REGULATION OF MITOCHONDRIAL GENE EXPRESSION IN MULTIPLE SCLEROSIS CORTEX

A dissertation submitted
to Kent State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May, 2012
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DEDICATION

I dedicate this work to my parents, Dr. Vidyadhar P. Pandit and Dr. Mrs. Shailaja V. Pandit, who are a constant source of inspiration and courage for me. To my mother whose positivity and love has helped me become a better person and my father who embodies all that I aspire to be. I humbly dedicate this work to both of you.
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List of Abbreviations

AD - Alzheimer’s disease
ALS - Amyotrophic Lateral Sclerosis
ATP - Adenosine triphosphate
ATP5 - ATP synthase, $H^+$ transporting, F0 complex subunit b
ATP5G3 - ATP synthase, $H^+$ transporting, F0 complex subunit c
ATP5I - ATP synthase, $H^+$ transporting, F0 complex subunit e
ATP5J - ATP synthase, $H^+$ transporting, F0 complex subunit F6
BA - Brodmann Areas [of the cerebral cortex]
BPB - Bromphenol Blue
C - Control Brain
C II - Complex II
$Ca^{++}$ - Calcium
CAPS - Chromatin-associated proteins
CGI - CpG island
ChIP - Chromatin Immunoprecipitation
CKB - Creatine kinase B
CLB - Cell lysis buffer
COX II or COX 2 - Cytochrome oxidase subunit II
COXVa - Cytochrome c oxidase subunit Va
COXVb or COX5b - Cytochrome c oxidase subunit Vb
COXVIIb or COX7b - Cytochrome c oxidase subunit VIIb
COXVIIc - Cytochrome c oxidase subunit VIIc
COX11 - cytochrome c oxidase assembly protein
COX17 - Cytochrome c oxidase assembly protein 17
CpG - Cytosine – phosphate – guanine dinucleotide
CREB - C reactive element binding protein
CSF - Cerebrospinal fluid
Cu/Zn-SOD - copper/zinc superoxide dismutase
Cyto - Cytoplasmic
DAB - 3,3’-diaminobenzidine
DBD - DNA binding domain
DIG-ddUTP - Digoxigenin bound to Uridine Triphosphate
DNA - Deoxyribonucleic Acid
DNAM - DNA methylase
DNMT - DNA methyltransferase
DOXO - Doxorubicin
DTT - Dithiothreitol
EAE - Experimental Autoimmune Encephalomyelitis
ECL - Enhanced chemiluminescence
ETC - Electron transport chain
EMEM - Eagles Minimum Essential Medium
EMSA - Electrophoretic Mobility Shift Assay

FBS - Fetal Bovine Serum

GABA - Gamma Amino Butyric Acid

GABPα - GA-binding protein α

GAD67 - Glutamic acid decarboxylase

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GPx - Glutathione peroxidase

H₂O₂ - Hydrogen Peroxide

HBB - Hemoglobin-β

HD - Huntington’s disease

HDAC - Histone deacetylase complex

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA - Human Leukocyte Antigen

HLA-DRB1 - Major Histocompatibility Complex, Class II, DR beta 1

HRP - Horseradish peroxidase

IL-2α - Interleukin-2α

IL-7α - Interleukin-7α

IP - Immunoprecipitation

MATCH - Matrix Search for Transcription Binding Sites

MBD - Methyl binding domain/Methylated DNA binding domain

MBP - Myelin basic protein

MHC - Major Histocompatibility Locus
Mito - Mitochondrial

Mn-SOD - Manganese superoxide dismutase

MRI - Magnetic Resonance Imaging

mRNA - Messenger Ribonucleic Acid

MS - Multiple Sclerosis

MOG - Myelin oligodendrocyte glycoprotein

NA - Data not available

NAA - N-Acetylaspartate

NAGM - Normal Appearing Grey Matter [cortical brain]

NDAR - No diagnostic abnormality recognized

NDUFA6 - NADH dehydrogenase (ubiquinone) 1α subcomplex 6

NDUFB1 - NADH dehydrogenase (ubiquinone) 1β subcomplex 1

NDUFB2 - NADH dehydrogenase (ubiquinone) 1β subcomplex 2

NDUFB7 - NADH dehydrogenase (ubiquinone) 1β subcomplex 7

NDUFB8 - NADH dehydrogenase (ubiquinone) 1β subcomplex 8

NDUFS2 - NADH dehydrogenase (ubiquinone) Fe-S protein 2

NDUFS4 - NADH dehydrogenase (ubiquinone) Fe-S protein 4

NDUFS2L - NADH dehydrogenase (ubiquinone) 1α subcomplex 9

NDUFV2 - NADH dehydrogenase (ubiquinone) flavoprotein 2

NF - Neurofilament

NRF-1 - Nuclear Respiratory Factor-1

NRF-2 - Nuclear Respiratory Factor-2
NLB - Nuclear lysis buffer
PD - Parkinson’s disease
PGC1A or PGC1α - Peroxisome proliferator-activated receptor gamma coactivator 1-α
PLP - Myelin proteolipid protein
PMI - Post Mortem Interval
PP MS - Primary Progressive Multiple Sclerosis
PVDF - Polyvinylidene fluoride
QP-C - now known as UQCRQ, ubiquinol-cytochrome c reductase, complex III subunit
VII
qRT-PCR [or QRT-PCR] - Quantitative Reverse Transcriptase - Polymerase Chain Reaction
ROS - Reactive oxygen species
RPM - Revolutions per minute
RR MS - Relapsing Remitting Multiple Sclerosis
SAM - S-adenosyl-L-methionine
SELDI-TOF-MS - Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SEM - Standard error of the mean
SP MS - Secondary Progressive Multiple Sclerosis
SNP - Single Nucleotide Polymorphisms
SH-SY5Y - Human Neuroblastoma Cell Line
TAD - Transcriptional activation domain
TF - Transcription factor
TFAM - Transcription Factor A - Mitochondrial
TRD - Transcription repression domain
TSA - Trichostatin A
UCSC - University of California, Santa Cruz
UQBP - Ubiquinol-cytochrome c reductase binding protein
UQCR - Now known as UQCR11, ubiquinol-cytochrome c reductase, complex III subunit XI
UQCRC2 - Ubiquinol-cytochrome c reductase core protein II
VEP - Visual Evoked Potential
Acknowledgements

I would like to thank my Advisor Dr. Jennifer McDonough for her tireless efforts at guiding me in my scientific endeavor. She has encouraged me to be a critical thinker and has provided unconditional support throughout my graduate training, making it a useful and rewarding experience. I would like to thank Dr. Ernest Freeman for his invaluable guidance and support during the course of my work. It was under the tutelage of Dr. McDonough and Dr. Freeman that I learned all of my molecular biology skill sets as well as associated skills such as scientific presentations and I am indebted to them for their time and effort in imparting me with these skill sets. They have also been instrumental in guiding my body of work towards meaningful and scientifically sound conclusions and I am grateful to them for allowing me to be a part of their vision. I have learnt everything I know about scientific investigation and critical thinking from them; an invaluable asset for my future, and I am thankful to them for that.

I take this opportunity to thank Dr. James Blank for being my teacher and for supporting, believing in and guiding me in my research. I thank my committee members Dr. Soumitra Basu for his insight and patience in me and my work, Dr. Derek Damron and Dr. Diane Stroup for being on my committee on short notice and sparing their valuable time for my cause. Dr. Dorman has been
one of my first teachers here in Kent and has provided support and guidance to me since then and I am thankful to him for that.

Special thanks to Judith Wearden for being a constant and tireless source of help and wisdom. Her kind patience is exemplary.

I would also like to thank Laurie Broadwater who has been a great friend and a source of encyclopedic knowledge for me time and again. I thank all my labmates, past and present for their enthusiasm and friendship which made this work enjoyable. I also thank Dr. Sausan Azzam for helping me with the EAE mice as well as Sara Houston for her help with the cell cultures. Special thanks to Will Huffman and Sabina Bhatta for providing me support in my work whenever needed.

I also thank all the donors and their families. In their selfless act they have helped further the cause of others. I also thank the Rocky Mountain MS Center, the Kathleen Price Bryan Brain Bank, and the Brain and Spinal Fluid Resource Center at UCLA for tissue as well as NIH and a Grant from the Ohio Board of Regents which funded this research.

Finally I would like to thank my family for their tireless support. My wife Savita, who is my best friend and with whom I have seen our wonderful life unfold over the last many years. It has been quite a journey and there is more to come. I cherish her presence in my life and look forward to the times ahead with her. My daughters, Ishani and Aishwarya, who are my life and who give purpose to everything I do. I thank Krishnna my son, who taught me acceptance and who encourages me every day to make the most
of life. Last but not the least, I thank my parents without whose blessings I would not have been able to accomplish anything in life.

Everybody mentioned here and others who may not have been named directly, have enriched my life and helped me become a better person and I wish to convey my deepest gratitude to all of them even as I try to reach for the stars. I would have achieved little without your support, mentoring, guidance, wisdom, friendship and love. I thank you all.
REGULATION OF MITOCHONDRIAL GENE EXPRESSION IN
MULTIPLE SCLEROSIS CORTEX

Introduction

1.1 Multiple Sclerosis [MS]:

MS was first described as a distinct neurological disorder in 1868 by Jean-Martin Charcot, a French neurologist (Talley 2005) and until 1920 it was considered a rare disease. In fact, until the 1950’s it was confused with a host of other disorders including, but not limited to, hysteria especially because of the relapsing-remitting nature of the disease progression. While the understanding of MS has improved, it is far from complete. MS is characterized as a complex
disorder of the central nervous system in which there is progressive and irreversible demyelination of axons, inflammatory lesions, axonal degeneration and associated physical disability (Slavin and others 2010). The complexity of the etiology of MS is underscored by the fact that it is polygenic and modestly heritable but does not follow Mendelian laws of inheritance as well as the fact that the disease shows a varied manifestation, suggesting a heterogeneous cause. Genetic linkage studies have shown that the Major Histocompatibility Locus [MHC] confers susceptibility to MS (Bertrams and Kuwert 1976; International Multiple Sclerosis Genetics Consortium and others 2007). Microarray profiling for genome wide single nucleotide polymorphisms [SNP] between unrelated MS subjects has highlighted those polymorphisms common in MS [and not genotypic expression] (Hoffjan and Akkad 2010). Over 100,000 SNPs can be simultaneously analysed using the microarray chip assay. The only consistent and strong genetic linkage is seen in the major histocompatibility complex, class II, DR beta 1 [HLA-DRB1] gene which accounts for between 20-60% of risk conferred. Other genes such as the interleukin-2α and the interleukin-7α have also been recognized as playing a role however their contribution is modest at best [>0.4%]. In addition to genetic linkage studies showing that MS is an autoimmune disease, expression microarray studies have also suggested changes in expression of the pro-inflammatory genes in MS. In an expression microarray study on peripheral blood monocytes from control and Relapsing Remitting MS [RR MS] patients, several genes involved in T and B cell activation were identified (Ramanathan and others 2001). Due to the results of these studies, MS has
traditionally been considered an autoimmune disease involving demyelination in white matter.

Characterizing MS has been difficult and contentious and to date, MS cannot be identified by one single symptom or laboratory test, hence a combination of clinical findings along with laboratory tests is used for a definitive diagnosis (Compston 2001; Fangerau and others 2004). Today’s diagnostic criteria have focused on laboratory/investigative tests to aid in the final diagnosis (Polman 2008), with a special emphasis on Magnetic Resonance Imaging [MRI] findings (Poser 2005). The overall approach however, has always been to identify signs and symptoms indicating progressive demyelination and accompanying disability with respect to time.

In 2000 an international panel chaired by Dr. W. Ian McDonald proposed that for diagnosing MS, there should be two or more ‘relapses’ along with evidence of at least two lesions typical of MS on an MRI. A ‘relapse’ was defined as a clinical presentation of neurological symptoms of demyelination, and had to last for at least 24 hours and there must be at least 30 days of remission of symptoms from the end of one attack to the beginning of the next. Analysis of the cerebrospinal fluid [CSF] and visual evoked potential [VEP] was recommended (Poser and Brinar 2002). At a minimum, clinical evidence of two attacks along with the demonstration of two lesions on an MRI are considered enough to make a diagnosis of MS. These criteria came to be known as the McDonald criteria and were extensively used since 2001 (Compston 2001) and later revised in 2005.
There are no known laboratory tests or biomarkers to differentiate between the various clinical sub-types of MS and the best indicator of the type of MS is the progression of disease itself. Currently, there are 4 sub-types of MS; namely relapsing-remitting MS [RR MS], secondary-progressive MS [SP MS], primary-progressive MS [PP MS] and benign MS. Figure 1 shows the salient features of the two most common types of MS.
Figure 1: Clinical Course and types of MS

(Compston and Coles 2002)
**Figure 1: Clinical Course and types of MS**

Schematic showing the most common presentation of MS. Relapsing Remitting [RR MS] and Secondary Progressive MS [SP MS], which are the most common presentations (Compston and Coles 2002). In RR MS, there are episodes of neurological deficit which resolve over weeks to months. Gradually, there is increasing residual deficit after every attack and recovery is incomplete. This phase evolves into the SP MS phase where the attacks become rarer but the neurological deficit progresses in spite of remission of the attacks. Shown on a comparative time scale in the lower half of the figure is the degree of inflammation and axonal loss. The inflammation correlates with the acute attacks and the axonal loss correlates with loss of brain volume and neurological deficit as measured by the Expanded Disability Status Score. Also shown is the threshold, which is a point in the progression of the disease after which axonal loss as well as the neurological deficit progress with or without any acute attacks.
1.2 Types of MS

About 80% of those diagnosed with MS present with the RR MS type in which sudden neurological deficit is seen over hours to days lasting 2-6 weeks. Recovery occurs over weeks to months. An important distinguishing feature of this type is that there is no increase in the residual disability between attacks. However, over time the RR MS pattern of transient disability is overlapped with the SP MS pattern of accumulating disability and as the disease slowly progresses from the RR MS phase to the SP MS phase, recovery is less and less complete and residual permanent deficits accumulate. An attack or episode has to last for 48 hours to be called as a relapse. Remissions are variable and can be from months to years. Typically, patients relapse every 6-12 months for the first 5 years after which the relapses are less frequent. After about 10 years this evolves into the SP MS variety.

The SP MS type of MS follows RR MS and is characterized by progressive worsening of disability over time with or without acute attacks. There can be minor improvements or plateaus but overall the baseline disability worsens over time. Axonal loss and progression of disability is essentially independent of acute attacks. Importantly, since patients with RR MS usually move on to SP MS there is a large time frame where features of both types [such as repeated relapses along with neurological deficit along with residual accumulation of the deficits seen during remissions] can be seen to coexist. However progressive worsening of baseline disability over time between acute relapses is characteristic of SP MS which is understood as a phase where acute attacks are no longer
a requirement for progression of disability. In fact, as SP MS progresses, relapses are seen to decrease over time while the disability worsens concomitantly.

PP MS is a rarer type of MS. In this type of MS, disability progressively worsens from the beginning without a return to a normal. Acute relapses if any are less frequent than in other types and do not alter the progression in any noticeable fashion. The progression of disability can plateau for some time but never improves and over time the disability is always progressive. The rate of progression of disability could vary between patients and even in the same patient over time. This type of progression shows an apparent disconnect between clinically manifested relapses and progression of disability.

Benign MS is a type of MS in which the attacks are mild in intensity and spread out over time with little to no residual disability. This type is very rare but has the potential to transform into one of the other three forms over the years. Diagnosis of benign MS is difficult. It is difficult to separate it from a milder onset of PP MS for eg. Until recently, normal MRIs were considered important for diagnosing benign MS. However recent evidence shows that with milder forms of residual disability, the MRI and clinical findings may be misleading. Additionally, people with what seems to be benign MS and normal MRIs have some degree of cognitive impairment which goes unnoticed. Finally studies have shown that people with benign MS for 10 years or more can then evolve into SP MS.

About 80-85% of patients demonstrate characteristics of RR MS which is the most common clinical presentation while about 10-15% present with PP MS in which the disease and disability progresses without remission (Slavin and others 2010). PP MS is
rare and is typically seen in the elderly (Poser and Brinar 2001). Another variable due to age is that sometimes transient attacks of disability consistent with RR MS are overlooked as age related weakness by these patients and not reported, further confusing the diagnosis in favor of PP MS. Over time, RR MS progresses to SP MS in which residual clinical disability increases with or without relapses (Confavreux and others 2000). While there is a wide range over which the disease landmarks are usually reached, research has shown that the frequency of initial relapses do not influence progression of disability, especially in SP MS (Confavreux and others 2000; Reynolds and others 2011).

1.3 Grey Matter involvement in MS

Extensive studies on lesioned white matter have been done in MS since the prevailing thought was that MS is a white matter disease. Most of the current research focus in MS has been on understanding mechanisms of immune mediated disease pathology in white and grey matter lesions. In contrast, the primary focus of my dissertation is to elucidate mechanisms involved in MS neuropathology in normal appearing grey matter [NAGM]. Because MS is both an autoimmune and a neurodegenerative disease (Nave and Trapp 2008) and grey matter pathology appears to involve a distinct mechanism from that which occurs in white matter (Filippi and Agosta 2009), my research is focused on analysis of primarily NAGM.
Even though MS was thought to be a white matter disease it is now known that there is extensive grey matter involvement as well (Reynolds and others 2011). While white matter lesions are typically associated with inflammatory exudates, grey matter lesions in contrast show relatively little to no signs of inflammation. The involvement of an autoimmune component to MS has not been found to be responsible for the progression of disability (Confavreux and others 2000). There is also a neurodegenerative component which is believed to be the major contributor to disability. Cortical grey matter lesion load has been found to be extensive (Bö and others 2006) and damage to neurons and axons correlates with progression and cognitive impairment (Bjartmar and others 2000; Mathiesen and others 2005; Rudick and others 2009).

Several studies have implicated mitochondrial dysfunction as a possible mechanism leading to grey matter pathology. While MRI studies have reported widespread grey matter damage and brain atrophy in MS (Cader and others 2007; De Stefano and others 2001; Fisher and others 2008; Ge and others 2004; Inglese and others 2004), molecular approaches including microarray and proteomics analyses have analyzed primarily white matter lesioned brain tissue or immune cells (Avasarala, Wall, Wolfe 2005; Chiasserini and others 2008; Han and others 2008; Quintana, Farez, Weiner 2008; Sarkijarvi and others 2006; Selmaj and others 2008; Westman-Brinkmalm and others 2009; Zetterberg and others 2008). Therefore studies on normal appearing grey matter are necessary to provide additional insight into disease manifestation and progression as it relates to the neurodegenerative component of MS pathology.
A previous microarray study of mRNA from normal appearing grey matter identified a decrease in 26 electron transport chain gene expression and also in GABAergic synaptic transmission (Dutta and others 2006) as shown in figure 2. 30% of the transcripts for electron transport chain genes were decreased in MS in this microarray comparison, a finding that was confirmed by Western blot. The overall number of mitochondria was not decreased but a subset of mitochondrial genes was decreased in MS. The decrease was shown to be in neurons in MS cortex by in situ hybridization studies. The decreases in electron transport chain genes had functional consequences as the function of the electron transport chain was decreased for Complex I and Complex III. The study was carried out with non-lesion areas of the motor cortex from postmortem control and MS samples.

The possibility that mitochondrial dysfunction may play a role in the pathology of MS is supported by previous studies showing that disability in MS is correlated with decreased neuronal mitochondrial metabolite N-acetylaspartate [NAA] (Ge and others 2004) (De Stefano and others 2001; Gonen and others 2000; Pendlebury and others 2000). Nuclear magnetic resonance spectroscopy has confirmed decreases in NAA, in MS brain and the decreases appear to precede neuronal atrophy indicating that a primary dysfunction in the mitochondria may precede neurodegeneration (Cader and others 2007; Inglese and others 2004). Decreased energy metabolism has also been found in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS (Qi and others 2006). The mouse EAE is a tried and trusted model for MS for many years. It has allowed precise elucidation of the inflammatory pathways, de- and re-myelination,
neuronal injury and apoptosis as well as therapeutic pathways and their effects. It is well
documented that the EAE mouse model is capable of precisely modeling many aspects of
the pro-inflammatory pathology seen in MS. However the modeling of cortical changes
seen in MS remains a challenge in the EAE model. As a part of the studies reported here,
the EAE model was studied to see if it could also replicate the changes seen in gene
expression in normal appearing grey matter.
Figure 2: Previous Studies: mRNA microarray of MS cortex

(Dutta and others 2006)
Figure 2: Previous Studies: mRNA microarray of MS cortex.

Mitochondrial electron transport chain gene transcripts are decreased in multiple sclerosis motor cortex. Color coded expression of Microarray data shown here. Red denotes an abundance of gene expression while blue represents a relative deficiency in gene expression. Twenty-six electron transport chain transcripts are decreased significantly (p ≤ 0.05) in multiple sclerosis motor cortex. These include NDUFB7 (NADH dehydrogenase (ubiquinone) 1β subcomplex 7), NDUFB8 (NADH dehydrogenase (ubiquinone) 1β subcomplex 8), NDUFB2 (NADH dehydrogenase (ubiquinone) 1β subcomplex 2), NDUFS2L (NADH dehydrogenase (ubiquinone) 1α subcomplex 9), NDUFV2 (NADH dehydrogenase (ubiquinone) flavoprotein 2), NDUFS2 (NADH dehydrogenase (ubiquinone) Fe-S protein 2), NDUFA6 (NADH dehydrogenase (ubiquinone) 1α subcomplex 6), NDUFS4 (NADH dehydrogenase (ubiquinone) Fe-S protein 4), NDUFB1 (NADH dehydrogenase (ubiquinone) 1β subcomplex 1), QP-C (now known as UQCRQ, ubiquinol-cytochrome c reductase, complex III subunit VII), UQRC2 (ubiquinol-cytochrome c reductase core protein II), UQBP (ubiquinol-cytochrome c reductase binding protein), UQCR (now known as UQCR11, ubiquinol-cytochrome c reductase, complex III subunit XI), COXVb (cytochrome c oxidase subunit Vb), COXVIIb (cytochrome c oxidase subunit VIIb), COXVIIc (cytochrome c oxidase subunit VIIc), COX17 (cytochrome c oxidase assembly protein 17), COXVa (cytochrome c oxidase subunit Va), COX11 (cytochrome c oxidase assembly protein), ATP5I (ATP synthase, H⁺ transporting, F0 complex subunit e), ATP5 (ATP synthase, H⁺ transporting, F0 complex subunit b), ATP5J (ATP synthase, H⁺ transporting, F0 complex subunit F6).
ATP5G3 (ATP synthase, H\(^+\) transporting, F0 complex subunit c). Expression levels of NDUFS4, QP-C, COX5a, and ATP5G3 mRNA decreased when measured by real time polymerase chain reaction. Mean fold change +/- SEM, p < 0.05.
My research has focused on investigating the transcriptional mechanisms which are involved in regulating the electron transport chain [ETC] and other genes involved in mitochondrial respiration, which are altered in MS grey matter. With this aim in mind, post mortem brain tissue was analyzed from Control and MS brains to better elucidate the expression and the regulating factors of the ETC genes as well as the epigenetic mechanisms that affect this regulation. Of particular interest was understanding if DNA methylation played a role in the transcriptional repression of the ETC genes in MS. Also of interest was knowing if the changes seen in NAGM were also seen in the EAE mouse model of MS.
CHAPTER 2

MATERIALS and METHODS

2.1 Tissue preparation and protein extraction

Multiple sclerosis and control brains were matched for brain region, age, sex, and post mortem interval (PMI) as closely as possible. Frozen human brain samples from matched Brodmann Areas [BA], (parietal cortex BA1, 2, 3 and frontal cortex, BA9) were obtained from The Rocky Mountain MS Center Tissue Bank (Englewood, CO), the Brain and Spinal Fluid Resource Center (UCLA) and The Kathleen Price Bryan Brain Bank (Durham, NC). Donor demographics are described in Table 1. Grey matter was obtained from the previously identified non-lesion cortical areas by cutting 60 μm section from frozen blocks and separating the grey and white matter with a scalpel as the sections were cut. Reagents used in the preparation of all buffers were obtained from Sigma-Aldrich.
Tissue was homogenized on separate dates as per study requirements using identical protocols. Five controls and eight MS donors were used for the initial studies on nuclear respiratory factor-2 [NRF-2] binding. Seven controls and five MS donors [including one control and one MS not used in the Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) studies] were used in the western blotting for confirmation of the SELDI data. EAE was induced in C57BL/6 mice by subcutaneous injection of myelin oligodendrocyte glycoprotein [MOG35-55] (Dasilva and Yong 2008). EAE brains and brains from control littermates were prepared at The University of Calgary and sent frozen to Kent State University for analysis by Western blotting. Protein was isolated from whole brains and from cortex for Western analyses. EAE-afflicted mice were at peak clinical severity (Grade 4 of the 5-point scale) when sacrificed.

Tissue was homogenized using a Wheaton homogenizer with a Teflon® pestle in whole cell homogenization buffer (20 mM KCl, 3 mM MgCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.9, 0.5% NP-40, 5% glycerol with protease inhibitors (P2714, Sigma-Aldrich, St. Louis, MO) in forty strokes. The homogenate was centrifuged for 10 minutes at 500 g at 4 °C. The pellet contains the nuclear fraction which was lysed in the nuclear lysis buffer (20mM Hepes pH 7.9, 0.225 M NaCl, 1mM EDTA, 3mM MgCl2, 0.5% Nonidet P-40, 10% glycerol and protease inhibitors). The supernatant was removed and centrifuged at 10,000 g for 30 minutes at 4°C. Now the pellet contains the mitochondrially enriched fraction and the supernatant contains the cytoplasmic fraction. The pellet containing the mitochondrially enriched
fractions was further purified by washing twice in 20 mM phosphate buffered saline (PBS), pH 7.4. The mitochondrial pellet was lysed in mitochondrial lysis buffer (50 mM Tris, 7 M urea, 3% CHAPS with protease inhibitors) by vortexing for 1 minute and then incubating for 20 minutes at room temperature. The mitochondrial lysate was centrifuged for 10 minutes at 10,000g at 4 °C. A modified Lowry assay was used to quantify the fractionated protein concentrations. All samples were stored at −80 °C until further analysis.

2.2 Cell Culture

The Human neuroblastoma cells [SH-SY5Y] were obtained from ATCC. They were cultured in Complete Media [45% Eagles Minimum Essential Medium [EMEM] + 45% F-12K medium + 9% Fetal Bovine Serum [FBS] + 1% Pen-Strep Antibiotic]. Cells were cultured on 100 mm sterile petridishes in 5% CO₂ at 37°C. Once they reached 70-80% confluency, they were treated with 5, 10 and 15 μM of hydrogen peroxide [H₂O₂] [once-a-day treatment] over a 2 and 7-day period. Untreated cells were taken as a control. After the peroxide treatment, cells were harvested and fractionated into nuclear, cytoplasmic and mitochondrially enriched fractions respectively.
2.3 Immunohistochemistry

Frozen sections 30 μM thick were immunostained using the Vectastain ABC staining kit [Vector labs] for the myelin proteolipid protein (PLP) to ensure the absence of demyelinated lesions as shown in figure 3. Briefly, blocks of frozen tissue were sectioned in a temperature controlled [-20C] microtome. The frozen sections were fixed in 70% ethanol for 30 minutes followed by a 1 ml PBS wash. They were then incubated in 1% hydrogen peroxide for 30 minutes at room temperature to quench endogenous peroxidases followed by a 1ml PBS wash and were allowed to air dry on a positively charged glass slide. Tissue sections were then encircled with a hydrophobic barrier [ImmEdge pen] and incubated in about 500 ul of the Myelin PLP antibody [1:200, MAB 388 Millipore] + 3% donkey serum in PBS + 0.5% Triton X-100 overnight at 4C. Every step was followed by 3 x 5 minute PBS washes. Sections were incubated in the secondary antibody, biotinylated donkey anti-mouse IgG [1:100, Vector Laboratories] in 3% donkey serum in PBS + 0.5% Triton X-100 overnight at room temperature followed by incubation in the Vector Elite ABC solution (1 drop A and 1 drop B per 5 ml of solution) for 1 hour at room temperature. Sections were then treated with Avidin:Biotinylated enzyme Complex [Vector Laboratories] with 3,3’-diaminobenzidine [DAB] [Vector Laboratories] as the chromogen.
Figure 3: Immunohistochemistry of MS tissue

In order to identify NAGM, 30 μM sections were immunostained with an antibody to PLP in order to identify non-lesion tissue. **A.** A block of frozen parietal MS cortex containing a gyrus is shown. **B.** A PLP stained section from the parietal block. The white circular spot seen is an artifact.
<table>
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C = Control Brain, MS = Multiple Sclerosis Brain, SPMS = Secondary Progressive MS, NDAR = No diagnostic abnormality recognized, NA = Data not available, * = Tissue sourced from the right hemisphere between the superior frontal gyrus and the precentral sulcus [Frontal Lobe].
2.4 Electrophoretic Mobility Shift Assay [EMSA]

Electrophoretic mobility shift assays are done to detect DNA [or RNA] - protein interactions. In principle, a short double stranded oligonucleotide sequence with the DNA binding site for the gene of interest is introduced to the proteins [nuclear fractions] in their native state. Complimentary proteins [typically transcription factors] will bind to the oligonucleotide [known as the ‘probe’]. The entire complex is then loaded onto a polyacrylamide gel and an appropriate electric current is applied. In one well, only the probe is loaded, which is the control, while the probe-protein samples of interest are loaded on the other wells. Double stranded DNA behaves as a rodlike entity and migrates in a predictable pattern across the polyacrylamide gel and is seen as a distinct band [or a smear] at the lower end of the gel [the side near the positive electrode]. When proteins bind on their binding sites to the double stranded DNA they ‘retard’ or slowdown the migration of the protein-DNA complex, partly because of their larger size. This is seen as a band, which has ‘shifted up’ or migrated less than the probe-only band. The rate of migration of the protein-DNA complex is inversely proportional to its size. The intensity of the band is directly proportional to the amount of protein-DNA complexes formed as well as the binding affinity of the protein to the probe and can be compared for binding affinity studies between two or more samples of interest. Visualization of the bands is achieved by 3’ end labeling of the probe with Digoxigenin bound to Uridine Triphosphate [DIG-ddUTP].
Oligos containing a consensus transcription factor binding site to NRF-2, 5’ ACCGACCCGGATGTTAGCAGA 3’ were synthesized by Invitrogen, (Carlsbad, CA). Single stranded oligos were annealed to produce double stranded DNA binding sites and 3-4 pmol was subsequently 3’ end labeled with DIG-ddUTP and 400U terminal transferase in a total volume of 20 ul at 37°C for 15 minutes using the DIG gel shift kit (Roche Diagnostics, Indianapolis, IN). Electrophoretic mobility shifts were done with 15 ug nuclear extracts. Binding was done in [100mM Hepes, pH 7.6, 5mM EDTA, 50mM (NH₄)₂SO₄, Tween 20, 1% (w/v), 150mM KCl, 0.8mM dithiothreitol (DTT)], in a total volume of 20 ul. Complexes were resolved on a nondenaturing 6% polyacrylamide gel, transferred to a nylon membrane at 30V for 1 hour in 0.5X TBE, and then complexes were fixed onto the membrane by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). The membranes were incubated with an alkaline phosphatase conjugated antibody to Digoxigenin 1:10,000, washed, and chemiluminescence detected. Quantitation of the complex formed on the probe was done by densitometry and was standardized by measuring the amount of the neuronal specific marker HuD on separate Western blots. Quantitation is the average from three separate mobility shifts for each sample pair. Data from nuclear extracts isolated from five pairs of parietal and two pairs of frontal control and MS blocks was analyzed with the T-Test for significance (p<0.05).
2.5 The Polymerase Chain Reaction:

The polymerase chain reaction or PCR is a widely used method to amplify RNA or DNA from a few initial copies to millions of copies. The idea behind using a PCR is that the ratio of the number of copies of DNA or RNA between two samples before and after the PCR amplification is more or less constant. In this way two samples can be compared for differences in amplified DNA after real-time PCR. In order to do this comparison accurately, measurements of amplification between two or more samples have to be made in the linear amplification phase. The advantage of real-time PCR is that by using sophisticated softwares we can measure the amount of product DNA at any stage or cycle of the experiment. Measurement of double stranded DNA product is achieved with the help of a reporter dye such as SYBR green. SYBR green intercalates between the two strands of DNA and fluoresces. This fluorescence is directly proportional to the number of double stranded DNA and can be compared across samples of interest. The main advantage of PCR is also its main disadvantage. Because the PCR can amplify minute amounts of DNA, even the smallest of contamination is equally amplified. Hence, great care must be taken to avoid introduction of contaminating DNA. Another disadvantage of PCR is the fact that the fluorescent dye SYBR green binds to any double stranded DNA. Hence, spurious amplifications are a problem for measurement. However, these issues can be solved by conducting the experiments carefully and with proper experimental design. Overall, the advantages of the PCR far outweigh its potential disadvantages.
RNA was made from about 200 mG of control and MS grey matter [RNAgent Total RNA Isolation System, Promega]. Tissue was homogenized in 3 ml denaturing solution on ice for 15-30 seconds. 300 ul of a 2M sodium acetate was added to it and mixed thoroughly after which 3ml of phenol:chloroform:isoamyl alcohol was added and mixed vigorously and incubated on ice for 15 minutes. The mixture was centrifuged at 14,000 revolutions per minute [RPM] for 20 minutes at 4C and the top aqueous phase containing RNA was separated. An equal volume of isopropanol was added to it and it was incubated at -20C for about 30 minutes to precipitate the RNA which was pelleted by centrifugation at 14,000 RPM for 10 minutes at 4C. The pellet was washed in 1 ml of 75% ice cold ethanol, centrifuged as before, air dried and reconstituted in nuclease-free water. RNA concentration and quality were determined using the Agilent 2100 Expert Bioanalyser. The RNA was then DNAse treated using the DNA free Kit from Ambion (Austin, TX). Primers were custom made for the mitochondrial electron transport chain Complex I gene, NADH dehydrogenase ubiquinone Fe-S protein 4, 18kDa (NDUFS4), FWD 5’ CTGTAGCTGCCCTTTCCGTTT 3’, REV 5’ CCATGTGGAAGTCCTCAACGA 3’, for the Complex III genes, low molecular mass ubiquinone binding protein (QP-C), FWD 5’ GGAGTCTTTCTTTCGGGTGGT 3’, REV 5’ TCGAACTCTTCAGTCCCCCAT 3’, and ubiquinol-cytochrome c reductase core protein II (UQCRC2), FWD 5’ TTGCTGCGTCTCTACATCCAG 3’, REV 5’ CATGAGTCTGCGGATTCTGA 3’, for the Complex IV genes, cytochrome c oxidase assembly protein (COX11) FWD 5’ CCCTTTATCGGCTCTATGCCC 3’, REV 5’ TGACCTGCAACTGCTCCTCCT 3’, and cytochrome c oxidase subunit Va (COX5A)
FWD 5’ CCGTGGCTATCCAGTCAGTT 3’, REV 5’ TGAGGTCTGCTTTGTCCCTT 3’, for the Complex V genes, ATP synthase H+ transporting F0 complex subunit c (ATP5G3), FWD 5’ TTGGAACAGTCTTTGTCAGCC 3’, REV 5’ TCAGACAAGGCAAATCCAGG 3’ and ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1 (ATP5F1), FWD 5’ TAGGTCCAGGGGTATTGCAG 3’, REV 5’ GTCTGCAACAAAGGGACCAT 3’, and primers were also made to cytochrome c, FWD 5’ TTGGCAATCCGTCATCAGTA 3’, REV 5’ CCCGACAGTGCTAGAAAGA 3’ by Integrated DNA Technologies (Coralville, IA). The 18S Primer pair from Ambion was used as a standard for all experiments. RT-PCR was performed using the FullVelocity SYBR Green QRT-PCR Master Mix Kit from Stratagene (La Jolla, CA) and their recommended protocol was followed. Five control samples were compared with five MS samples (three control and three MS parietal cortex samples and two control and two frontal cortex samples). Quantitative RT-PCR was performed on RNA of each pair three times and results were calibrated using the ABI PRISM 7000 SDS Software (Applied Biosystems, Foster City, CA). Data from the three pairs of parietal and two pairs of frontal control and MS samples was individually pooled for genes in each Complex and for cytochrome c and was analyzed with the T-Test for significance (p<0.05).
2.6 Western Blotting

Western blotting is perhaps the most widely used procedure in molecular biology. It is used for separation and detection of proteins based on their size. Typically, proteins are given a uniform negative charge in proportion to their size and run on a gel which is a matrix with small pores. A current is applied to one end of the gel and the proteins migrate to the positive side in the gel. The rate of migration of the proteins is determined by the size. Smaller proteins move faster and larger proteins will move slower. Since the proteins are colorless, a dye which migrates faster than the proteins is also included in the mix. The current is stopped when the dye is seen to approach the end of the gel. A preformed mix of proteins color-coded according to size, known as a ‘ladder’ is also run side-by-side with the proteins of interest to identify the size of the protein bands for comparison of their expression between samples of interest. Once the proteins are separated on the gel they are transferred onto a nitrocellulose or Polyvinylidene fluoride [PVDF] membrane. The proteins can now be detected by a system of primary and secondary antibodies tagged with a reporter enzyme such as horseradish peroxidase [HRP]. When a chemiluminescent substrate such as luminal in ECL [enhanced chemiluminescence, Amersheim] is added, the HRP oxidizes it to produce a detectable light signal which is captured on an x-ray film as dark bands against a light background. The area and intensity of these bands can be measured by softwares such as ImageJ by a process known as densitometry. Densitometry readings can be compared between the samples of interest and they are an indirect representation of the amount of protein of
interest in the respective protein samples. 10–30 µg of protein was used per sample for the Western blots.

Nuclear, mitochondrially enriched and cytoplasmic extracts were prepared from non lesion grey matter from frozen parietal and frontal blocks as described earlier. Protein concentrations were determined using the Bio-Rad protein assay kit [modified Lowry protocol]. Western blots were run on Nupage Novex 4-12% Bis-Tris gels, (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Signal was detected by chemiluminescence (ECL Western Blotting system, Amersham International, Arlington Heights, IL or ECL from Santa Cruz Biotechnology, Inc. Santa Cruz, CA). The blots were exposed to X-ray film (Kodak MR-1) for 1-30 minutes. The film was developed and the immunoreactive bands quantified by densitometry (3D Doctor or Image J Software). For the NRF-2 data, nuclear fractions with antibodies to GA-binding protein α [GABPα] were run on Western blots. Antibodies to HUD were used as the loading control. Also, for detecting oxidative stress, cytoplasmic fractions were run on Western blots with antibodies to Nitrotyrosine and actin, which was used as the loading control. Similarly, for the MS and EAE data, mitochondrially enriched or cytoplasmic samples from either human [C, MS] or mouse [C, EAE] were separated on NuPage® 4–12% Tris gel (Invitrogen, Carlsbad, CA) for 35 minutes at 200 V, transferred to nitrocellulose paper for 1 hour at 45 V and incubated with the appropriate antibodies. The blots were exposed to the corresponding secondary horseradish peroxidase conjugated secondary IgG (sc2005-mouse/sc2001-rabbit Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Densitometry was performed from three MS and control comparisons
or EAE and control brain comparisons from at least two experiments. Quantitation was
standardized with antibodies against either Complex IV subunit 2 (COX2, 1:2000, MS405, Mitosciences, Eugene, OR), a mitochondrially encoded protein, or the Complex II SDHA subunit (Molecular Probes, Eugene, OR) for mitochondrial fractions, and Glyceraldehyde 3-phosphate dehydrogenase [GAPDH] (Chemicon, Temecula, CA) for cytoplasmic fractions. A Student's t-test was performed to determine statistical significance.

2.7 Antibodies

Antibodies used for Western blots were nitrotyrosine (Upstate, Lake Placid, NY) and either actin or HuD (Santa Cruz Biotechnology Inc., Santa Cruz, CA). For Western blots and in electrophoretic mobility shift competitions, an antibody to GABPα C-20 and an antibody to mitochondrial transcription factor A (TFAM) were obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Confirmation of the SELDI data was done using either mouse MBP (AbCam, Cambridge, MA), human MBP (Chemicon, Temecula, CA), creatine kinase (AbCam, Cambridge, MA), COX5b (Invitrogen, Carlsbad, CA), hemoglobin (Thermo Scientific, Rockford, IL), or neurofilament (Chemicon, Temecula, CA). Antibodies used for the Immunoprecipitation [IP] were an IP specific antibody to MeCP2 [Abcam] and the negative control non specific antibody to IgG [Agilent technologies, supplied with the IP kit].
2.8 Chromatin Immunoprecipitation [ChIP] and DNA-PCR

2.8.1 Chromatin Immunoprecipitation [ChIP]

I performed chromatin immunoprecipitation [ChIP] with Millipore kit which was designed for chromatin fixation, extraction and shearing from cultured cells [preferably monolayer cells] as shown in figure 4. The kit protocol had to be modified so as to use it for solid brain tissue. PBS was used as the substrate for all solutions and washes. Chromatin was extracted and the DNA concentrations were measured. The total amount of chromatin taken for shearing was standardized across all the samples before shearing them so as to be able to compare the relative yields after Immunoprecipitation [IP]. At every stage that I had to change the media/reagents over the culture dish in the kit protocol, I instead centrifuged at 2400 RPM for 10 mins @ 4C. The kit provides enough reagents for processing from 5 culture plates, which can be translated to process 5 samples of tissue. I used two controls and three MS samples. Per sample I did a negative control IgG antibody and the antibody of interest, the MeCP2 antibody. The shearing efficiency was optimized to give us sheared fragments between the 400-2000 bp range.
Figure 4: Chromatin Immunoprecipitation

http://www.motifolio.com/6111187.html
Figure 4: Chromatin Immunoprecipitation [ChIP].

The chromatin immunoprecipitation procedure allows us to study DNA-protein interactions as they would occur in vivo. Fig 4 delineates the essential steps involved in the ChIP protocol. In the first step intact cells or tissue of interest is cross-linked or fixed using formaldehyde. In the case of solid tissue, it is gently homogenized to tease it apart for better fixation before introducing the formaldehyde. Once the tissue is fixed, it is homogenized in the appropriate lysis buffer to release the chromatin. This chromatin is then sonicated to get chromatin fragments typically between 500 to 1500 base pairs. This chromatin still has the various bound transcription factors fixed to it. It is then incubated, usually overnight, along with an antibody to the transcription factor of interest. The whole complex is bound to protein G coated magnetic beads. After the incubation, the beads along with their bound complex of antibody and chromatin is separated from the rest of the lysate with the help of a magnet. The beads are then separated from the complex and the complex is treated with proteinase K to remove all the proteins as well as with RNase A to remove any RNA present, leaving behind our DNA fragments of interest. In this way we can enrich for fragments of DNA with promoter regions [binding sites] for the transcription factor of interest. These fragments of DNA can then be amplified by PCR using appropriate primers and compared between the samples of interest. A difference in the binding ability of the transcription factor of interest in vivo between two samples is translated into a difference in their relative enrichment after chromatin immunoprecipitation and can be measured by the difference in the amplified product after PCR.
Control parameters were at the level of the IP and the PCR. In the IP, the negative control was the non specific mouse IgG antibody and the positive control was the Input DNA. The antibody of interest was antibody to neuron specific methyl binding protein MeCP2. For the PCR, GAPDH was considered the negative control. GAPDH is a housekeeping gene and is constitutively active. Therefore it should be hypomethylated. Glutamic acid decarboxylase [GAD67] was the positive control since it was seen in other studies to be methylated (Kundakovic and others 2009).

Chromatin was extracted from 2 controls and 3 NAGM MS samples. The tissue was gently homogenized in the fixation solution [20 ml of 1X PBS with 550 ul 37% formaldehyde] by using a manual homogenizer with a Teflon pestle. The fixation was stopped by 2 ml of 10X glycine. This was then centrifuged @ 2400 RPM for 10 mins @ 4C and the supernatant was discarded. The pellet was then washed with 20 ml PBS and centrifuged @ 2400 RPM for 10 mins @ 4C and the supernatant was discarded. The pellet was reconstituted in 2 ml 1X PBS supplemented with protease inhibitors and 1 ml of cell lysis buffer [CLB]. This was incubated on ice for 30 minutes, vortexing every 5 minutes. After incubation, it was centrifuged as before and the supernatant was discarded. The pellet was reconstituted in 1 ml of nuclear lysis buffer [NLB] and the DNA concentrations were measured. After equilibrating for the lowest concentration of DNA among the five samples, 500 ul of the equilibrated stock chromatin was sonicated on ice to generate sheared chromatin from 300 to 1200 bp after sonicating for 10 sets, where each set = 5 cycles of 15 second sonication followed by 50 seconds of cooling on ice. Shearing was confirmed on an agarose gel following which the sheared samples
were centrifuged at 11,000 RPM. The supernatant was saved and the DNA concentration assay was repeated. The DNA concentrations before and after shearing were different across samples, so they were re-equilibrated before the IP. Two sets of IP reactions per sample were made, one for the negative control IgG antibody and one for the MeCP2 antibody. 900 μl of the kit Dilution Buffer [450 μl*2 IPs per sample] along with 4.5 μl PIC were added to 100 μl [50 μl * 2] of the sheared chromatin. From this, a 1% [10 μl] aliquot per sample was removed and stored as INPUT DNA. The remaining volume was divided equally between the two IP incubations per sample. All the samples were immunoprecipitated with similar amounts of chromatin [18.1 μg]. For each IP [per antibody per sample] 50 μl sheared chromatin + 450 μl Dilution buffer from kit + 2.25 μl PIC+ 20 μl magnetic beads + Antibody were incubated overnight on a rotary shaker at 4 C. 1 μl [=1 μg] of the IgG antibody [provided in the kit] for the negative control or 25 μl [=25 μg] of the MeCP2 antibody [Abcam, ChIP grade MeCP2 A/b, Cat # ab2828] was added per reaction as required. Post-incubation, the Protein G bead-antibody/chromatin complex was pelleted using a bar magnet and the supernatant was discarded. The beads were washed serially with 0.5 ml of each of Low Salt Immune Complex Wash Buffer, one wash; High Salt Immune Complex Wash Buffer, one wash; LiCl Immune Complex Wash Buffer, one wash and TE Buffer, one wash. 100 μl ChIP Elution Buffer and 1 μl Proteinase K was added to each reaction as well as the Input DNA. This was incubated at 62°C for 2 hours with shaking, followed by incubation at 95°C for 10 minutes to remove all the DNA-bound proteins. The beads were re-pelleted with the magnet and discarded. The supernatant, which contains the immunoprecipitated or input DNA was purified
using spin columns provided in the kit to yield a final volume of ~50 ul of immunoprecipitated and Input DNA.

2.8.2 DNA-PCR

For the PCR I was testing 5 samples and three types of DNA per sample: MeCP2 immunoprecipitated, IgG immunoprecipitated [negative control] and Input DNA [positive control]. PCR on DNA immunoprecipitated with the MeCP2 and the IgG antibodies as well as on the Input DNA was done using the Stratagene Brilliant III Fast SYBR® Green QPCR master mix from Agilent Technologies. Primers for the GAPDH, GAD67, PGC1A, ATP5G3, ATP5J and ATP5I genes were made by IDT Technologies and Invitrogen. All the DNA PCRs were run for 40 cycles. Primers used were as follows: GAPDH FWD 5'-CGGCTACTAGCGGTTTTACG-3', REV 5'-AGGAGGAGCAGAGAGCGGAAG-3'; GAD67 FWD 5'-CTCGTGCGTGTCAACCTTCA-3', REV 5'-ACGGAGCTGGATTGTTGTA-3'; PGC1A FWD 5'-AAGGGAAATGTGCTGGTG-3', REV 5'-GCTGTGAGAGCTGCAACAAG; ATP5G3 FWD 5'-GTAGGGGCAGGATTCAGGAT-3', REV 5'-CACGTGGCGACCTTGTGAAGA-3'; ATP5J FWD 5'-AGGGTCTGCAATTCTGACTT-3', REV 5'-ATTTCGTTCTGGAGACTT-3'; ATP5I FWD 5'-GCAGTCTGGGTGTGGAC-3', REV 5'-CACAAAGGCTGGAGTG-3'. Differences in the expression of the PCR
products for the MeCP2 and the IgG immunoprecipitations were measured as fold changes. Fold change for the expression of either MeCP2 or IgG immunoprecipitated DNA between samples was determined by using the formula $2^{\Delta Ct}$ where $Ct$ is the number of PCR cycles required to express fluorescence beyond a threshold level in the linear phase of amplification. Values of amplification of DNA immunoprecipitated with MeCP2 over and above that of the DNA immunoprecipitated with IgG per sample were calculated and expressed as a percent of the respective input DNA.

2.9 Agarose Gel

An agarose gel is commonly used to visualize DNA or RNA separation based on the length of the fragment [separation by size]. The process is essentially similar to that of a polyacrylamide gel in that the negatively charged DNA strands are pulled through the agarose gel matrix by applying a positive current from the opposite end of the gel. Agarose gel-pores are larger than polyacrylamide gel-pores to suit the migration of the longer strands of DNA. Shorter strands migrate faster and longer strands migrate slower on the gel. A mixture of color coded DNA strands of known base pairs [length] is also run along side the DNA of interest to identify the sizes of the bands seen after running the gel. I used a 1.6% agarose gel in 1X TBE. 0.8 gms or 1.6 gms of agarose was dissolved in 50 ml or 100 ml 1X TBE respectively, depending on the size of the gel required. The mixture was boiled and 0.5-1 ug/ml of 1% ethidium bromide was added and swirled to
mix. A comb was added and the gel was allowed to set for 30-45 minutes. 16 ul of DNA + 4 ul loading dye Bromphenol Blue [BPB] [20% of the volume] was added per lane of the gel as required. The gel was run at 100 V for 45-60 minutes in 1X TBE, until the BPB has run more than 3/4th of the gel and the migration of DNA observed and photographed under UV light. Agarose gels were run to confirm the sizes of the sheared DNA as well as to confirm the sizes of the amplified DNA PCR products.
CHAPTER 3

GLOBAL DECREASE OF mRNA EXPRESSION IN ELECTRON TRANSPORT CHAIN PROTEINS IN MS CORTEX

3.1 Introduction

Neurons have an extremely high energy requirement in order to maintain ion homeostasis as a result of the ion fluxes which are a part of neurotransmission. Therefore they are particularly vulnerable to perturbations in energy production (Davey, Peuchen, Clark 1998; Stys 2005). Mitochondria are
the primary energy generators in the cell and mitochondrial dysfunction can lead to both apoptosis and necrotic injury. Understanding the mechanisms involved in regulating energy metabolism is necessary to prevent these deleterious events.

Previous studies have shown a decrease in the expression of the ETC genes in the motor cortex. As discussed in the introduction, I wanted to see if the changes seen in the motor cortex were localized or more global in nature. I also wanted to study the promoter regions of the ETC genes whose expression had been repressed in MS in an effort to better understand the mechanism of their regulation. I found NRF-2 binding sites in the promoter regions of 21 of the 26 ETC subunit genes whose gene transcripts were found to be decreased previously [Table 2]. NRF-2 is also known to play a role in the coordinate regulation of nuclear encoded ETC genes (Dhar, Ongwijitwat, Wong-Riley 2008; Kelly and Scarpulla 2004; Ongwijitwat and Wong-Riley 2005; Scarpulla 1997) and is redox regulated (Chinenov and others 1998; Martin and others 1996). For this reason I decided to study the expression levels of NRF-2 in MS cortex.

NRF-2 or GABP belongs to the ETS family of transcription factors. The ETS family of transcription factors is involved in the regulation of a wide variety of cellular functions in different cell-types, including but not limited to cell cycle control, hormonal regulation, hematopoiesis, apoptosis and expression of housekeeping genes. Their DNA binding domain [DBD] binds preferentially to the core GGAA/T sequence on the DNA. There are about 30 known ETS transcription factors in mammals. Each tissue type expresses a specific pattern of the different ETS factors and any one tissue type can simultaneously express different ETS factors all at the same time. Since they all bind to
similar core sequences it is unclear how different tissue types can achieve their individual signature expressions. The ETS family of transcription factors is also known to form complexes on the DNA with other transcription factors and/or enhancers and it is possible that different tissue types are able to control binding of different ETS transcription factors by modulating the relevant transcriptional co-activator complexes.

As the name suggests, the GA binding protein transcription factor was so named because of its affinity for binding to the G-A rich sequences on the DNA. GABP was first isolated in rat liver in 1991. It is now known to take part in, among other things, the transcription of nuclear genes encoding for the mitochondrial electron transport chain protein subunits. Hence it is also referred to as NRF-2. The GABP transcription factor complex includes two unrelated proteins known as GABPα and GABPβ. GABPα belongs to the ETS family while GABPβ does not. GABPα contains the DBD while the GABPβ contains the transcriptional activation domain [TAD]. Hence, by virtue of this configuration, it is unique among the ETS family as being the only factor requiring the formation of a heterotetramer composed of two GABPα subunits and two GABPβ subunits for functional activity. In fact, the presence of the DBD and the TAD on separate proteins distinguishes GABP from most other transcription factors and from all the other ETS transcription factors. GABPα binding to the DNA is a prerequisite for recruiting GABPβ to the target genes. Interestingly, GABPα is the only protein in the ETS family that can recruit GABPβ to bind to the DNA. Also interesting is the fact that while the GABPα/GABPβ can form heterodimers in solution they form a heterotetramer only when bound to the DNA. While GABPα does not homodimerize, GABPβ does so
and this property is a requirement for the formation of the GABPα/GABPβ heterotetramer on the DNA. Binding of GABPβ to GABPα is a necessary requirement for transcriptional activation and it also stabilizes GABPα binding to DNA by over 100 fold.

In the event of an acute stress such as exercise, exposure to cold or even oxidative stress due to an increase in ROS, the cell will initiate a calcium dependent PGC1-α [PGC1A] pathway which will stimulate nuclear respiratory factor-1 [NRF-1], TFAM and NRF-2. This can increase the number of mitochondria, the expression of mitochondrial genes, and anti oxidative enzymes such as peroxidases and catalases (Gutsaeva and others 2006; Lee and Wei 2005; St-Pierre and others 2006). Although every instance of mitochondrial and ETC protein biogenesis increases the possibility of increasing the amount of ROS in the cellular environment, the PGC1-α pathway also stimulates antioxidant enzymes and studies have shown that inspite of the potential increase in ROS due to mitochondrial and ETC protein biogenesis, the overall effect is to reduce the amount of ROS and is beneficial for the cell. Also, if in response to increased ATP production there is also an increase in ROS to unacceptable levels, then the ROS in turn will have a negative feedback to inhibit excess ATP production. This provides a mechanism to maintain a balance between the metabolic rate of cells and the production of reactive oxygen species [ROS] which results as a byproduct of mitochondrial activity and can be deleterious to cells. A balance between energy demands/ATP generation and controllable ROS production is healthy for the cell. It is when the oxidative stress is
chronic that the cell crosses a threshold, the compensatory mechanisms fall short and the cell enters an apoptotic pathway.

It has been previously shown that an increase in ROS can have an adverse effect on the binding capacity of NRF-2 and by consequence on the transcription of the ETC proteins. The ETC proteins are responsible for generating ATP, which is the energy currency of the cell. Hence a decrease in the transcription of these proteins could directly impact the energy balance in the cell which, if severe enough, could lead to cell death [neurodegeneration] with or without the contribution of an inflammatory autoimmune response. In order to understand how the ETC genes were coordinately regulated in MS, I analysed the expression and binding ability of endogenous NRF-2 in nuclear fractions isolated from Control and MS grey matter.

3.2 Results

3.2.1 mRNA RT-PCR of ETC gene transcripts in MS

Microarray profiling studies have shown previously that the expression levels for transcripts encoding many subunits of the ETC are decreased in postmortem MS motor cortex (McDonough and others 2003). In order to determine how global these changes are in MS cortex, the expression of a subset of the nuclear encoded ETC subunit genes
which were previously found to be decreased in the motor cortex, were quantified by RT PCR in the parietal and frontal cortices. I quantified the expression of seven subunits of the electron transport chain and also another nuclear encoded mitochondrial transcript involved in respiration, cytochrome c. The expression levels of the Complex I NDUFS4 gene, the Complex III QP-C and UQCRC2 genes, the Complex IV COX11 and COX5A genes, the Complex V ATP5G3 and ATP5I genes, and cytochrome c were quantified from mRNA isolated from non lesion grey matter from parietal cortex from three MS and three control brains (C1 compared with MS2, C2 with MS4 and C4 with MS8), and from frontal cortex from two MS and two control brains (C3 compared with MS1 and C5 with MS8). Patient demographics are shown in Table 1. The expression levels of ETC subunit genes from each complex were decreased in the MS samples in parietal cortex and these decreases were statistically significant (T-Test, p<0.05) except for the Complex III UQCRC2 gene as shown in figure 5. In the frontal cortex, Complex I, III, and V genes were decreased in MS and these decreases were statistically significant (p< 0.05) except for the Complex III QP-C gene. In contrast to the parietal cortex, neither of the Complex IV subunit genes were decreased significantly in the frontal cortex. The expression of cytochrome c was not significantly changed in either the parietal or frontal cortex.
Figure 5: Decrease in expression of ETC gene transcripts in MS cortex

ETC gene expression is decreased in parietal and frontal cortex in MS. Changes in transcript expression were determined by RT-PCR for the Complex I NADH dehydrogenase ubiquinone Fe–S protein 4 (NDUFS4) gene, the Complex III genes, low molecular mass ubiquinone binding protein (QP-C) and ubiquinol-cytochrome c reductase core protein II (UQCRC2), the Complex IV genes, cytochrome c oxidase assembly protein (COX11) and cytochrome c oxidase subunit Va (COX5A), the Complex
V genes, ATP synthase H\(^+\) transporting subunit c (ATP5G3) and ATP synthase, H\(^+\) transporting, mitochondrial F0 complex, subunit B1 (ATP5F1), and for the cytochrome c gene. A significant decrease in the expression of all the genes was seen in the parietal cortex with the exception of the UQCRC2 subunit of Complex III and Cytochrome C. In the frontal cortex, a significant change was seen in the gene transcripts for Complexes I, III and V with the exception of the QPC subunit of Complex III. Complex IV genes were decreased but not significantly and no change in the expression of Cytochrome C was noted. Data are the average % decrease for message isolated from three MS compared to three control samples for parietal cortex and two MS compared to two control samples for frontal cortex. Expression was standardized with levels of expression obtained with 18S RNA primers. Levels of mRNA from MS samples are expressed as % control. Error bars represent SEM and are from three separate experiments. *p < 0.05.
3.2.2 Analysis of the promoter regions of select ETC genes in MS

Since there was a significant decrease in gene transcripts in the parietal and frontal cortices, I analyzed the promoter regions of the down regulated electron transport chain genes with the Transfac database MATCH [Matrix Search for Transcription Binding Sites] program (Kel and others 2003) to identify potential transcription factor binding sites which may coordinately regulate the expression of these genes. This analysis was done on the 2 Kb sequence upstream of the start site of transcription for each gene. Binding sites for NRF-2 in the 500 bp promoter regions are shown in Table 2.

I studied the sequence data for each altered gene from the University of California Santa Cruz [UCSC] human genome database [http://genome.ucsc.edu/]. They have a downloadable online database of the entire verifiable human genome sequence. I downloaded the ‘2kb upstream’ file from ‘upstream2000.fa.gz’ which has the 2 kb upstream sequences of all the verifiable human genes along with their accession numbers and retrieved the 2 kb upstream sequences of the transcription binding sites for each of the 26 altered gene using the genes’ accession numbers. MATCH lists out all the possible transcription factors that have a matching complementary sequence to the base pairs of interest [it can show all the transcription factors that will bind to the base pairs of interest] (Kel and others 2003). Consensus binding sites for NRF-2 were identified in all 26 of the promoter regions of the twenty six electron transport chain genes which were shown to be decreased previously in motor cortex in MS (Dutta and others 2006; McDonough and others 2003) including the seven electron transport chain genes
analyzed in parietal and frontal cortex in this analysis. NRF-2 sites were not identified in the cytochrome c promoter and the expression of this gene was not decreased in MS samples in my RT PCR analysis. Consistent with this observation, cytochrome c has been reported to be regulated by NRF-1 as opposed to NRF-2 (Evans and Scarpulla 1989).
Table 2

Analysis of the promoter regions of mitochondrial genes decreased in MS

NRF-2 Transcription Factor Binding Sites. C/A GGA A/T A/G consensus binding site.

<table>
<thead>
<tr>
<th>Name of ETC Subunit encoded</th>
<th>Nearest Sequence from Start</th>
<th>Distance from Start of Transcription site in base-pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDUFB7</td>
<td>CGGAAG</td>
<td>-15, -53, -341</td>
</tr>
<tr>
<td>NDUFB8</td>
<td>GCCTTT</td>
<td>-13, -143, -350, -370</td>
</tr>
<tr>
<td>NDUFB2</td>
<td>GCCTAT</td>
<td>-495</td>
</tr>
<tr>
<td>NDUFA9 (formerly NDUFS2L)</td>
<td>TCCTTC</td>
<td>-146, -162, -206, -215, -322, -337, -393</td>
</tr>
<tr>
<td>NDUVF2</td>
<td>CGGAAA</td>
<td>-207</td>
</tr>
<tr>
<td>NDUFA6</td>
<td>CGGATA</td>
<td>-222, -409, -465</td>
</tr>
<tr>
<td>NDUFS2</td>
<td>GCCTTT</td>
<td>-60, -335, -350, -456</td>
</tr>
<tr>
<td>NDUFB1</td>
<td>TCCTTT</td>
<td>-342, -365</td>
</tr>
<tr>
<td>NDUFS4</td>
<td>AGGAAG</td>
<td>-50</td>
</tr>
<tr>
<td>UQCRQ (formerly QPC)</td>
<td>AGGAAA</td>
<td>-38, -55</td>
</tr>
<tr>
<td>UQCRRC2</td>
<td>CGGAAG</td>
<td>-217, -305</td>
</tr>
<tr>
<td>UQCR (formerly UQBP)</td>
<td>GCCTTT</td>
<td>-47, -131, -339, -451, -477</td>
</tr>
<tr>
<td>COX5A</td>
<td>GCCTAC</td>
<td>-95, -247, -328, -337</td>
</tr>
<tr>
<td>COX5B</td>
<td>CGGAAG</td>
<td>-32, -74</td>
</tr>
<tr>
<td>COX7B</td>
<td>CGGAAA</td>
<td>-123, -336</td>
</tr>
</tbody>
</table>
Table 2: Analysis of the promoter regions of mitochondrial genes decreased in MS

The aim here was to find which transcription factor/s bind to a gene of interest. The transcription factors will start binding to the gene upstream of the actual transcription site. University of California, Santa Cruz [UCSC] has an online database of the entire verifiable human genome sequence free to download [http://genome.ucsc.edu/]. The MATCH software identified NRF-2 binding sites in the 500 bp promoter regions of 23 genes decreased in MS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Motif</th>
<th>NRF-2 binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX7C</td>
<td>TCCTTC</td>
<td>-136, -260, -430</td>
</tr>
<tr>
<td>COX11</td>
<td>AGGAAA</td>
<td>-244</td>
</tr>
<tr>
<td>COX17</td>
<td>TCCTTT</td>
<td>-62, -398, -408, -435</td>
</tr>
<tr>
<td>ATP5I</td>
<td>GCCTTT</td>
<td>-125, -209</td>
</tr>
<tr>
<td>ATP5F1 (formerly ATP5)</td>
<td>AGGATA</td>
<td>-169, -414</td>
</tr>
<tr>
<td>ATP5J</td>
<td>CGGAAA</td>
<td>-36, -67, -223</td>
</tr>
<tr>
<td>ATP5G3</td>
<td>GCCTTC</td>
<td>-20, -94, -307, -320</td>
</tr>
</tbody>
</table>
To test whether the decrease in expression of the electron transport chain subunit genes could be explained by a decrease in the NRF-2 transcription factor, I performed Western blots using nuclear protein extracts and incubated them with antibodies for GABP$\alpha$, which is the DNA binding subunit of NRF-2. I found that there was no change in the concentrations of GABP$\alpha$ between control and MS sample pairs (Figure 6). Even though the expression of the NRF-2 DNA binding subunit was not altered in MS other factors such as posttranslational modifications and compartmentalization could be regulating the availability of NRF-2 to activate transcription. For this reason I also quantified the levels of protein for both of the NRF-2 subunits, GABP$\alpha$ and GABP$\beta$, in cytoplasmic extracts isolated from MS and control cortex and did not find any significant changes in protein expression (data not shown).
Figure 6: Expression of GABPα in Control and MS cortex

The expression of NRF-2 protein is not altered in nuclear extracts isolated from MS cortex. Western blots were done with the nuclear extracts with an antibody to GABPα, which is the DNA binding subunit of NRF-2. Western blots were also done with the nuclear extracts with an antibody to HuD, a neuron specific marker, to control for cellularity. These data show that the amount of the GABPα subunit of NRF-2 is not decreased in MS nuclear extracts.

(Pandit and others 2009)
I then tested the ability of endogenous NRF-2 in nuclear extracts isolated from MS and control tissue to bind a consensus NRF-2 binding site by performing electrophoretic mobility shift experiments. I found that a shifted complex was formed on a labeled DNA probe containing the NRF-2 transcription factor consensus binding site containing the NRF-2 transcription factor consensus binding site 5’ ACCGACCGGATGTTAGCAGA 3’ when incubated with nuclear extracts denoted by the arrow in Figure 7. Addition of an antibody to GABPα blocked the formation of the shifted complex as shown in lane 3 of Figure 7, but the complex still formed in the presence of an antibody to TFAM. These data indicate that the complex formed on the probe contains GABPα, the DNA binding subunit of NRF-2. The fact that there is a decrease in the formation of the complex when incubated with the nonspecific TFAM antibody (lane 4 in Figure 7) indicates that there is some level of nonspecific binding to the probe.
Figure 7: NRF-2 competitive inhibition mobility shift assays

Electrophoretic mobility shift showing endogenous NRF-2 binds the NRF-2 site in the probe. Lane 1 is the labeled NRF-2 transcription factor binding site probe alone. In Lane 2 nuclear extract prepared from non-lesion grey matter from parietal cortex from a control donor was incubated with the labeled NRF-2 binding site probe. The upper arrow denotes the complex formed on the probe when incubated with nuclear extract. The lower arrow denotes unbound probe. The binding of the complex is blocked by addition

(Pandit and others 2009)
of a NRF-2 subunit antibody, GABPα, (Lane 3), but is not blocked by an antibody to mitochondrial transcription factor A, TFAM (Lane 4).
I then performed mobility shift assays to compare the amount of endogenous NRF-2 which could bind DNA in nuclear extracts isolated from grey matter from seven MS compared to four control parietal cortex blocks (C1 compared with MS1 and MS2, C2 compared with MS3 and MS4, C3 compared with MS5 and MS7 and C4 compared with MS6) and from grey matter from two MS compared to two frontal cortex blocks (C5 compared with MS8 and C3 compared with MS1). A decrease in the amount complex containing NRF-2 was found in nuclear extracts isolated from all parietal and all frontal cortices in MS when compared with controls (Figure 8). The same samples were also separately run on a Western Blot and incubated with antibodies to the neuronal marker HuD to control for cellularity. Densitometry was performed from three separate experiments to quantify for the amount of complex formed and was standardized with levels of HuD for each nuclear extract seen in Figure 9. The amount of the NRF-2 complex formed was found to be significantly reduced (p < 0.05) in nuclear extracts isolated from MS cortex on average by 60% in parietal cortex and by 50% in frontal cortex.
Figure 8: Electrophoretic mobility shift assay of the Control and MS cortex with the NRF-2 probe

Electrophoretic mobility shift assays performed with the NRF-2 binding site probe and nuclear extracts from MS and control parietal and frontal cortex show a decrease in the shifted complex containing the NRF-2 transcription factor in MS samples. The shifted complex is denoted by the upper arrow. Shown above are representative gel shifts from different assays. We cannot run too many samples at the same time because once loaded, the samples have to be run immediately or they will dissociate in the buffer. Each panel is a representative mobility shift containing a control on the left and either one or two MS samples on the right. Lane 1 is probe alone, not incubated with nuclear extract, in lanes

(Pandit and others 2009)
2, 5, 8, 10, 12, 14, and 16 nuclear extracts isolated from control parietal or frontal cortex were incubated with the NRF-2 probe, and in lanes 3, 4, 6, 7, 9, 11, 13, 15, and 17 nuclear extracts isolated from MS parietal or frontal cortex were incubated with the labeled NRF-2 probe.
Figure 9: Decreased binding of NRF-2 in MS

Quantitation of the amount of the complex formed was done and standardized to HuD for three separate gel shift experiments. The control samples in each comparison were set to 100% (black bars) and for MS samples data are expressed as % control (open bars). The amount of the NRF-2 complex was significantly reduced in the MS samples in all nine comparisons. Significance was determined using the T-test. *p < 0.05.
The binding activity of NRF-2 is redox regulated, so one explanation for the results in Figures 6-9 could be that there is an overall increase in oxidative damage to proteins, including NRF-2, in the MS samples as compared to controls. Increased oxidative damage to NRF-2 could be inhibiting binding to DNA in MS samples. To analyze the overall differences in the amount of oxidative damage between control and MS samples I performed Western blots on cytoplasmic fractions, isolated from the same tissue the nuclear extracts were isolated from, using an antibody to nitrotyrosine. The levels of nitrotyrosine immunoreactivity were standardized to actin on the same blots to control for protein loading. I found an increase in both the amount of protein nitration and the number of nitrated proteins in all of the MS samples tested as compared with their respective controls as shown in figure 10. These data suggest that there is an increase in oxidative damage to proteins in the MS samples. I then performed mobility shift experiments in which I incubated nuclear extracts from a control and three MS samples with the reducing agent dithiothreitol (DTT) in an attempt to reverse the sulfhydryl modifications to cysteines in the DNA binding domain of NRF-2 which prevent binding (Chinenov and others 1998).

Shown in Figure 11, NRF-2 binding was not altered by increased DTT for Control 1 (C1), but NRF-2 binding was increased by incubation with 1.5mM DTT in mobility shifts done with nuclear extracts isolated from MS2, MS6, and MS7. The results from these experiments indicate that DTT can reverse the oxidative modifications to NRF-2 and lead to an increase in the binding of NRF-2 to the probe in MS samples.
Figure 10: Protein nitration in MS

Increased oxidative damage in MS tissue is correlated with decreased binding of NRF-2. Cytoplasmic fractions were isolated from non-lesion grey matter from parietal and frontal cortex from MS and control donors and Western blots were performed with an antibody to nitrotyrosine. Lanes 1, 3 and 5 are control protein; Lanes 2, 4, and 6 are protein isolated from MS cortex. MS samples contain both increased number and intensity of nitrotyrosine immunoreactive (NT) proteins denoted by arrows in each comparison as compared to controls. The same blots were subsequently incubated with an antibody to actin to control for loading.
Electrophoretic mobility shift experiments were done with the labeled NRF-2 probe and nuclear extracts isolated from control and MS cortex with the reducing agent DTT. Shown above are gel shifts from different assays. We cannot run too many samples at the same time because once loaded, the samples have to be run immediately or they will dissociate. Lane 1 is probe alone, in lane 2 is Control 1 (C1), lane 3 is C1 + DTT, lane 4 is MS2, lane 5 is MS2 + DTT, lane 6 is MS6, lane 7 is MS6 + DTT, lane 8 is MS7, lane 9 is MS7 + DTT. Gels were run separately, hence are shown separated. Lanes indicating ‘+ DTT’ were incubated in binding buffer containing 1.5 mM DTT for 15 minutes. The experiment was aimed to reverse the sulfhydryl modifications to cysteines in the DNA
binding domain of NRF-2 which prevent binding of NRF-2. As shown, there is no increase in binding in the control C1 after incubation with 1.5 mM DTT. I did see an increase in binding in MS 2, MS 6 and MS 7 [Lanes 5, 7and 9] when incubated with DTT.
3.2.3 SH-SY5Y Human Neuroblastoma cell line with hydrogen peroxide treatment:

In order to better understand the effects of oxidative damage on MS pathology I used the human neuroblastoma cell lines [SH-SY5Y] as a model to study the effects of oxidative damage on NRF-2. So as to correctly model the oxidative damage seen in MS the SH-SY5Y cells must mimic the changes seen in the properties of NRF-2 in MS. To ascertain this, both the MS and the SH-SY5Y cells were separately studied for the expression and functionality of NRF-2 and the results compared.

The initial aim of the studies on the SH-SY5Y cell line was to confirm that the effects of oxidative stress and the consequent decrease in NRF-2 binding on all of its downstream transcripts in MS were also seen in the SH-SY5Y cells. For this, I will have to demonstrate that there is an increase in protein nitration and a decrease in NRF-2 binding after oxidative stress but no change in the amount of NRF-2 available for binding in the SH-SY5Y cell lines in cells cultured with an oxidative stressor. Only when that is established, would I be able to investigate other genes for changes in their transcriptional status.

Shown in Fig 12, the 7-day H₂O₂ treated cells were fractionated into nuclear, cytoplasmic and mitochondrially enriched fractions and tested for protein nitration on Western Blots. There was evidence of increasing nitration with H₂O₂ treatment. While there is some expected physiological nitration seen in the control group, there is a visible increase in nitration in the H₂O₂ treated groups as compared to the control group as seen in both blots. Also seen across both the blots is the fact that the increase in the number
and intensity of nitrated proteins is proportional to the increasing concentrations of H₂O₂ treatment. This suggests that there is an intracellular oxidative environment in the SHSY-5Y cells after H₂O₂ treatment and that the levels of intracellular oxidation are proportional to the concentration of H₂O₂ treatment. As seen in Fig. 13, a 2 day exposure to 0, 5, 10 and 15 μM H₂O₂ proportionally increases NRF-2 binding, while the binding decreases proportionally over a 7 day period of exposure. A lower non-specific band is also seen in both the gel shifts in all the lanes in Fig. 13, including the probe-only lane, which is not of any known significance. It should be non-specific binding as it appeared repeatedly in every lane during experimentation, even after the probe was remade. I also saw a middle band in the control lane in the gel shift in Fig. 13-B [7 day treatment with H₂O₂]. This band also fades away in the H₂O₂ treated lanes, and behaves similar to the upper NRF-2 band. It is possible that it is an NRF-2 dimer and the upper band is a tetramer of NRF-2. One way of knowing if it is significant to my experiment is by doing competitive inhibition assays for NRF-2. However, since it behaves almost exactly like the upper band, it might perhaps be superfluous to do so. I saw that as the H₂O₂ concentrations increase, there is a marked decrease in binding of NRF-2 as demonstrated by the fading away of the band. The gel shifts demonstrate that NRF-2 binding increases after an acute exposure to oxidative environments but decreases when the oxidative stress is chronic. As can be seen Fig 14, the amount or expression of GABPα, a subunit of NRF-2, decreases after a 7-day exposure to H₂O₂, which is not the case with MS, where oxidative stress decreases the binding of NRF-2 but not the expression of NRF-2. Hence,
The SH-SY5Y cell line may not be appropriate for studying the effects of alterations in NRF-2 function on MS pathogenesis.
Figure 12: Effect of H$_2$O$_2$ treatment on protein nitration in Human Neuroblastoma cells.

SHSY-5Y cell lines were cultured in sterile Petri dishes. Once they reached 70-80% confluency, they were separated into four labeled groups of Control, 5 μM, 10 μM, and 15 μM H$_2$O$_2$ treatment. The control group was allowed to grow without any treatment. The other three groups were treated daily with a final concentration in the growth media of 5 μM, 10 μM and 15 μM H$_2$O$_2$ respectively for 7 days. All other parameters such as changing media, passing the cells etc was kept constant across groups. All the groups were harvested after 7 days and fractionated using differential centrifugation into Nuclear, Cytoplasmic and Mitochondrial fractions. Shown above are two separate western blots [A and B]. Both have been incubated with antibody to Nitrotyrosine. Each treatment was done on the Nuclear, Cytoplasmic and the Mitochondrial fractions in that
order.  

**A.** The treatments done on the blot A were: Control, 5 μM H₂O₂ and 10 μM H₂O₂.  

**B.** The treatments done on the blot B were: Control and 15 μM H₂O₂.
As mentioned previously, SHSY-5Y cell lines were cultured, treated with H$_2$O$_2$ for 2 and 7 days respectively, harvested and fractionated into Nuclear, Cytoplasmic and Mitochondrial fractions. As before, the treatments were once a day. Shown above are two separate gel shifts [A and B] using the nuclear fractions of the SH-SY5Y cells. Both have correspondingly similar concentrations of H$_2$O$_2$ treatment. The gel shift ‘A’ has
been treated with H₂O₂ for 2 days while the gel shift ‘B’ has been treated for 7 days. The proteins are incubated with binding sites for NRF-2 to test the ability of endogenous NRF-2 to bind to its promoter regions. I saw three separate bands in Fig.13-B and two bands in Fig. 13-A. The lowest band [*] is perhaps some non-specific binding as it was observed repeatedly in all the experiments, even in the probe only lane. It persisted even after the probe was remade, hence it might be a probe artifact. The fact that the middle band [**] in Fig. 13-B behaves exactly like the uppermost band leads us to believe that it might be a dimer of NRF-2. Of interest to us are the bands seen on the top [marked by the top arrow] in both the gels. They probably represent an NRF-2 tetramer. In Fig. 13-B, the middle and the topmost bands behave in the same manner in response to H₂O₂; hence they both should be representative of NRF-2 binding. A: In the 2-day treatment, the binding increases progressively and in proportion to increasing concentrations of H₂O₂, denoted by an increase in the size and intensity of the bands. Lane 1 is the probe-only lane, lane 2 is the control with no H₂O₂ treatment, lane 3 has 5 μM H₂O₂, lane 4 has 10 μM H₂O₂ and lane 5 has 15 μM H₂O₂. B: In the 7-day H₂O₂ treatment, I saw a decrease in binding from lane 2 to lane 5. Again, lane 1 is the probe-only lane, lane 2 denotes control with no H₂O₂ treatment, lane 3 has 5 μM H₂O₂, lane 4 has 10 μM H₂O₂ and lane 5 has 15 μM H₂O₂.
Contrary to previous findings in MS showing no change in the expression of NRF-2, in the SH-SY5Y cells, I saw a decrease in expression of GABPα, a subunit of NRF-2 with increasing doses of oxidative stress caused by 7-day H₂O₂ treatment. This makes the SH-SY5Y cells as inappropriate candidates to study the role of NRF-2 and its downstream transcripts in MS. The nuclear transcription factor C reactive element binding protein [CREB] is the loading control.
3.3 Conclusion

These studies demonstrate that there is an oxidative environment in the MS brain and that this change is more global than previously determined. There is also seen to be a decrease in the binding capacity of NRF-2 in MS, although its expression is unchanged. A significant decrease in the ETC gene expression was observed concurrent with the decrease in NRF-2 binding. Since NRF-2 is known to play a role in the transcription of the ETC genes, it suggests that a decrease in NRF-2 may be responsible for the decrease in the expression of the ETC genes. A decrease in the ETC genes can have a deleterious effect on the production of ATP and the overall energetic balance of the cell, tilting it towards neurodegeneration.

SH-SY5Y cell lines do not mimic the observation that there is no change in the expression of NRF-2 in MS and hence may not be suitable for studies related to effects of oxidative damage on transcription factor NRF-2. I will look at the EAE mouse model of MS and study it to see if it mimics the changes seen in MS cortex.
Figure 15: Proposed model for the impaired binding of NRF-2
Figure 15: Proposed model for the impaired binding of NRF-2.

When in a reduced state, GABP binds well to its promoter regions and activates transcription of gene transcripts for mitochondrial ETC proteins. However, under conditions of increased oxidative stress, the cysteines in the DNA binding domain of NRF-2 are oxidized and reduce DNA binding of GABP is decreased (Chinenov and others 1998). This in turn decreases transcription for mitochondrial ETC proteins. This negative feedback loop works well for a normally functioning cell. During normal ATP production, there is some inevitable production of ROS, which is harmful to the cell. As more and more ATP is generated, the levels of ROS also increase, effectively inhibiting GABP binding to DNA and reducing transcription of ETC proteins, which in turn limits the rate of ATP production. It is hypothesized that in MS a predominant and prolonged oxidative stress can inhibit production of ETC protein subunits to an extent where the cell suffers from energy depletion because of a lack of adequate ATP generation. This will eventually disturb the ion homeostasis and lead to a cascade of destructive events causing cell death.
Chapter 4

Are changes in mitochondrial and respiratory genes seen in MS brain modeled in EAE mouse?

Introduction

4.1 EAE and MS

The mouse model of EAE has been a popular and useful tool for studying MS pathogenesis, in part because the mouse species comes closest to mimicking the different stages of MS. The EAE mouse model is generated by inoculation of specific epitopes of myelin protein such as myelin basic protein [MBP], myelin oligodendrocyte glycoprotein [MOG] or myelin proteolipid protein [PLP] (Mix
An additional dose of the pertussis toxin boosts the immunogenicity by breaking down the blood brain barrier. The degree of disability is graded on a scale of 0-5 with 0 being normal, 1 corresponding with tail limpness, 2 corresponding with hind limb part-paresis with gait abnormalities, 3 with complete hind limb paralysis, 4 with quadriplegia and 5 with terminal paralysis [near death state]. The most widely used EAE models are those induced with epitopes of the PLP or the MOG protein, including the MOG$_{35-55}$ epitope used in my study. The antigen introduced determines the type of EAE generated. The PLP antigen induces EAE symptoms similar to RR MS while the MOG antigen induces chronic progressive EAE similar to SP MS or PP MS. The EAE mouse model has been useful in delineating several aspects of MS pathology such as demyelination, remyelination and degeneration of neurons, the immunogenic response in MS along with some of the heritable genetic risk factors such as the Interleukin gene [IL-2α and IL-7α] and some Human Leukocyte Antigen genes [HLA] responsible for the major histocompatibility complex [MHC] class II type proteins. The EAE mouse model has also been exceedingly useful in the generation of therapeutic targets for MS, the analysis of their animal trials and more recently the attempts at enhancing remyelination by stem cell transplants or growth factors. It is an invaluable tool for in vivo experiments such as knock down or knock up studies of the suspected pathological genes in MS. However, no disease model including the EAE is perfect. EAE cannot replicate the age of onset or the chronicity of MS progression in humans. It is also not well suited to study cortical lesions or primary neurodegeneration in MS. EAE generates global neuronal damage while being unable to replicate the
primary demyelination seen in MS (Farooqi, Gran, Constantinescu 2010). The fact that even aggressive immunosuppression cannot halt the progression of MS is evidence that other mechanisms have a hand in the pathogenesis of MS. I wanted to compare the changes seen in the expression of the ETC genes in MS with the EAE mouse model. Also, a proteomic study on MS had demonstrated changes in the proteomic expression profiles in MS and I wanted to know if these changes were mirrored in the mouse model of EAE.

Mitochondrial dysfunction has also been implicated in disease pathology in the EAE mouse model of MS (Mao and Reddy 2010). It has been reported that increased nitration of electron transport chain components leads to decreased mitochondrial activity in EAE (Qi and others 2006) and that cyclophilin D, a modulator of the mitochondrial permeability transition pore, is protective (Forte and others 2007). Similarly, analyses of postmortem MS white matter have identified alterations to mitochondrial enzyme activity and damage to mitochondrial DNA in lesions (Kalman, Laitinen, Komoly 2007; Mahad and others 2008; Witte and others 2009). Other studies have demonstrated defects in mitochondrial electron transport gene expression and function in normal appearing grey matter in postmortem MS cortex (Dutta and others 2006; Pandit and others 2009). My previous experiments demonstrated that oxidative damage is involved in the altered transcription of mitochondrial genes in the MS brain.

These studies have identified transcriptional changes in important mitochondrial genes; however, translational or post-translational alterations in a multitude of other proteins may also impact mitochondrial function and energy production. Because studies
of proteins originating from MS brain tissue may provide new insights into the pathogenesis and etiology of the disease, recently the SELDI-TOF-MS technique was applied to analyze mitochondrial fractions derived from MS cortex so as to obtain a better understanding of protein expression changes that could contribute to the mechanisms of mitochondrial dysfunction in MS (Broadwater and others 2011).

This study was a collaborative effort involving our laboratory, the core Imaging facility of the Bio-Medical department where the confocal microscopy studies were conducted, the department of Clinical Neurosciences and Oncology, University of Calgary and the Chemistry department at Kent State University; specifically the Proteomics core facility, where the Mass Spectrometry studies [SELDI] and some other aspects of the study were conducted. In order to better understand the context of my work in this study, I am providing an overview of the salient features of this study, followed by an introduction to my work therein.

Proteomic analyses of MS tissue have focused on white matter, lesioned tissue, and CSF (Avasarala, Wall, Wolfe 2005; Chiasserini and others 2008; Han and others 2008; Quintana, Farez, Weiner 2008; Selmaj and others 2008; Westman-Brinkmalm and others 2009; Zetterberg and others 2008). However, there is evidence from magnetic resonance imaging studies that the mechanisms involved in tissue damage are different in grey matter and white matter in MS (Fisher and others 2008). Therefore studies detailing protein differential expression in normal appearing grey matter are necessary to provide additional insight into disease manifestation and progression as it relates to the neurodegenerative component of MS pathology.
With the aim of trying to better understand the possible differences in protein expression in control and MS human brains in the NAGM, studies based on the SELDI technique were performed by others (Broadwater and others 2011). These studies were able to more thoroughly and efficiently elucidate potential alterations in the expression of mitochondrial and related proteins in postmortem MS and control cortex than previously known. Most of these differentially expressed proteins are involved in energy metabolism in MS grey matter and include cytochrome c oxidase subunit 5b (COX5b), the brain specific isozyme of creatine kinase [CKB], hemoglobin β-chain [HBB] and MBP.

I performed Western blots on human control and MS brain samples and the EAE mouse whole brain and cortex in order to see if the changes seen in the expression of the ETC proteins in MS were also seen in the EAE mouse model. Also, the SELDI studies had indicated changes in protein expression in MS and I wanted to know if this was also seen in EAE. I found that the EAE mouse model did not mirror changes seen in ETC complex IV subunit COX5b. Expression of other ETC proteins was inconsistent on Western blots probably since the protein subunits were very small or because of weak antibodies. In my studies on EAE mice brains, MBP was seen to be altered in a similar fashion to MS. These data indicate that while the EAE mouse model may mimic aspects of MS neuropathology which result from inflammatory demyelinating events, there is another distinct mechanism involved in mitochondrial dysfunction in grey matter in MS which is not modeled in EAE. In the other part of my studies, my data with the human control and MS brain samples concurred with the previous SELDI findings.
4.2 Results

4.2.1 Results from proteomic analysis of MS cortex by others:

Mitochondrial samples from either parietal or frontal cortex were obtained from MS NAGM as confirmed using PLP staining done previously. For the SELDI analysis mitochondrial fractions from eight control and MS donors, paired primarily for brain regions, and also for age, sex and PMI, were studied. The SELDI-TOF mass spectra demonstrated the over expression of HBB, MBP, and CKB, and decreased expression of COX5b in MS relative to controls.

4.2.2 Analysis of EAE and MS by Western blots:

Whole brains and cortex from frozen EAE mice brains were fractionated separately for mitochondrially enriched and cytoplasmic fractions. For the Western blots on MS and controls, 7 controls were compared with 5 MS samples. These included 3 parietal and 4 frontal controls and 2 parietal and 3 frontal MS samples. The samples were matched for brain region, age sex and PMI. Donor demographics are described in
Table 1. My main aim was to investigate if the mouse model for MS, namely EAE mice, would mirror the changes seen in the ETC proteins in my earlier studies in MS, and to what extent. SELDI had previously demonstrated the over expression of HBB, MBP and CKB, and decreased expression of COX5b in MS relative to controls. I also wanted to see if the changes seen by SELDI could be confirmed on Western blots.

Western blots were performed on EAE whole brains and cortex as well as on paired human control and NAGM MS brains. The frozen EAE mice brains [stage 4] were kindly provided by Dr. V. Wee Yong, Departments of Clinical Neurosciences and Oncology, University of Calgary, Calgary, Alberta, Canada. Each of the control and MS human brain samples, and the EAE whole brains and the cortices were separately fractionated into cytoplasmic and mitochondrial fractions.

In order to demonstrate the purity of the mitochondrial fractionation procedure, Western blots were performed for expression of neurofilament, which is a neuronal marker, and the mitochondrial encoded protein cytochrome oxidase subunit II (COX2), in MS and control cytoplasmic and mitochondrial fractions (Fig. 16). The results were as expected, with neurofilament showing up in the cytoplasmic fractions and the COX2 showing up in the mitochondrial fractions.
Figure 16: Testing the purity of the subcellular fractions

Representative western blot demonstrating the relative purity of the cellular fractionation. Western blots were performed on cytoplasmic (cyto) and mitochondrial (mito) fractions isolated from MS and control cortex, run side by side and blotted with an antibody to the neuron specific cytoplasmic protein, neurofilament (NF), and an antibody to the mitochondrial encoded COX2 protein. Arrows denote multiple NF immunoreactive proteins present in cytoplasmic fractions.

(Broadwater and others 2011)
Once the purity of the samples had been established, Western blots were done with antibodies to COX5b, CKB, HBB and MBP on the mitochondrial and cytoplasmic fractions separately. To control for loading accuracy Western blots with the mitochondrial fractions were reblotted with antibodies to either Complex II or COX2, and Western blots with the cytoplasmic fractions were reblotted with antibodies to GAPDH for both the human as well as EAE mitochondrial and cytoplasmic fractions respectively. The altered expression of proteins including COX5b, CKB, HBB and MBP in MS mitochondrial fractions was similar to that seen by SELDI (Fig. 17-19). Statistical significance was calculated using the T-test [1 Tailed, Type 1, p<0.05].
Figure 17: Expression of COX5b and creatine kinase in Control, MS and EAE

Shown above are representative Western blots for COX5b and CKB expression in human and EAE brain samples. Mitochondrial fractions from three pairs of matching control and MS samples as well as control and EAE whole brain samples were run on Western blots and tested for expression of COX5b and creatine kinase. The blots were rebotted with antibodies to either Complex II [CII] or COX2 to control for loading discrepancies. There was a significant decrease in the expression of COX5b in the MS samples when compared with their corresponding controls. There was also a significant increase in the expression of CKB in the MS samples. However, no corresponding significant change was observed in the expression of either COX5b or CKB in the EAE samples [whole brain and cortex].
Figure 18: Expression of Hemoglobin-beta in Control, MS and EAE

Shown above are representative Western blots for HBB expression in human and EAE brain samples. Mitochondrial fractions from three pairs of matching control and MS samples as well as control and EAE whole brain samples were run on Western blots and tested for expression of HBB. The blots were reblotted with antibodies to CII to control for loading discrepancies. There was a significant increase in the expression of HBB in the MS samples when compared with their corresponding controls. However, no significant change was observed in the expression of HBB in the EAE samples [whole brain and cortex].
Figure 19: MBP expression in Control, MS and EAE

Shown above are representative Western blots for MBP expression in human and EAE brain samples. Mitochondrial fractions from three pairs of matching control and MS samples as well as control and EAE whole brain samples were run on Western blots and tested for expression of MBP. The blots were rebotted with antibodies to CII to control for loading discrepancies. There was a significant increase in the expression of MBP in the MS as well as the EAE samples [whole brain and cortex] when compared with their corresponding controls. As can be seen from further illustrations, this appears to be the only aspect of MS pathology that is seen to be mirrored in the EAE mouse model in all the proteins investigated in this study.
Quantitation of western blotting experiments confirmed in the mitochondrial fractions of the human control and MS samples a significant increase in CKB, HBB as well as MBP in MS over control samples (Fig. 20). On the other hand, COX5b shows a significant decrease in MS as compared to the control samples. These changes were observed across samples from both the cohorts as well as additional samples [C3 and MS11] not included in the SELDI studies.

Cytoplasmic fractions from three pairs of matching control and MS samples as well as control and EAE whole brain samples were run on Western blots [data not shown] and tested for expression of HBB and CKB. The blots were reblotted with antibodies to GAPDH to control for loading discrepancies. There was no significant change seen in the CKB the cytoplasmic fractions of either the MS or the EAE [whole brain and cortex] samples. HBB does appear to have an increased expression in MS, however this increase is not statistically significant. There is no change seen in the expression of HBB in the cytoplasmic fractions of the EAE samples [whole brain and cortex].

Western blots were also performed for these proteins in mitochondrial fractions isolated from either whole brain or cortex from EAE and control mice. Representative western blots are shown in Fig. 17-19 and quantitation in Fig. 20. No significant change was seen in the expression of HBB, COX5b or CKB in the mitochondrial fractions of EAE brains. There was a statistically significant increase in MBP in the mitochondrial fractions of the EAE samples indicating that only increased MBP expression is modeled in the EAE mouse. I did observe more than one isoform of MBP increased (two bands
~18–20 kDa) in the EAE mitochondrial fractions rather than the one isoform increased in human MS tissue at ~18 kDa (Fig. 19).
Figure 20: Quantification of MS and EAE studies using Western blot

(Broadwater and others 2011)
Figure 20: Quantification of MS and EAE studies using Western blot

Shown above are HBB, CKB, MBP and the COX5b, in MS and EAE, mitochondrial fractions, as a percent of their expression in the respective control samples. EAE data shown above is from the densitometry for the Westerns using EAE cortex. Western blots for studying the expression profiles of all the proteins under consideration in this study were repeated at least three times. The resulting images were scanned and densitometry was done using the ImageJ software. For statistical calculations data generated by densitometry for the expression of the proteins in the mitochondrial fractions was divided by that of either Complex II, or COX2 to give us an output unbiased by errors in loading. Similarly, densitometry data generated from the expression of proteins in the cytoplasmic samples was divided by that of GAPDH to generate an unbiased output with respect to errors in loading. Statistical significance was calculated using the T-test of significance with p<0.05.
In contrast to the results seen in the mitochondrial fractions of the human brains, no significant changes are seen in CKB, COX5b or HBB in EAE. MBP and COX5b did not show adequate expression in control or MS samples in the cytoplasmic fractions and are therefore not shown.

One control sample not included in the SELDI analysis but which was analyzed by western blot, C9, contained consistently increased mitochondrial content as indicated by the increase in COX2 expression (Fig. 17). MS samples still expressed increased levels of creatine kinase B relative to this control sample in spite of this increase in mitochondrial expression in C9. Further, COX5b expression was significantly decreased in MS samples even when C9 was not included in the analysis. COX5b and MBP were only expressed in mitochondrial fractions. Hemoglobin and creatine kinase B were detected in both mitochondrial and cytoplasmic fractions, but were only significantly increased in mitochondrial fractions in MS.

4.3 Conclusion

A significant increase in the expression of CKB, HBB and MBP was noted in the mitochondrial fractions of the MS samples. There was also a significant decrease in the expression of COX5b in the MS mitochondrial fractions. No significant change in expression was noted in the expression of CKB or HBB in the cytoplasmic fractions of the MS samples. There was no detectable expression of MBP OR COX5b in the human
cytoplasmic samples. In the EAE brains there was a significant increase seen only in the expression of MBP in the mitochondrial fractions which leads us to conclude that the changes seen in the expression of the ETC genes in MS may not be due to pro-inflammatory changes associated with MS and other factors may play a role in the evolution of MS. In the context of proteomic expression profiles, MBP is the only protein amongst those investigated in EAE in this study to mimic changes seen in MS.
Analysis of the role of Epigenetic Mechanisms in MS

Introduction

5.1 Epigenetics and MS

MS is a disease of heterogeneous etiology. There is an autoimmune component contributing in terms of inflammatory demyelinating changes in MS white matter. While the EAE mouse model is traditionally considered a good animal model for MS, my studies indicate that EAE may be predominantly mimicking the pro-inflammatory changes seen in MS and that there are other factors that contribute to MS pathology, especially in grey matter. Proteomic studies demonstrate a differential protein expression between EAE and MS. While COX5b, HBB, CKB
and MBP were found to have a changed expression profile in MS, only MBP was seen to have been changed in EAE. EAE is induced in mice as an autoimmune reaction and hence by corollary, something other than what can be explained by EAE also contributes to the generation of MS pathology. In other words, something other than an inflammatory autoimmune attack on the neurons may be playing a significant role in the pathogenesis of MS. In line with this thought, it is possible that another mechanism, namely, epigenetic modification of DNA may play a role in the pathogenesis of MS as shown in figure 21. Epigenetic changes are caused by ‘extra-genetic’ influences [such as environmental factors, diet, etc.] on the genome and they change the expression profile of the genome.

Conrad Waddington first defined epigenetics in 1942 as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington C 1942). This broad spectrum definition was able to encapsulate the essence of epigenetics as we know it today exceptionally well, given the fact that DNA and its properties were just being discovered around that time and a consensus over the fact that the DNA is responsible for the genotype had not yet been formed (Cyr and Domann 2011). Since there was no way then to prove [or disprove] the existence and/or effects of epigenetic factors on the phenotype, for the next two to three decades, the definitions of ‘epigenetics’ became more subjective, dissimilar, numerous and varied. Each researcher gave his or her own version of what epigenetics might be, and a unified definition or though on the matter did not materialize until the 1980s. By then DNA methylation was already known to be a heritable regulatory process in
eukaryotic cells. In 1987, Robin Holliday refocused the attention of the scientific community on epigenetics with his description of epigenetics which was “the strategy of genes in unfolding the genetic program for development”. While this description too lacked specifics, it marks a monumental shift in the interest of the scientific community towards epigenetics, an interest which has been growing and intriguing us till date.

A more contemporary description of epigenetics is that epigenetic mechanisms such as DNA methylation, histone acetylation etc modulate gene expression in the cell without an actual change in the DNA sequence. They are heritable and induce changes in gene expression and function, changes which are not encoded directly in the DNA (Bock and Lengauer 2008). Epigenetic influences induce a phenotypic change in gene expression without a corresponding change in the genotype. Epigenetics has also been described as a chromosomal adaptation “so as to register, signal or perpetuate altered activity states” by Bird (Bird 2007). If epigenetic modifications are an adaptive response, then a non-genetic influence could also influence the phenotype via epigenetic modifications (Cyr and Domann 2011). Epigenetics could be the link integrating environmental influences with genetic traits to yield the final phenotype. While the number of environmental, cellular and other non-genetic stimuli may vary greatly, epigenetic modifications in response to such influences are hypothesized to integrate them and somehow weigh in their relative contributions thus giving us the final phenotype.

It is thought that the fact that the epigenome has a marked influence on the expression of the genome may be advantageous for survival. For example, the brain is
one of the most complex organs and once it is hardwired postnatally it loses to some extent its potential for plasticity of response. Epigenetic modulation of a hardwired brain allows it more plasticity in responding to either rapid or long-term changes in its environment, thus increasing its chances of survival. During the course of evolution, epigenetic influences have become not only useful but also critical for normal and stable brain development and maturation. Aberrations in physiological epigenetic mechanisms are now known to be responsible for an array of disorders, including but not limited to cancer, developmental disorders and psychiatric disorders (MacDonald and Roskams 2009).
Figure 21: Genetic and environmental factors influence the final phenotype

Schematic showing how the final phenotype is influenced by genetic and environmental interactions. Effects of gene-gene interactions also influence the final phenotype. Environmental factors can also be intracellular and factors such as oxidation can influence intracellular genomic expression in much the same way as diet etc can influence the organism as a whole. Also shown are other factors such as age, health etc which may be decisive on the amount of impact a certain epigenetic factor will have on the modulation of genomic expression.

(Hoffjan and Akkad 2010)
DNA methylation and histone deacetylation have come to be recognized as the two major epigenetic signals for the modulation of gene expression. As the name suggests, DNA methylation occurs on the DNA nucleotide while histone deacetylation occurs on the histone proteins responsible for compacting the nucleosome. Epigenetic modifications can be either covalent [such as methylation, deacetylation, phosphorylation] or non-covalent modifications [such as chromatin remodeling] and all of these modifications will result in a modulation of the phenotype.

In DNA methylation, a methyl group is covalently added at the 5’ position on the pyrimidine ring of the cytosine residues as shown in figure 22. In eukaryotes, this modification occurs within the cytosine – phosphate – guanine [CpG] dinucleotides. In the human genome, most of the CpG dinucleotides are methylated. Methylated cytosines tend to spontaneously deaminate to thymines, except at the ‘CpG-islands’. CpG islands [CGIs] are short sequences of about 1 Kb length which are rich in CpG repeats. They are found to be in the promoter regions of most of the human genes and are mostly unmethylated. A computational study aimed at detecting CGIs in the human genome found that ~ 72% genes had classical CpG islands while the rest had a background level of CpG occurrence. The methylated cytosines in the CGIs resist the spontaneous deamination to thymine by an as yet unknown mechanism. The classical CpG islands were significantly seen in the promoter regions of housekeeping genes while the promoter regions with low CpG island density represented genes for specific differentiated cell-type functions. Another study demonstrated that most of the methylated CGIs were located in the intergenic regions. Interestingly, these regions also
contained RNA markers of transcription start sites, suggesting that they could be influencing alternate transcripts. Thus alternate transcripts could be initiated or repressed based on cell-type and required function. Thus epigenetic regulation of CGIs could play a larger role than merely suppressing gene transcription.

DNA methyltransferases [DNMTs] are responsible for catalyzing the transfer of the methyl group on the cytosine residues from S-adenosyl-L-methionine [SAM]. There are two important types of DNMTs. They are the maintenance methyltransferases and the De Novo methyltransferases. The maintainence methyltransferases such as DNMT1, play a role in the primary methylation of a replicating DNA while the de novo methyltransferases such as DNMT3a and DNMT3b are responsible for establishing new methylation patterns in a post-mitotic cell. While the DNAMTs are well known, surprisingly little is known about the types of DNA methylases [DNAMs] and the mechanism of their action. DNAMs putatively de-methylate the DNA. TET1 protein is currently being considered as a potential DNAM. TET1 is a hydroxylase which oxidises the methyl group [CH3] on the cytosines to a hydroxyl-methyl group [CH$_2$OH]. This may be a first step in the process of removal of the methyl group from the cytosine. However, the complete pathway to the removal of the methyl group is as yet unclear.

MeCP2, a monomer, contains two main domains, the methyl binding domain [MBD] and the transcription repression domain [TRD] and is about 75-80 KDa in size. The methyl binding domain, as the name suggests, is responsible for binding to methylated DNA while the TRD is responsible for recruiting other enzymes such as chromatin-associated proteins [CAPS] which in turn function to condense or compact the
nucleosome. The TRD serves as a guide or co locator for other chromatin compacting proteins and helps them stabilize at their target location. The binding of MeCP2 to methylated DNA is independent of its ability to recruit proteins and condense chromatin into higher order structures. MeCP2 is a mostly unstructured protein with protease resistant domains. While it is an ubiquitous protein, the brain has the highest levels of MeCP2 (Gonzales and LaSalle 2010). In the brain, the neurons contain the highest amounts of MeCP2. Around 60% of the protein is unstructured and has the ability to form a high variety of secondary structures on associating with binding partners and DNA (Ghosh 2010). The N-terminal has the highly conserved methylated DNA binding domain [MBD]. Two unstructured domains near the MBD modulate the binding of the MBD to methylated DNA. The TRD is associates with a variety of partners such as Sin3A and histone deacetylase complexes [HDACs] to repress gene expression. MeCP2 acquires its secondary structure upon DNA binding and it varies according to its binding partners. MeCP2 binds to symmetrically methylated CpG dinucleotides and aids in their compaction, thereby preventing gene transcription. While the exact molecular mechanisms are still unclear, it is known that absent or inadequate MeCP2 expression in a developing nervous system causes Rett Syndrome and autism probably because of mutation of MeCP2 interacting surfaces which would hamper the binding of partner proteins leading to expression of genes usually repressed.

Methylation of DNA at a particular locus is usually a signal for gene repression mechanisms to act on and suppress gene expression in that region. It is now known to be an important epigenetic regulator of gene expression. MeCP2 binds to symmetrically
methylated CpG dinucleotides and suppresses gene expression in three known ways: by physically inhibiting promoters from recognizing and therefore binding at their binding sites, by recognizing methylated DNA sites and then either covering up the methylated loci or/and recruiting corepressors at that site to suppress transcription or by recruiting a host of other proteins together known as methylated DNA binding CAPS to compact the nucleosome, thus making it inaccessible [figure 22] (Adkins and Georgel 2011). Many of the target genes for MeCP2 show a change in expression in relation with neuronal activity. It is postulated that MeCP2 is centrally poised to integrate signals from neuronal activation with epigenetic landmarks on target genes and modulate gene expression (Gonzales and LaSalle 2010).
Figure 22: Schematics of transcriptional repression of DNA by methylation

(DNA Methylation and Transcriptional Repression)

1. Direct interference with transcription activator factor binding
   a. Active transcription

2. Specific transcriptional repressors
   a. Active transcription

3. Inactive chromatin structure formation

   Examples: Methylation sensitive TF: AP-2, E2F, NFkB
   Methylation insensitive TF: Sp1

   methylated CpG:
   unmethylated CpG:

(Singal and Ginder 1999)
Figure 22: Schematics of transcriptional repression of DNA by methylation

Transcriptional repression of DNA due to methylation occurs in three known ways. 1. Methylation of the cytosine residues of DNA directly inhibits binding of the transcription factor to its binding site. 2. The methylated cytosine residues will attract DNA methyl binding domain [MBD] proteins, such as MeCP2, which in turn will recruit gene repressor complexes such as Sin3 and HDACs to again prevent the transcription factor and the DNA polymerase II from binding to DNA and effecting transcription. 3. The transcriptional repressor complex of the MBDs and the HDAC will effect chromatin compaction which makes the transcription factor binding site inaccessible for transcription.
5.2 Contribution of methylation in MS

I would like to explore the effects of the changes in the methylation patterns of the promoter regions of the ETC genes in MS and studying the effects on their expression. Just as oxidative stress can decrease the overall efficiency of NRF-2 binding activity, epigenetic factors such as methylation of cytosine residues on the promoter sites can also decrease the rate of transcription. Epigenetic factors such as DNA methylation, histone acetylation etc modulate gene expression in the cell without an actual change in the DNA sequence. They induce changes in gene function which are not encoded directly in the DNA (Bock and Lengauer 2008). While an autoimmune component is suspected in the etiology of MS, demyelinating lesions are seen even without any inflammatory cells in MS cortex. Genetic linkage studies are not by themselves able to account for the heterogeneity of occurrence of MS either. Monozygotic twins have a concordance of only about 20-30% for acquiring MS (Ebers 2005; Hansen and others 2005b). The above studies support the hypothesis that MS is associated with complex and multiple etiologies (Borchelt D.R. and others ; Gonzalez-Scorano and Rima 1999; Hansen and others 2005a) which may perhaps exert a combinatorial effect on an individual’s susceptibility [to MS].

Methylation is an important epigenetic influence on the genome and in the majority of the cases it serves to repress gene expression. Epigenetic factors such as DNA methylation, histone acetylation etc serve to translate the effects of non-genetic factors such as oxidative stress into epigenetic changes which modulate gene expression in the cell without an actual change in the DNA sequence. They are responsible for
‘tweaking’ the genetic output in response to changes in its environment by inducing changes in gene function without a corresponding change in the genome (Bock and Lengauer 2008). In this way they allow an additional level of plasticity to an already versatile genome.

I was interested in studying the effects of methylation on the regulation of electron transport chain gene expression, particularly the Complex V gene because I found that their promoter regions were CG rich, suggesting that they may be regulated by methylation. I also studied the effects of methylation on the PGC1A transcription factor because it regulates many mitochondrial genes and is regulated by methylation. It does not bind DNA however, and is a co-factor, binding other transcription factors such as NRF-2 to activate transcription.

5.3 Results

5.3.1 Chromatin Immunoprecipitation and DNA-PCR:

I have begun analyzing the methylation status of PGC1A and Complex V genes by chromatin immunoprecipitation with the MeCP2 antibody. The results of this study should be considered as preliminary data. It would be helpful and necessary to add more control and MS samples to this data set in order to get a better idea of the differential
methylation patterns that may exist between them as well as to enhance the validity of the statistical tests used. Furthermore, the results of the changed methylation patterns in MS as observed by the ChIP would have to be verified, either by demonstrating corresponding changes in protein expression by Western blots or by demonstrating changes in mRNA transcripts for the corresponding genes by PCR. More samples have been available for this study and were not used due to time constraints.

The chromatin immunoprecipitation had a few challenges of its own. The commercially available kits for ChIP were all tailored to suit extraction of chromatin from cultured cells [typically a monolayer], while I needed to extract chromatin from chunks of solid tissue. This resulted in a fair bit of modifications to the original protocol as suggested by the kit used by us [EZ-Magna ChIP™ G Chromatin Immunoprecipitation Kit, Millipore], before I could carry out the ChIP experiments successfully. The modifications made to the original protocol to adapt it for chromatin extraction from solid tissue are detailed in the methods section. Three MS and two controls were selected for the initial study from an available larger pool of samples. Unfortunately, I later realized that one of the controls was a cancer patient who had undergone extensive chemotherapy. Chemotherapy is known to alter methylation patterns and hence that particular control may yield unpredictable results. Chemotherapeutic drugs have a variety of modes of action including [but not limited to] demethylation, which might help if required genes such as the Tumor Suppressor gene are hypermethylated, and hypermethylation which would act on demethylated genes in cancer, such as oncogenes, which are physiologically silenced by methylation (Franco and others 2008). This particular study is a work in
progress, especially since more samples are available to be analyzed. Having said that, it is important to note that the results so far are encouraging.

As mentioned before, three MS and two control samples were used in this study. Chromatin extracted from each sample was sheared by sonication, cleaned of proteins and RNA and divided into three parts. One part was immunoprecipitated with the MeCP2 antibody [Abcam], in order to precipitate methylated DNA bound by MeCP2, which as discussed previously is a methyl CpG binding protein predominately expressed in neurons. The second part was immunoprecipitated with an antibody to IgG [Millipore], which is the negative control. The third part was left as it is [Input DNA] and was the positive control for the PCR reaction. In order to ascertain that the immunoprecipitation was successful, PCR products from the linear phase of amplification for the input DNA, the MeCP2 immunoprecipitated DNA and the IgG immunoprecipitated DNA for both the positive control GAD67 and the negative control GAPDH primers were run on an agarose gel and compared for relative expression of enrichment for methylated DNA [Figures 23 and 25]. As expected, amplified DNA is seen in the input DNA, but not in the MeCP2 or IgG immunoprecipitated DNA for the GAPDH control while the amplification with the GAD67 primers demonstrates amplification in the input DNA and the MeCP2 immunoprecipitated DNA but not in the IgG immunoprecipitated DNA. GAPDH was the negative control primer. GAPDH is a housekeeping gene and is constitutively active. Therefore it should be hypo methylated. The GAD67 gene is responsible for the decarboxylase enzyme [glutamic acid decarboxylase] that converts Glutamate to GABA. It has been previously shown that
GAD67 is significantly hyper methylated as compared to GAPDH in cell cultures (Kundakovic and others 2009). GAD67 is also shown to be decreased in MS (Clements, McDonough, Freeman 2008; Dutta and others 2006). GAD67 expression is upregulated by drugs which disrupt DNA methyl transferases and histone deacetylases. Hence GAD67 was considered as the positive control for the PCR.

Other promoter regions were analyzed for methylation levels by PCR including Complex V ATP synthase subunit genes ATP5G3, ATP5J, and ATP5I. Of the electron transport chain genes decreased in MS cortex, I found that the promoter regions of the Complex V genes are CG rich suggesting that they may be regulated by methylation. I also analyzed the promoter region of the mitochondrial transcription factor PGC1A which is a transcriptional co-activator in the regulation of the ETC genes and is also known to be regulated by methylation (Barres and others 2009). Primers for the PCR were selected from the 500-2000 bp promoter region upstream of the genes of interest. Shown in Figures 24 and 26-30 are the promoter regions of the respective genes under scrutiny with the potential methylation sensitive CG-rich regions highlighted in yellow and the primer pairs for PCR in bold and underlined. Also shown are bar graphs for the relative enrichment for methylated DNA per control or MS sample. The levels of methylated DNA immunoprecipitated by the MeCP2 antibody for each primer set were determined by the amount of PCR product obtained from the MeCP2 immunoprecipitated DNA compared to the level of amplification obtained from input DNA. This was determined by quantitative PCR with the $2^{-\Delta\Delta Ct}$ method, so that a difference in amplification of one cycle is considered a two fold difference in expression. Levels of
PCR products from MeCP2 immunoprecipitated DNA are shown as a percent of input DNA for each primer set for MS and control samples [Figures 24, and 26-30]. Results in Figure 31 reflect the differential immunoprecipitation of methylated DNA between control and the MS samples for the above mentioned genes of interest. Data were pooled for control and MS samples for each primer set. These results show that the GAD67 and ATP5I promoter regions appear to be more methylated in MS as compared to control as shown by a statistically significant increase in the percentage of PCR product in the MeCP2 immunoprecipitated DNA as compared to input DNA in MS [Figure 31].
Figure 23: Representative expression of GAPDH PCR product on an agarose gel

Shown above are the PCR products of MS 12 amplified by the negative control GAPDH [product size 164 bp]. The DNA ladder [Bioline, Hyperladder II] shows a band at 200 bp. Input DNA demonstrates a PCR product at the expected size while the MeCP2 and the IgG do not show enrichment for GAPDH promoter regions.
Figure 24: Levels of methylation in the GAPDH promoter region by ChIP
Figure 24: Levels of methylation in the GAPDH promoter region by ChIP

Shown above are the results of the PCR with GAPDH primer. GAPDH is the negative control PCR primer. The immunoprecipitation was done on five samples [two controls and three MS] with the MeCP2 antibody. GAPDH is a housekeeping gene and is constitutively active. Therefore it should be hypo methylated. Shown above are the 500 base pairs of the promoter region of the GAPDH gene. In bold and underlined are the two primers selected for PCR. The PCR product was 164 base pairs. Highlighted in yellow are the C-G regions which are prone to methylation by DNA methyltransferases. Also shown is a graph for the PCR products of the GAPDH primer for all the five samples under consideration. PCR products for immunoprecipitations using the MeCP2 antibody are shown as a percent of the PCR products of Input DNA. IgG is the negative control. Results are the average percentile results from 3 runs of PCR. From the results, it is apparent that the MeCP2 antibody did not result in enrichment of methylated DNA over and above that seen in the input DNA except for C 11 which shows an exaggerated pattern of methylation for the GAPDH promoter region as compared to the rest of the samples studied.
Figure 25: Representative expression of GAD67 PCR product on an agarose gel

Shown above are the PCR products of MS 13 amplified by the positive control GAD67 [product size 259 bp]. Input DNA demonstrates the largest amplification with the MeCP2 lesser than it and no PCR product in IgG, which is the negative control for the IP.
Figure 26: Levels of methylation in the GAD67 promoter region by ChIP
Figure 26: Levels of methylation in the GAD67 promoter region by ChIP

Shown above are the results of the PCR with GAD67 primer. GAD67 is the positive control PCR primer because physiologically GAD67 has been shown to be significantly methylated when compared with GAPDH, which was hypo methylated (Kundakovic and others 2009). The immunoprecipitation was done on five samples [two controls and three MS] with the MeCP2 antibody. Shown above are the 600 base pairs of the promoter region of the GAD67 gene. In bold and underlined are the two primers selected for PCR. The PCR product was 259 base pairs. Highlighted in yellow are the C-G regions which are prone to methylation by DNA methyltransferases. Also shown is a graph for the PCR products of the GAD67 primer for all the five samples under consideration. PCR products for immunoprecipitations using the MeCP2 antibody are shown as a percent of the PCR products of Input DNA. IgG is the negative control. Results are the average percentile results from 3 runs of PCR.
Figure 27: Levels of methylation in the PGC1A promoter region by ChIP
Figure 27: Levels of methylation in the PGC1A promoter region by ChIP

Shown above are the results of the PCR with PGC1A primer. PGC1A plays a role in mitochondrial biogenesis and is therefore important when comparing methylation patterns between control and MS brains. The immunoprecipitation was done on five samples [two controls and three MS] with an antibody to MeCP2. Shown above are the 2000 base pairs of the promoter region of the PGC1A gene. In bold and underlined are the two primers selected for PCR. The PCR product was 145 base pairs. Highlighted in yellow are the C-G regions which are prone to methylation by DNA methyltransferases. Also shown is a graph for the PCR products of the PGC1A primer for all the five samples under consideration. PCR products for immunoprecipitations using the MeCP2 antibody are shown as a percent of the PCR products of Input DNA. IgG is the negative control. Results are the average percentile results from 3 runs of PCR.
Figure 28: Levels of methylation in the ATP5G3 promoter region by ChIP
Figure 28: Levels of methylation in the ATP5G3 promoter region by ChIP

Shown above are the results of the PCR with ATP5G3 primer. ATP5G3 is a subunit of the mitochondrial ATP synthase enzyme which catalyzes ATP synthesis. The immunoprecipitation was done on five samples [two controls and three MS] with an antibody to MeCP2. Shown above are the 500 base pairs of the promoter region of the ATP5G3 gene. In bold and underlined are the two primers selected for PCR. The PCR product was 108 base pairs. Highlighted in yellow are the C-G regions which are prone to methylation by DNA methyltransferases. Also shown is a graph for the PCR products of the ATP5G3 primer for all the five samples under consideration. PCR products for immunoprecipitations using the MeCP2 antibody are shown as a percent of the PCR products of Input DNA. IgG is the negative control. Results are the average percentile results from 3 runs of PCR.
Figure 29: Levels of methylation in the ATP5J promoter region by ChIP
Figure 29: Levels of methylation in the ATP5J promoter region by ChIP

Shown above are the results of the PCR with ATP5J primer. The immunoprecipitation was done on five samples [two controls and three MS] with an antibody to MeCP2. The ATP5J gene encodes for the F6 subunit of the membrane spanning F0 complex of ATP synthase. Shown above are the 500 base pairs of the promoter region of the ATP5J gene. In bold and underlined are the two primers selected for PCR. The PCR product was 265 base pairs. Highlighted in yellow are the C-G regions which are prone to methylation by DNA methyltransferases. Also shown is a graph for the PCR products of the ATP5J primer for all the five samples under consideration. PCR products for immunoprecipitations using the MeCP2 antibody are shown as a percent of the PCR products of Input DNA. IgG is the negative control. Results are the average percentile results from 3 runs of PCR.
Figure 30: Levels of methylation in the ATP5I promoter region by ChIP
Figure 30: Levels of methylation in the ATP5I promoter region by ChIP

Shown above are the results of the PCR with ATP5I primer. The ATP5I gene encodes for the ‘e’ subunit in the membrane spanning FO complex of the ATP synthase enzyme. The immunoprecipitation was done on five samples [two controls and three MS] with the MeCP2 antibody. Shown above are the 500 base pairs of the promoter region of the ATP5I gene. In bold and underlined are the two primers selected for PCR. The PCR product was 186 base pairs. Highlighted in yellow are the C-G regions which are prone to methylation by DNA methyltransferases. Also shown is a graph for the PCR products of the ATP5I primer for all the five samples under consideration. PCR products for immunoprecipitations using the MeCP2 antibody are shown as a percent of the PCR products of Input DNA. IgG is the negative control. Results are the average percentile results from 3 runs of PCR.
Figure 31: Quantification of methylation in Control and MS
Figure 31: Quantification of methylation in Control and MS

Shown above is the average pooled fold change seen in the C and MS samples after PCR for all the primers used in this study. PCR was done for 40 cycles. All the primers were run at least three times with all the samples in this study. Three MS and two controls were used. For the graph above, data for controls and MS from all the three runs per primer was pooled together [for control and MS, respectively]. Results are expressed as a pooled percent of immunoprecipitated DNA compared to input DNA for Control and MS samples. A T-Test for significance [p<0.05] was done comparing percent of immunoprecipitated DNA for pooled MS samples with percent of immunoprecipitated DNA for controls. Error bars represent the Standard Error of the Mean [SEM]. A statistically significant change over controls is denoted in the graph by an * on top of the error bars. There is a statistically significant increase in methylation in MS in the regions studied by PCR in the genes encoding for GAD67 and ATP5I. No significant change in methylation is seen in GAPDH, PGC1A, ATP5G3 and the ATP5J genes in this study.
Of the two controls used in the study, C 11 was suffering from cancer and undergoing extensive chemotherapy. It is possible that the chemotherapy may have altered the methylation patterns for some of the genes studied in that control. In this regard it would be necessary to include more samples in this ongoing study to enhance the scientific validity of this study and to enable a more robust statistical analysis. This data should be regarded as preliminary data in the light of the information given above. In the pooled comparisons between the MS and control samples there was no significant change observed in the expression of GAPDH, as would be expected. GAD67, the positive control for the experiment, demonstrated a statistically significant increase in methylation in MS over and above that of the Controls. In the individual comparisons for the methylation status of GAPDH and PGC1A between control and MS samples, a large increase in methylation was observed in C 11. The reasons for this hyper methylation are unclear at this time. When the control and MS data for PGC1A was pooled, no significant change in the methylation status of the MS was seen. Individual comparisons for the methylation status of ATP5G3 show an increase in methylation in MS, however this is not significant. Statistical analysis of the pooled data for ATP5G3 also does not show any significant difference in the methylation status in MS as compared to the controls. Individual comparisons show that the control sample C 11 is the most methylated between all the samples for the ATP5J gene while C 10 is the least methylated. The MS samples are consistently in between these two. No significant change in the overall methylation status of the MS samples over controls was seen in the ATP5J gene. There is a higher degree of methylation in MS seen in the individual
comparisons for the ATP5I genes. As would be expected, analysis of the pooled data also reveals a significant hyper methylation in the ATP5I gene in MS as compared to the controls.

5.4 Conclusions

For this preliminary study, I chose three MS and two control samples for analysis. However, one of the two controls demonstrated a strange pattern of excessive hyper methylation for the GAPDH, PGC1A and the ATP5J genes, the reasons for which are still unclear. More samples, especially more controls, will have to be included in the study in the future to let us better understand the comparable methylation patterns for all the genes under consideration between control and MS. This would also give us a better understanding of the predicted methylation patterns in controls for the genes under consideration, which in turn would let us conclude with more certainty if the methylation patterns seen in C 11 are aberrant or the norm. Based on the data generated by this study, since I have studied only two controls with differing methylation patterns, it is difficult to say with any degree of precision if one or the other demonstrates an aberrant pattern of methylation. It is interesting to note that GAD67 is significantly methylated in MS which leads credence to the possibility of a decrease in GABAergic influence on the brain, thereby enhancing the excitotoxic effect of Glutamate. Also an increase in methylation is seen in ATP5I methylation which would contribute to imbalancing the
energy homeostasis in the cell. However, since I do not have adequate number of stably methylated controls, this data should be considered preliminary at this stage.
Chapter 6

6.1 Discussion

The main conclusions of my work are, first, transcripts for ETC genes are decreased more globally not only in the motor cortex but also in the frontal and parietal cortex in MS. Secondly, I confirmed that the COX5b subunit of Complex IV was decreased at the protein level in MS as well as other proteins involved in respiration founds to be altered in MS in a collaborative proteomic study (Broadwater and others 2011). I also found that the EAE mouse does not model the respiratory deficits observed in MS. I showed that oxidative damage to NRF-2 transcription factor and aberrant methylation of the promoter of PGC1A transcription factor or to the promoter regions of electron transport chain subunit genes themselves may be involved in their decreased transcription reported in MS. The major cause of irreversible disability in MS is due to progressive damage to neurons and axons. Previous studies have implicated mitochondrial dysfunction as playing a role in this pathology. NAA is present in high
concentrations in neurons (Clark 1998; Moffett and others 1991; Simmons, Frondoza, Coyle 1991) and is produced by neuronal mitochondria (Patel and Clark 1979). The levels of NAA are decreased by inhibition of the ETC (Bates and others 1996) suggesting that decreased NAA in the MS brain may be not only indicating a loss of axonal integrity but may also be explained in part by dysfunctional mitochondria. This hypothesis is supported by studies which have shown that decreases in NAA are reversible (Khan and others 2005) and precede neuronal atrophy indicating that dysfunctional mitochondria may precede neurodegeneration (Cader and others 2007; Ge and others 2004). A role for dysfunctional mitochondria in MS pathology is also supported by what is known about the molecular mechanisms involved in axonal and neuronal pathology in MS. These mechanisms are similar to events which occur during ischemia including a loss of ion homeostasis in axons which activates a Ca$^{++}$ dependent cascade and deleterious enzymes which injure axons (Micu and others 2006; Stys 2004; Stys 2005).

It has been previously shown by microarray analysis that the expression of twenty six nuclear encoded electron transport chain subunit genes was decreased in MS cortex and this decrease occurred in neurons (Dutta and others 2006; McDonough and others 2003). These changes led to functional alterations in metabolism as indicated by significant reductions in the activities of Complex I and Complex III of the electron transport chain in MS cortex compared to control cortex by 61% and 40% respectively. In this study I have extended my analysis of mitochondrial electron transport chain gene expression and have analyzed other areas of the cortex to see if the changes observed in motor cortex are
unique or occur in other areas of the brain. I have found that in fact there is also a statistically significant reduction in expression of transcripts for subunits of nuclear encoded electron transport chain genes in the parietal and frontal cortex in MS. I found that the promoters of these subunit genes share a common regulatory element, NRF-2 binding sites, suggesting that alterations to NRF-2 may explain the down regulation of these genes. While there was no change in the expression of the NRF-2 transcription factor in MS cortex, the ability of endogenous NRF-2 to bind a consensus NRF-2 transcription factor binding site was decreased in nuclear extracts isolated from MS cortex as compared to controls. Further the decrease in binding was reversible by increasing the amount of the reducing agent DTT, indicating that oxidative modifications to NRF-2 were inhibiting binding in MS samples. A decrease in the binding capacity of NRF-2 to the promoter region of the electron transport chain genes would lead to a decrease in transcription and downstream production of protein subunits for the electron transport chain complexes. The two Complex IV subunit genes analyzed were significantly reduced in the parietal cortex but not in the frontal cortex. This result could be due to either a regional difference in the levels of oxidative damage to NRF-2, or compensatory regulation by another transcription factor. Many mitochondrial genes, including some electron transport chain genes and all ten Complex IV subunit genes, have been shown to be regulated by NRF-1 and NRF-2 (Dhar, Ongwijitwat, Wong-Riley 2008; Kelly and Scarpulla 2004; Ongwijitwat and Wong-Riley 2005; Scarpulla 1997; Virbasius, Virbasius, Scarpulla 1993). While both NRF-1 and NRF-2 may be important for the regulation of mitochondrial gene expression, they may regulate expression in
response to different physiological signals. My data and that of others (Chinenov and others 1998; Martin and others 1996) suggest that NRF-2 is a redox sensitive regulator of electron transport chain gene transcription while other studies show that the regulation of transcription by NRF-1 is responsive to growth signals such as increased serum, which cause phosphorylation of NRF-1 and upregulation of gene expression (Herzig, Scacco, Scarpulla 2000). Cytochrome c has been reported to be regulated by NRF-1 as opposed to NRF-2 (Evans and Scarpulla 1989). I did not identify NRF-2 sites in the promoter region of cytochrome c, nor was this transcript decreased in MS. These data point to NRF-2 and not NRF-1 as being involved in the decrease in electron transport chain gene expression observed in the MS samples.

I attempted to test the SH-SY5Y cell line as a model for studying the effects of NRF-2 down regulation on its downstream transcripts due to the fact that they can be subjected to different treatments such as oxidative stress, reducing agents etc. MS samples had shown a reversal of oxidation-induced decrease in NRF-2 binding after treatment with a reducing agent and I was interested in studying it further. Initially, the SH-SY5Y cells reacted to the 2-day peroxide treatment by upregulating the binding of NRF-2. Not surprisingly, a short and therefore relatively mild exposure to oxidative stress should increase the binding of NRF-2 and consequently increase the production of its downstream transcripts, some of which are for potent antioxidant enzymes. This would be the physiological protective response against an acute oxidative stress by a living cell and was not unexpected. It has been documented before that a mild oxidative stress does increase mitochondrial biogenesis via the stimulation of PGC-1α, which
stimulates the NRF-1 and NRF-2 pathway, among others (Lee and Wei 2005). An acute increase in demand for and production of ATP will generate more ROS [which is expected to mimic the acute peroxide treatment], which will stimulate NRF-2 to generate more downstream transcripts, some of which are for antioxidant enzymes. Within physiological limits, the increased ROS is adequately neutralized by antioxidant enzymes such as mitochondrial manganese superoxide dismutase [Mn-SOD], cytosolic copper/zinc superoxide dismutase [Cu/Zn-SOD], glutathione peroxidases (GPx), and catalase. Mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD convert superoxide anions to \( \text{H}_2\text{O}_2 \). This \( \text{H}_2\text{O}_2 \) can then either be converted to water by GPx or to water and oxygen by catalase (Lee and Wei 2005). When the production of ROS exceeds physiological limits or persists for a longer time [dose and duration dependent], then it affects the binding of NRF-2 negatively and decreases it’s binding. It is a sign of the failure of the intracellular anti-oxidant mechanisms to contain the damaging ROS. Concurrent with this hypothesis, I saw an increase in nitrated proteins; a key hallmark of intracellular oxidative stress, with increasing concentrations of \( \text{H}_2\text{O}_2 \), an oxidative stressor, when human neuroblastoma cells [SH-SY5Y] were cultured and treated with 5, 10 and 15 µM of \( \text{H}_2\text{O}_2 \) [once-a-day treatment] over a 7-day period. I also saw that the binding of NRF-2 increases proportionately with increasing concentrations of \( \text{H}_2\text{O}_2 \) with a 2-day peroxide treatment but decreases rapidly with the same concentration gradient over a 7-day period of treatment. However, since there is a decrease in the expression after oxidative insult in SH-SY5Y cells, they differ from the MS tissue in that respect and could not be used as a
model for studying the impact of oxidative stress on NRF-2 and its downstream transcripts.

Inflammatory cells can induce collateral oxidative damage in the neuronal environment in the form of ROS, which has been shown to reduce mitochondrial enzyme activity and cause damage to mitochondrial DNA in MS lesions (Lu and others 2000; Radi and others 1994; Sarti and others 2003). I have analyzed non lesion areas in MS and my data suggests that there is another mechanism by which ROS can decrease energy metabolism. This is by a transcriptionally mediated mechanism which feeds back to modify NRF-2 and thereby decrease NRF-2 binding when ROS becomes high. I have demonstrated that the ability of endogenous NRF-2 to bind DNA is decreased in MS grey matter in the same blocks of tissue in which electron transport chain gene expression is decreased. This mechanism may normally be protective to maintain a balance between mitochondrial activity and levels of ROS which is mainly a byproduct of mitochondrial respiration. I found an increase in protein nitration in MS grey matter compared to controls suggesting an oxidative environment exists in MS cortex despite the lack of any obvious inflammatory involvement. These data suggest that in MS cortex there may be a global inflammatory environment. Interestingly, a short term increase in the oxidative environment in a cell actually up regulates the expression of NRF-2 and other mitochondrial transcription factors which can increase the number of mitochondria, the expression of mitochondrial genes, and anti oxidative enzymes such as peroxidases and catalases (Gutsaeva and others 2006; St-Pierre and others 2006). While these compensatory mechanisms work well against an acute oxidative event, they do not seem
to be active in the event of prolonged oxidative stress which may occur in a chronic disease such as MS. Increased nitrination of electron transport chain subunits, CKB, and MBP in MS cortex has been detected previously (Broadwater and others 2011). This is indicative of increased ROS and peroxynitrite mediated oxidative damage in MS and is consistent with previous studies (Bizzozero and others 2005; Pandit and others 2009) which reported increased levels of nitrated proteins in MS tissue when compared with controls. Nitration of mitochondrial electron transport chain subunits has also been identified in Alzheimer’s disease [AD] postmortem brain and in the spinal cord in EAE (Qi and others 2006; Sultana, Perluigi, Butterfield 2006). Increased protein nitration suggests an inflammatory mechanism is involved as inflammatory microglial cells express inducible nitric oxide synthase (iNOS) and release nitric oxide (NO) in MS lesions (Colton and Gilbert 1993; Liu and others 2001). This NO can diffuse and react with superoxide formed as a byproduct of mitochondrial respiration and create peroxynitrite which can also diffuse through tissue (Beckman 1991) and may account for the increased protein nitrination seen even in NAGM in MS (Pandit and others 2009). Either direct nitrination of electron transport chain proteins by peroxynitrite or competitive inhibition of electron transport chain Complex IV by NO can lead to mitochondrial dysfunction (Radi and others 1994; Sarti and others 2003).

In addition to showing that the electron transport chain gene transcripts are decreased in MS cortex, I confirmed alterations of protein expression for COX5b and other respiratory proteins in MS. The loss of this subunit may play a role in mitochondrial dysfunction since the nuclear encoded subunits function in the assembly
and maintenance of Complex IV (Galati and others 2009). Indeed, in null yeast mutants of subunit 4, 5a, 5b, 6c and 7a, Complex IV failed to assemble (Capaldi 1990). Additionally, Complex IV dysfunction may impact the production of free radicals; since it has been shown that various cytochrome c oxidase subunits provide protection from highly reactive peroxynitrite (Fontanesi and others 2006). In neurons it has been suggested that CKB may serve to buffer fluctuations in ATP and levels of inorganic phosphate, as well as serve as a shuttle for high energy phosphate bonds between mitochondria and cytoplasm (Andres and others 2008). Also hemoglobin may serve as a storage for oxygen. I confirmed the changes in COX5b, HBB and CKB in the MS brain but these were not modeled in EAE.

The results of the immunoprecipitation studies need more data for further validation. GAPDH is a housekeeping gene and as such is expected to remain active [and hypo methylated]. In keeping with this thought, no significant change in GAPDH methylation was observed in the Immunoprecipitation studies in the pooled data for the MS samples. Previous studies have also shown GAPDH to be hypo methylated in cultured human NT2 cell lines (Kundakovic and others 2009). In the individual comparisons however, I did see an increase in methylation in control sample C11 and it could be effected by aberrations generated in the methylation patterns because of cancer or because of chemotherapy. For eg, studies on human breast cancer and prostate cancer cell lines show that treatment with Trichostatin A [TSA], a Histone Deacetylase Inhibitor [HDACi], modulate the expression of GAPDH (Mogal and Abdulkadir 2006).
GAD67 was taken as the positive control primer for the PCR. It is the key enzyme responsible for the conversion of Glutamate [the main excitatory neurotransmitter in the brain] to GABA which is the main inhibitory neurotransmitter in the brain. Previous studies on human NT2 cell lines have shown that GAD67 is significantly methylated as compared to GAPDH (Kundakovic and others 2009). Further, treatment with either a DNAMT1 inhibitor Doxorubicin [DOXO] or a HDAC1 inhibitor [MS-275] up regulated the expression of GAD67 transcription indicating that the promoter regions for the GAD67 gene are methylated and that epigenetic mechanisms play a role in the expression of GAD67 (Kundakovic and others 2007; Kundakovic and others 2009). GAD67 was seen to be significantly more methylated in MS in my studies. Marked downregulation of GAD67 expression with hypermethylation over and above that normally seen is a hallmark feature seen in post mortem brains of Schizophrenia patients as well (Costa and others 2006). A decreased expression of GAD67 [and therefore GABA] in the MS brain would result in a decrease in its inhibitory effect on neurotransmission. Previous studies show that there is a decrease in transcripts for GABA receptors as well as GAD67 in the MS grey matter (Clements, McDonough, Freeman 2008; Dutta and others 2006). This decrease in GABAergic inhibition might contribute to a possible excitotoxic effect either due to accumulating glutamate not converted to GABA or by accentuating the excitatory effect of normal glutamate concentrations due to a deficiency of GABA. In either case it would contribute to an energetic imbalance, priming the neuron for degeneration.
In the individual comparisons between control and MS in the chromatin immunoprecipitation studies, control sample C 11 shows a manifold increase in PGC1α methylation, which again could possibly be due to chemotherapy, although the exact cause is unclear. However, control sample C 10 is hypo methylated as compared to all the MS samples. When pooled, the control samples show an increase in their methylation status as compared to MS, possibly due to the fact that one of the two controls is strongly hyper methylated. It is difficult to determine at this stage of the study if the methylation of the controls represents a universal increase or not. C 10 is hypo methylated as compared to all the MS samples, while C 11 is hypermethylated manifold. More samples have to be studied to make a definitive conclusion. However, my previous work, in which a significant decrease in the transcripts for the ETC proteins was seen in MS, suggests that there may be an increase in the methylation of PGC1A in MS. PGC1α or PGC1A is a key transcriptional co-activator of NRF-2, which, as we have seen before, plays an important role in the transcription of the ETC proteins. Along with NRF-2, PGC1α also co-activates TFAM which in turn translocates to the mitochondria to induce mitochondrial biogenesis. Thus, while PGC1α itself has no DNA-binding domain, it plays an important role in mitochondrial biogenesis and cellular respiration [OX-PHOS] (Handschin and Spiegelman 2006; Liu and Lin 2011). Expression of PGC1α is upregulated in tissues with a high metabolic rate such as heart, kidneys, skeletal muscles, brain and brown adipose tissue [BAT]. PGC1α is essential for cellular respiration, and studies show that PGC1α knockout animals suffer from neurodegeneration. Repression of PGC1α expression in the MS brain due to possible hyper methylation of its gene in
MS along with a decreased binding of NRF-2 as indicated by my mobility shift assay studies could adversely affect the balance of cellular energetics and predispose the neuron to degeneration. Interestingly, PGC1α is hyper methylated at non-CpG dinucleotides on cytosine residues in the skeletal muscles of type-2 diabetic patients and the degree of methylation is inversely proportional to the levels of PGC1α mRNA transcripts as well as number of mitochondria (Barres and others 2009).

Three genes encoding for subunits of the ATP synthase enzyme [Complex V] were also tested for their methylation status. These were ATP5G3, ATP5J and ATP5I. The ATP5G3 gene was seen to be more methylated in MS but not significantly so. In the individual comparisons between control and MS for ATP5J, control sample C11 was once again observed to be strongly hyper methylated, while control sample C10 was hypo methylated as compared to all the MS samples. In the pooled comparisons for ATP5J, no significant change in methylation is seen. The averaging out of the methylation expression in controls for the ATP5J gene could possibly be masking a potential difference as both the controls again show polar differences in their methylation status. Studying more samples would help resolve this issue. For ATP5I, all the MS samples were hyper methylated as compared to both the controls in the individual comparisons and statistical tests confirmed a significant increase in the methylation status in the MS samples in the pooled comparisons as well.

ATP synthase along with the entire chain of ETC proteins work under a tightly regulated structure to produce ATP while minimizing the production of ROS. Under normal circumstances about 2 to 4% of the electrons transferred via the ETC are lost and
are responsible for generating ROS. Any increase or decrease in the rate of oxidative phosphorylation would increase the rate of ROS formation. An increase in the rate of mitochondrial respiration would increase the rate of ROS formation in proportion, which could possibly overwhelm the functioning capacity of the anti-oxidative enzymes and cause oxidative damage. Deficiencies in the production of the ETC subunits would adversely impact the rate of oxidative phosphorylation. Should the rate of oxidative phosphorylation slowdown, there is a back up of energized electrons which are lost more readily to other targets (Lee and Wei 2005). This would also increase the production of ROS beyond the physiological limit and cause oxidative stress. As in any other physiological process, maintaining a balance between the rate of oxidative phosphorylation to meet the energy demands and the inevitable generation of ROS is necessary in a healthy cell. Deficiencies in the expression of key ETC protein subunits can disturb this delicate balance and cause oxidative damage due to excessive production of ROS. Additionally, deficient production of ATP would contribute to an energetic imbalance in the cell. Studies have shown that a change in the mitochondrial functioning also induces a change in mitochondrial morphology (Kucharczyk and others 2009). For example, oligomerization of the ATP synthase plays an important role in the structural organization of the inner mitochondrial membrane cristae. A dysfunctional mitochondrial respiration would not only generate more ROS, but also generate an energy deficiency in the cell due to a decreased supply of ATP. In addition to that, changes in mitochondrial morphology may also be contributing to the pathological processes by an as yet unknown mechanism.
Mitochondrial dysfunction and neuroinflammation contribute to neuropathology not only in MS (van Horssen and others 2011), but are also common to other complex neurodegenerative diseases such as Alzheimer’s Disease (AD), Parkinson’s Disease (PD) (Di Filippo and others 2010; Parker, Boyson, Parks 1989; Smith and others 1997) and Huntington’s Disease. The expression of mitochondrial electron transport chain proteins has also been shown to be decreased in PD in the brain with Complex I most consistently reported (Hattori and others 1991; Mizuno and others 1989; Schapira 1993). In contrast, in AD, fewer mitochondria but increased cytochrome c oxidase (C IV) and mitochondrial DNA have been observed in neurons (Hirai and others 2001). In AD, reactive oxygen species (ROS) may be a primary event mediating mitochondrial dysfunction whereas in Parkinson’s disease [PD], a defect in mitochondria may mediate increased ROS as mitochondrial toxins can elicit neuronal pathology similar to PD (Betarbet and others 2000; Langston and others 1983). In MS, mitochondrial damage similar to what has been reported in both AD and PD has been observed. Damage to mitochondrial DNA and altered cytochrome c oxidase immunoreactivity similar to what has been observed in AD have been reported in axons in chronic active and inactive lesions (Lu and others 2000; Mahad and others 2009) while grey matter pathology in MS is more consistent with PD, including reductions in electron transport chain subunits and mitochondrial activity (Dutta and others 2006; Pandit and others 2009). In Huntington’s disease [HD] a mutation in the huntingtin gene causes a decrease in the expression of the mitochondrial transcriptional regulator PGC1α. Mutant huntingtin interferes with the transcriptional activation of the PGC-1α promoter (Cui and others 2006; Weydt and others 2006). This
transcriptional cofactor is important for mitochondrial biogenesis and upregulates antioxidant systems (Rohas and others 2007). In Huntington’s disease this decreased expression of the transcriptional coactivator PGC-1α leads to mitochondrial dysfunction and neurodegeneration in striatal neurons.

A decrease in the expression of GAD67 as seen by my ChIP studies could contribute to accumulation of Glutamate. Glutamate excitotoxicity has been related to neuronal dysfunction and chronic neurodegeneration in diseases such as Amyotrophic Lateral Sclerosis [ALS], MS, Parkinson's disease, etc. (Lau and Tymianski 2010). Glutamate excitotoxicity causes neurodegeneration in two ways. Excitation of the NMDA receptors induces an influx of calcium, which leads to prolonged depolarization depleting the intracellular ATP (Gonzalez-Zulueta and others 1998). This causes the mitochondria to generate more ATP and therefore more ROS which in a negative cascade of events damages the mitochondria, depleting the ATP reserves further. An excessive influx of calcium can, of itself, induce apoptosis. All of these mechanisms result in neurodegeneration.

While the underlying mechanisms and events involved in mitochondrial dysfunction differ between these neurodegenerative diseases, the end result of these mitochondrial perturbations is similar resulting in increased ROS, loss of Ca^{2+} homeostasis, and necrotic or apoptotic cell death (Celsi and others 2009). In MS these processes are exacerbated by demyelination which increases energy demand to maintain ion homeostasis through the energy dependent Na^{+}/K^{+} ATPase in neurons (Waxman 2006).
6.2 Global Conclusions and Future Directions

This study has implicated oxidative damage to a mitochondrial transcription factor, NRF-2, as a mechanism contributing to neuropathology. It has demonstrated that the transcriptional repression of the ETC genes seen in previous microarray studies on the human motor cortex are more global in nature. Evidence of an increase in the oxidative stress was seen in MS corresponding with a decrease in the functionality of NRF-2 and this effect was reversible. Future studies are necessary to understand the mechanisms which up regulate the expression of NRF-2 and strengthen antioxidant defense pathways, both of which may be beneficial in MS. The study data also suggest that alterations in protein nitration and MBP expression in MS cortex involve an inflammatory mechanism shared with the EAE mouse model. The EAE mouse model is an autoimmune disease model, in which lesions similar to those observed in MS are observed upon immunization with myelin protein antigens. This model has been helpful in understanding inflammatory disease mechanisms in MS but does not reflect all aspects of MS neuropathology (Croxford, Kurschus, Waisman 2011). MS is both an autoimmune and a neurodegenerative disease, and while protein nitration and altered MBP expressions are consistent in the EAE mouse model and in MS, similar alterations were not observed in the analysis of the expression levels of other proteins involved in respiration including COX5b. These proteins were identified to be differentially expressed between Control
and MS by SELDI and confirmed by Western blots, but not seen to be changed in EAE. Thus there are components of MS pathology different from those of EAE indicating that a separate mechanism is also involved in MS pathology. Since one of the two controls in the IP studies had received chemotherapy, the results are less conclusive than desired and more samples should be included in the study. My studies till date indicate a significant increase in the methylation of the GAD67 and the ATP5I genes in MS and they both can potentially contribute to an imbalance in the energy homeostasis in MS if repressed. This study can be extended to include hybridization of the immunoprecipitated product on a whole genome microarray to reveal difference in gene expression, which would give an indication of the extent of methylation the genes of interest have. These results could be confirmed with PCR of mRNA transcripts of those genes or by identifying differential protein expression on Western blots. Bisulfite sequencing of the genes of interest can also reveal the true extent of their methylation. My data examined non-lesion grey matter and has confirmed conclusions from previous studies which found decreased expression of mitochondrial electron transport chain subunit genes in MS (Dutta and others 2006; Pandit and others 2009) and has extended these findings and implicated an imbalance in the regulation of additional respiratory proteins in MS disease pathology. The changes seen in hemoglobin expression in MS, if further investigated, could add a new dimension to MS pathology and future targets for therapy. These findings have important implications for the development of new neuroprotective therapies for MS.
7. References


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