
</tr>
<tr>
<td>mGlu 5 R</td>
<td>Glutamate mGlu 5 receptors</td>
</tr>
<tr>
<td>GP</td>
<td>Globus Pallidus</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus Pallidus externa</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus Pallidus interna</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber’s Hereditary Optic Neuropathy</td>
</tr>
<tr>
<td>LS</td>
<td>Leigh Syndrome</td>
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<tr>
<td>LLS</td>
<td>Leigh Like Syndrome</td>
</tr>
<tr>
<td>LDYT</td>
<td>LHON with prominent dystonia</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>MSNs</td>
<td>Medium-sized Spiny Neurons</td>
</tr>
<tr>
<td>MMA</td>
<td>Methylmalonic Aciduria</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometers</td>
</tr>
<tr>
<td>mt-DNA</td>
<td>Mitochondrial-DNA</td>
</tr>
<tr>
<td>MTDPS5</td>
<td>Mitochondrial DNA depletion syndrome 5</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondral Myopathy Encephalopathy Lactic acidosis and Stroke-like episodes.</td>
</tr>
<tr>
<td>MRCD</td>
<td>Mitochondrial Respiratory Chain Disorders</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine Oxidase</td>
</tr>
<tr>
<td>MERRF</td>
<td>Myoclonus Epilepsy with Ragged-Red fibers</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NDPK</td>
<td>Nucleotide Diphospho Kinase</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-Methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation System</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PAS</td>
<td>Periodic Acid-Schiff</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PPN</td>
<td>Pedunculopontine Nucleus</td>
</tr>
<tr>
<td>QD</td>
<td>Quality depth</td>
</tr>
<tr>
<td>RC</td>
<td>Respiratory chain Complexes</td>
</tr>
<tr>
<td>RF</td>
<td>Reticular Formation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia Nigra</td>
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<tr>
<td>SNc</td>
<td>Substantia Nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia Nigra pars reticulata</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic Nucleus</td>
</tr>
<tr>
<td>SB</td>
<td>Strand Bias</td>
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<tr>
<td>SUCLA2</td>
<td>Succinyl-CoA Ligase 2</td>
</tr>
<tr>
<td>SCS</td>
<td>Succinyl-CoA Synthetase</td>
</tr>
<tr>
<td>SC</td>
<td>Superior Colliculus</td>
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<tr>
<td>Acronym</td>
<td>Term/Mnemonic</td>
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<td>SMA</td>
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<td>VTT</td>
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<td>VMAT2</td>
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Contributions

Whole Exome Sequencing was performed at The Center for Applied Genomics, The Hospital for Sick Children. The data analysis was performed by the author.

Mitochondrial Respiratory Chain Assay was performed at Dr. Brian Robinson’s laboratory, The Hospital for Sick Children. SUCLA2 functional enzyme assay was performed at the Laboratory of Genetic Metabolic Diseases, Enzyme Diagnostic section, Academic Medical Center, Amsterdam, The Netherlands.
Chapter 1
Introduction to the mitochondria and the basal ganglia

Movement of the human body involves complex processes that are orchestrated in part by important interactions between the cerebral cortex and the basal nuclei, to produce coordinated outputs in response to inputs received from central and peripheral afferent neurons. Many types of movement disorders are known to be due to a defect in a single gene and are referred to as monogenic (Lohmann and Klein 2013), manifesting autosomal recessive, autosomal dominant, mitochondrial, or X-linked inheritance patterns. Mitochondria are important cellular components that are responsible for energy generation. The pivotal role of mitochondria in controlling cell life, and death, are established (Lee and Wei 2000). Disruption of electron transport results in oxidative phosphorylation deficits with a consequent impairment of ATP production and decline in energetic capacity (Green and Reed 1998). This cascade of events triggers cell death via necrosis and neurodegeneration. Mitochondrial dysfunction is often suspected in neurodegenerative disorders with basal ganglia involvement, because of a poorly understood selective vulnerability of neurons in this location to the metabolic stress resulting from mitochondrial insufficiency (Nishino et al., 2000). A combination of factors have been proposed to be a cause of the selective striatal vulnerability, including glutamatergic excitotoxicity, and dopamine cytotoxicity (Nishino et al., 2000). The striatum is the only region where massive glutamatergic inputs and massive dopaminergic inputs converge. Glutamate and dopamine are important neurotransmitters in the brain, but when concomitantly over released they can act as neurotoxins (Olney and de Gubareff 1978; Ben-Shachar, Zuk, and Glinka 1995).

The human brain has been called the most complex structure of the known universe. Over 90% of human genes participate in the formation and function of the brain. Study of divergences from normal, i.e., of disease, helps us understand the normal state. Study of such a complex system, generally, is most informative when a single variable is modified and effects on the system analyzed. In this vein, studying the brain of a person who has a naturally occurring single brain function related gene mutation is crucial to uncovering the basic mechanisms of how the nervous system functions. In the present work, I describe a new mitochondrial movement disorder inherited in an autosomal recessive Mendelian fashion. I identify its causative single
gene defect. I initiate an understanding of its pathogenesis and I explore possible therapies. Knowledge granted from this study opens avenues for novel therapeutic approaches in a number of mitochondrial diseases.

1.1. Mitochondrion structure and function

Mitochondria are double membrane-enveloped intracellular organelles that are the principal source of energy in human cells. This energy is released by the oxidation of nutrients to produce adenosine triphosphate [ATP] via the oxidative phosphorylation system [OXPHOS pathway]. This pathway comprises reactions carried out by five enzymatic complexes within the mitochondria [respiratory chain complexes or RC].

Mitochondria are present in almost all eukaryotic cells. Their structure is comprised of both proteins and lipids and they measure between 0.5 to 1.0 micrometers [µm] in size. The number of mitochondria that are present in any particular cell varies. Highly energy-consuming tissues such as brain, skeletal muscle, and heart muscle may contain up to hundreds or thousands of mitochondria, while other cells may contain only a single mitochondrion.

In addition to the mitochondrion’s role as the main supply of chemical energy in the form of ATP (Jiang and Wang 2012), it also has a vital role as the main intracellular calcium reservoir. Finally, the mitochondrion has a significant role in controlling the cell cycle, by promoting both cell growth and cell death (Chipuk, Bouchier-Hayes, and Green 2006; McBride, Neuspiel, and Wasiak 2006).

An individual mitochondrion is composed of several compartments, namely the intermembrane space, the inner and outer membranes, the matrix and the cristae, each compartment having a unique function (Chipuk, Bouchier-Hayes, and Green 2006; Herrmann and Neupert 2000) [Figure 1-1].
Mitochondria have their own genetic material, and generate their own RNA and proteins. Mitochondrial DNA contains over 16,569 base pairs encoding 37 total genes, including 13 peptide genes. The 13-mitochondrial peptides in humans are integrated into the inner mitochondrial membrane, along with proteins encoded by genes present in nuclear DNA.

1.1.1. Mitochondrial structure

1.1.1.1. The outer mitochondrial membrane

The outer mitochondrial membrane encloses the entire organelle. It is composed of phospholipids and contains a large number of membrane integral proteins called porins. They form channels that allow molecules of small weight [<12kDa] to freely diffuse between the intermembrane space and the cytoplasm. The transport of larger proteins is coordinated by the outer membrane translocase. This is a large multisubunit protein that spans the mitochondrial outer membrane and coordinates the active movement of proteins of large size [>12kDa] across
the outer membrane (Herrmann and Neupert 2000). Significant disruptions in the structure and function of the outer membrane permit proteins in the intermembrane space to leak into the cytosol, leading to cell death (Chipuk, Bouchier-Hayes, and Green 2006).

1.1.1.2. The intermembrane space

The intermembrane space is the space between the outer membrane and the inner membrane. It is also known as perimitochondrial space. The concentration of small molecules including ions [mainly potassium and chloride] and glucose in the intermembrane space, known as intermembrane ionic strength, is similar to the cytosol (Cortese, Voglino, and Hackenbrock 1991) Chemiosmosis is the process by which there is a significant increase in the concentration of protons within the intermembrane space. Whenever the concentration of protons is high, a strong electrochemical gradient develops within the inner membrane of the mitochondrion and protons are able to re-enter the matrix via the ATP synthase complex where their energy is used to synthesize ATP.

1.1.1.3. The inner mitochondrial membrane and the cristae space

The inner mitochondrial membrane has a high protein-to-phospholipid ratio. Enfolding of the inner mitochondrial membrane forms the cristae spaces provides a high level of surface area for chemical reactions to occur and enhance the capability to produce ATP (Mannella 2006).

The Cristae contain protein components of the RC complex, which is a set of enzymatic complexes that are embedded into the inner membrane, and form the core of the OXPHOS machinery. The protein subunits that form the RC chain are under dual genetic control. They contain polypeptides that are encoded by both mtDNA and nDNA, except for complex II which is composed of four subunits, all nuclear coded (Zeviani 2004). These proteins have different types of complex functions. There also are specific transport proteins that regulate metabolite passage into and out of the matrix, protein import machinery and mitochondria fusion and fission proteins. In addition to the proteins, this compartment has an unusual phospholipid known as cardiolipin, which contains fatty acids that contribute to inner membrane impermeability.
The inner membrane is highly impermeable to all molecules. Therefore almost all ions and molecules require membrane transporters to enter or exit the matrix. Proteins are transported into the matrix via the translocase of the inner membrane [TIM].

1.1.1.4. The matrix

The matrix is the space enclosed by the inner membrane. Approximately 2/3 of mitochondrial proteins are contained in the matrix (Bruce et al., 1994). The matrix is important in the production of ATP, which is generated by the ATP synthase contained in the inner mitochondrial membrane. The matrix contains a concentrated mixture of enzymes, mitochondrial ribosomal tRNA, and several copies of the mitochondrial DNA genome. The major functions of mitochondrial enzymes include oxidation of pyruvate and fatty acids and the citric acid cycle [TCA] reactions. The matrix houses 2-10 copies of a small, 16.6-kb double-stranded, circular DNA the mtDNA encoding 37 genes.

1.1.2. Mitochondrial Function

1.1.2.1. Energy conversion

Mitochondria plays a major role in ATP production. ATP production is an energy consuming reaction, made possible by a set of oxidation and reduction reactions that create a buildup of an electrochemical gradient. The dissolution of this electrochemical gradient is necessary for the synthesis of ATP. The ETC maintains this gradient by continuing to generate hydrogen ions [protons] as the electrons flow through the ETC complexes. Glucose is considered the major source of cellular energy, the process of energy production starts through glycolysis of glucose, which yields 32 ATP. When this production comes from glucose metabolism, the mitochondrion can produce 13 times higher energy supply during aerobic respiration as compared to anaerobic fermentation. The process of aerobic respiration [or cellular respiration] is very much dependent on the available supply of oxygen (Stoimenova et al., 2007). When there is an inadequate supply of oxygen, the glycolytic products undergo metabolism via anaerobic fermentation, and a net of only four ATP molecules are generated from each glucose molecule.
1.1.2.2. Citric acid cycle

Citric acid cycle or TCA is a series of chemical reactions used by aerobic organisms to generate energy. In the citric acid cycle, acetyl-CoA derived from carbohydrates, fats and proteins; is oxidized into carbon dioxide. As a result of this oxidation process, electrons are generated and are carried by the electron carriers Nicotinamide Adenine Dinucleotide [NAD] and Flavin Adenine Dinucleotide [FAD]; a total of three molecules of NADH and one molecule of FADH2 are produced in each round of the TCA cycle. The electrons carried by these molecules are transported into a series of protein complexes in the inner mitochondrial membrane known as the Electron Transport Chain [ETC] for a final production of chemical energy in the form of ATP. [Figure 1-2]. The enzymes that catalyze key steps in TCA are normally present within the matrix of the inner mitochondrial membrane (King 2006).

Figure 1-2. The Citric Acid Cycle; TCA cycle, or Krebs cycle.
1.1.2.3. NADH and FADH2: the electron transport chain

ATP synthesis requires energy. This energy is derived from the oxidation of the energy rich molecules NADH and the FADH$_2$ by four protein complexes of the electron transport chain (ETC). NADH and FADH$_2$ are produced by the process of glycolysis in the cytoplasm or the citric acid cycle taking place in the matrix of the inner mitochondrial membrane (Berg, 2008, p. 85). The process of electron transport involves NADH and FADH$_2$, which act as electron transporters as they flow through the inner mitochondrial membrane space. In complex I NADH is oxidized to NAD. In complex II FADH$_2$ is oxidized generating more electrons to flow through the ETC. At complex III [cytochrome c reductase] the flow of the electrons from complex I and II takes place without generation of new electrons. Cytochrome oxidase occurs in complex IV. After catalyzing the movement of electrons from the cytochrome c to oxygen [O$_2$], a total of four electrons is required to produce 2 H$_2$O from O$_2$ (Insel et al., 2013).

While these oxidation and reduction reactions take place, another set of reactions occurs in the ETC. The movement of electrons through complexes I-IV causes protons [hydrogen atoms] to be pumped out of the intermembrane space into the cell cytosol. As a result, a net negative charge from the electrons builds up in the matrix space while a net positive charge from the proton pumping builds up in the intermembrane space. This differential electrical charge establishes an electrochemical gradient on either side of the inner mitochondrial membrane. The outside is positive while the inside is negative. The positive hydrogen ions are allowed to flow back across the inner membrane via specialized channels. ATP synthase, also known as complex V, uses the energy created by this proton transport to synthesize ATP from ADP and phosphate [Figure I-3].
**Figure 1-3.** The electron transport complex located in the inner mitochondrial membrane. The inner membrane contains protein components of the respiratory chain complex, which is a set of enzymatic complexes that are embedded into the inner membrane. Complex I. Complex II. Complex III. Complex IV. Complex V; ATP synthase. complex I (NADH-ubiquione oxidoreductase (UQ), complex II (succinate-ubiquinone oxidoreductase), complex III (decylubiquinone-cytochrome c reductase), or complex II+II (succinate-cytochrome c reductase).

### 1.1.2.4. ATP synthesis and ATP function

ATP is described as the currency of energy with respect to life by many biologists. It is a high-energy molecule that is responsible for the storage of the energy required to carry out daily activities. All the physiological mechanism in the body acquire energy for operation directly from ATP. Cells maintain a supply of Adenosine phosphate through the oxidization of food, as this releases energy. A remarkable ability of ATP is that it may be involved in many reactions simultaneously, extracting and releasing energy at the same time.
ATP is a formed from adenosine and inorganic phosphate (Piekutowska-Abramczuk et al., 2009). The reaction sequence is:

\[
\text{Adenosine} + \text{Pi} \rightarrow \text{AMP} + \text{Pi} \rightarrow \text{ADP} + \text{Pi} \rightarrow \text{ATP}.
\]

Catalyzed by ATP synthase. A small amount is also formed in the TCA cycle by Succinyl CoA ligase, here the energy released from the breakdown of glucose is used to attach phosphate group to an ADP molecule, thus producing ATP.

ATP is a purine nucleoside and has a role in not only various biochemical processes in the body but also serves as an inhibitory neurotransmitter when dephosphorylated to adenosine (Fredholm et al., 2005). It is abundant in cells, and mainly serves as an energy rich molecule, providing energy to vital processes within the cell by breaking away the energy-rich phosphate group. ATP is vital for living cells, processes within the cell cannot use the energy stored in the bonds of a glucose molecule, and they have to rely on ATP. Thus it is present in all living cells, and is particularly highly concentrated in the presynaptic terminals. This is because presynaptic terminals require a lot of energy for the maintenance of the resting membrane potential and the release of neurotransmitters (Burnstock, 2009). ATP fulfills multiple intracellular functions. It is described as a molecule with dual function, intracellular and extracellular.

With its intracellular function it acts as the central cellular energy source, ATP supports the synthesis of biological compounds, energizes transport and generates mechanical force and movement (Khakh and Burnstock 2009). As a phosphate donor it is a comediator of numerous regulatory functions. ATP and acetylcholine serve as extracellular signaling substances in the nervous system and in other tissues. ATP can be co-stored within synaptic vesicles and co-released from cholinergic and from gabaergic neurons (Hara and Nakaya 1997).

ATP was reported to be a transmitter in non-adrenergic, non-cholinergic inhibitory nerves in the guinea-pig taenia-coli (Mackenzie and Burnstock 1980). It was then identified as a co-transmitter in sympathetic and parasympathetic nerves (Burnstock, 2009). Currently ATP is known to act as a sole transmitter or a co-transmitter in most nerves in both the peripheral and the central nervous systems. ATP as well as adenosine mediate the neurotransmitter action. And attempts to differentiate between the role of ATP and adenosine on post-synaptic terminals have
been ineffective so far. Although their mechanism of action is different, they seem to have similar role in the postsynaptic cell.

ATP is present in high concentration within the brain, varying from 2mM/kg in the cortex to 4mM/kg in the putamen and hippocampus (Kogure and Alonson 1977). It acts as a fast excitatory neurotransmitter and has a potent long-term role in cell proliferation, growth and development, and in disease and cytotoxicity. In addition to its functions as a sole transmitter, and a co-transmitter with various neurotransmitters, it has an important prejunctional and postjunctional neuromodulator function, often after breakdown to adenosine. Extracellular adenosine induces cellular responses through activation of P1 receptors (Matsuoka and Ohkubo 2002). P1 receptor subtypes recognize adenosine (Burnstock, 2009), while ATP acts via the ionotropic P2X and metabotropic P2Y receptors on the postsynaptic cell, and uses Ca++ as the second messenger. This ATP is hydrolyzed to AMP and then to Adenosine by enzymes present on the surface of the plasma membrane close to the adenosine receptors (Dasgupta et al., 1996). Ecto-nucleotidase is thought to be the major enzyme in the formation of adenosine in the synaptic cleft. Adenosine then activates the different Adenosine receptors receptors. A1 and A2 receptors act on Adenylyl cyclase, cyclic Adenosine Monophosphate cAMP is the second messenger inside the cells.

Since the classification of the purinergic receptors has been established, considerable efforts have been invested to explain the diverse pharmacological profiles of the adenine nucleotide induced response in tissues of the central and peripheral nervous system (Barajas-López et al., 1995.; Forsyth, Bjur, and Westfall 1991; Shinozuka, Bjur, and Westfall 1988). In these experiments the action exerted by the adenine nucleotides did not fit the P2X or P2Y receptor mediated action. A possible involvement of metabolically generated adenosine was excluded by demonstrating that the addition of adenosine deaminase [ADA] did not inhibit the effect (Anwar et al 1999.; Bennett and Boarder 2000; Barajas-López et al.,1995). The addition of adenosine uptake inhibitor did not augment the observed reaction (Forsyth, Bjur, and Westfall 1991; Shinozuka, Bjur, and Westfall 1988), in addition, metabolically stable ATP analogues produced a similar effect on the postsynaptic cells as did the adenine nucleotide. Further studies reported ATP-induced cyclic AMP accumulation in cells, and subsequent detailed analysis along with experimental measures of extracellular ATP catabolism concluded that ATP indirectly activates
adenosine receptors through a rapid and localized adenosine formation (Ohkubo, Kimura, and Matsuoka 2000; Ohkubo et al., 2001).

This was confirmed by subsequent studies that demonstrated the ATP-induced cellular response via adenosine receptor activation was very similar to the Adenosine-induced cellular response (Matsuoka et al., 2002), which implies that adenine nucleotides are totally hydrolyzed to adenosine on the membrane surface. And suggests the significant role of local adenosine formation in the ATP-induced cellular response (Dunwiddie, Diao, and Proctor 1997; Cunha, Sebastião, and Ribeiro 1998). On the basis of these findings, scientists have hypothesized that adenosine generating enzymes [ecto-nucleotidases] are co-localized with adenosine receptors on the membrane surface, where adenosine converted from ATP can stimulate adenosine receptors (Matsuoka I and Ohkubo S 2004). Adenosine A1 and A2 receptors have high affinity to adenosine, thus maintaining adenosine at a very low level at the synapse is favorable, and that takes place by the orchestrated function of active uptake through adenosine transporter and conversion to inosine by ADA, which co-localizes with A1 and A2b receptors (Herrera et al., 2001) [Figure 1-9].

Adenosine release under the physiological state is ambiguous; it is markedly accumulated in the extracellular space under pathophysiological conditions, namely inflammation and ischemia. ATP is actively released into the extracellular space under physiological conditions. Adenosine transporter and ADA do not exert an action on ATP, ATP can distribute to the adenosine receptor site as a pro-agonist. The close association of the ecto-nucleotidase to adenosine receptor may function as an ATP receptor system, and determine the cellular response to extra cellular ATP (Matsuoka I and Ohkubo S 2003). ATP has a complex mode of action that utilizes both ATP and Adenosine to activate receptors in the postsynaptic cell. The hydrolysis of ATP doesn’t render it inactive, and the adenosine continues to exert its physiologic role until it is absorbed into the cell.

11.1.2.5. Heat production

A fascinating role of the mitochondria in the cell is its involvement in heat production or thermogenesis, in addition to its complex function in oxidative phosphorylation and metabolic pathways. This role is controlled by multiple internal and external factors; for example inner
membrane transporter proteins and thyroid hormone. As described earlier in this chapter; the mitochondrial electrochemical proton gradient, generated as electrons are passed down the respiratory chain, and this is the primary source of cellular ATP synthesis. In addition to reentry of protons through ATP synthase, proton leak represents another mechanism consuming the mitochondrial proton gradient. Any proton leak not coupled with ATP synthesis would provoke uncoupling of respiration and be used for thermogenesis. Respiration and mitochondrial ATP synthesis are coupled. This process is regulated by a feedback mechanism that links mitochondrial ATP synthesis and cellular ATP demand, the observation that decreased ATP utilization inhibits oxygen consumption and that respiratory rate increases when mitochondria synthesizes more ATP lead to the concept of respiratory control by ADP phosphorylation (Rousset et al., 2004). This coupling may be interrupted by the uncoupling proteins (UCPs). UCPs are mitochondrial transporters of the inner membrane that control the level of respiration coupling. Uncoupling of respiration is vital for the cell, as it allows continuous reoxidation of coenzymes that are essential to metabolic pathways. It also prevents an exaggerated increase in ATP level that would inhibit respiration. This way mitochondria consumes the proton gradient via two reentery processes; one is through ATP synthesis and the other is via proton leak; each exchange of electrons across the mitochondrial membranes results in a loss of some energy as heat.

1.1.2.6. Storage of Calcium ions

The endoplasmic reticulum [ER], which serves as a storage site for calcium interplays with the mitochondrion in regulating intracellular calcium. Calcium is transferred from the ER to the mitochondrial matrix via a calcium uniporter, which is present within the inner mitochondrial membrane (Pizzo and Pozzan 2007; Miller 1998). In line with this, the sodium-calcium exchange process or the pathway of the calcium-induced-calcium-release is responsible for releasing the stored calcium back into the cell.

The mitochondrion is capable of storing calcium to maintain homeostasis in the cells’ required calcium supply. For this reason, several authors consider mitochondria as reliable cytosolic Ca^{2+} buffers (Ducreux, Gregory, and Schwaller 2012; Rossier 2006).
1.1.2.7. Other Mitochondrial functions

Other mitochondrial functions include regulating the membrane potential, regulating cellular metabolism (McBride et al., 2006), facilitating cell death caused by apoptosis (Chipuk et al., 2006; McBride et al., 2006), facilitating calcium-signalling-mediated apoptosis (Hajnóczky et al., 2006), synthesizing steroids (Rossier 2006) and participating in heme reactions (Oh-hama 1997). Considering the vital functions of mitochondria, disruption in their functions can understandably lead to health problems including rapid ageing (Jiang and Wang 2012), cardiac-related problems (Lesnefsky et al., 2001).

1.2. The Basal Ganglia

The basal ganglia consist of masses of neurons, which contain cell bodies. Cell bodies are surrounded by white matter comprised of their incoming and exiting processes. The nuclei are divided into input and output nuclei. These ganglia are arranged in separate parallel circuits. This arrangement, coupled with the somatotopic representation of each cortical motor area wherein each area corresponds to a particular part of the body, is illustrative of the complexity of the role of basal ganglia in modulating movement. The wiring of this system consists of networks formed by finely arranged parallel motor loops between the cortex and basal ganglia, which provides positive or feed forward signaling for movement preparation and execution and feedback from affected body parts that has a stabilizing function. This role is not limited to the movement plan, as the basal ganglia are also involved in complex executive tasks and in motor learning processes.

1.2.1. Basal Ganglia Anatomy and Circuitry Modules

The basal ganglia comprise the striatum, globus pallidus [GP], substantia nigra [SN] and subthalamic nucleus [STN]. The striatum consists of the caudate nucleus, putamen and nucleus accumbens. The globus pallidus consists of an external segment [GPe], located laterally adjacent to the striatum, and an internal segment [GPi] located medially. The substantia nigra is composed of a cell-dense pigmented region, the substantia nigra pars compacta [SNc], and an unpigmented region, the substantia nigra pars reticulata [SNr][Figure 1-4].
Figure 1-4. Basal Ganglia Anatomy. globus pallidus [GP], substantia nigra [SN]; pigmented region, substantia nigra pars compacta [SNC], and an unpigmented region, the substantia nigra pars reticulata [SNr]. Subthalamic nucleus [STN]. The striatum consists of the caudate nucleus [C], putamen [P], and nucleus accumbens (not shown here). The globus pallidus consists of an external segment [GPe], located laterally adjacent to the striatum, and an internal segment [Gpi].

The current functional basal ganglia model was introduced in the 1980s, following observations made on animal models of neurodegenerative diseases and patients with hypo- or hyper-kinetic movement disorders (Albin, Young, and Penney 1989; DeLong 1990). While this model is a simplification of more complex underlying anatomical connections, it has proven valuable in providing a simple model of basal ganglia function that helps understand the mechanism of Parkinson’s disease and other common movement disorders and their therapies. The two output nuclei of the BG are the GPi and the SNr, whereas the main input nucleus is the striatum [caudate and putamen]. The input and output regions of the basal ganglia are connected via either a direct or an indirect network. The direct pathway arises from medium-sized spiny neurons
[MSNs] in the striatum, and projects directly to the output regions of the basal ganglia [Figure 1-5]. The indirect network, also arising from striatal MSNs, takes a circuitous route to the GPi and SNr, involving the GPe. MSNs of the indirect pathway project from the striatum to the GPe. The GPe influences basal ganglia output via a GPe to GPi/SNr connection, and via the intermediary of the STN, via a GPe to STN connection, and then a projection from the STN to the GPi and SNr [Figure 1-6]. Neurons of the GPi and SNr that receive inputs from the direct and indirect pathways constitute the output of the basal ganglia and project to the ventral thalamus, superior colliculus [SC], pedunculopontine nucleus [PPN], and reticular formation [RF]. It is known that the BG influence motor, sensory and cognitive cortical information processing through the two output nuclei (Middleton and Strick 1997). Through the SC target, the BG influence movements of the head and eyes (Hikosaka, Takikawa, and Kawagoe 2000). Through the PPN target, the BG influence spinal cord processing, locomotion (Garcia-Rill et al., 1983) and postural control (Takakusaki et al., 2003).

Figure 1-5. Direct Basal Ganglia Circuit Pathway: Dopamine D1 receptor facilitates the direct pathway. GABA: Gamma Amino Butyric Acid. Gpe: Globus Pallidus externa. GPI: Globus Pallidus interna. SMA: Supplementary Motor Area. Sp: Substance P. VTT: Ventral Tier Thalamic nuclei.
**Figure 1-6.** Indirect Basal Ganglia circuit pathway: Inhibited by Dopamine via D2 action. A2 antagonizes D2 receptor. ENK: enkephalin. GABA: Gamma Amino Butyric Acid. Gpe: Globus Pallidus externa. GPi: Globus Palidus interna. SMA: Supplementary Motor Area. VTT: Ventral Tier Thalamic nuclei.

Parallel to the direct/indirect pathway organization is a loop involving the striatum and SNc. The striatum projects to the SNc, and the SNc projects back to the striatum, in what is the well-known dopaminergic pathway underlying Parkinson’s disease (Takakusaki et al., 2003) [Figure 1-8]. Over time, the circuit model of the basal ganglia has undergone significant changes. The basal ganglia were believed to receive input from large areas of the frontal cortex, to process and integrate information, and to send output to the motor cortex via the thalamus; early models emphasized a selection function of the basal ganglia (Kemp and Powell 1971). It was proposed that the basal ganglia selected the appropriate input based on the context and sent the appropriate command to the motor cortex. Cerebellar and basal ganglia outputs were believed to converge at
the thalamic level. This model has been replaced by the segregated circuit model in which the basal ganglia are seen as components of a family of parallel re-entrant loops, over which information sent from individual cortical areas is processed in specific and non-overlapping territories, and then returned to the respective frontal lobe area of origin via the thalamus.

**Figure 1-7.** Dopaminergic pathway. Involving the striatum and SNC. The striatum projects to the SNC, and the SNC projects back to the striatum, in what is the well-known dopaminergic pathway underlying Parkinson’s disease. Globus pallidus [GP], substantia nigra [SN]; pigmented region, substantia nigra pars compacta [SNC], and an unpigmented region, the substantia nigra pars reticulata [SNR]. Subthalamic nucleus [STN]. The striatum consists of the caudate nucleus [C], putamen [P]. The globus pallidus consists of an external segment [GPe], located laterally adjacent to the striatum, and an internal segment [Gpi]. Adenosine receptors [A1]&[A2a]. Dopamine receptors [D1] &[D2].
In 1981 DeLong and Geogropoulos described a precursor of this model, based on physiologic and anatomic observations in primates, which revealed segregated motor and non-motor areas in the basal ganglia. This model was further expanded in the mid-1980s with the description of five functionally distinct circuits, a motor, oculomotor, a dorsolateral prefrontal, a lateral orbitofrontal and an anterior cingulate [limbic] circuit. In this model, the motor circuit is centered on the supplementary motor area, with inputs from the motor cortex and the premotor areas. These cortical motor areas project topographically to the post-commissural putamen, which sends efferents to GPi and the Caudolateral SNr. The motor areas of GPi/SNr project, to portions of the ventral anterior and ventrolateral thalamus [VA/VL] also referred to as the ventral tier nuclei, which then return output to the supplementary motor area SMA, with lesser projections to premotor and motor cortices. Neuronal specificity and somatotomy are maintained throughout the circuit.
While the original model emphasized the separation of basal ganglia and cerebellar cortical-subcortical circuits, evidence indicates that there are connections between the basal ganglia and the cerebellum. Cerebellar output reaches the striatum via the thalamus (Hoshi et al., 2005), and that basal output from the STN reaches the cerebellum (Bostan, Dum, and Strick 2013). There is also evidence that cerebellar and basal ganglia outputs converge on the same cortical areas (Hoover and Strick 1999; Clower, Dum and Strick 2005; Akkal 2007). These findings suggest that the basal ganglia and cerebellar circuits may be more closely inter-related than previously thought.

Direct and indirect pathways originate from different populations of striatal projection neurons, whose activity is strongly dependent on their cortical inputs (Albin, Young and Penney 1989; Crossman 1989; DeLong 1990; Alexander, Crutcher and DeLong 1990). The activity at corticostriatal synapses and the activity of the direct and indirect pathways are regulated by striatal dopamine supplied by the nigrostriatal projection from the SNc. In the classic model of the basal ganglion, the different polarities [Glutamatergic stimulatory vs. GABA inhibitory effect] of the direct and indirect pathways are thought to lead to different effects at the level of the basal ganglia output nuclei. Activation of direct pathway neurons leads to neuronal inhibition, while activation of neurons of the indirect pathway leads to disinhibition of GPi/SNr neurons. Because basal ganglia output is inhibitory, reduction of basal ganglia output through activation of the direct pathway would disinhibit thalamocortical activity, while activation of the indirect pathway would increase the inhibition of thalamocortical projections.

The direct and indirect pathways also differ in other aspects. Direct pathway neurons express substance P, while indirect pathway neurons contain enkephalin. In addition, direct pathway neurons express D1-family Dopamine receptors, while indirect pathway neurons express D2-family receptors (Gerfen et al.,1990).

Attempts have been made to explain the BG’s proposed movement-related functions, including movement sequencing on the basis of the direct/indirect pathway organization of the basal ganglia. It is hypothesized that the basal ganglia serve roles in controlling the speed and amplitude of movement, by allowing movements to occur via activation of the direct pathway, and by terminating them through activation of the indirect pathway. Evidence for a role of the basal ganglia in such movement function was demonstrated in studies of the activity of pallidal
neurons in monkeys trained to perform movements of different amplitudes (Georgopoulos, DeLong, and Crutcher 1983).

Denny-Brown, proposed an alternative module to explain the movement related function of the BG. In this module the basal ganglia act by selecting the most appropriate action for a given situation (Denny-Brown 1963). Kemp and Powel incorporated this view into their model (Kemp and Powell 1971). A related concept was proposed by another group, who suggested that the basal ganglia act to select which movements should be carried out in response to competing sensory stimuli and to suppress unwanted movements (Albin, Young, and Penney 1989). This model was further developed by Mink, who suggested that the selection of specific movements involved activation of the direct pathway, while the inhibition of competing movements involved activation of the indirect pathway (Mink 1996).

A problem with these hypotheses is that there is relatively slow conduction along the indirect pathway, as compared to that in the direct pathway, which would in theory result in premature activation of the movement compared to the inhibition of competing movements (Nambu 2008). This suggests that there may be a non-striatal route for cortical inputs to the BG, a hyper direct cortico-subthalamic pathway. Rapid activation of the STN-GPi route via a hyper direct pathway would generate an inhibitory surround environment in which a specific movement can be activated. [Figure 1-8].

**Figure 1-8.** Hyper Direct Basal Ganglia pathway: ENK: enkephalin. GABA: Gamma Amino Butyric Acid. Gpe: Globus Pallidus externa. GPi: Globus Palidus interna. SMA: Supplementary Motor Area. VTT: Ventral Tier Thalamic nuclei.
This hypothesis has created debates and arguments; one of the difficulties with it is that it requires an active role of the basal ganglia in the action selection process. The basal ganglia modules are directly tied to their specific cortical inputs, which may support the idea that action selection, activation or inhibition is primarily a cortical rather than BG processes, and that the BG do not play a role in the initiation or selection of movement. Instead they likely have vital roles in the training or conditioning of the cortical modules.

To better understand the function of the cortico-thalamocortical loops, and the ways by which basal ganglia and cerebellar output regulate them, the role of the connections between the basal ganglia and cerebellum needs to be defined. New models need to incorporate the intrinsic
processing within the basal ganglia nuclei, and the role of inter-neuronal processing in the striatum need to be explored and defined.

1.2.2. **Neurotransmitters in the Basal Ganglia**

The main cell-signaling neurotransmitters in the BG are Gama-aminobutyric acid [GABA] and glutamate. They exert their effects through inotropic and metabotropic receptors. Inotropic receptors form an ion channel pore while metabotropic receptors are indirectly linked with ion channels on the plasma membrane and act through a secondary messenger system, which uses messenger molecules, one example of a secondary messenger systems; synthesized and activated by enzymes, is cyclases [adenylcyclase] that synthesize cyclic nucleotides [cAMP], another example of a secondary messenger system acts by opening ion channels to allow influx of metal ions, like Ca2+ signaling. Small molecules bind and activate protein kinases to initiate a signaling cascade. The activation of these receptors in response to released neurotransmitter depends on their precise localization within synapses and on the mitigating effects of other modulatory neurotransmitters. In the classic BG model, the STR, which constitutes the input stage of the basal ganglia, receives the glutamatergic excitatory input from all areas of the cerebral cortex. The GPi and the SNr, which represent the major output nuclei of the basal ganglia exert tonic GABAergic inhibitory influence on the excitatory neurons located in the ventral tier thalamic nuclei. Most neurons within the basal ganglia use the inhibitory neurotransmitter GABA. The direct pathway originates from striatal neurons that contain GABA plus SP and project via a single cellular connection to the GPi/SNr. The indirect pathway arises from striatal neurons that contain GABA and ENK and whose influence is conveyed to the GPi/SNr by way of a sequence of connections involving the GPe and the STN. This sequence is composed of:

1. A GABAergic inhibitory projection from the STR to GPe.

2. An inhibitory GABAergic projection from the GPe to STN.

3. An excitatory glutamatergic projection from the STN to GPi/SNr.

At the STR level, dopamine appears to facilitate transmission along the direct pathway and inhibit transmission along the indirect pathway. This has the effect of stimulating movement.
These two opposite effects of dopamine are mediated by D1 excitatory and D2 inhibitory dopamine receptors.

Huntington’s disease (HD) represents an example of the consequences of disruption of the neurotransmitter symphony orchestrated by the basal ganglia nuclei, it is a dominantly inherited neurodegenerative disorder characterized by progressively worsening chorea. The degenerative process primarily involves medium spiny striatal neurons; GABAergic encephalin neurons of the basal ganglia are the most vulnerable in HD (Mitchell et al., 1999). HD is caused by expansion of a polymorphic CAG trinucleotide repeat encoding a polyglutamine tract within the Huntingtin protein. The mechanism responsible for pathogenicity is unknown; the most puzzling question is why are the striatal neurons most vulnerable despite the ubiquitous expression of mutant and normal Huntingtin.

In rats, it has been estimated that 98.9% of basal ganglia neurons are GABAergic. GABA<sub>α</sub>-positive synapses are formed by terminals enriched in GABA (Fujiyama, Stephenson, and Bolam 2000), but in the rat striatum, about half of these terminals present no detectable GABA levels (Fujiyama et al., 2002). Currently the significance of this finding is not clear, but this could represent non-GABAergic terminals associated with GABA<sub>α</sub> receptors.

Another group of neurotransmitters in the basal ganglia includes the purinergic neurotransmitters, Namely Adenosine Triphosphate [ATP] and Adenosine. ATP is synthesized by mitochondrial complex V of the ETC; also known as ATP synthase. The diverse physiological actions of ATP were recognized as early as 1929 when the potent action of extracellular ATP and Adenosine on heart and coronary blood vessels was described (Druy and Szent- Gyorgyi 1929). ATP has multiple intracellular as well as extracellular functions as summarized in the previous section. Increasing evidence suggests that ATP functions as an extracellular signaling molecule. Electrophysiological experiments provide strong evidence to indicate that the net effect of ATP at basal ganglia interneurons is a depolarization that leads to increased GABA<sub>α</sub> receptor-mediated synaptic inhibition through activation of the purinergic P2Y<sub>1</sub> receptors (Zimmermann 1994). In addition to the direct action of the ATP, the effect of ATP at the basal ganglia is also mediated through adenosine, which is generated at adenosine receptor level by dephosphorylation of ATP that is released together with the classical neurotransmitters; this reaction is catalyzed by the enzyme ecto-nucleotidase [Figure 1-9].
The purinergic neurotransmitters interact with purinergic receptors that are divided into two large categories: P1 and P2 receptors. P1 receptors are metabotropic receptors subdivided into four adenosine receptors, A₁, A₂a, A₂b and A₃ [Figure I-10]. The A₂ subtypes are Gₛ/Golf/Gq coupled, stimulating adenylyl cyclase, whilst A₁ and A₃ couple to Gᵢ/Gₒ and inhibit adenylyl cyclase (Benarroch, Smith, and Shuman 2008; Boison 2010; Fredholm et al., 2005).

**Figure 1-9.** Adenosine synthesis and hydrolysis. Adenosine is generated at the site of the Adenosine receptors by dephosphorylation of ATP.

**Figure 1-10.** Purinergic receptors classification. The purinergic receptors are divided into two large categories P1 and P2 receptors; they are subdivided into multiple subtypes.
Under physiologic conditions the effect adenosine imposes on brain function is greatly dependent on the activity of A1 and A2a receptors. These receptors have high affinity to adenosine; they are activated by nanomolar adenosine concentrations. Adenosine receptors are expressed and located presynaptically, and postsynaptically on neurons, the position of these receptors predetermines the activity of adenosine. The role of presynaptic A1 is to restrict excitatory synaptic transmission mediated by N-type and P/Q-type calcium channels (Ambrósio et al., 1997; Gundlfinger et al., 2007). The neuron’s responsiveness to excitatory stimuli is influenced by postsynaptic A1 receptors, which restricts N-Methyl-D-aspartate [NMDA] receptors. A1 receptors represent the natural antiepileptic agent within the brain; it reduces excitatory neurotransmitter release through the activation of presynaptic A1 receptors, which activates K+ channels resulting in neuronal hyperpolarization. (Birnstiel et al., 1992). Activation of A1 receptor can exert neuroprotective actions; it protects neurons against ischemia-reperfusion damage in the brain through the activation of the defense mechanism called "ischemic preconditioning" (von Lubitz et al., 1996). A1 receptors are found on the SP/direct pathway.
neurons. This allows inhibitory A₁ receptors to modulate the action of excitatory D₁ receptors (Fink et al., 1992; Schiffmann and Vanderhaeghen, 1993; Svenningsson et al., 1997; Schiffmann, Jacobs, and Vanderhaeghen, 1991). In contrast A₂a receptor is specifically localized in the soma and dendrites of enkephalinergic-GABAergic-MSN of the corpus striatum (Rosin et al., 1998; Schiffmann et al., 2007). Adenosine A₂a receptor is the only transmitter receptor presenting with such a restricted localization (Martin, 1984). Pre-synaptically it is found on the cortico-striatal glutamatergic projections (Hettinger et al., 2001). Adenosine A₂a and dopamine D2 receptors colocalize on the dendritic spines of the striatopallidal neurons, there is a protein-protein interaction between the two receptors that leads to the formation of a receptor heteromer (Canals et al., 2003). The activity of A₂a receptors does not influence synaptic transmission significantly, but it does control synaptic plasticity (Canas et al., 2009; Gomes et al., 2011; Mills et al., 2011). A₂a receptor also has a very important role in controlling microvascular function; their expression is specific and mostly traced in endothelial cells of brain capillaries in the corpus striatum (Mills et al., 2011; O'Regan, 2005). A₂a receptor is the only one with a selective localized expression in the cochlear blood vessels; it is also expressed with higher density in the root region of the spiral ligament, and in the organ of Corti (Vlajkovic et al., 2007). [Table I-1].

A₂b and A₃ are the low affinity receptors; they need higher adenosine concentrations to be activated, for example in pathological conditions where there is a leak of adenosine from cells.

**Table I-1.** Localization of Dopamine and Adenosine receptors, and signaling cascade.
Adenosine $A_{2A}$ receptor density is found to be decreased in the basal ganglia of Huntington’s disease patients but unaltered in Parkinson’s disease patients. This suggests that these receptors are located on neurons having their cell body in the striatum rather than on terminals of afferents to the striatum (Martin 1984). $A_{2a}$ receptor regulation in Huntington’s disease is complex and remains controversial.

The interaction between the dopaminergic and purinergic neurotransmitter systems in the basal ganglia is a complex process; this process may represent the underlying control of movement of the human body. A high density of dopamine D 2 receptors [D 2 R] is observed in the striatal neurons. $A_{2a}$ receptors [$A_{2a}$ R] make two reciprocal antagonistic interactions with D2R (Ferr. et al., 2008). In the $A_{2a}$R–D2R interaction; $A_{2a}$R resists the D2R-mediated inhibitory change of NMDA receptor mediated effects leading to the alteration of Ca$^{++}$ influx, and subsequent change to the up-state and neuronal firing (Higley and Sabatini 2010). The ability of $A_{2a}$ R and D2R to heteromerize is the most important part of the receptor-receptor interaction; this interaction is
accountable for the locomotor depressant and stimulating influences of \( A_{2a} \)R agonists and antagonists correspondingly (Ferr. et al., 2007; Ferr. et al., 2008).

In the second type of D2R–A2aR interaction, D2R activation resists A2aR signaling through adenylyl cyclase (Chen et al., 2001). Second Messenger level is a place, where D2R–A2aR interaction happens. Activation of G i/o -coupled D2R restrains the influence of G s/olf -coupled A2aR activation (Ferr. et al., 2007, 2008). Great tonic effect of endogenous dopamine on striatal D2R makes such interaction prevent A2aR signaling. In spite of this, dopamine reduction or pharmacological D2R blocking makes it possible to release A2aR-mediated signaling through the cAMP-PKA cascade.

A2aR–D2R and D2R–A2aR happen simultaneously in the same cell. This fact emphasizes the presence of different populations of postsynaptic striatal A2aR in the enkephalinergic MSN (Ferr. et al., 2007). The task of one of such populations is to form heteromers with D2 and define that A2aR activation restrains D2R-mediated signaling. At the same time the other population does not form any heteromers with D2R. This does not mean that second population would not create heteromers; it may create them with glutamate receptor subtypes (Ferr. et al., 2002).

The localization of the A2A receptor on the ENK cells has led to the suggestion that A2a antagonists may be useful in the treatment of Parkinson’s disease by reducing the imbalance between direct and indirect pathways (Schwarzschild et al., 2006). The crystal structure of the A2a receptor bound to an antagonist has been determined (Jaakola et al., 2008), knowledge of this structure should lead to development of more selective adenosine receptor compounds.

1.3. Mitochondrial disease presenting with movement disorder

Mitochondrial medicine emerged in 1988 to 1989 with the discovery of mt-DNA deletions as the cause of Kearn-Sayre syndrome and Leber hereditary optic neuropathy (Holt et al., 1988.). Since then the ability to rapidly sequence mtDNA has allowed the description and biochemical characterization of many mitochondrial disorders whose mutations are reported at Mitomap (www.mitomap.org). Mitochondrial diseases occur due to defects in the mitochondrial respiratory chain [RC], the terminal pathway of OXPHOS, where most cellular ATP is generated. This is the only metabolic pathway that depends on two genomes: mtDNA associated
with strictly maternal inheritance and nDNA Mendelian inheritance. Due to its dual genetic control, RC defects can be due to mutations in both mtDNA or in nDNA.

The largest representation of nuclear mutations directly affecting the ETC subunits are the complex I defects, which typically result in the clinical picture of Leigh disease. Mutations affecting nonstructural nuclear genomes are also a cause of mitochondrial disease (Horvath et al., 2006). Mitochondrial genes implicated in mtDNA stability represent the largest group of nuclear mutations involved in mitochondrial cytopathies. In the past decade, a growing number of patients with OXPHOS dysfunction due to depletion of mtDNA have been reported (Vu et al., 1998). This syndrome is characterized by a quantitative defect of mtDNA resulting from dysfunction of one of several nuclear-encoded factors responsible for maintenance of mitochondrial deoxyribonucleoside triphosphate [dNTP] pools or replication of mtDNA. (Moraes et al., 1992; Tritschler et al., 1992; Elpeleg et al., 2005; Carrozzo et al., 2007; Morava et al., 2009).

The phenotype of diseases in which mitochondrial dysfunction is the primary defect is extremely heterogeneous. Such diseases may be mild, restricted to only one organ, or may be lethal in infancy and childhood [e.g., Leigh syndrome or LS]. Evidence is accumulating that mitochondrial dysfunction may also play a significant role in normal aging and late-onset neurodegenerative disorders such as Parkinson’s disease (Reeve, Krishnan, and Turnbull 2008). Postmitotic tissues that are highly dependent on oxidative metabolism, including neural, muscular, and cardiac, appear to be vulnerable to energy depletion (Schapira 1993). Common neurological features of mitochondrial disorders are seizures, ataxia, stroke-like episodes, psychomotor retardation, migraine-like headaches, and movement disorders (DiMauro et al., 2002). Cardiovascular involvement has been reported with mitochondrial disorders in the form of dysrhythmia, and cardiomyopathy (Anan et al., 1995). Mitochondrial disorders rarely present as an isolated movement disorder, and if so they are less likely to remain as such. Mitochondrial disorders are suspected in the setting of a complex progressive clinical picture where the movement disorder is part of a more generalized nervous system dysfunction. LS and LLS are the mitochondrial syndromes associated with the widest spectrum of movement disorders, dystonia being the most common manifestation.

1.3.1. Leigh syndrome LS and Leigh-like syndrome LLS
Leigh Syndrome [sub acute necrotizing encephalomyelopathy] was described by Denis Leigh in 1951. The case reported was a 7month old boy with encephalopathy, bilateral hearing impairment, bilateral optic atrophy, and spasticity. Postmortem examination revealed bilateral symmetric spongiform necrotic lesions and demyelination involving the brainstem, thalami, cerebellum, spinal cord, and optic nerves. The similarity of these pathological findings with Wernicke’s encephalopathy suggested a nutritional deficiency as an etiology of this disease (Leigh 1951).

In 1996 specific criteria for the diagnosis of LS were suggested including: 1. A progressive neurological disease including motor and intellectual developmental delay; 2. Signs and symptoms of brainstem and basal ganglia disease; 3. Raised lactate levels in blood or CSF; and at least one of the following: a. characteristic findings on neuroimaging; b. typical postmortem neuropathological changes; or c. typical neuropathology in an affected sibling. A proportion of patients have a strongly suggestive clinical presentation but do not fulfill the criteria; they are classified as having Leigh-Like Syndrome LLS (Rahman et al., 1996).

### 1.3.1.1. LS and LLS caused by nDNA mutations

The first mutation in nDNA-encoded respiratory chain subunits leading to LS was reported in 1995 (Bourgeron et al., 1995). The patients were two Tunisian sisters, homozygous for an Arg554Trp substitution in the flavoprotein subunit of succinate dehydrogenase, resulting in complex II deficiency. The first nuclear-encoded complex I mutation was reported in 1998 in the NDUFS8 gene (Loeffen et al., 1998). Since then, mutations in other complex I subunits have been found (Smeiyink and van den Heuvel, 1999;Petruzzella 2001;Benit 2004). A more common cause of LS is SURF1 mutation, a complex IV assembly factor (Tiranti et al., 1998; Zhu et al., 1998). Movement disorders described in patients with LS include tremor (Piekutowska-Abramczuk et al., 2009) and dystonia (Bénit et al., 2001).

### 1.3.1.2. LS and LLS caused by mtDNA mutations
More than 20 mtDNA point mutations have been reported to date to be associated with LS or LLS (Finsterer 2008). Dystonia has been described to be part of the phenotype in many cases of LS and LLS caused by mtDNA mutations. In most such cases dystonia is not the presenting or predominant symptom, the exception being two cases of LS-associated childhood-onset progressive dystonia caused by point mutations in genes encoding complex I subunits (Sarzi et al., 2007). The first one is in the mitochondrial ND3 gene, and the second mutation is in the mitochondrial ND6 gene (Ugalde et al., 2003; Lebon et al., 2003; Solano et al., 2003). Both mutations are associated with complex I deficiency.

1.3.2. Leber’s hereditary optic neuropathy

This was the first maternally inherited disease to be genetically linked to a point mutation in mtDNA (Wallace et al., 1988). The clinical features of this disease were described in 1871 (Leber, 1871). Patients usually present between the second and fourth decade with subacute painless loss of central vision, initially monocular, followed by involvement of the other eye over a period of weeks or months (Eichhorn-Mulligan and Cestari 2008). Recovery of some vision has been reported to occur years after visual loss and recurrence is extremely rare, the mechanism of recovery of vision is not clear (Newman 2005).

Movement disorders of many types have been reported in patients with LHON; postural tremor, cortical myoclonus, chorea, tics, parkinsonism and dystonia have all been reported with specific mutations (Ostergaard et al., 2007). A variant of LHON associated with prominent dystonia [LDYT] has been reported to occur with rare mtDNA point mutations. Patients with LDYT usually present in childhood with progressive dystonia associated with striatal necrosis, and some later develop optic neuropathy. All the mtDNA mutations presenting with LHON and movement disorders lie in genes encoding complex I subunits and result in severe complex I deficiency.

1.3.3. Myoclonus Epilepsy with Ragged-Red fibers (MERRF)
MERRF is one of the major mitochondrial syndromes and a common cause of progressive myoclonic epilepsy. Mitochondrial abnormalities in skeletal muscle of patients with inherited myoclonic epilepsy were first reported in 1973 (Tsairi, 1973). In 1980 the first description of MERRF syndrome was reported (Fukuhara et al., 1980). Symptoms may begin at any age; the cardinal features include cerebellar ataxia, epilepsy, and myoclonus (Berkovic et al., 1989). Other clinical manifestations include myopathy, deafness, cognitive impairment, neuropathy and optic atrophy. The syndrome is described with significant variability. Diagnosis is made based on the combination of the clinical features and a maternal pattern of transmission. Serum lactate may be raised and muscle biopsy shows typical ragged red fibers with Gomori Trichrome staining (Hammans et al., 1995; Mancuso et al., 2004). The EEG reveals background slowing, 2-5 Hz generalized spike-and-wave or polyspike and wave discharges, and photosensitivity. Brain imaging may be normal or show nonspecific findings, such as cerebral or cerebellar atrophy. Myoclonus is usually the presenting symptom of MERRF. It is typically generalized and stimulus sensitive. Tremor is another common movement disorder in MERRF (Mancuso et al., 2004). In the majority of cases it is an intentional tremor due to cerebellar dysfunction. Dystonia has been reported with MERRF, but it is a rare phenomenon (Wiedemann et al., 2008).

1.3.4. Mitochondral Myopathy, Encephalopathy, Lactic acidosis and Stroke-like episodes (MELAS)

MELAS is a mitochondrial syndrome characterized by seizures, lactic acidosis, and stroke-like episodes. It was first described in 1984 and identified as a mitochondrial disease (Pavlakis et al., 1984). Brain MRI usually shows symmetric lesions with a predilection for the parieto-occipital cortex and relative sparing of the deep white matter and the basal ganglia. These lesions do not conform to well-defined vascular distribution. Biochemical testing of mitochondrial function in muscle biopsy reveals complex I deficiency with relative sparing of complex IV; this pattern is not typically seen in other mitochondrial disorders such as MERRF (DiMauro et al., 2002). The most common movement disorder described in patients with MELAS is myoclonus. Dystonia has been reported but it is a rare phenomenon (Hammans et al., 1995). There has been a case report of dystonia as the presenting symptom of a specific mutation causing MELAS (Sudarsky
et al., 1999). Chorea has been reported in combination with other symptoms and can occur acutely, triggered by hypoglycemia (Kang et al., 2005; Nakagaki et al., 2005). The pathophysiology of MELAS is not well-understood, The mechanisms underlying the pathogenesis of stroke-like episodes are also not fully understood, multiple different factors being involved in the cascade of events occurring during these episodes, including neuronal hyperexcitability and mitochondrial angiopathy (Sakuta and Nonaka, 1989;Iizuka and Sakai 2005;Iizuka 2007).

1.3.5. Mitochondrial depletion syndrome V caused by SUCLA2 gene mutation

This syndrome was described in 2005 (Elpeleg et al., 2005), and reportedly occurred in the Faroe islands with an incidence of 1 in 1700, due to a founder effect (Ostergaard et al., 2007). Patients present with a distinct phenotype, consisting of hypotonia, muscle atrophy, deafness, and dystonia (Carrozzo et al., 2007; Ostergaard et al., 2007). Brain MRI shows symmetric T2-hyperintense lesions in the basal ganglia in many patients. The underlying genetic defect lays in the SUCLA2 gene, which encodes the subunit of the adenosine diphosphate-forming succinyl-coenzyme A synthetase, an enzyme participating in the Krebs cycle. This disorder is associated with mitochondrial depletion seen on electron microscopic examination of muscle biopsy. The pathophysiology of this disease is not clearly understood but there are several recent theories based on scientific findings that are elaborated on in chapter 2.

1.3.6. Mitochondrial DNA depletion secondary to mutations in DNA polymerase Gamma POLG and Twinkle

Mitochondrial DNA replication and maintenance is a complex process that depends on nuclear-encoded proteins. Defective function of these proteins can lead to diseases that are inherited in a mendelian fashion, and are associated with multiple mtDNA depletion. Two very important proteins in this process are POLG and TWINKLE. POLG is the only active DNA polymerase within the mitochondria (Clayton 1982), Twinkle is a helicase responsible for unwinding the mtDNA replication fork (Korhonen, Gaspari, and Falkenberg 2003). In 2001 the first link
between mutations in the genes encoding those proteins and human disease was made. The phenotype described was progressive external ophthalmoplegia associated with multiple DNA deletions (Spelbrink et al., 2001; Van Goethem et al., 2003). Over 100 mutations have been described in the POLG gene (Chinnery and Zeviani 2008).

A variety of movement disorders have been described in association with POLG mutations. Myoclonus was reported to occur with the most common homozygous mutation in this gene (Van Goethem et al., 2003; Wong et al., 2008; Tzoulis et al., 2006). Facial dyskinesia, choreoathetosis, head and limb tremor were also observed (Hakonen et al., 2005). One of the most interesting discoveries in mitochondrial medicine was the association of POLG mutations with Parkinsonism (Luoma et al., 2004). Parkinsonism has also been reported in association with the Twinkle mutations (Baloh et al., 2007; Vandenberghe et al., 2009). This confirmed the earlier hypothesis that mitochondrial dysfunction has an implication in the pathophysiology of Parkinson’s disease (Schapira et al., 1993).

1.4. The role of Next Generation Sequencing in Disease Gene Discovery

Momentum for the implementation of personalized medicine in clinical practice is gaining. The catalog of disease-associated genetic variations, produced from genome-wide association studies, array comparative genomic hybridization [array CGH], and next generation sequencing, is rapidly increasing. The rapid expansion in the field of medical genetics, following sequencing of the human genome and the more recent development of next-generation sequencing technologies, has led to identification of specific genes associated with human diseases. By 2011 the molecular basis of nearly 40% of known Mendelian disorders had been identified (Bamshad et al., 2011), giving rise to more accurate diagnostic testing and the ability to research novel treatments. With current advances in whole genome and whole exome sequencing methods, it is likely that the majority of variants underlying monogenic diseases will be mapped within the next few years. Identification of genes of monogenic Mendelian diseases classically proceeds through mapping of the disease gene locus through linkage analysis and in consanguineous families homozygosity mapping followed by positional and functional candidate gene
sequencing. The candidate gene approach selects a genetic variant of potential interest and looks for this gene in cases and controls.

Linkage studies are useful for studying disorders occurring in multiple members of families, including in families where there is consanguineous relations in the parents. The aim is to identify genetic markers that are inherited by family members with the disease and not by those who lack the disease. Single nucleotide polymorphisms [SNPs] are examples of such genetic markers. SNPs are first genotyped across the genome, and distribution of haplotypes [sets of consecutive SNPs] among members of the family is studied. The disease mutation must be linked to haplotypes segregating with affected members, and not unaffected members. This type of analysis reduces the subsequent search for the disease mutation to a fraction of the genome, i.e., to that portion of the genome segregating with the disease. Special populations provide specific opportunities to pioneer this task, and provide unique advantages for these study designs. The larger the number of affected members in a family the fewer the haplotypes shared among them, and thus the smaller the candidate parts of the genome to be considered. The process of linkage is further aided when consanguinity exists in parents of affected patients. Here, not only shared haplotypes provide information, but also shared homozygosity in haplotypes. In these families, the same mutation, and thus the same haplotype flanking it, has descended through the consanguineous parents to the affected offspring. As such, the disease gene will be located only in segments of genomic homozygosity, allowing exclusion of all other genome regions from consideration.

Analyses of the complete spectrum of genome variants will create new analytical challenges; tackling of these challenges requires large, well-documented data collections so that a large spectrum of risk combinations can be tested.

Automated Sanger sequencing was the workhorse of genome sequencing until recently. With the advent of a number of improvements; automated Sanger sequencing now is considered to be the “first generation technology” while the newer, more advanced and recently developed methods are termed next generation sequencing (Metzker 2010). [whole exome or whole genome sequencing] which can be utilized to sequence, simultaneously, all genes in the genome. Next generation sequencing is a high-throughput sequencing platform, which enables massively parallel sequencing of the coding regions of the human genome and has immensely
revolutionized the field of biology (Hou et al., 2013). It enables faster and less laborious sequencing of DNA. The greatest advantage of next generation sequencing is that it is time saving and relatively inexpensive. This technology involves various steps such as preparation of template, sequencing and imaging, and analysis of data. One of its drawbacks is that it is capable of generating very complex multilayer data; with the current lack of prognostic or functional understanding of most of the variants that are being identified; the relevance of these variants in health and disease is not known, this makes interpretation of genomic sequence data significantly limited in many occasions, another limitation is the ethical issues that govern data interpretation and reporting.

Reference libraries for nuclear and mitochondrial DNA that consists of more than thousands of samples belonging to people of various ethnicities can be used to evaluate and compare the variants whose clinical significance is unknown. In addition, there are online databases organizing and cataloguing the variations. Combining the classical methods with the modern next generation methods, allows a rapid focusing on candidate positional variants in candidate functional genes.

In the following I will utilize a combination of classical linkage methods and whole-exome sequencing to identify the causative defects in the new diseases identified in the clinic.

**1.5. Problem Statement**

Most clinical disorders with a genetic basis result from complex interactions between multiple genes [multigenic] and environmental factors, termed multifactorial inheritance. Monogenic disorders are rare and are first recognized clinically by their predictable patterns of inheritance in families. The present research project aims at investigating the genetic etiology of a rare novel neurological disorder: Autosomal recessive non-progressive dystonia-myoclonus with sensorineural hearing loss.

**1.6. Thesis overview**
A rapidly increasing number of mitochondrial diseases that are inherited in Mendelian fashion have been recognized in recent years, in part due to the greater awareness of the clinical presentation, and in part due to the utilization of homozygosity mapping in consanguineous families and of genome wide or MitoExome sequencing in patients with mitochondrial diseases. These discoveries have immediate practical influence, as they allow us to uncover the fascinating facts to explain pathophysiology, and subsequently explore treatment options; in addition to providing prenatal diagnosis to families in whom a child has succumbed to one of these devastating diseases.

I hypothesize that:

The syndrome I am describing is a novel autosomal recessive inherited Mendelian disorder, and that it is caused by a mutation that leads to dysfunction in the basal ganglia leading to the movement disorder. I predict that the defect is in a mitochondrial protein. Clinically this hypothesis is supported by the symptom combination of sensorineural hearing impairment and movement disorder, and the biochemical finding of increased serum lactate in addition to the MRI finding of abnormal signal symmetrically involving the basal ganglia. The data provided in this thesis offers important insights into the scientific understanding of the role of the TCA cycle in energy production and ATP synthesis. It helps us understand the role of ATP deficiency in mitochondrial diseases presenting with movement disorders, and the selective vulnerability of the basal nuclei to mitochondrial insufficiency. The data provided in this thesis also provides support to earlier hypotheses of complex enzyme-enzyme interactions and their role in mitochondrial maintenance and function.

My aim is to identify the genetic mutation causative of this rare childhood onset neurological disorder. It is a novel disease, the clinico-radiologic picture is consistent with mitochondrial disease, but no further clinical supportive evidence confirms mitochondrial dysfunction. Therefore, search for cause via the genetic approach using a combination of linkage analysis, homozygosity mapping and whole-exome sequencing is an appropriate approach. Combining the information of candidate chromosomal loci with candidate variants, I expect to be able to identify and then validate the causative mutation. Once I identify the disease-causing mutation, we may be able to tailor a treatment to the disease. Identifying the affected protein will also
open avenues of research to understand disease pathogenesis and the underlying normal neurobiological functions.

To answer the research question, the study is divided into the following objectives:

- Identification of the gene locus and defect in this disorder.
- Analysis of the biology of the protein encoded by this gene and the effects caused by the mutation.
- Analysis of possible pathogeneses addressing the following two questions:
  - Why is there a selective involvement of the corpus striatum?
  - Why is there sensorineural hearing impairment?
  - How can the phenotype of a hyper kinetic movement disorder be explained?
- Explore possible therapies, based on the nature of the defective protein.
Identification of a new mutation in SUCLA2 presenting with a novel phenotype

2.1. Introduction

Mitochondrial cytopathies represent a heterogeneous group of disorders with clinical onset at any age; and with abnormal mitochondrial structure or function as a defining feature. The prevalence of mitochondrial disorders is not known, but there is data to support that its prevalence is higher in patients with Lebanese ancestry. The minimum birth prevalence for respiratory chain disorders in children was significantly higher, with a figure of 58.6/100 000 (95% CI 34.7±92.6) noted for Australian families of Lebanese origin compared to 5.0/100 000 [95% confidence interval (CI) 4.0±6.2] for the general Australian population. The Lebanese patients included in that study were not homogeneous with respect to their region of origin within Lebanon or in religious background, clinical presentation or enzymatic diagnosis (Skladal et al., 2003).

Consensus criteria have been developed based on review of patients referred for the diagnosis of mitochondrial respiratory chain disorders [MRCD] and reevaluation of the previously proposed diagnostic criteria (Walker, Collins, and Byrne 1996; Bernier et al., 2002; Haas et al., 2008). These criteria are not routinely used by practicing physicians as concluded in a study that looked at the practice patterns of mitochondrial disease physicians in North America (Parikh et al., 2013). The modified diagnostic criteria are separated into major and minor criteria [table 2-1] [table 2-2] (Haas et al., 2008).

Table: 2-1. Mitochondrial cytopathy, major diagnostic criteria. Adopted from Haas et al., 2008.
Clinical

Fulfilling three of the following conditions:

Unexplained combination of multisystem symptoms that is pathognomonic for a mitochondrial disorder.

Symptoms that may include at least three of the following organ system presentations described: neurologic, muscular, cardiac, renal, hepatic, endocrine, hematologic, otologic, ophthalmologic, or dermatologic.

A progressive clinical course with episodes of exacerbation (following intercurrent illness).

A family history that is strongly indicative of a mtDNA mutation.

Other possible metabolic or nonmetabolic disorders have been excluded.

Histology:

>2% ragged red fibers in skeletal muscle.

Enzymology:

>2% COX-negative fibers if <50 years of age.

>5% COX-negative fibers if >50 years of age.

<20% activity of any RC complex in a tissue.

<30% activity of any RC complex in a cell line.

Functional:

Fibroblast ATP synthesis rates>3 SD below mean.

Molecular:

Identification of a nuclear or mtDNA mutation of undisputed pathogenicity.

Clinical:

Symptoms compatible with a RC defect.

Histology:

Ragged red fibres.

>2% subsarcolemmal mitochondrial accumulations in a patient <16 years of age.

Widespread electron microscopic abnormalities in any tissue.

Enzymology:

Antibody-based demonstration of a defect in RC complex expression.

20-30% activity of any RC complex in a tissue.

30-40% activity of any RC complex in a cell line.

30-40% activity of the same RC complex activity in > two tissues.

Functional:

Fibroblast ATP synthesis rates 2-3 SD below mean.

Fibroblasts unable to grow on media with glucose replaced by galactose.

Molecular:

Identification of a nuclear or mtDNA mutation of probable pathogenicity.

Metabolic:

One or more metabolic indicators of impaired RC function.

If a patient’s clinical, histological, enzymological, functional, molecular, and metabolic evaluations meet two major criteria or one major and two minor criteria, then a definite diagnosis
of MRCD can be made. If the patient meets one major and one minor, or three minor criteria, then a probable diagnosis is made.

Mitochondrial disorders are complex and a large number of genes are involved. The disease diagnosis is challenging, requiring the incorporation of clinical, biochemical, and histochemical evaluations, followed by final confirmation of the diagnosis by the identification of mutations in causative genes. While recent advances in genomics have allowed for an increased ability to diagnose primary mitochondrial disease, many patients remain who are found to have biochemical abnormalities suggesting mitochondrial dysfunction but in whom no genetic etiology is identified.

Treatment of mitochondrial disease is multifactorial and supportive; it includes optimization of the nutritional deficiencies, vitamin and cofactor supplementation, exercise and more recently anaplerosis, namely bypassing the metabolic block through activation of circumventing pathways. Despite the relatively high prevalence of mitochondrial disease, only a few selective studies of vitamins and xenobiotics were deemed acceptable by the Cochrane Collaborative study conducted by (Pfeffer et al., 2012).

Here I describe a family with three affected children suffering from symptoms suggestive of mitochondrial dysfunction, namely lifelong sensorineural hearing impairment and a movement disorder. The diagnostic evaluation of these children, including, the biochemical profile and MRI study, supports the clinical suspicion of mitochondrial disease even though a muscle biopsy structure and enzymology do not. In this chapter, I describe the clinical presentation, the workup performed, and the in-depth attempt to explain the clinical and biochemical presentation through the understanding of cellular physiology pertinent to this particular disorder.

2.2. The Clinical Syndrome

The index case is a 22-year-old girl, who presented to clinic at the age of 15. She had a lifelong history of sensorineural hearing impairment and non-progressive dystonia with myoclonic jerks that manifested at age one. In review of the past history, pregnancy was normal, birth was uncomplicated, and early milestones were acquired at age appropriate level except for no language acquisition. She walked at the age of one but had frequent fall due to frequent jerking
and abnormal transient recurrent posturing of the trunk and arms, she appeared to be dancing, this movement was persistent during movements and during rest and there was no diurenal variations associated with it. She is the youngest of three children, she has two siblings in their early thirties who share the symptoms of hyperkinetic movement disorder and hearing impairment, they have all followed an identical non-progressive clinical course. All three are cognitively intact and use the full panel of sign language for normal communication. They are Lebanese in origin. Their parents are consanguineous, from a small village in the south of Lebanon. The pedigree is summarized in [Figure 2.1].

**Figure 2-1:** Pedigree of consanguineous family with autosomal recessive non-progressive dystonia and sensorineural hearing impairment. Square is male, circle is female. Three affected children shown in black; white is unaffected; double line indicates consanguinity, crossed box indicates deceased. Arrow pointing to index case.

On physical examination, they are thin built, but have a good muscle mass with a normal tone, a normal power and normal deep tendon reflexes of +2/4 symmetric bilateral upper and lower extremities, plantar reflexes were extensors indicating a central nervous system disease, clonus was absent. It was evident on neurological examination that they have dystonia with sudden myoclonic jerks that can be mistaken for chorea, the movement disorder lead to frequent falling.
The disease has been maintaining a non-progressive course over the last three decades. An extensive metabolic workup, including serum amino acids, serum lactate, pyruvate, urine organic acids, and muscle biopsy from the gastrocnemius muscle was performed in patients IV: 1 and IV: 2 at the age of 5 and 3 respectively, it was reported to be normal; the muscle was examined histologically, it was reported to be normal; blood smear was examined for acanthocytes in the three affected patients IV: 1, IV: 2 and IV: 3, it was negative.

An MRI of the brain of patient IV: 3 showed signal hyperintensity selectively involving the corpus striatum bilaterally similar to the MRI features of Leigh’s syndrome. The rest of the brain anatomy is normal; Magnetic Resonance Spectroscopy [MRS] was normal with normal metabolite peaks [Figure 2.2]. The clinico-radiologic picture in this case is consistent with mitochondrial disease.

**Figure 2-2.** MRI head of affected patient with autosomal recessive non-progressive dystonia and sensorineural hearing impairment. Note the hyper-intense T2 signal affecting the putamen symmetrically.
While a combination of dystonia, sensorineuronal hearing impairment and progressive Leigh like encephalopathy is described in mitochondrial depletion syndrome V, where the nuclear DNA encoded SUCLA2 gene mutation leads to a mitochondrial depletion phenotype, the patients with mitochondrial depletion syndrome V have biochemical evidence of mitochondrial dysfunction in the form of elevated serum lactate and urine Methyramalonic Acid [MMA]. As well, they show mitochondrial structure and function abnormalities evident on muscle biopsy examination. In contrast, the patients I am describing have a non-progressive disease course, with no biochemical evidence of mitochondrial dysfunction in blood and urine testing. Interestingly in 1977 Damasio reported two siblings with sensorineural hearing impairment and chorea with no other associated neurological symptoms. Those patients also followed a non-progressive disease course and the causative gene defect for this disorder was not identified (Damasio, Antunes and Damasio 1977). [Table 2-3].

**Table 2-3.** Summary table of similar patients in literature

<table>
<thead>
<tr>
<th>Age of onset</th>
<th>Current family</th>
<th>Damasio et al., 1977</th>
<th>Mitochondrial depletion type V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonia</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Developmental delay</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Progressive encephalomyopathy</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Movement disorder</td>
<td>Dystonia and myclonus</td>
<td>Chorea</td>
<td>Dystonia</td>
</tr>
<tr>
<td>Sensorineural deafness</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Muscle biopsy</td>
<td>Normal</td>
<td>Not reported</td>
<td>Mitochondrial depletion</td>
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<tr>
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<td>SUCLA2</td>
<td>Unknown</td>
<td>SUCLA2</td>
</tr>
</tbody>
</table>
2.3. Materials and Methods

2.3.1. Clinical Investigation

Blood tests consisting of Complete Blood Count (CBC), blood urea nitrogen (BUN), creatine, serum lytes and liver enzymes were done. Metabolic tests consisting of serum lactate, pyruvate, serum acylcarnitine, total and free carnitine, serum copper and ceruloplasmin. Urine organic acid was also tested. Muscle biopsy was obtained from the right deltoid muscle on patient IV:3. Specimens were obtained and immediately processed for biochemical assay of mitochondrial function in the form of respiratory chain functional assay, and histologic examination including electron microscopy.

2.3.2. Muscle histology and electron microscopy

Tissues collected at biopsy from the right deltoid muscle of patient IV:3 were divided into three batches, one snap frozen by liquid nitrogen, one fixed in 10% neutral buffered formalin and one minced into pieces approximately 1mm³ in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Formalin-fixed materials were embedded in paraffin, sectioned, and mounted on glass slides. They were then stained with periodic acid-Schiff [PAS] reagent following diastase treatment, cover-slipped, and examined under the light microscope. Material collected in glutaraldehyde was rinsed thoroughly in phosphate buffer, post-fixed in OsO4 and dehydrated and infiltrated in Embed 812. The tissues were then embedded and polymerized at 60 degrees C overnight in flat embedding molds to insure proper orientation. Areas of interest were then chosen using 0.5µm toluidine blue stained sections. Ultrathin sections were then cut, mounted on grids and stained with uranyl acetate and lead citrate prior to examination in a transmission electron microscope (JEOL 1011, JEOL USA, Peabody, MA).

2.3.3. Respiratory chain complexes assay

The RC enzymes were assayed in homogenates prepared from the frozen muscle tissue. To prepare the homogenates, the tissue was dissected out, weighed and placed in ice-cold medium
A. It was trimmed of fat and connective tissues, cut finely with scissors, and rinsed in medium A to remove any blood; the tissue was cut further by a scalpel blade then homogenized on ice using T25 Ultra-Turrax homogenizer [500rpm for 5 sec] in a total volume of 1ml of medium A. The homogenates were centrifuged at 600Xg$_{av}$ for 10 minutes at 4 degrees C to remove nuclear debris. The supernatant was centrifuged again at 600Xg$_{av}$ for 10 minutes at 4 degrees C. Enzyme measurements were performed on this supernatant.

Mitochondrial fractions were prepared by differential centrifugation of the homogenates. The muscle tissue was made up to 20 volumes with respect to the original wet weight of tissue with Medium A and homogenized with the T25 Ultra-Turrax homogenizer [9500 rpm for 5 sec]. The homogenate were centrifuged at 600 X g$_{av}$ for 10 min and the supernatant was filtered through four layers of cheesecloth to remove fat and fibrous tissue. The pellet was re-suspended in 8 volumes of Medium A with a hand-held Teflon-glass homogenizer [Potter-Elvehjem] [5–6 strokes] and centrifuged at 600 g$_{av}$ for 10 min. The second supernatant was filtered through four layers of cheesecloth and combined with the first. The pellet obtained at this stage was retained. The combined supernatants were centrifuged [17,000 X g$_{av}$ for 10 min], and the pellet, which contains the mitochondria, was re-suspended in 10 volumes of Medium A and centrifuged at 7000 X g$_{av}$ for 10 min. The pellet was re-suspended in 10 volumes of Medium B and centrifuged at 3500 X g$_{av}$ for 10 min, and the mitochondrial fraction was finally suspended in a small volume of Medium B [25–50 mg protein/ml].

Mitochondrial RC activities were assayed by spectrophotometric assay of the ETC enzyme complexes: complex I [NADH-ubiquione oxidoreductase], complex II [succinate-ubiquinone oxidoreductase], complex III [decylubiquinone-cytochrome c reductase], or complex II+III [succinate-cytochrome c reductase]. The measurement of ETC complex activities is based on the absorbance change of the substrate, either NADH or cytochrome c, depending on the complex being assayed. The enzyme assay was performed at the Mitochondrial Disease Lab at the Hospital for Sick Children following their standard protocol.
2.3.4. Genome analysis, and mutation screening

Blood was collected from the five individuals in the nuclear family via venipuncture of the left arm using sterile technique, 3-5ml of blood was collected in EDTA tubes, it was stored for 4 hours at room temperature, DNA was extracted using the Qiagene DNA extraction kit.

2.3.4.1. Linkage study

High resolution [2,443,177 markers] SNP microarray genotyping analysis was done using the Infinium HumanOmni2.5-Quad v1.0 BeadChip at The Center for Applied Genomics, The Hospital for Sick Children. SNP data were analyzed using GenomeStudio (Illumina Inc., San Diego, USA); the CNV Partition v.3.1.6 module was used for copy number variation [CNV] and loss of heterozygosity [LOH] [copy neutral] analysis.

2.3.4.2. Whole Exome Sequencing

Whole exome sequencing was performed at The Center for Applied Genomics TCAG, The Hospital for Sick Children, in parallel with the above assay-based genotyping. Exome sequencing to a minimum mean target depth of 50X was performed using Life Technologies SOLiD5500xL (Life Technologies, Foster City, CA, USA) sequencing platform with target enrichment using Agilent SureSelect 50Mb human all exon capture kit v4 (Agilent Technologies, Santa Clara, CA, USA). Briefly, 3ug of genomic DNA was sheared by sonication to a target size of 200bp using a Covaris-S2 instrument (Corvaris Inc., Woburn, MA, USA). Fragmented DNA was end-repaired using end polishing reagents and purified using Agencourt Ampure XP beads (Beckman Coulter, Inc.). Target fragments of approximately 200bp were size selected by agarose gel electrophoresis with SOLiD library size selection (2%) gel and the recovered DNA amplified using SureSelect Pre-capture primers and Herculase II Fusion Enzyme (Agilent Technologies, Santa Clara, CA, USA) with conditions of 72°C for 20 minutes, 95°C for 5 minutes followed by 12 cycles of 95°C for 15 seconds, 54°C for 45 seconds, 70°C for 60 seconds and a subsequent final extension of 70°C for 5 minutes. Hybridization with biotinylated RNA baits corresponding to target regions was carried out using 500ng of prepared library for 24 hours at 65°C and captured DNA recovered with streptavidin coated magnetic beads. The recovered library was
amplified and barcode sequencing tag incorporated by amplification with Herculase II Fusion Enzyme and AB-specific SureSelect barcoding primers under conditions of 95°C for 5 minutes, then 95°C for 15 seconds, 54°C for 45 seconds, 70°C for 60 seconds for 12 cycles and a subsequent final extension of 70°C for 5 minutes. Equimolar quantities of six barcoded exome libraries were pooled for preparation of templated beads by emulsion PCR using the Life Technology EZ Bead system according to the manufacturer’s instructions. Beads were loaded on a 5500 flowchip and paired-end sequencing carried out on a SOLiD 5500xl instrument.

The second step is preparing read maps, variant calls and annotation; variant calls were made with GATK after aligning with BFAST. Paired end reads for samples were combined and mapped to the reference human genome (UCSC hg19) using BFAST (http://bfast.sourceforge.net) (Homer et al., 2009). Indel and SNP calls were made using GATK version 1.0.5506 and recommended parameters (Depristo et al., 2011). Base quality score recalibration optimized to SOLID 5500xl is used to remove reference bias. All no-call reads are removed from the analysis and if a reference base was inserted the base quality will be set to zero and the inserted reference nucleotide was set to “N”. Variant recalibration is used before using GATK implemented BEAGLE to refine the genotypes. Variant calls are annotated using SNPeff (http://snpeff.sourceforge.net/). SNPeff is a program used for annotating and predicting the effect of SNVs in the genome, for example it predicts the effect of variants on amino acid change. To differentiate common polymorphisms from novel variants SNVs were overlapped with public SNP databases (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/; 1000genomes, www.1000genomes.org) (1000GenomesProjectConsortium, 2010) and the exome variants server database (http://evs.gs.washington.edu/EVS/).

2.3.4.3. Polymerase Chain Reaction (PCR)

Once I identified the candidate gene, I designed the primers to amplify it using the online program Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) as follows:

**SUCLA2**;

Forward primer exon 9 (9F): ggtttggtctgaaaatctttctatc.

Reverse Primer exon 9 (9R): ctgaactccatggaatgtgaa.
Product size: 312 bp. The following PCR conditions were used:

**Table 2-4. PCR conditions.**

<table>
<thead>
<tr>
<th></th>
<th>Initial concentration/quantity</th>
<th>Final concentration/quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10X</td>
<td>2.4ul</td>
<td>10X</td>
</tr>
<tr>
<td>MgCl2</td>
<td>1.5ul</td>
<td>25mM</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.5ul</td>
<td>10mM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.7ul</td>
<td>10uM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.7ul</td>
<td>10uM</td>
</tr>
<tr>
<td>H2O</td>
<td>17.975ul</td>
<td></td>
</tr>
<tr>
<td>Taq</td>
<td>0.125ul</td>
<td>5U/ul</td>
</tr>
<tr>
<td>DNA</td>
<td>1ul</td>
<td>50ng/ul</td>
</tr>
<tr>
<td>Total volume</td>
<td>25ul</td>
<td></td>
</tr>
</tbody>
</table>

**PCR cycles**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>94 C</td>
<td>3’</td>
<td></td>
<td>1 cycle</td>
</tr>
<tr>
<td>94C</td>
<td>45”</td>
<td></td>
<td>35 cycles</td>
</tr>
</tbody>
</table>

PCR amplification was verified by gel electrophoresis in 1.2% agarose gel. PCR purification was performed with microClean as follows: 1 volume PCR + 1 volume microClean (ex. 20ul PCR+20ul uClean) mixed well by vortexing, then left at room temperature for 5 minutes, the mixture was spined at maximum speed on a bench to centrifuge for 7 minutes, supernatant was removed, spined again, last drops were removed and let to dry, then resuspend in 15µl H2O.
2.3.4.4. Sanger Sequencing

Fredrick Sanger developed Sanger sequencing technique in the 1970s, in this technique labeled dideoxy trinucleotide triphosphates (ddNTPs) are utilized as DNA chain terminators. Terminated chains of various lengths are separated by gel electrophoresis to determine base order in the sequence.

For sequencing I mixed 50ng purified PCR product+ 0.5ul primer (from 10uM working aliquot)+H2O= 7.7ul. Sequencing was performed at The Center for Applied Genomics, The Hospital for Sick Children, following the standard protocol summarized in [Figure 2-3]. The sequencing graphs were generated using 4 peaks program.

**Figure 2-3:** Sanger method for DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA.

![Diagram of Sanger Sequencing](http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/D/DNAsequencing.html)
2.3.4.5. Control studies

I screened 40 ethnically matched healthy controls from the South of Lebanon, the DNA samples were provided by Dr. Rosemary Boustani from the American University of Beirut AUB. The region of interest was amplified by PCR and then Sanger sequencing was performed.

2.4. Results

2.4.1 Clinical investigation

Initial blood and urine tests summarized in the table below were normal, these tests were performed in the 1980’s at The American University in Beirut (AUB) samples were collected from individuals IV: 1 and IV: 2 ages were 5 and 3 respectively, repeated testing after the molecular genetic information was obtained was done on all three affected patients at age 35, 33 and 22, it showed evidence of TCA disruption, namely elevated serum lactate, Acyl carnitine, and urine MMA as summarized in [Table 2-5].
Table 2-5. Summary of biochemical investigation in patients with novel SUCLA2 mutation.

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient IV: 1</th>
<th>Patient IV: 2</th>
<th>Patient IV: 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>5year</td>
<td>32 year</td>
<td>3year</td>
</tr>
<tr>
<td>Serum. Lactate (0-2.4mmol/L)</td>
<td>Normal</td>
<td>Not done</td>
<td>Normal</td>
</tr>
<tr>
<td>Serum, Acylcarnitine (C3&lt;1.08umol/L)</td>
<td>Normal</td>
<td>Not done</td>
<td>Normal</td>
</tr>
<tr>
<td>Urine MMA</td>
<td>Negative</td>
<td>Elevated</td>
<td>Negative</td>
</tr>
<tr>
<td>Muscle biopsy</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>RC assay</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>


2.4.2. Pathological findings

In the affected patient muscle RC complex activity was normal. Muscle histology and Electron Microscopic assessment confirmed normal muscle structure and normal mitochondria.

**Figure 2.4.** Muscle Electron Microscopic Exam showing normal mitochondrial structure.
2.4.3. Gene locus

Using homzygosity mapping. I identified multiple regions of homozygousity shared between the three affected members of the family; the largest regions were two homozygous regions of 19Mb and 8Mb, both on chromosome 13q14, the coordinates were; Hg19: Ch13: 45,245,670-64,202,461 and Ch13: 97,513,234-105,666,880. [Figure 2-6].

**Figure 2-5.** Homozygosity map. Shared region of homozygosity among the affected individuals. Image from Illumina’s Genome Studio Genotyping Module software v.2011.

In total 30,803 Single Nucleotide Variants SNVs were identified via the exome sequencing. I analyzed the data based on the information I gathered from the homozygosity mapping focusing on the largest region of shared homozygosity, which was on chromosome 13. I applied a series of exclusion steps to uncover the causative mutation, considering the autosomal recessive mode of inheritance; I rejected variants that were heterozygous, synonymous and predicted to have a tolerated no damaging effect on the protein. I also eliminated variants that were reported in the SNP database dbSNP, the 1000 Genomes project and the in house database. On chromosome 13 there were 1959 calls, of which a total of 407 calls were in the two shared region of
homozygosity. 115 variants were non-synonymous that represents 28.5% of the shared region on chromosome 13, 48 (11.5%) were synonuymous and 244 (60%) were of unknown effect on the amino acid. Of the 115 non-synonymous variants 83 were novel, of which 33 variants were rejected because they have a tolerated non-damaging effect on the protein, and 48 variants were also rejected because they were heterozygous variants within the shared region. Only 2 of a total of 51 novel, non-synonymous and predicted damaging variants were homozygous, one is a mutation in MED4 gene that codes for mediator of RNA polymerase II transcription subunit 4, this is a subunit of vitamin D3 receptor-interacting protein complex. The mutation was of low confidence; the number of reads for that particular variant is only 2 (Total read depth). This is one good reason not to consider this variant as real, and not even homozygous, with only 2 reads one cannot really be sure if both alleles are being sequenced. Another reason to reject this call is; it had a quality depth (QD) of 11 and strand bias (SB) of 0. The other homozygous variant had the highest confidence of all variant calls, with a QD of 33 and a SB of -1,044. This variant was in the SUCLA2 gene, whose product is known to be involved in a mitochondrial related biosynthesis pathway [Figure 2-7 A and B].
Figure 2-6. Panel A depicts total number of variants detected by Whole Exome Sequencing and filtering the variants detected on whole exome sequencing based on the information obtained from homozygosity mapping. Panel B depicts whole Exome Data analysis, proportion of the homozygous variants that contained non-synonymous novel variants.
The variant SUCLA2 c.1219 C>T (R407W) is a missense mutation in exon 9 of SUCLA2 gene, it leads to amino acid arginine (R) change to tryptophan (W), The presence of arginine in this location is conserved [Figure 2.10]. Sanger sequencing confirmed this mutation and its segregation [Figure 2-12]. Sanger sequencing in controls from the same ethnic background confirmed that the variant is not present in control population [Figure 2-13]. The amino acid R change to W at position 407 leads to a major conformational change in the structure of the protein as predicted by Blosum Matrix (BLOck Substitution Matrix); a substitution matrix used for sequence alignment of proteins [Figure 2-13]. The SUCLA2 gene codes for succinate-CoA ligase [ADP forming] beta subunit. This mitochondrial matrix enzyme is made of 463 amino acids, which is a part of the succinate/malate CoA ligase beta subunit family. It is an important constituent of the TCA cycle.

**Figure 2-7.** Identification of the disease causing mutation in SUCLA2 gene. The presence of arginine amino acid at position 407 is conserved as indicated by the red box.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Gene</th>
<th>Bed start</th>
<th>Bed end</th>
<th>Codon</th>
<th>Substitution</th>
<th>SNP type</th>
<th>QD</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>SUCLA2</td>
<td>48,523,62</td>
<td>48,523,62</td>
<td>CGG</td>
<td>R407W</td>
<td>Nonsynonymous</td>
<td>33</td>
<td>-1,044</td>
</tr>
</tbody>
</table>

![Fig](image.png)
Figure 2-8. Sanger sequencing confirmed the mutation in *SUCLA2* and its segregation with the disease. The mother and father are heterozygous while the affected three children are homozygous.
Figure 2-9. Sanger sequencing in controls from the same ethnic background confirmed that the variant is not present in the control population, shown below is the sequence in 36 individuals.
Figure 2-10. The effect the mutation at protein level. A. The amino acid arginine change to tryptophan at position 407. B. The aminoacid change leads to a major conformational change in the structure of the protein as indicated in the orange box, predicted by Blossom Matrix. C. Swiss model 3D structure of the SCS-β domain, it shows the conformational change in the protein caused by the amino acid change, the amino acids of interest are represented by the red balls.
Arginine

Tryptophan
2.4.4. Succinyl-CoA Synthase functional enzyme assay

The succinyl-CoA synthase activity was determined in fibroblasts, after permeabilization of fibroblasts with digitonin to remove the cytosol, and subsequent centrifugation in two independent experiments. The formation of succinyl-CoA from succinate plus ATP or GTP and CoA was determined by quantification of the succinyl-CoA as produced over time using High Performance Liquid Chromatography [HPLC]. Succinyl-CoA ligase activity was expressed as a ratio. As described earlier by (Wanders, Ruiter, and Wijburg 1993).

2.4.5. Functional enzyme assay result

The Succinyl-CoA ligase activity with GTP was normal compared to the activity in the control cell lines measured in the same experiment. The activity with ATP was not detectable in the fibroblasts of the patient in contrast to the abundant activity in the control fibroblasts.

Ratio in control fibroblasts [with GTP]: 95 and 130. Ratio in patient fibroblasts: 118.


2.5. General Discussion

2.5.1. Succinyl-CoA Synthetase Biology

Succinyl-coenzyme A synthase is a mitochondrial inner membrane-integral enzyme associated with the Krebs cycle. It catalyzes the reversible conversion of succinyl-coenzyme A to succinate, which is coupled to the phosphorylation of guanosine diphosphate [GDP] to guanosine triphosphate [GTP] or adenosine diphosphate [ADP] to adenosine triphosphate [ATP]; it has two subunits; α and β. The α subunit, encoded by the SUCLG1 gene [also referred to as SUCLA1] is constant, whereas the β subunit, encoded by SUCLA2 for the ADP-specific subunit and by SUCLG2 for the GDP-specific subunit, determines substrate specificity for GDP or ADP.
2.5.1.1. Succinyl-CoA Synthetase in Prokaryotes

The molecular, biochemical, and structural characteristics of SCS in the prokaryote *Escherichia coli* [*E.coli*] have been studied. In *E. coli* the genes that encode the α and β subunits of SCS are encoded by sucD and sucC, respectively (Bailey et al., 1999). The detailed structure has been described (Fraser et al., 1999). Examination of SCS structure by x-ray crystallography has elucidated the enzyme active site and nucleotide diphosphate-binding site. The SCS active site is formed at the interface of the α and β subunits. The polypeptide of each subunit is folded into distinct N-terminal and C-terminal domains. One molecule of CoA is bound to each α-subunit at a nucleotide-binding motif in the N-terminal domain consisting of a Histidine amino acid located at 246α. Two glutamate residues [Glu 208α and Glu 197β] are critical for SCS enzyme activity and are required for the phosphorylation and dephosphorylation of His 246α amino acid, which is located within the active site of the α-subunit. Within each αβ-dimer both C and N domains of the α-subunit and the C-domain of the β-subunit contribute structural elements that form an active site. The two active sites in the tetramer are equivalent deep pockets arranged on the same face of the whole enzyme. At each active site, the molecule of CoA is bound. The binding sites for the substrates succinate and ADP remain to be identified. There are a total of three nucleotide-binding motifs associated with each active site. One of these (in the N-domain of the α-subunit) is accounted for as the CoA-binding site. The second motif is in the C-domain of the α-subunit, and the third is in the C-domain of the β-subunit. Experiments have shown that ADP is phosphorylated in the presence of α-subunit and the absence of the β-subunit; this suggests that the ADP-binding site is entirely on the α-subunit (Pearson and Bridger 1975), the crystal structure of the SCS enzyme suggests a possibility that binding of the nucleoside part of ADP is to a region of the β-subunit. The nucleotide-binding motif in the C-domain of the β-subunit is an appropriate distance from the phospho-histidine (Wolodko et al., 1994). [Figure II-14]
Figure 2-11: The detailed crystal structure of SCS in e-coli.

A SCS alpha-subunit

CoA bound to N-terminus of SCS alpha-subunit

Phosphorylated histidine
2.5.1.2. Succinyl-CoA Synthetase in Eukaryotes

SCS is conserved throughout eukaryotes (Fraser et al., 2002). After the initial purification of eukaryotic SCS in the 1950s (Sanadi, Gibson, and Ayengar 1954; Ayengar, Gibson, and Sanadi 1954), the ADP-specific SCS was purified from multiple sources, including pigeon (Severin SE et al., 1976; Allen et al., 1986) in the 1970s and 1980s.
In the 1990s, with the application of PCR based technologies, the identification and cloning of GDP and ADP β gene isoforms was demonstrated across multicellular eukaryotes (Johnson et al., 1998).

Eukaryotic SCS is catalytically active as a αβ-dimer. The residues of the α- and β-subunits that are involved in forming a αβ-dimer and the residues located in key active sites motifs are conserved, this implies that the catalytic mechanisms of the two forms of the enzyme are similar.

**Figure 2-12**: simplified illustration of the two domains that constitute β-subunit of SCS.
Figure 2-13: Crystal structure of SCS-β subunit, the red ribbon constitutes the CoA-ligase domain while the blue ribbon constitutes the ATP-grasp domain.
The tissue specific expression of the ADP and GDP forming subunits are summarized in the figure below [Fig 2-14] (Johnson et al., 1998).

**Figure 2-14.** Real time-PCR Expression of Succinyl-CoA Synthetase in 16 different human tissues. Note that the brain and the liver have a reverse expression pattern of the A and G-specific β SCS, labeled by the orange box.

2.5.2. Mitochondrial Depletion Syndrome caused by *SUCLA2* mutation

2.5.2.1. Literature review and summary of previously reported *SUCLA2* mutations

Mitochondrial DNA depletion syndromes are autosomal recessive disorders with a broad genetic and clinical spectrum characterized by a reduction in mtDNA content in affected organs. Adequate amounts of mtDNA are required for the production of mitochondrial respiratory chain complex [RC] subunits and subsequent energy production. Depletion of mtDNA results in organ dysfunction due to insufficient synthesis of respiratory chain components needed for energy production (Sarzi et al., 2007; Spinazzola et al., 2009).
Mitochondrial depletion syndrome V results from mutations in *SUCLA2*, the same gene mutated in our family. Our patients do not have mitochondrial depletion, nor do they have the progressive severe course of patients with mitochondrial depletion syndrome V. The reasons for this are clarified below.

Mitochondrial depletion syndrome V manifests with a wide range of disease phenotypes, all severe and progressive, including infantile lethal lactic acidosis, encephalomyopathy with sensorineural hearing loss, and BG dysfunction manifesting as a hyperkinetic movement disorder, mainly dystonia. Neuroimaging findings are those of Leigh’s disease namely; bilateral, symmetrical lesions in the basal ganglia, thalamus and/or brainstem (Sofou et al., 2013; Elpeleg et al., 2005).

In 2005 Elpeleg et al. described the first case of mitochondrial depletion syndrome V caused by mutations in *SUCLA2*. Two first-degree cousins from a consanguineous middle eastern family presented with an encephalomyopathic phenotype including Leigh’s disease features on brain MRI, multiple respiratory chain deficiencies in muscle (sparing complex II), and depletion of mtDNA in muscle (Elpeleg et al., 2005). Homozygosity mapping identified a complex indel mutation spanning the exon 6-intron region. This mutation caused aberrant mRNA splicing affecting exon 6 and exon 7 resulting in a frame shift and truncated transcript. Many more mutations in the *SUCLA2* gene have been identified at different splice sites confirming the role of *SUCLA2* as an important human disease gene [Table III-1].

Various studies have investigated mutations in the *SUCLA2* genes and the disorders associated with these mutations. Carrozzo et al. investigated mutations in the *SUCLA2* gene in 14 patients from eleven families, eight of which were from the Faroe Islands, and three of which were from southern Italy (Carrozzo et al., 2007). Three novel mutations in the *SUCLA2* gene were found. These mutations were also associated with mtDNA depletion. Clinically the patients described suffered from a progressive encephalomyopathy, and early onset deafness and dystonia. MRI abnormalities primarily affecting the caudate nuclei and the putamen, described as Leigh-like changes. In 2008 Ostergaard reported *SUCLA2* mutation in twelve patients from the Faroe Islands. Symptoms of the disorder were hyperkinesia, growth retardation, hypotonia, and hearing impairment. Brain imaging consisted of basal ganglia atrophy; there also were both cortical as well as central atrophy. The patients had high levels of methylmalonic acid in the plasma and
urine. The two studies independently conducted by Carrozzo et al. 2007 and by Ostergaard et al. 2008 identified the same homozygous founder splice site mutation in the SUCLA2 gene through different approaches. The mutation [c.534 + 1G>A] alters exon splicing, resulting in the introduction of a stop codon, and causing premature stopping of the mRNA and subsequent truncation of the final mRNA transcript. Exon skipping is observed in the cDNA, however, different transcripts by the same mutation were observed by the two different groups (Chinnery 2007). The carrier and disease frequencies in this population were estimated to be 2% (Ostergaard et al., 2007; Carrozzo et al., 2007).

Another reported mutation in SUCLA2 gene was a homozygous 850C-T transition in exon 7 of the gene, resulting in a G118R amino acid; identified in a boy from southern Italy with encephalopathy and methylmalonic aciduria (Carrozzo et al., 2007). Subsequently, a distantly affected relative of this patient was identified with delayed motor milestones and failure to thrive noticed from the fourth month of life. At the age of 3 years he presented with poor growth, axial hypotonia, and dystonic movements. Brainstem Auditory Evoked Potentials [BAEP] revealed sensorineural deafness. Laboratory investigations showed increased blood lactate (3.0 mmol/l, normal 52). He was found to be compound heterozygous for G118R and R284C (Carrozzo et al., 2007).

In 2012 two different investigators’ groups identified other mutations in SUCLA2, one study reported a patient who presented with severe encephalopathy onset at age 4 months, she also had dyskinesia and sensorineural hearing impairment confirmed by BEAR. She had elevated MMA in urine, biochemical testing and muscle biopsy confirmed mitochondrial dysfunction and mtDNA depletion. Brain MRI revealed mild cerebral atrophy. This patient carried two novel heterozygous missense mutations in SUCLA2, c.1048G>A and c.1048 G>T, affecting the same amino acid residue, which is a highly conserved glycine that is changed to serine and valine, respectively p.G350S/p.G350V, both parents were heterozygous (Navarro-Sastre et al., 2012). The second study described two female siblings who presented with a similar phenotype of hypotonia at birth and severe psychomotor retardation, Dystonia, and ptosis, the older sibling died at age 15 due to respiratory illness, and the younger was age 7 at the time of description, she was found to have an elevated MMA in urine, also biochemical testing and muscle biopsy confirmed mitochondrial dysfunction and mitochondrial DNA depletion. At age 14 months MRI brain revealed severe bilateral abnormalities in the corpus striatum and atrophy of the cerebellum.
and the medulla oblongata, mtDNA sequencing failed to identify mutations in mitochondrial; WES helped detect a c.308C>A variant that leads to a p.A103N substitution in SUCLA2 gene. The amino acid change affects a highly conserved residue (Lamperti et al., 2012).

In 2013, a novel mutation (p. D 251 N) in the SUCLA2 ATP grasp unit was reported in a patient who presented with encephalopathy and mitochondrial depletion syndrome, with elevated succinylcarnitine in urine and a lack of the usually observed Methylmalonic aciduria [MMA]. It is not understood why this patient with the SCS ATP grasp domain mutation did not have the elevated MMA in urine (Jaberi et al., 2013). It had been hypothesized that the observed elevated urine MMA in many patients might result from the secondary inhibition of Methylmalonyl-CoA mutase by elevated Succinyl-CoA resulting from SCS deficiency (Ostergaard et al., 2007). Table 2-6 summarizes all the SUCLA2 gene mutations reported to date.

Table 2-6. Summary of previously reported mutations in SUCLA2.

<table>
<thead>
<tr>
<th>SUCLA2</th>
<th>SUCLA2 mutation</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch 13q14.2</td>
<td>g.32720del43ins5</td>
<td>Elpeleg et al 2005</td>
</tr>
<tr>
<td>Ch 13q14.2</td>
<td>c.850C&gt;T (p.R284C)</td>
<td>Carrozzo et al 2007</td>
</tr>
<tr>
<td>Ch 13q14.2</td>
<td>c.352 G&gt;A (p.352G&gt;A (p.G118R)</td>
<td></td>
</tr>
<tr>
<td>Ch 13q14.2</td>
<td>c.534+1G&gt;A</td>
<td>Ostergaard et al 2008</td>
</tr>
<tr>
<td>Ch 13q14.2</td>
<td>c.308C&gt;A (p. A103D)</td>
<td>Lamperti et al 2012</td>
</tr>
<tr>
<td>Ch 13q14.2</td>
<td>c.1049G&gt;T (p.G350V)</td>
<td></td>
</tr>
<tr>
<td>Ch 13q14.2</td>
<td>c.751G&gt;A (p.D251N)</td>
<td>Jaberi et al 2013</td>
</tr>
</tbody>
</table>
2.5.2.2. Novel SUCLA2 mutation

Mutations in the SUCLA2 gene result in mitochondrial DNA depletion syndromes type 5 [MTDPS5]. Here we have described a new phenotype that is allelic to MTDPS 5 due to SUCLA2 gene mutations. The most interesting part in this novel phenotype is the fact that SUCLA2 activity deficiency in our patient does not result in a mitochondrial depletion or structural mitochondrial pathology, and the patients follow a non-progressive disease course. The actual molecular mechanism behind this phenomenon is yet to be investigated; the explanation for the finding that all patients with SUCLA2 mutations to date also have mitochondrial depletion is as follows:

MtDNA maintenance is a complex process that requires different proteins encoded by nuclear genes to regulate DNA replication, and DNA repair. Unlike nuclear DNA, mtDNA replication is a continuous process and it is independent of cell division. The dNTPs used for DNA replication can be synthesized via either a de novo pathway, which is cell cycle regulated, or the salvage pathway in which dNTPs are produced by utilizing preexisting deoxynucleosides to synthesize DNA precursors. Due to the high turnover of mitochondria, mtDNA synthesis is continuous throughout the cell cycle and the salvage pathway becomes essential for mtDNA maintenance. The nucleotide sequence used for mtDNA replication is produced within the mitochondria, and the dNTP pools are maintained through the salvage pathway. There are several proteins that are involved in this pathway, one of which is the enzyme nucleotide diphosphokinase [NDPK], which catalyzes the transfer of terminal phosphates from nucleoside triphosphates to nucleoside diphosphate to yield a nucleotide triphosphate that is used for mtDNA replication. There are three isoforms of NDPK of which one is a mitochondrial isoform.

The pathomechanism of SUCLA2 deficiency-associated mitochondrial depletion is based on a demonstrated physical association between the mitochondrial form of NDPK and SUCL. The mitochondrial isoform of NDPK was recovered in the immune-precipitation of mitochondria using an antiserum directed against SUCL, suggesting that NDPK might be in a complex with SUCL (Kadrmas, Ray, and Lambeth 1991; Kavanaugh-Black et al. 1994; Kowluru, Tannous, and Chen 2002).

SUCLG2 is the hepatic isoform of SUCL. SUCLA2 is the brain isoform. The brain does not
express SUCLG2. Similarly the liver expresses SUCLG2, but not SUCLA2. Other tissues, such as fibroblasts, express both isoforms [Figure 2-14] Loss of SUCLG2 has not been reported yet as a disease causing mutation, possibly due to lethality in the absence of hepatic SCS activity. Loss of SUCLA2, as mentioned above, causes mitochondrial depletion syndrome type V. The mutation described in the current family is unique; it is located near the C-terminal of the β-subunit in the CoA-ligase domain. Protein stability is linked to protein folding. For proteins to be stable they have to be folded into, and maintained in, their final active conformation. A folded protein consists of a core of hydrophobic amino acids surrounded by hydrophilic amino acids that interact with themselves and the exterior aqueous environment through hydrogen bonding. In the current mutation there is a major conformational change of the SCS protein changing a hydrophilic amino acid [Arginine] to a hydrophobic one [Tryptophan] [Fig2-13]. This influences the folding of this enzyme thus affecting its function, because the amino acid change is near the C-terminal which is one of the three parts that form the SCS active site, it is possible that the major conformational change caused by the mutation affected the function of the catalytic site of the enzyme; sparing the complex formed between the SCS and NDPK [Table 2-6]. As such, the current model is that the mitochondrial depletion syndrome associated with SUCLA2 deficiency is not directly linked to the succinyl CO-A ligase function, instead it is a secondary phenomenon due to the loss of NDPK stability and activity when its interacting/stabilizing partner, SUCL, is lost (Elpeleg et al.2005).

In this project, we describe a novel neurological disorder with a new clinical phenotype. This disorder is allelic to the known mitochondrial depletion syndrome V caused by mutation in SUCLA2 and presenting with symptoms caused by a combination of TCA defect, plus mitochondrial depletion, this dual pathology makes it difficult to treat. In contrast, in this new disease we are describing; the patients do not present with a mitochondrial encephalomyopathy or other symptoms and signs of mitochondrial depletion, the presentation in the family I am describing differs significantly from the clinical presentation of patients with mitochondrial depletion syndrome V, while both disorders share the combination of sensorineural hearing impairment, dystonia, and the neuroradiological feature of Leigh’s disease, namely; T2 signal hyperintensity involving the corpus striatum bilaterally. In the current disease the patients follow a non-progressive disease course, the oldest sibling is in her third decade and has a similar clinical phenotype like the index case who is in her early 20’s, they have normal mitochondrial
histology and biochemistry, patients with \textit{SUCLA2} related mitochondrial depletion syndrome V follow a progressive disease course and have evidence of mitochondrial depletion on histology and abnormal mitochondrial biochemical profile. It appears that the novel mutation described in the current family affects only the TCA cycle without the mitochondrial depletion component. This allows for exploring treatment options to overcome the block in the TCA cycle and reactivate this process.

**Figure 2-15:** Panel A depicts up to date summary of amino acid changes reported due to \textit{SUCLA2} mutation. Panel B shows crystal structure of SCS-β subunit domains, the amino acids that were previously reported are labeled.
Table 2-7. Summary of previously reported SUC2LA2 mutations.

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<tr>
<th>Damasio et al., 1976</th>
<th>Epeleg et al., 2005</th>
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<th>Ostergaard et al., 2008, and Carrozzo et al., 2007</th>
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**Hypotonia**
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2.5.3. Treatment

Mitochondrial cytopathies are difficult to treat. Early attempts to develop treatments for mitochondrial disorders have focused on enhancing respiratory chain complexes functions. This was done through different approaches, one example used supplements aimed at increasing respiratory chain substrate availability, including carnitine, which facilitates the transfer of fatty acids, increasing the availability of metabolites from the citric acid cycle. These metabolites include niacin, the precursor to NADH, which transfers electrons from intermediate metabolites to the respiratory chain, and thiamine, which enhances pyruvate dehydrogenase activity. The first case report of a treatment benefit in mitochondrial disease was published in 1981. This was a report of correction of increased serum pyruvate and lactate after using large doses of thiamine in patients with Kearns-Sayre syndrome (Lou 1981). To enhance electron transfer within the respiratory chain; supplementation with riboflavin was tried; riboflavin is the precursor for flavin adenine dinucleotide [FAD], an electron carrier bound to complexes I and II (Bernsen et al., 1993). Another way to enhance the electron transfer is the use of coenzyme Q10 [CoQ10], which is also known as ubiquinone. This is an electron carrier from complexes I and II to complex III (Rauchová, Drahota, and Lenaz 1995). Synthetic agents based on CoQ10 and vitamin E such as the drugs Idebenone have also been designed to increase the penetration into mitochondria and/or CNS tissue and act as an electron carrier (Orsucci et al., 2011; Shrader et al., 2011).

Another strategy to treat mitochondrial diseases includes biochemical bypass of specific respiratory chain complexes, such as with the use of a combination of vitamins C and K to bypass complex III (Eleff et al.,1984). Anaplerosis is the most recent trend in therapeutic approach in patients with mitochondrial diseases; it is the process of replenishing depleted TCA cycle intermediates that have been extracted for biosynthesis, to maintain a dynamic state of equilibrium of the cycle’s biochemical reactions. One such example is the use of succinate in patients with MELAS, with favorable outcome (Oguro et al., 2004) as described below. Finally, exercise therapy is thought to produce adaptations in mitochondria that improve oxidative capacity and/or reduce muscle deconditioning (Cejudo et al., 2005).

The currently described new disorder occurs as a result of a defective function of SUCLA2 enzyme, a TCA cycle enzyme, the defect leads to depletion of succinate; an intermediate in the TCA cycle that also directly donates electrons to the ETC by the reaction: succinate + FAD →
fumarate + FADH2. This reaction is catalyzed by the enzyme succinate dehydrogenase, also known as succinate-ubiquinone oxidoreductase or complex II of the mitochondrial ETC. Complex II is a membrane-bound lipoprotein, which couples the oxidation of succinate to the reduction of ubiquinone. It is the only enzyme that participates in both the TCA cycle and the ETC. A block in TCA cycle at the step of succinate production leads to depletion of the succinate and a subsequent depletion of the electrons produced in the steps downstream of this reaction, and carried by the electron carrier FAD [Figure 2-15]. As a result the electron transport in the ETC will be slowed down and there will be a net reduction in the synthesis of ATP, which is synthesized by complex V of the ETC.

2.5.3.1. Treatment implemented in this family

There are multiple treatment approaches that can be explored and implemented in this family. The most obvious treatment approach to consider is the supplementation of the product that is deficient due to the defective SCS enzyme function anaplerosis. Due to the fact that the mitochondria is intact in these patients and they are not suffering from the dual pathology described in mitochondrial depletion syndrome V due SULA2 mutation, theoretically, reactivating the TCA cycle by supplying succinate will lead to more electrons generated and carried by FAD, then fed into the ETC to produce ATP. When ATP is dephosphorylated to adenosine, it interacts with the adenosine receptor in the BG as an inhibitory neuromodulator of dopamine, and improves the hyperkinetic movement disorder that is disabling the patients.
Figure 2-16. Figure showing the consequences of a block in TCA cycle at the step of succinate production. This leads to depletion of the succinate and a subsequent depletion of the electrons produced in the next step of the cycle, and carried by the electron carrier FAD.

In mitochondrial cytopathies secondary to complex II deficiency, there is a block of the TCA cycle just downstream to the Succinate synthesis step catalyzed by SUCLA2 and there is also a reduced amount of electrons generated and carried to the ETC. The difference in our case is the tissue specificity in SUCLA2 mutations. In the disorder I am describing the defect affects brain and skeletal muscle ATP production, while complex II deficiency affects all organ systems. If succinate is supplemented the cycle can be reinstated and the block can be overcome. Theoretically, the same number of electrons will be produced and ATP synthesis will be reactivated. To obtain good efficacy in the treatment of mitochondrial encephalopathy, the drug needs to cross the blood brain barrier [BBB], and needs to be delivered to the mitochondrial
matrix after crossing the outer membranes. Succinate has a low molecular weight of 116.07 Da; it is small enough to be transported into the mitochondrial matrix by passive diffusion.

Succinate has been used for the treatment of MELAS. In 2004 a 27-year-old patient with confirmed MELAS who was successfully treated with succinate for two years at a dose of 6 g per day. With this treatment his encephalopathy improved and using Hasegawa Dementia Rating Scale to document response to treatment he showed an increase from 21 points to 26 points over the treatment period (Oguro et al., 2004).

Despite the defect in complex I in this patient with MELAS, the respiratory chain complex function could be restored by administration of succinate, through activation of complex II system. It has been reported that succinate increases electron flow from complex II to complex III and complex IV, enabling these two complexes to operate normally (Ichiki et al., 1988).

In fact we have started treating the patients in the current family with food grade succinate and opted to start the patients on 3 g twice a day. Our plan is to monitor their response by obtaining pretreatment blood and urine work to be repeated in 4 weeks, including serum lactate, acyl carnitine, and urine MMA, in addition to close clinical monitoring of the movement disorder via follow up visits to the clinic. The plan is to treat for a period of 3 months guided by the biochemical test results, if there is no improvement in the biochemical values of the metabolites mentioned above in blood and urine and there is no noticeable improvement in the clinical presentation then the plan is to start treatment with option 2, which is predicted to correct the hyperkinetic movement disorder based on a scientific background as will be explained shortly.

2.5.3.2. Other potential treatment approaches assessed

The second plan of treatment aims at correcting the movement disorder. This can be achieved by using agents that reduce the amount of dopamine available at the neuronal synapse by blocking dopamine neurotransmission since there is a short fall of ATP synthesis, leading to a deficiency of Adenosine, which is an inhibitory neuromodulator of Dopamine receptors.

ATP, in addition to being the cell’s energy currency, is converted to adenosine; a neuromodulator that acts on adenosine receptors. Adenosine A1 and A2A receptors antagonistically modulate the binding, transduction and functional characteristics of D1 and D2
dopamine receptors, respectively. In our disorder there is potentially a net deficiency of brain ATP production, thus leading to a potentiation of the dopaminergic neurotransmission. Therefore, treating these patients by inhibiting dopaminergic neurotransmission may reverse the hyperkinetic movement disorder. Tetrabenazine (TBZ) a reversible inhibitor of vesicular monoamine transporter 2 (VMAT2). This VMAT2 monoamine vesicular transporter inhibitor was first introduced in the 1970s for the symptomatic management of hyperkinetic movement disorders. Blocking the transport of dopamine into the presynaptic vesicles will lead to its degradation in the cytosol and subsequent reduction of its availability at the synapse [Figure 2-16].

**Figure 2-17.** Mode of action of Tetrabenazine (TBZ); a reversible inhibitor of vesicular monoamine transporter 2 (VMAT2). Blocks the transport of Dopamine into the synaptic vesicles for storage. Thus, less Dopamine is available for neurotransmission at the synapse.
TBZ is an effective oral therapy for chorea of Huntington disease and may be considered as an alternative agent for the management of dystonia, Tardive dyskinesia [TDk], and tic disorders (Chen et al., 2012; Zai et al., 2013; Bhidayasiri et al., 2013). The drug possesses an acceptable tolerability profile and has been used in pediatric and adult populations (Jain, Greene, and Frucht 2006).

A third possible and novel approach to treating the movement disorder would be to use adenosine receptor agonist. Substantial efforts have been made in the development of adenosine receptor agonists for different conditions namely cardiac arrhythmia, pain, myocardial ischemia, and malignancy (Gao and Jacobson. 2007).

Trials are in progress at different phases for some conditions, for example selective A1 adenosine receptor agonists are being trialed as a pain-relieving agent. A1 receptors are expressed in the spinal cord and other neuronal tissue and activation of A1 receptors produced pain-relieving effects in a number of preclinical animal models. Selective A1 agonists are currently in phase II trials for patients with neuropathic pain (Gao and Jacobson 2007). Several compounds that act as A1 receptor agonists have been withdrawn from trials due to insufficient Central Nervous System [CNS] penetrance, or unfavorable side effects (Giorgi and Nieri 2013).

This requires in-depth pharmacological experiments to create an agonist that can cross the BBB and with selective A1, A2 affinity, because adenosine receptors are ubiquitously expressed, and activating it in the heart or other organ systems may lead to serious side effects.

Through the knowledge obtained from uncovering the genetic cause of this disease and exploring the pathophysiology, one may postulate that this disease symptom is caused by purinergic neurotransmitter deficiencies. This is also applicable to the other mitochondrial diseases, a possibility that to my knowledge has not been considered in mitochondriopathies. With this idea in mind, and through the knowledge attained from the above research of this disease, theoretically, one could treat other mitochondrial diseases similarly, i.e., by decreasing dopamine neurotransmission. Perhaps this approach would be beneficial to the movement disorder component of mitochondrial diseases beyond our new SUCLA2 phenotype here.

Despite a major lack of clinical research, there is scientific research to support the concept of this disease and other mitochondrial diseases symptoms caused by purinergic neurotransmitter
deficiency, combining knowledge from the scientific and clinical literature, and researching this in depth may help shed the light on those not well understood pathophysiologic mechanisms. And at the same time open avenues for innovative therapeutic approaches and treat mitochondrial diseases with agents that can compensate the purinergic neurotransmitter deficiency; including the use of novel adenosine receptor agonists that are presently in various stages of clinical trials for various non-mitochondrial movement disorders.
Chapter 3

Summary and Future Directions

Since the completion of the human genome project and the development of next generation sequencing, a rapidly increasing number of disorders and potential treatment targets have been identified and characterized. The identification of gene mutations serves as a magnifying lens into cellular biology and physiology. This is a key step into understanding the human body and researching novel ways of overcoming disease-causing defects. Pieces of puzzles are solved and the complete picture becomes clear at the end of this process of disease characterization. Investing in disease characterization is particularly fruitful when understanding the pathomechanism of the disease opens up avenues for exploring novel treatment options.

This study was undertaken to characterize a disorder inherited in an autosomal recessive fashion as a result of a consanguineous union. Three affected siblings present with a phenotype that is consistent with a probable mitochondrial disorder based on the clinical presentation and radiological evidence, but the clinical course does not fit with the already described syndromes. Therefore the use of the available advanced technology in molecular genetic investigation was used as an approach to identify the underlying genetic etiology causing this disorder. The objectives that this study was aimed at were achieved; I have identified a novel mutation in SUCLA2. As anticipated from the clinical and radiologic phenotype the causative mutation is in a mitochondrial enzyme encoded by the nuclear genome, however defect of this gene have been associated with a different mitochondrial encephalopathy known as mitochondrial depletion syndrome V. This mutation leads to a single nucleotide change that causes a non-synonymous amino acid change and subsequently leads to a major conformational change in the ATP specific domain of CoA ligase subunit of Succinyl-CoA synthase (SCS-A) sparing the ATP grasp domain that forms a complex with another enzyme Nucleotide Diphospho Kinase (NDPK), thus leaving the NDPK function intact. The later enzyme catalyzes a key step in mitochondrial DNA replication and mitochondrial maintenance. Because of this, the patients with this novel mutation do not suffer from mtDNA depletion. This supports earlier hypotheses that mtDNA depletion in mitochondrial depletion syndrome V is a secondary phenomenon due to a secondary dysfunction in NDPK. The explanation that was proposed by Elpeleg in 2005 is obtained from the reported
finding that SCS-A copurifies and is tightly associated in a complex with nucleoside diphosphate kinase [NDPK] in both prokaryotes and eukaryotes (Kadmas, Ray, and Lambeth 1991; Kavanaugh-Black et al., 1994; Kowluru, Tannous, and Chen 2002). The mitochondrial isoform of NDPK catalyzes the exchange of terminal phosphates between tri- and diphosphoribonucleosides and are crucial for maintaining the homeostasis of ribonucleotides and deoxyribonucleotides (Senft et al., 1973). Mitochondrial deoxyribonucleotide diphosphates are phosphorylated by a mitochondrial NDPK (Milon et al., 2000). The importance of this salvage pathway step for mtDNA synthesis is mainly due to the lack of de novo synthesis of dNTPs in this organelle and by the constant need for building blocks for mtDNA synthesis throughout the cell cycle (Bogenhagen and Clayton. 1976). SCS and NDPK are in physical proximity, the functional significance of their coexistence in a complex is less clear. It was proposed that this proximity signals the involvement of NDPK in energy metabolism, possibly by directly activating the SCS by phosphorylation (Kowluru, Tannous, and Chen 2002). The data reported by Elpeleg suggest that SCS activity is essential for mtDNA synthesis and that SCS deficiency results in mtDNA depletion, possibly because of secondary NDPK dysfunction, and that is confirmed in this study by identifying a novel mutation that is in a domain that leads to a TCA cycle dysfunction without affecting mitochondrial quantity. Whether such interference involves the nucleotide triphosphate donation or a conformational change induced by the SCS protein is presently unknown. But the finding we are reporting here favors the second hypothesis of conformational change, to prove this; further experiments are needed.

Some of the long-standing historic questions in mitochondrial diseases generally, which would also apply to the disorder described here are addressed in this study. The literature on this subject specifically in the context of pathophysiologic mechanisms that explain the phenotype of most mitochondrial disorders and the treatment of these disorders is inconclusive on several vital questions; in this study I was able to propose alternative explanations to some of these questions. The first two question addressed are; why is there a selective involvement of the corpus striatum? And why is there sensorineural hearing impairment?

The historic answer to these questions is a speculative explanation that relate to the greater energy requirements of those particular neurons. I am proposing an alternative explanation that links the selective vulnerability of the corpus striatum and the cochlea in this and other mitochondrial diseases, presenting with movement disorder to the specific and selective
localization of a subtype of adenosine receptors in these two tissues. In recent years a great effort has been made to describe and characterize the purinergic neurotransmitter system. An interesting finding reported is the specificity of the expression of the adenosine A$_{2a}$ receptors in the corpus striatum neurons, striatal microvasculature, and in the inner ear cochlear root region of the spiral ligament and cochlear microvasculature (Srdjsn et al., 2009). Reduced purinergic neurotransmission secondary to a shortfall in ATP production leads to a decreased availability of adenosine. Adenosine is synthesized by dephosphorylation of ATP at the receptor level upon demand; this deficiency leads to a cascade of changes in signaling pathways. Perhaps the specific presence of adenosine receptors on corpus striatal nuclei, striatal vessels and the cochlear microvasculature is the explanation for specificity of disease in these nuclei in mitochondrial diseases.

The next question is; how can the phenotype of a hyper kinetic movement disorder be explained? The observed symptoms of basal ganglia (BG) dysfunction in the form of hyperkinetic movement disorder can be explained by deficiency of adenosine, which acts as a modulatory inhibitor of dopamine acting on D2 receptors, via an allosteric interaction between the A$_{2a}$ and D2 receptors. Loss of inhibition leads to the hyperkinetic movement disorder.

Answering the questions related to pathophysiology allowed to explore novel therapeutic approaches, studies on mitochondrial disease treatment have been increasing in the past decade, some treatment benefits are seen in open label studies, but a recent systematic review results suggest a publication bias towards small, non-blinded studies that report positive effects of treatment for mitochondrial disease, despite the fact that these findings are not supported by larger randomized studies. This lack of reproducibility could be due to the fact that these small studies are carried out without a clearly defined end point, and without a defined statistical analysis plan. One such example that reflects this point is the CoQ10 and carnitine treatment experience (Bresolin et al., 1988; Glover et al., 2010). The positive outcome in the open-label studies was contradicted by the results of the higher-quality trials that showed no treatment effect, despite the use of higher doses (Glover et al., 2010). In the patients I am describing, the defect results in TCA block and thus treatment with anaplerosis and supplying succinic acid is a possible treatment option, similar to what has been implemented with promising results in an adult patient with MELAS syndrome (Oguro et al., 2009).
In the central nervous system, adenosine is generated by dephosphorylation of ATP in relation to activity, or by the action of the enzyme ectonucleotidase on ATP released together with the classical neurotransmitter (Duarte-Araújo et al., 2009) [Figure I-5]. The central adenosine deficiency likely leads to loss of the inhibitory neuromodulatory action on the dopaminergic neurons, leading to a potentiation of the dopamine effect on the cells and clinically resulting in movement disorders. Reversing this deficiency may cure the hyperkinetic movement disorder. Exploring this idea more may also lead to effective therapies of mitochondrial cytopathies more broadly by looking at this new disorder as a disease that leads to a neurotransmitter deficiency.

ATP has been used as a treatment in patients with MELAS who presented in refractory status epilepticus where it helped control the seizures acutely (Oguro et al., 2004). The role of the mitochondria is to produce ATP molecules. Mitochondrial cytopathy leads to a disrupted mitochondrial function and a final deficiency of ATP production. Adenosine the neurotransmitter is synthesized from ATP. Therefore, with mitochondrial cytopathy there is a net reduced production of the neurotransmitter adenosine, which leads to multisystem involvement. This idea has not been explored in the literature to date. Other mitochondrial cytopathies that present with movements disorder and selective basal ganglia signal hyper intensity on MRI, like Leigh syndrome and Leigh-like syndrome, may benefit from this approach.

With this in mind another treatment approach is proposed in my current study and not yet implemented, this is aimed at correcting the secondary neurotransmitter deficiency that occurs in these patients due to TCA block and reduced ATP and adenosine production specific to the basal ganglia. Two different strategies can be implemented to correct this purinergic transmission deficiency; one approach would be by using a compound that can imitate the function of the inhibitory neuromodulator adenosine, since its deficiency leads to a potentiation of the dopamine effect; Inhibiting this process by a compound that reduces dopamine neurotransmission would be expected to correct this defect at least to an extent. Tetrabenazine is a reversible inhibitor of Dopamine. It has been used in hyperkinetic movement disorder for years with favorable results. The second approach is novel, and requires further research before being implemented; this is the use of adenosine receptor agonists.

The identification of this new disorder, which appears to be a novel neurotransmitter disease not previously described, has added to the depth of the literature. Knowledge gained here helps support some early postulations about a very important enzyme that has a complex and dual
function: SCS. To support novel approaches to treatment, further research is required. For example creating *SUCLA2* knock out mice and measuring CSF or brain levels of adenosine would yield valuable insights. These approaches would help characterize the precise sites and extent of neurotransmitter defects in this disorder and would allow implementation of novel therapeutic approaches that could transform the current management of mitochondrial cytopathies and alter their prognoses.

In conclusion the work presented here has helped characterize a new disease, and explore different pathophysiologic mechanisms of this disease and many other mitochondrial disorders. In addition, novel treatment approaches are being implemented; suggested and explored that may benefit the patients in the current family and patients suffering from mitochondrial cytopathies without depletion in general. The discussion presented here will open up potentials for further research that may impact mitochondrial disease prognosis.
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