Investigating the Role of Nuclear Pore Complex and Nucleocytoplasmic Transport Alterations in Multiple Sclerosis and Related Neurodegenerative Diseases

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In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Anatomy, Physiology, and Pharmacology
University of Saskatchewan
Saskatoon

By

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Abstract

Multiple sclerosis (MS), classically known as an autoimmune, demyelinating disease of the central nervous system, has a significant neurodegenerative component which underlies permanent disability in MS patients. Current MS treatments are immunomodulatory and can decrease relapses, but no treatments target neurodegeneration, which drives disease progression. Dysfunctional RNA binding proteins (RBPs) are a common mechanism of many neurodegenerative diseases, including MS. The nuclear pore complex (NPC) and nucleocytoplasmic transport are critical to the proper functioning of cells, but are altered in neurodegenerative diseases, including Huntington’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease. Previous RNA sequencing data from our lab identified differentially expressed genes related to the NPC and nucleocytoplasmic transport in cells with knockdown of an RBP. The function of the NPC and nucleocytoplasmic transport has yet to be studied in MS.

I hypothesized that the NPC is perturbed in MS and a related model of RBP dysfunction, affecting normal nucleocytoplastic transport, potentially contributing to neurodegeneration. Knockdown of the RBP heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in Neuro2a cells (a neuronal-like cell line), a model of RBP dysfunction, significantly affected the structure of the nuclear envelope. Next, I generated a novel method to classify Lamin B staining patterns of the nuclear envelope to improve analyses and reduce bias. Using this method, I replicated the finding that hnRNP A1 loss alters the nuclear envelope. The structure of the NPC itself was significantly affected and the expression of two NPC proteins was decreased. Then, using a previous RNA sequencing dataset of hnRNP A1 knockdown, I performed gene ontology analysis and found alterations in pathways related to the NPC and nucleocytoplasmic transport, providing support to the role of the NPC and nucleocytoplasmic transport play in the pathogenesis of MS. The nuclear envelope remained intact, but active nucleocytoplasmic transport was perturbed, leading to mislocalization of a protein undergoing continuous transport through the NPC. I then validated my findings in human tissues and determined that Lamin B staining was significantly altered in MS.

These results demonstrate that dysfunctional RBPs can affect the nuclear envelope and the NPC. Perturbations in nucleocytoplastic transport can lead to further protein mislocalization, exacerbating RBP dysfunction and nuclear RNA accumulation thereby affecting protein synthesis. Dysfunctional nucleocytoplasmic transport, which can lead to neuronal cell death, is a novel mechanism implicated in the pathogenesis of MS.
Acknowledgements

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A special thanks goes to my parents, who encouraged me to apply myself and pursue university education. This master’s thesis would not have occurred without their motivation and support.

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# Table of Contents

Permission to Use.........................................................................................i
Abstract...........................................................................................................ii
Acknowledgements........................................................................................iii
Table of Contents............................................................................................iv
List of Tables.....................................................................................................vii
List of Figures...................................................................................................viii
List of Abbreviations........................................................................................x

1.1 Multiple Sclerosis.....................................................................................1
  1.1.1 History of Multiple Sclerosis.............................................................1
  1.1.2 Current Multiple Sclerosis Diagnosis and Subtypes.......................1
  1.1.3 Initiation and Possible Causes.........................................................3
  1.1.4 Immunopathology and Pathogenesis.............................................5
  1.1.5 Axonal and Neuronal Damage.......................................................6

1.2 RNA Binding Proteins............................................................................7
  1.2.1 Common RNA Binding Protein Structure......................................7
  1.2.2 RNA Binding Protein Function......................................................8
  1.2.3 Heterogeneous Nuclear Ribonucleoprotein A1..............................9

1.3 Nuclear Envelope and Nuclear Pore Complex......................................10
  1.3.1 Structure of the Nuclear Envelope and Nuclear Pore Complex.......10
  1.3.2 Function of the Nuclear Pore Complex: Nucleocytoplasmic Transport...14
  1.3.3 Nuclear Pore Complex Damage....................................................16

1.4 RNA Binding Protein and Nuclear Pore Complex Dysfunction Consequences...17
  1.4.1 Functional Consequences of RNA Binding Protein Dysfunction......17
  1.4.2 Changes in the Nuclear Pore Complex Alters Nucleocytoplasmic Transport..........................................................18
  1.4.3. Altered Nucleocytoplasmic Transport Affects RNA and Protein Localization..................................................19

1.5 RNA Binding Proteins and the Nuclear Pore Complex in Neurodegeneration...20
  1.5.1 Huntington's Disease (HD)...........................................................20

CHAPTER 1: Introduction..............................................................................1
1.5.2 Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) ..........................................................20
1.5.3 Multiple Sclerosis (MS) ..........................................................21

1.6 Hypothesis and Specific Aims ....................................................22
1.6.1 Explore Potential Links Between hnRNP A1, Lamin B, and NPC Structure. 22
1.6.2 Investigate the NPC Function Through Assessing Active Nucleocytoplasmic Transport .........................................................23
1.6.3 Establish a Link Between Multiple Sclerosis and Nuclear and NPC Structure .........................................................23

CHAPTER 2: Methodology .................................................................24
2.1 Cell Culture and Transfection ..................................................24
2.2 siRNA Oligonucleotides and Plasmids .......................................24
2.3 Protein Isolation From Cells ....................................................25
2.4 Western Blotting .................................................................25
2.5 Immunohistochemistry ..........................................................26
2.6 Immunocytochemistry .........................................................27
2.7 Fluorescence Quantification ...................................................27
2.8 Abnormal Staining Quantification ..........................................28
2.9 Three-Dimensional Quantification .........................................29
2.10 Gene Ontology Analysis ......................................................29
2.11 Experimental Design and Statistical Analysis .........................30

CHAPTER 3: Results ....................................................................31
3.1 Lamin B and the Nuclear Pore Complex Structure is Affected by hnRNP A1 Knockdown ..............................................................31
3.1.1 Knockdown of hnRNP A1 Significantly Alters the Nuclear Envelope ......31
3.1.2 Qualitative Phenotypes of Lamin B Staining Can Be Identified and Quantified Using Three-Dimensional Images ..................35
3.1.3 The Novel and Manual Methods of Lamin B Phenotype Identification Are Equivalent .........................................................37
3.1.4 hnRNP A1 Knockdown Significantly Alters the Nuclear Pore Complex Structure .........................................................40
3.2 Structural Changes in the Nuclear Pore Complex Impact Nucleocytoplasmic Transport ........................................................46
3.2.1 hnRNP A1 Knockdown Alters Gene Expression Related to Nucleocytoplasmic Transport……………………………………………………46
3.2.2 hnRNP A1 Knockdown Alter Active Nucleocytoplasmic Transport……..46
3.2.3 Nuclear Envelope Integrity Remains Intact with hnRNP A1 Knockdown….49

3.3 Nuclear Structure Alterations Are Present in MS Tissue Establishing Clinical Relevance………………………………………………………………………..52
3.3.1 Lamin B is Significantly Altered in Neurons From MS as Compared to Healthy Controls……………………………………………………………52

CHAPTER 4: Discussion………………………………………………………………………………55
4.1 Loss of hnRNP A1 Function is Sufficient to Perturb the NPC and Nuclear Envelope…………………………………………………………………………………………55
4.2 Active Nucleocytoplasmic Transport is Altered Affecting Protein Localization……..58
4.3 Changes in the Nuclear Envelope Are Recapitulated in Multiple Sclerosis…………..61

CHAPTER 5: Conclusion and Future Directions……………………………………………………63
CHAPTER 6: References…………………………………………………………………………67
APPENDIX A……………………………………………………………………………………78
List of Tables

Table 1.1. Location of NPC Proteins.................................................................12

Table 3.1. Summary of postmortem tissue characteristics.................................52

Table A.1. Select Differentially expressed genes identified by RNA sequencing in cells with hnRNP A1 knockdown.................................................................78
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Schematic overview of the nuclear pore complex</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Nucleocytoplasmic transport cycles</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Summary of the normal structural processes present in healthy neurons related to this thesis</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>siA1 treatment significantly decreased hnRNP A1 expression compared to siNEG treatment in cells labelled with Lamin B</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Cells treated with siA1 demonstrate abnormal Lamin B staining</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>3D quantification and method of automatic Lamin B phenotyping of siNEG and siA1 treated cells</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Quantification of the manual and computer-based phenotyping approaches</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Contingency tables and graphs reveal no difference between the automatic and manual Lamin B phenotyping methods</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Abnormal phenotypes of Nup98 staining significantly increased with siA1 treatment</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>RanGAP1 staining phenotypes showed a significant increase in abnormal phenotypes with hnRNP A1 knockdown</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>The prevalence of abnormal POM121 significantly increased with siA1 treatment</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>The prevalence of abnormal Nup214 phenotypes was the same between siNEG and siA1 treated cells</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>Nup98 and RanGAP1 protein levels were significantly decreased with hnRNP A1 knockdown</td>
</tr>
<tr>
<td>Figure 3.11</td>
<td>hnRNP A1 knockdown in cells altered mRNA transcripts related to pathways of nucleocytoplasmic transport and nuclear structure</td>
</tr>
<tr>
<td>Figure 3.12</td>
<td>hnRNP A1 knockdown leads to deficits in active nucleocytoplasmic transport</td>
</tr>
<tr>
<td>Figure 3.13</td>
<td>The nuclear envelope is not compromised by siA1 treatment</td>
</tr>
<tr>
<td>Figure 3.14</td>
<td>Lamin B was significantly altered in postmortem MS tissue compared to controls</td>
</tr>
</tbody>
</table>
Figure 5.1. Summary of the main conclusions of the thesis ...............................66
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>CIS</td>
<td>Clinically isolated syndrome</td>
</tr>
<tr>
<td>CLIPseq</td>
<td>Cross linking and immunoprecipitation followed by RNA sequencing</td>
</tr>
<tr>
<td>CLIPdb</td>
<td>CLIP database</td>
</tr>
<tr>
<td>cNLS</td>
<td>Classical nuclear localization signal</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>FG</td>
<td>Phenylalanine glycine</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
</tr>
<tr>
<td>hnRNP A1</td>
<td>Heterogeneous nuclear ribonucleoprotein A1</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>ncNLS</td>
<td>Non-classical nuclear localization signal</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>Nlp</td>
<td>Nucleoporin-like protein</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear import signal</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>NTF2</td>
<td>Nuclear transport factor 2</td>
</tr>
<tr>
<td>Nup</td>
<td>Nucleoporin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NXF1</td>
<td>Nuclear RNA export factor 1</td>
</tr>
<tr>
<td>PPMS</td>
<td>Primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>Rae</td>
<td>Ribonucleic acid export</td>
</tr>
<tr>
<td>RanBP2</td>
<td>Ran binding protein 2</td>
</tr>
<tr>
<td>RanGAP1</td>
<td>Ran GTPase-activating protein 1</td>
</tr>
<tr>
<td>RanGEF</td>
<td>Ran guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA binding protein</td>
</tr>
<tr>
<td>RIS</td>
<td>Radiologically isolated syndrome</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing remitting multiple sclerosis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SPMS</td>
<td>Secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>S-tdTomato</td>
<td>Shuttle-tdTomato</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA binding protein 43 kilodaltons</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T cell intracellular antigen 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Tpr</td>
<td>Translocated promotor region</td>
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This thesis contains results from the manuscript by Stang TE, Salapa HE, Clarke, J-P WE, Popescu BF, and Levin MC, as it addresses the main aims of the thesis. It has been reformatted to fit within the thesis. TES assisted with the design of the experiments, performed the research, analyzed the data, created the computer script, and wrote the manuscript with support from HES and MCL. BFP assisted with the human pathology and JPC assisted with the bacterial cloning. This manuscript is currently under review in *Frontiers in Neuroscience*. Once published, it will be an open access article distributed under the Creative Commons Attribution License (CC BY), in which it is not necessary to obtain permission to reuse content, given that full acknowledgement is made.

CHAPTER 1: Introduction

1.1 Multiple Sclerosis

1.1.1 History of Multiple Sclerosis

Multiple Sclerosis (MS) was well characterized in the 19th century, but the disease dates much further back in history. The first documented case of MS may have affected Saint Lidwina of Schiedam sometime between 1396 and 1399 after breaking a rib during a fall and developing facial paralysis, blindness, and sensibility disturbances, which developed slowly over 37 years\(^1,2\). A better described and more convincing historical case is that of Sir Augustus D’Este, grandson of a United Kingdom king, King George III, between 1822 and 1848, where he chronicled his symptoms until his death\(^1,3\). These cases demonstrated that MS is indeed a historical disease that existed before it was clinically characterized.

Jean-Martin Charcot is often credited with describing MS in the 1860s, but Friedrich Theodor von Frerichs described and diagnosed a patient in 1849\(^1,3,4\). Charcot did make the disease more well-known and described three symptoms: nystagmus, intention tremor, and scanning speech to diagnose the disease, which became known as Charcot’s triad\(^1,3,4\). Due to Charcot’s detailed characterization and inclusion of microscopic and postmortem observations, his diagnostic criteria and descriptions were upheld for a long time.

1.1.2 Current Multiple Sclerosis Diagnosis and Subtypes

Currently, MS is diagnosed based on the 2017 revisions of the McDonald criteria for the diagnoses of MS\(^5\). The criteria states that there must be at least one clinical attack and at least one MRI lesion with cerebrospinal fluid specific oligoclonal bands, one clinical attack with at least two lesions, or at least two clinical attacks with a lesion demonstrating dissemination in space\(^6\).
Practically, MS is often diagnosed through differential diagnosis and ruling out other possible diseases. Symptoms that often lead towards a possibility of MS include fatigue, paresthesias (numbness and tingling), pain, decreased cognition, muscle spasms, stiffness and weakness, vision problems and Lhermitte’s sign, which is a shock-like sensation down the back when the neck is flexed. Another indicator of MS is Uhthoff’s sign, which is a temporary worsening of symptoms in response to increased heat. There are a variety of symptoms due to differences in the brain and spinal cord areas that are affected. Patients will not experience all symptoms, and each patient will have different symptoms, complicating the diagnosis of MS.

There are two types of clinical syndromes that put patients at high risk for developing MS prior to a formal MS diagnosis. They are clinically isolated syndrome (CIS) and radiologically isolated syndrome (RIS). RIS occurs when a patient receives an MRI for a reason unrelated to MS symptoms, and multiple lesions consistent with MS are present in the brain or spinal cord. This fulfills the McDonald criteria for dissemination in space [at least two different areas of the central nervous system (CNS) have lesions], however MS is not formally diagnosed because of a lack of clinical symptoms. The risk of RIS converting to MS depends on the location of the MS lesion, with spinal cord lesions significantly increasing the risk of developing MS. However, in most cases, approximately only 10% of people with RIS will develop MS; therefore, RIS is usually not treated with MS disease modifying therapies due to the low risk of developing MS and the side effects of medications. CIS is defined as a single episode of clinical symptoms indicative of a focal or multifocal inflammatory demyelinating event in the CNS consistent with an MS relapse, lasting at least 24 hours, and in the absence of a fever or infection, but in a patient not known to have MS. The risk of developing MS with CIS is greater than with RIS, but the 2017 revisions allowed some previously diagnosed CIS patients to reach the threshold for MS diagnosis and decreased the time of CIS patients to develop MS with CIS due to the expanded diagnostic criteria. Patients with CIS and RIS are typically monitored more closely due to the increased risk of developing one of the main subtypes of MS.

MS affects more than 2.8 million people worldwide, with its prevalence increasing in recent years. In younger adults, MS usually presents as relapsing-remitting MS (RRMS) and accounts for approximately 85% of initial diagnoses. RRMS is characterized by distinct attacks of new or recurrent neurological symptoms with periods of complete or incomplete recovery between attacks. Generally, recovery is complete earlier in the disease and the time between
attacks is greater. RRMS affects females at a ratio between 2-3 to 1 male and is diagnosed at approximately 30 years of age\textsuperscript{19–21}. Most MS medications are approved for RRMS and can reduce the number of relapses, increase the time between relapses, and delay disease progression. However, as the disease progresses, permanent disability in MS patients also increases, which is often independent of relapses. RRMS eventually progresses into another category of MS called secondary progressive MS (SPMS). SPMS usually occurs around 10 to 15 years after the initial diagnosis and is characterized as an increasing severity of disability with or without overlapping relapses\textsuperscript{22}. The last main type of MS is called primary progressive MS (PPMS), and it accounts for the remaining 15% of initial diagnoses. PPMS is distinguished by the continuous development of unremitting neurological dysfunction from disease onset\textsuperscript{22,23}. The sex ratio of females to males is closer to 1:1, and the age of onset is approximately 10 years later than RRMS\textsuperscript{19,21,24,25}. The age where continuous progression occurs is similar in PPMS and SPMS. MS disease modifying therapies, which are highly effective in treating RRMS, are significantly less effective in the two progressive forms of MS, and progressive MS patients have a poorer prognosis compared to RRMS.

1.1.3 Initiation and Possible Causes

There are two main hypotheses that describe the mechanism of MS initiation: the ‘outside-in’ and the ‘inside-out’ hypotheses. The outside-in hypothesis proposes that the disease begins in the periphery through autoimmune inflammation and later gains access to the CNS, where myelin degradation and axonal damage occur. This pathway suggests that autoimmunity initiates the disease, and demyelination and neurodegeneration occur secondarily. The inside-out hypothesis suggests that MS begins in the CNS with myelin or axonal damage, which causes an inflammatory response, leading to autoimmunity. In this latter model, neurodegeneration is primary, and autoimmunity is secondary. Both models include neurodegeneration and autoimmunity, but the argument is which comes first and initiates the disease. While there is evidence to support both theories, it is possible that both pathways can initiate MS in different patients and that the two models are not mutually exclusive\textsuperscript{26}.

While there are theories about the initiation of MS, the cause is unknown. However, there is research supporting the involvement of different genetic and environmental factors. Genetically, MS can cluster in families compared to the general population, and an individual’s risk of MS increases if a family member has the disease\textsuperscript{27,28}. Further support for the genetic involvement in
MS, is a Canadian study, which found that the concordance of MS was 25% in monozygotic twins but only 5% for dizygotic twins and 3% for nontwin siblings. Genome wide association studies (GWAS) have identified more than 200 genes involved in the risk of developing MS. Many of the genetic factors that can increase the risk of developing MS are related to the immune system, specifically in T-cell, B cell, and major histocompatibility complex (MHC) genes. While genetics are involved in MS risk, other factors play a more prominent role.

Environmental factors play a significant role in MS. Obesity increases the risk of developing pediatric MS, and adolescent obesity increases the general risk of MS. Interestingly, childhood obesity does not increase the risk of developing MS as an adult. In addition to increasing the risk of developing the disease, obesity is associated with greater disease severity and poorer outcomes. Possible explanations of obesity associated with MS include nutrition, the gut microbiome, adipokines, and inflammation. Smoking is another risk factor for MS, with smokers having a relative risk of 1.5 compared to nonsmokers. Obesity and tobacco use are more prevalent in more developed countries, which may partially explain the higher incidence of MS in countries further from the equator. Canada specifically has one of the highest rates of MS worldwide. A more robust explanation for this phenomenon is the vitamin D hypothesis, as there is less sunlight exposure and lower serum vitamin D levels in people living farther from the equator. Low vitamin D levels are overrepresented in people with MS compared to the general population. Greater exposure to sunlight in childhood and adolescence is associated with a decreased risk of developing MS. Higher vitamin D levels predict reduced MS activity and slower progression compared with MS patients who have low vitamin D. Vitamin D regulates the immune system, can directly impact the CNS, and act as a neuroprotective agent. While vitamin D is related to the risk of developing MS, multiple clinical trials have shown that vitamin D supplementation does not decrease MS progression or severity. It is likely that vitamin D is not disease-modifying but may be involved in the initial risk and initiation of the disease, and supplementation in people with MS is too late to be beneficial.

Epstein-Barr virus (EBV) is also associated with MS, and it is the strongest known risk factor. In the 1970s and 80s, an association between EBV and MS was first discovered. However, with over 90% of adults having been infected with EBV, research on EBV in MS was challenging and progressed slowly. A recent, seminal study utilizing serum samples obtained through the United States Department of Defense was the strongest and largest study conducted related to the
potential for EBV infection to cause MS\textsuperscript{52}. In the study, the authors found only one case of MS out of 801 cases where an individual was EBV negative\textsuperscript{52}. The other main finding was a 32-fold increased risk of MS in people initially EBV negative but seroconverted during the study, demonstrating that initial EBV infection in young adults significantly increases risk\textsuperscript{52}. Together, these findings provide strong evidence of EBV as a leading cause of MS. EBV infects B cells and can alter the immune system, however molecular mimicry between EBV antigens and CNS autoantigens is thought to be the predominant mechanism of MS initiation\textsuperscript{53,54}.

1.1.4 Immunopathology and Pathogenesis

While the innate immune system plays a previously under-recognized role in MS, the adaptive immune system plays a more significant role. The immune system in the CNS is also crucial. Microglia, CNS-resident phagocytes, play a role in both the innate and adaptive immune systems. Under homeostatic conditions, microglia protect the CNS from foreign attack and infection. However, in MS, microglia become ‘activated’ and exhibit a damaging phenotype contributing to inflammation and cellular degeneration\textsuperscript{55}. Microglia secrete inflammatory cytokines in MS and can produce free radicals, increasing the immune system’s potency leading to demyelination\textsuperscript{56,57}. Microglia can also present antigens to other cells, including T-cells, to generate autoimmunity in the adaptive immune system\textsuperscript{58}. MS was historically considered a T-cell mediated disease as many distinct T-cell populations are present in the CNS of MS patients. CD4\textsuperscript+ T\textsubscript{H}1 and T\textsubscript{H}17 cells, which both produce interferon-gamma (IFN\textgreek{g}) and tumour necrosis factor-alpha (TNF\textalpha), two inflammatory cytokines, act on microglia and astrocytes to promote further inflammation and damage\textsuperscript{59}. They can also act directly on neurons and lead to axonal damage\textsuperscript{60}. CD8\textsuperscript+ T-cells have been found in the CNS and cerebrospinal fluid of RRMS patients early in disease, demonstrating the importance of these cells in MS pathogenesis\textsuperscript{61,62}. B-cells also play a recently discovered, significant role in MS, as demonstrated by the efficacy of B-cell depleting therapies in relapsing and early progressive forms of MS. Together, this autoimmunity leads to neuronal and myelin damage.

Oligodendrocytes wrap around axons to myelinate them, increasing nerve conduction speed, allowing neural signalling, and supporting axons. In MS, the myelin sheath becomes damaged, and oligodendrocytes die through immune-independent and immune-mediated mechanisms\textsuperscript{63}. Areas of demyelination, known as ‘MS plaques’ can be visualized as lesions on
MRIs and postmortem tissue. MS plaques occur throughout the MS disease course and are responsible for the transient disability, which occurs in RRMS, as oligodendrocytes and oligodendrocyte precursor cells can remyelinate some areas with damaged myelin. While demyelination is a significant component of MS, permanent disability correlates poorly with demyelination, but more strongly with neurodegeneration (axonal and neuronal damage).

1.1.5 Axonal and Neuronal Damage

There is yet to be an effective treatment for progressive forms of MS, which is caused by neuronal damage and death and can be independent of demyelination. While neuronal damage and death occur throughout MS, the brain can compensate for loss early in the disease and during RRMS, when permanent disability is minor in MS patients. However, in later stages and progressive forms of MS, neuronal reserves are depleted, and disability accumulates more rapidly than in the initial stages of the disease. The discovery of the importance of grey matter pathology and axonal damage in MS has only occurred in the last 25 years and has since lagged behind research on white matter and immune system pathology. However, multiple mechanisms of neuronal damage and death have been discovered in MS.

First, the immune system can act on neurons and cause damage through myelin damage, cytokine secretion, and microglial dysfunction. Second, oxidative stress related to iron deposition may play a role in the pathogenesis of MS as it is increased in the brains of people with MS compared to controls. Iron reacts with hydrogen peroxide in the Fenton reaction and generates reactive oxygen species, which induces oxidative damage. Oxidative damage can lead to neuronal damage through lipid, protein, and DNA oxidation, causing degradation and cell death when damage is severe. Neurons appear to have a limited ability to cope with oxidative stress as mitochondria are vulnerable to reactive oxygen species damage, and neurons have high energy requirements and, therefore, are highly dependent on the proper functioning of mitochondria. Mitochondrial defects are present in MS and play a substantial role in neuronal damage and death. Mitochondrial-induced neuronal damage can also be caused by mitochondrial DNA damage and mitochondrial rupture, which initiates a calcium imbalance and calcium-mediated apoptosis.

Third, abnormal axonal transport may play a role in neurodegeneration in MS. Axonal transport is crucial in neurons to allow synaptic proteins to reach the axon terminal and retrograde transport of feedback messengers and protein degradation. Defective axonal transport appears to
be a common mechanism in multiple neurodegenerative diseases, including Parkinson’s disease, Alzheimer’s disease, Huntington’s disease (HD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS), occurring early in disease where it causes axonal degeneration. In an MS mouse model, axonal transport defects preceded changes in the axons, demonstrating both the presence of axonal transport defects and their impact on axonal degeneration. Axonal transport defects lead to axonal swellings and Wallerian degeneration, the destruction of an axon distal to a lesion in an anterograde manner. This mechanism of neuronal degeneration relates MS to other neurodegenerative diseases where similar processes may be occurring. Finally, RNA binding protein (RBP) dysfunction is another common mechanism of neurodegeneration in CNS diseases, including MS.

1.2 RNA Binding Proteins

1.2.1 Common RNA Binding Protein Structure

RBPs represent hundreds of proteins that bind and interact with RNA, resulting in a dynamic interactome called the ribonucleoprotein complex. These interactions may be transient or long-term depending on the RBP, RNA, and the role of the RBP. The most common domains in RBPs are RNA recognition motifs (RRMs) and zinc finger domains, which both recognize and bind RNA. Some RBPs have multiple RRMs that bind to multiple RNA molecules or support the binding of RNA to the first RRM, depending on the flexibility of linkers between the domains. Other characterized regions of RBPs include low complexity domains, sometimes called prion-like domains, and intrinsically disordered regions, which consist of amino acid repeats enriched for glycine, arginine, lysine, and tyrosine. Intrinsically disordered regions are present in other proteins, but RBPs are highly enriched for these domains compared to the human proteome. These regions do not have a constant structure and are free-form regions that are highly flexible. The enrichment of intrinsically disordered regions in RBPs suggests that these domains are critical to the function of RBPs.

RBPs are predominantly found in either the nucleus or cytoplasm, where location is dependent on RBP structure and function. Some RBPs contain either a nuclear localization signal (NLS), a nuclear export signal (NES), or both. These sequences allow the protein to travel between the nucleus and cytoplasm and to maintain its physiological location. There are several different NLS sequences on RBPs that allow specific control of their transport across the nuclear envelope and localization. One of the two main types of NLSs is the classical NLS, which includes a
monopartite and bipartite category, which are generally four or more positively charged residues and two clusters of 2-3 positively charged residues separated by a linker of approximately 10 amino acids\textsuperscript{88}. The other main type of NLS is the non-classical nuclear localization signal, which includes the proline-tyrosine NLS characterized by 20-30 amino acids with a disordered structure used for nuclear import\textsuperscript{88}. There is one main nuclear export pathway for proteins with the NES sequence approximately 8-15 residues long and includes a high proportion of hydrophobic amino acids\textsuperscript{89}. The NLS and NES transport proteins between the nucleus and cytoplasm and allow RBPs to perform their various functions.

1.2.2 RNA Binding Protein Function

RBPs perform a variety of critical functions in a cell, the most common being stabilizing and splicing mRNA\textsuperscript{84}. For example, RBPs can differentially splice transcripts by either promoting the exclusion or inclusion of exons in the final transcript. Additionally, RBPs can be involved in the processing, modification, localization, and translation of RNA\textsuperscript{90,91}. In RBPs with an NLS, NES, or both, transport between the nucleus and cytoplasm is crucial for their proper function. RBPs with both an NLS and an NES often bind RNA in the nucleus, stabilize it, and transport it across the nuclear envelope into the cytoplasm. It is critical that the RBP returns to the nucleus to maintain normal RNA processing. Thus, the proper import, export, and localization of RBPs are paramount to their proper functioning. However, in some cases, RBPs do not function properly. Such is the case when intrinsically disordered regions and low complexity domains in RBPs lead to misfolding and improper protein-protein interactions, which can cause fibrilization and aggregation. Misfolding can occur because of abnormal post-translational modifications, aberrant protease truncation, and changes towards a $\beta$-sheet structure\textsuperscript{83,92}. The aggregation of RBPs prevents their proper functioning and can negatively impact cell health. Neurons have a complex RNA metabolic system due to the presence of long axons and their importance in signaling, making them more dependent on the processing and stability of RNA and highly susceptible to RBP dysfunction\textsuperscript{83,93}. RBPs involved in neurodegenerative diseases, including ALS, FTD, HD, and MS, are often nuclear in homeostatic conditions but mislocalize to the cytoplasm in disease\textsuperscript{83,94}. Transient cellular stress in these diseases causes the formation of transient cytoplasmic stress granules as a healthy response. RBPs are involved in these stress granules, which can bring stress granules adjacent to RBPs and lead to misfolding and aggregation\textsuperscript{83,95}. Aggregation can lead to subsequent nuclear
depletion of RBPs and mislocalization to the cytoplasm thereby altering RBP functions. This dysfunction can alter RNA metabolism, which negatively impacts cellular health and is a mechanism of neurodegeneration\textsuperscript{82}.

1.2.3 Heterogeneous Nuclear Ribonucleoprotein A1

Heterogeneous nuclear ribonucleoprotein (hnRNP A1) is an RBP found in many tissue types, but it is highly expressed in neurons of the CNS\textsuperscript{96,97}. There are two isoforms of hnRNP A1, where isoform A is 320 amino acids in length and expressed 20 times greater than isoform B, which is 372 amino acids and contains an expanded prion-like domain\textsuperscript{97,98}. Both isoforms have two RRMs near the N terminal, called RRM1 and RRM2, separated by a short linker of 13 amino acids, followed by a glycine-rich prion-like domain on the C terminal. The structure of the two RRMs has been experimentally determined, but the structure of the prion-like domain remains undetermined due to its flexible and disordered nature. There is a proline-tyrosine NLS in hnRNP A1 within the prion-like domain and is referred to as the M9 region (amino acids 268 to 305), which also contains a sequence for nuclear export and, therefore, is responsible for shuttling hnRNP A1 between the nucleus and cytoplasm\textsuperscript{99}. As hnRNP A1 has a non-classical NLS, it binds to Importin \(\beta\)2, also called Karyopherin \(\beta\)2 or Transportin 1, to undergo nuclear import. The export of hnRNP A1 has not been studied as extensively and is still partially unclear, but hnRNP A1 does not need the exportin protein exportin 1 to undergo export\textsuperscript{100}. Instead, different post-translational modifications can modulate the import and export of hnRNP A1\textsuperscript{98}. The primary export mechanism, though, is likely that hnRNP A1 binds RNA in the nucleus, and nuclear RNA export factor 1 (NXF1) exports the bound RNA, resulting in the cytoplasmic localization of hnRNP A1\textsuperscript{101}.

The two RRMs within hnRNP A1 play an essential role in stabilizing the secondary and tertiary structure of hnRNP A1 and are responsible for binding RNA\textsuperscript{102}. While the sequence of the two RRMs is similar, they perform different functions and can bind distinct RNAs\textsuperscript{98,103}. hnRNP A1 binds to the loop on RNA hairpin loop structures and most commonly binds to a UAGG motif\textsuperscript{104,105}. The sequence and structure of RNA are both critical for hnRNP A1 binding. Post-translational modifications can also affect the affinity and binding of hnRNP A1 to its RNA targets\textsuperscript{101}. Once bound, hnRNP A1 can stabilize the RNA, transport it into the cytoplasm, splice the pre-mRNA to mRNA, or carry out a combination of the functions. HnRNP A1 is an essential spliceosome component that contributes to both constitutive and alternative RNA splicing\textsuperscript{98}, where
it can cause exon inclusion or exclusion in different RNAs\textsuperscript{106–109}. HnRNP A1 also plays a role in miRNA biosynthesis and regulation\textsuperscript{97,98}. HnRNP A1 binds to internal ribosomal entry site (IRES) sequences and is known as an IRES-transactivating factor. IRES binding allows hnRNP A1 to control protein translation by influencing cap-independent translation, which is translation dependent on the IRES present in specific mRNAs\textsuperscript{110,111}. Other functions of hnRNP A1 include transcriptional regulation and telomere maintenance. Given the critical functions of hnRNP A1 in RNA metabolism, hnRNP A1 is vital for cells, and particularly neurons, to maintain regular RNA expression and homeostasis.

In ALS and MS, dysfunction of hnRNP A1 caused by mutations, misfolding, inflammation, antibodies, or unknown causes has been observed\textsuperscript{60,94,112–114}. Although hnRNP A1 moves between the nucleus and cytoplasm, it is predominantly nuclear in homeostatic conditions. In neurodegenerative diseases, hnRNP A1 is depleted from the nucleus, where it mislocalizes to the cytoplasm and can form pathogenic fibrils\textsuperscript{113,114}. Dysfunctional hnRNP A1 correlates with neuronal damage, neuronal death, and patient disability\textsuperscript{115}. Loss of hnRNP A1 function results in altered RNA expression and neuronal damage\textsuperscript{116}. In MS, dysfunctional hnRNP A1 perturbs normal RNA expression and splicing, leading to neuronal cell death, which drives disease in MS patients\textsuperscript{82}. hnRNP A1 function is crucial in cell functioning, and dysfunctional hnRNP A1 is observed in neurodegenerative diseases, where dysfunction plays an active role in neuronal damage and disease progression. In order to remain functional, hnRNP A1 has to shuttle between the nucleus and cytoplasm through the nuclear pore complex (NPC).

1.3 Nuclear Envelope and Nuclear Pore Complex

1.3.1 Structure of the Nuclear Envelope and Nuclear Pore Complex

The nuclear envelope is composed of two lipid bilayer membranes called the inner and outer nuclear membranes, with a small space between them called the perinuclear space. The nuclear envelope is supported by peripheral and transmembrane proteins and is responsible for separating the contents of the nucleus and cytoplasm. Proteins embedded in the nuclear envelope bind to actin and other filaments in the cytoplasm and are associated with additional proteins that bind to Lamin proteins and chromatin in the nucleus\textsuperscript{117}. The nuclear lamina is composed of A-type Lamins (Lamin A and Lamin C) and B-type Lamins (Lamin B1 and Lamin B2) located on the inner nuclear membrane. In neurons, there are low levels of Lamin A/C, and Lamin B1 is more important for neuronal function and viability\textsuperscript{118–120}. The nuclear lamina binds to proteins embedded
in the nuclear envelope, including NPC proteins, to anchor them in place, and to chromatin\textsuperscript{121}. This provides a rigid structure of the nucleus and nuclear envelope. Alterations or loss of the nuclear lamina results in abnormal shape of the nuclear envelope\textsuperscript{121–124}. The nuclear Lamin proteins are important for the nuclear envelope, and Lamin B1 is important in neurons for providing the structure of the nucleus and supporting the NPC.

The NPC is a multi-protein complex present where the two nuclear membranes meet to create a pore between the nucleus and cytoplasm. The NPC has eight-fold rotational symmetry and consists of approximately thirty distinct protein types (Table 1.1), but there are numerous copies of these proteins in a single NPC, comprising hundreds to thousands of individual proteins\textsuperscript{125,126}. The total mass of the NPC in most vertebrates is approximately 125 MDa, making it one of the largest protein assemblies in a cell\textsuperscript{125,126}. The NPC has distinct regions due to its large size and position in the nucleus, cytoplasm, and nuclear envelope (Figure 1.1). On the cytoplasmic side of the NPC, there is an asymmetrical ring with filaments extending into the cytoplasm\textsuperscript{127,128}. On the nuclear side, an asymmetrical structure called the nuclear basket extends into the nucleoplasm\textsuperscript{127,128}. There are also symmetrical rings, often referred to as the Y-complex, located at the periphery of the pore, which allow attachment of the cytoplasmic filaments and nuclear basket\textsuperscript{129}. The central structure of the pore includes transmembrane rings embedded in the nuclear envelope and provides structural support and anchoring of the NPC. The Nup93 subcomplex provides further support and scaffolding for the central channel of the pore, which contains proteins with filaments extending into the center of the pore to create a permeability barrier\textsuperscript{127,130}. Approximately several hundred to a thousand individual NPCs are located on the nuclear envelope in a single cell\textsuperscript{126,131}. This number allows efficient and viable transport from the nucleus to the cytoplasm and vice versa.
<table>
<thead>
<tr>
<th>Table 1.1 Location of NPC proteins</th>
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<tr>
<td><strong>Cytoplasmic Ring and Filaments</strong></td>
</tr>
<tr>
<td>RanBP2(^{126,129,132,133})</td>
</tr>
<tr>
<td>Nup214(^{126,129,132,133})</td>
</tr>
<tr>
<td>Nup88(^{126,129,132,133})</td>
</tr>
<tr>
<td><strong>Outer Rings [Y-Complex, Nup107(-160) Subcomplex]</strong></td>
</tr>
<tr>
<td>Nup107(^{126,129,132,133})</td>
</tr>
<tr>
<td>Nup37(^{126,129,132,133})</td>
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<tr>
<td>Sec13(^{126,129,132,133})</td>
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<tr>
<td>Nup96(^{126,129,132,133})</td>
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<tr>
<td><strong>Central Pore (Nup93 Subcomplex)</strong></td>
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<tr>
<td>Nup93(^{126,129,132,133})</td>
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<tr>
<td>Nup53(^{132,133})</td>
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<tr>
<td><strong>Transmembrane Ring</strong></td>
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<tr>
<td>POM121(^{126,129,132,133})</td>
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<tr>
<td><strong>Central Channel</strong></td>
</tr>
<tr>
<td>Nup62(^{126,132,133})</td>
</tr>
<tr>
<td><strong>Nuclear Basket</strong></td>
</tr>
<tr>
<td>Tpr(^{126,132,133})</td>
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Abbreviations: Nup = Nucleoporin, RanBP2 = Ran binding protein 2, Nlp = Nucleoporin-like protein, Rae = Ribonucleic acid export, Tpr = Translocated promotor region
Figure 1.1 Schematic overview of the nuclear pore complex. The main structures of the NPC are indicated with arrows. Specific proteins that are studied further are included under the bolded structure they belong to. The nuclear lamina is located on the inner nuclear membrane, which provides support to the nuclear envelope and binds to the NPC. Made with BioRender.
1.3.2 Function of the Nuclear Pore Complex: Nucleocytoplasmic Transport

The NPC allows for the passive diffusion of small molecules, typically those 30-40 kDa and smaller\textsuperscript{131,134}. However, this transport is slow, especially with larger cargo, so these molecules can also bind transport molecules and undergo active transport. Larger molecules which utilize active transport (Figure 1.2), are excluded from diffusion across the pore by a permeability barrier formed by phenylalanine glycine (FG) repeat-containing NPC proteins\textsuperscript{135,136}. The FG-Nup proteins typically face the interior of the pore to form the barrier and include all the proteins of the central channel, the Nups of the nuclear basket, Nlp1, POM121, Nup214 and Nup98\textsuperscript{132}. The FG region varies in length but is usually located on a terminal end of a protein. It is a disordered region that creates a tangle of unfolded proteins to create a block in the pore but still allows transport through the entanglement of proteins. POM121, in the transmembrane ring, is a structural protein necessary for the maintenance and organization of the NPC\textsuperscript{137}. Ran GTPase-activating protein 1 (RanGAP1) is located in the cytoplasm and the cytoplasmic ring and filaments of the NPC, along with Nup214, and plays a role in active nucleocytoplasmic transport, while Nup214 plays a structural role\textsuperscript{127,128,138,139}. The NPC proteins, POM121, Nup214, RanGAP1 and Nup98, are important in this thesis, as they are studied in detail later.

The energy required for active nucleocytoplasmic transport comes in the form of GTP. A protein called Ran will bind GTP, which is hydrolyzed to GDP, and it is the concentration of Ran in the nucleus and cytoplasm that allows the transport of molecules across the NPC. Active nucleocytoplasmic transport also involves additional proteins, specialized for binding other molecules and shuttling them across the nuclear envelope, called importins and exportins. Nuclear protein export begins with a Ran-GTP molecule binding to exportin 1, also known as chromosomal maintenance 1 and the cargo protein, which results in the translocation of the export complex across the NPC and into the cytoplasm (Figure 1.2B)\textsuperscript{138}. As Ran-GTP passes the outer cytoplasmic NPC proteins, RanGAP1 hydrolyzes the GTP to GDP, which causes the Ran-GDP to release exportin 1 and the cargo in the cytoplasm\textsuperscript{139}. Exportin 1, now free in the cytoplasm, is able to cross back into the nucleus alone.

The two main mechanisms of nuclear import, classical and non-classical, involve classical NLS and non-classical NLS sequences, respectively. Classical nuclear import involves an importin $\alpha$ and importin $\beta 1$ complex binding to the cargo protein and transporting it into the nucleus (Figure 1.2Ai)\textsuperscript{140}. In non-classical nuclear import, the cargo protein is bound by one of the twenty known
importin βs, which translocates the protein into the nucleus (Figure 1.2Aii)\(^88\). Once in the nucleus, Ran-GTP will bind to the import complex, which releases the cargo and transports the import molecules into the cytoplasm\(^{138,139}\). Once the Ran-GTP molecule is hydrolyzed, the import molecules are released to undergo another round of import. The import and export cycle results in a buildup of Ran-GDP in the cytoplasm and depletion of Ran-GTP in the nucleus. To replenish the energy stores, nuclear transport factor 2 (NTF2) binds Ran-GDP and transports it into the nucleus, where Ran guanine nucleotide exchange factor (RanGEF) exchanges GDP for GTP, allowing continuous cycles of nuclear import and export (Figure 1.2B)\(^{141}\).

**Figure 1.2 Nucleocytoplasmic transport cycles.** (A) Overview of nuclear import depicting (i) classical NLS (cNLS) mediated nuclear import and (ii) non-classical NLS (ncNLS) mediated nuclear import. Ran-GTP transports the importin molecules back to the cytoplasm. (B) Nuclear export and Ran-GDP transport to replenish the energy stores of Ran-GTP required for nucleocytoplasmic transport. Made with BioRender.
1.3.3 Nuclear Pore Complex Damage

The turnover of the NPC greatly depends on the cell type and its location in the cell cycle. The nuclear envelope disappears in some mitotically dividing cells, meaning the NPC must disassemble. The turnover of the NPC in these dividing cells is very rapid because they need to quickly reappear as the nuclear envelope does to allow for transport across the reformed nuclear envelope\textsuperscript{142}. In contrast, in terminally differentiated and non-dividing cells, such as neurons, turnover of the NPC is likely absent\textsuperscript{143}. Instead, in these cells, individual proteins are exchanged with each other; more peripheral proteins are more likely to be replaced, while the core proteins have a longer life span\textsuperscript{144}. Some of the core proteins in the Y-complex have been found to not turnover throughout the lifespan of a cell\textsuperscript{143}. These proteins can accumulate damage over the lifetime of a cell and result in altered structure and function of the NPC if the damage is extensive and severe. Damage and altered functioning of the NPC have been linked with aging, Hutchinson-Gilford progeria syndrome (a pre-mature aging disease), and neurodegenerative diseases\textsuperscript{145,146}. A recent review has summarized damage to the NPC in various neurodegenerative diseases and depicted alterations in more than 20 different NPC proteins\textsuperscript{147}. Damage to the NPC can have different causes in cells. Two main hypotheses of NPC damage have been put forth.

One hypothesis is that oxidative stress, present in many neurodegenerative diseases, damages the proteins involved in the NPC and nucleocytoplasmic transport. Oxidative stress can also affect the solubility of proteins and lead to aggregation of NPC proteins, which is related to their mislocalization to the cytoplasm\textsuperscript{148}. Oxidative damage is hypothesized to have a more significant effect on the central channel since the proteins are more exposed, which increases the permeability of the pore\textsuperscript{143}. Additional proteins involved in nucleocytoplasmic transport can also be oxidatively damaged, leading to their dysfunction and altered nucleocytoplasmic transport\textsuperscript{149,150}. Oxidative damage can also lead to disruption of the nuclear lamina and nuclear structure, which is implicated in neuronal damage in Alzheimer’s disease\textsuperscript{118}. Alterations in the central channel of the NPC are sufficient to cause mislocalization of an RBP involved in ALS, TAR DNA-binding protein 43 kilodaltons (TDP-43)\textsuperscript{151}. Another study found that the repair of the NPC restored the localization and function of TDP-43\textsuperscript{152}. In this model, the structure and function of the NPC are first affected, which leads to RBP dysfunction and cellular damage.

The other hypothesis is that \textit{RBP dysfunction occurs first}, leading to NPC damage and alterations in nucleocytoplasmic transport. The FG domains of NPC are another form of low-
complexity domains, which can interact and aggregate with molecules that bind low-complexity domains, including RBPs, which induce the mislocalization of Nups from the NPC\(^{146}\). The formation of cytoplasmic protein aggregates can lead to the accumulation of NPC proteins and proteins involved in nucleocytoplasmic transport in the aggregates, which alters nucleocytoplasmic transport and exacerbates the nuclear loss of RBPs\(^{153,154}\). Importantly, it was the presence of the cytoplasmic granules and not cellular stress which led to the abnormal NPCs and nucleocytoplasmic transport\(^{153}\). Inhibiting nuclear protein export can reduce the cytoplasmic mislocalization of TDP-43 and rescue nucleocytoplasmic transport function\(^{155}\). Evidence also suggests that cytoplasmic aggregates are more toxic to nucleocytoplasmic transport than nuclear aggregates\(^{156}\). This could be because the NPC and transport proteins sequestered in granules, RanGAP1, import proteins, and NPC proteins of the central channel and cytoplasmic ring and filaments are more available in the cytoplasm. *This hypothesis suggests that dysfunctional RBPs are the cause of NPC alterations and damage.*

1.4 RNA Binding Protein and Nuclear Pore Complex Dysfunction Consequences

1.4.1 Functional Consequences of RNA Binding Protein Dysfunction

The effect of dysfunctional RBPs is considered multifactorial, with a loss of function in the nucleus due to mislocalization and a toxic gain of function in the cytoplasm due to the formation of RBP-induced protein aggregates\(^{157}\). The loss of nuclear function of hnRNP A1 can profoundly affect RNA due to its regulation of RNA\(^{82,116}\). The stability of RNA, its transport to the cytoplasm and its translation could all be affected by the loss of function of RBPs. This altered RNA metabolism may decrease the amount of RNA present in the cell, particularly in the cytoplasm. High RNA-to-protein ratios can prevent the aggregation of RBPs, whereas low RNA-to-protein ratios can promote aggregation\(^{158}\). Loss of RBP function may lead to decreased cytoplasmic RNA, which in turn leads to clustering of RBPs and other proteins with low complexity domains, including NPC proteins and proteins involved in nucleocytoplasmic transport.

The formation of harmful protein inclusions in the cytoplasm containing RBPs can confer a toxic gain of function since these aggregates can sequestrate additional proteins, such as those involved in the translation of specific mRNAs into proteins\(^{159,160}\). This new function of protein aggregates likely causes a change in mRNA translation into proteins. Our lab has recently studied the effects of RBP dysfunction, specifically hnRNP A1, on mRNA through RNA sequencing in MS and MS models\(^{82,116}\). Various transcripts were up- or down-regulated because of hnRNP A1
dysfunction. Some of the RNAs affected included those related to the NPC. Loss of hnRNP A1 function can potentially affect the turnover of exposed NPC proteins involved in nucleocytoplasmic transport, affecting the localization of hnRNP A1 and additional RBPs. This would lead to RBP aggregates, further damage to the NPC, and cell death. Support for this comes from data in a relevant mouse model of MS, experimental autoimmune encephalomyelitis (EAE), where hnRNP A1 mislocalization positively correlates with neurodegeneration in the CNS and negatively correlates with the number of neurons in the affected CNS regions\textsuperscript{115}. While dysfunctional RBPs, especially hnRNP A1 in MS, are linked to cellular toxicity and neuronal cell death, the mechanism of the negative impact on cell health remains an active area of investigation.

1.4.2 Changes in the Nuclear Pore Complex Alters Nucleocytoplasmic Transport

The most immediate effect NPC disturbances will have on a cell is an alteration in nucleocytoplasmic transport. Many studies looking at NPC changes in neurodegenerative diseases have examined this in detail. Ran has been found to be a component of cytoplasmic inclusions of TDP-43 in an \textit{in vitro} neuronal-like ALS model\textsuperscript{154}. Altered localization of Ran has been also observed in induced pluripotent stem cell-derived neurons from patients with ALS\textsuperscript{161}. Entrapment of Ran in the cytoplasm likely decreases its nuclear presence, which affects active nucleocytoplasmic transport due to a loss of nuclear Ran-GTP. The disruption in the Ran gradient points to defects in nucleocytoplasmic transport.

One of the transmembrane proteins in the transmembrane ring of the NPC, POM121, is essential for linking the NPC to the nuclear envelope\textsuperscript{162}. A study in HeLa cells found that knockdown of POM121 ablated assembled NPCs on the nuclear envelope\textsuperscript{137}. In a clinically relevant study, the reduction of POM121 resulting from mutated RNA in ALS initiated a decrease of seven additional NPC proteins, leading to a change in the localization of Ran\textsuperscript{161}. Experimental reduction of POM121 initiates NPC injury and leads to TDP-43 dysfunction\textsuperscript{152}. Over-expression of POM121 is sufficient to restore NPC structure, nucleocytoplasmic transport, and TDP-43 function\textsuperscript{152,161}. Knockdown of RanBP2 in the motor neurons of mice disrupted nucleocytoplasmic transport and was sufficient to cause ALS-like syndromes\textsuperscript{163}. RanBP2 plays a crucial role in cells with nucleocytoplasmic transport, and disrupting its function has severe effects on cells. RanGAP1 is closely associated with RanBP2 in location and function. Decreases in the level of RanGAP1 increase the nuclear accumulation of Ran, which likely caused a decline in nucleocytoplasmic transport and quantitatively increased nuclear RNA\textsuperscript{164}. Thus, disrupting the structure of the nuclear
pore complex alters the function of normal nucleocytoplasmic transport and has severe effects on the homeostatic functioning of cells.

**1.4.3 Altered Nucleocytoplasmic Transport Affects RNA and Protein Localization**

The NPC allows for the bidirectional transport of molecules across the nuclear envelope. Alterations in the structure of the NPC change the transport and distribution of molecules that are shuttled between the nucleus and cytoplasm. Disruption of nucleocytoplasmic transport has been found to increase the presence of RNA in the nucleus in drosophila and a cell line both used to model ALS. The changes in nucleocytoplasmic transport affect RNA export, which leads to decreased cytoplasmic RNA and altered protein synthesis in the cell. This can cause severe downstream effects if essential proteins are no longer synthesized. In another study, nuclear RNA accumulation was associated with reduced cell viability and increased apoptosis. This finding confirms the toxicity of nuclear RNA accumulation caused by alterations in nucleocytoplasmic transport.

Disruptions in nucleocytoplasmic transport not only influence RNA distribution, but also affect protein distribution. TDP-43 cytoplasmic aggregates were found to induce alterations in nucleocytoplasmic transport, which led to the mislocalization of hnRNP A1 and another RBP, fused in sarcoma (FUS), and the nuclear depletion of TDP-43. The authors concluded that the nuclear depletion of TDP-43 was caused by nucleocytoplasmic transport disruption and not the cytoplasmic aggregates. Other studies have analyzed the localization of fluorescent proteins with an NLS and found significantly higher protein levels in the cytoplasm in cells with disrupted nucleocytoplasmic transport. This provides more robust evidence of cytoplasmic accumulation of homeostatically nuclear proteins downstream of nucleocytoplasmic transport dysfunction. Proteins, such as RBPs, that form insoluble clusters may be more susceptible to mislocalization due to their entrapment in the cytoplasm. Both increased nuclear RNA and decreased nuclear proteins have a negative impact on cell health. Defects in nucleocytoplasmic transport can reduce cell viability and increase cell death. Both RBP dysfunction and NPC alterations have been observed in neurodegenerative diseases, where the effect of both contributes to neuronal death and disease pathogenesis.
1.5 RNA Binding Proteins and the Nuclear Pore Complex in Neurodegeneration

1.5.1 Huntington’s Disease (HD)

HD is a genetic disease that results from a mutant form of the huntingtin protein, which has an expanding trinucleotide repeat section in the \( Htt \) gene\(^{167} \). Neuronal cell death occurs leading to impairments characteristic of the disease. A secondary feature of HD is dysfunctional RBPs. TDP-43 has been found to colocalize with the mutant huntingtin protein in cytoplasmic, but not intranuclear, inclusions in all 10 cases studied by Schwab et al.\(^{168} \). Another RBP, FUS, was also found to interact with mutant huntingtin aggregates in a cell line and a mouse model of HD\(^{169,170} \). Decreasing the expression levels of both FUS and TDP-43 increases the survival of neurons in HD models\(^{171} \). Together, this provides evidence that the aggregation of RBPs in HD reduces neuron viability and has a negative impact on cell health. Mutant huntingtin protein also resulted in the mislocalization and aggregation of NPC proteins, which impacted nuclear envelope integrity and altered nucleocytoplasmic transport\(^{172} \). Another study found nuclear pore defects and altered nucleocytoplasmic transport were present in neurons from induced pluripotent stem cells from patients with HD\(^{173} \). Although dysfunctional RBPs and altered nucleocytoplasmic transport are present in HD and likely contribute to neurodegeneration, their exact role still needs to be determined.

1.5.2 Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD)

ALS and FTD also referred to as frontotemporal lobar degeneration or Pick’s disease, are comparable neurodegenerative diseases in that similar patterns of RBP dysfunction occur in both diseases. In ALS, upper and lower motor neurons are primarily affected and degenerate, which leads to physical disabilities and loss of muscle control. In FTD, the brain’s frontal and anterior temporal lobes atrophy, leading to cognitive deficits\(^{174} \). Due to similar RBP mechanisms, the comorbidity of ALS and FTD is estimated to occur in approximately 50% of patients with ALS\(^{175–177} \). Both diseases offer a poor prognosis and have no effective treatments, which causes a loss of independence and eventually, death. The best studied RBP in ALS is TDP-43. However, many dysfunctional RBPs are linked to ALS, including FUS, hnRNP A1, T cell intracellular antigen 1 (TIA-1), and others\(^{177,178} \). Mutations in these proteins have been observed but are not found in all instances of the disease. Faulty RBPs mislocalize to the cytoplasm and can form protein and RNA inclusions, which are pathological and can form from the aggregative properties of the low
complexity and prion-like domain, post-translational modifications, or disrupted nucleocytoplasmic transport\textsuperscript{178}.

In ALS and FTD, the presence of NPC and nucleocytoplasmic transport disruptions have been studied in great depth. TDP-43-associated pathology in these diseases has been found to disrupt the NPC. For example, cytoplasmic aggregates of TDP-43 were associated with the mislocalization of Nups, and NPC proteins were a component of TDP-43 aggregates\textsuperscript{165}. One of the NPC proteins, Nup62 from the central channel, was found to be severely altered in postmortem human ALS spinal motor neurons that also displayed TDP-43 pathology\textsuperscript{179}. Neurons with nuclear depletion of TDP-43 had severely altered patterns of Nup62 immunoreactivity and a loss of Nup62 staining\textsuperscript{179}. A hexanucleotide repeat present in some ALS and FTD cases has been found to physically interact with RanGAP1 and lead to the mislocalization of RanGAP1\textsuperscript{166}. Another study found 18 genes associated with the NPC and nucleocytoplasmic transport that altered the phenotype of the hexanucleotide repeat in an ALS model\textsuperscript{165}. These studies support the hypothesis that dysfunctional proteins, including RBP, interact with NPC proteins and lead to their mislocalization and alterations in the structure of the NPC.

1.5.3 Multiple Sclerosis (MS)

Understanding the neurodegenerative aspects of MS is crucial to providing novel and effective treatments for the disease. RBP dysfunction has been discovered in oligodendrocytes and neurons from MS patients and linked to inflammatory plaques and neurodegeneration\textsuperscript{94,114,115,180}. The role of hnRNP A1 in MS pathogenesis has been more extensively studied than other RBPs. Similar to other neurodegenerative diseases, dysfunctional RBPs can aggregate in the cytoplasm and form pathological protein inclusions. The dysfunction of these RBPs in MS results in altered RNA metabolism and increased cell death and plays a critical role in the neurodegeneration occurring in the disease\textsuperscript{60,82,115,116}. Whether dysfunctional RBPs can affect the localization of NPC proteins in MS is unknown.

Research on the NPC and nucleocytoplasmic transport in MS is sparse, with only a few studies published. The expression of mRNA transcripts of multiple NPC targets is altered in the context of MS. Transcripts of two nuclear basket proteins are upregulated, and POM121 is downregulated\textsuperscript{133}. The study did not focus on MS, as 10 other inflammatory diseases were also investigated. Nuclear import of the signalling protein and transcription factor, Notch1-intracellular domain (cleaved Notch1 after binding to its ligand), was found to be nearly absent in
oligodendrocyte precursor cells, which prevented the maturation of the cells and remyelination of axons in MS\textsuperscript{181}. Another study found abnormal cytoplasmic localization of histone deacetylase 1 (HDAC1), leading to impaired mitochondrial and axonal transport which contributes to axonal damage\textsuperscript{182}. HDAC1 mislocalization was believed to be due to abnormal nuclear export, but impaired nuclear import could not be ruled out. These studies demonstrate that the NPC is potentially altered, and the nucleocytoplasmic transport of specific proteins is abnormal in MS. However, both studies focused on specific proteins, not broader nucleocytoplasmic transport as a common mechanism. As previously stated, dysfunction of hnRNP A1 alters RNA metabolism and transcripts in MS and relevant models. The transcripts include targets of the NPC and nucleocytoplasmic transport, and gene ontology (GO) analysis identifies pathways related to transport\textsuperscript{82}. This provides further evidence of the presence of alterations in the NPC and nucleocytoplasmic transport in MS. \textit{However, specific investigation of the NPC and nucleocytoplasmic transport, studied in other neurodegenerative diseases, is lacking in MS.}

1.6 Hypothesis and Specific Aims

Under homeostatic conditions, the NPC is anchored to the nuclear envelope and evenly distributed, nucleocytoplasmic transport is functional, and most RBPs are primarily located in the nucleus (Figure 1.3). RBP dysfunction is a significant mechanism in neurologic diseases. In addition, RBP dysfunction can reciprocally be caused by NPC alterations. An in-depth study of the NPC and nucleocytoplasmic transport, yet to be completed, is needed to better understand the pathogenesis of neurodegeneration in MS.

The hypothesis for this thesis is that the NPC is altered in MS and a related model, affecting normal nucleocytoplasmic transport, which has the potential to negatively impact neuronal viability. This hypothesis will be tested through the following three aims.

1.6.1 Explore Potential Links Between hnRNP A1, Lamin B, and NPC Structure

Using hnRNP A1 knockdown as a model of RBP dysfunction, the phenotypic staining pattern of Lamin B will be assessed in neuron-like cells. Current methods of Lamin B phenotypic pattern quantification are prone to bias and variation. Therefore, a novel method will be created to automatically assess the phenotypes based on quantified measurements collected from 3D analysis of Lamin B staining. Phenotypic staining and protein expression levels of additional NPC proteins will be evaluated following hnRNP A1 knockdown.
1.6.2 Investigate the NPC Function Through Assessing Active Nucleocytoplasmic Transport

RNA sequencing data previously published in our lab will be newly analyzed to establish a connection between dysfunctional hnRNP A1 and nucleocytoplasmic transport. Active nucleocytoplasmic transport will be assessed with a fluorescent protein containing an NLS and NES, which continuously travels between the nucleus and cytoplasm. Nuclear envelope integrity will be assessed with a different fluorescent protein that remains nuclear.

1.6.3 Establish a Link Between Multiple Sclerosis and Nuclear and NPC Structure

The phenotypes of Lamin B staining will be assessed in postmortem, age- and sex-matched, MS and control brains. Lamin B will be used as a readout of NPC structure as it binds the NPC in place and altered Lamin B staining provides evidence of NPC alterations in vitro.

Figure 1.3 Summary of the normal structural processes present in healthy neurons related to this thesis. The nuclear envelope is round, smooth and outlines the nucleus. NPCs are evenly distributed around the nuclear envelope and functional, allowing passive and active transport between the nucleus and cytoplasm. Proteins that undergo nucleocytoplasmic transport are located in their normal, homeostatic location. Made with BioRender.
CHAPTER 2: Methodology

2.1 Cell Culture and Transfection

Neuro-2a cells (Cedarlane Labs, CCL-131), which exhibit a neuronal phenotype upon differentiation, were grown in T-75 flasks at 37°C in a humidified environment containing 5% CO₂ and 95% normal atmosphere. Cells were passaged at 80% confluency in complete media, consisting of DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Neuro-2a cells were differentiated into a neuronal phenotype with differentiation media containing DMEM, 1% penicillin/streptomycin, 2% FBS, and 10 µM retinoic acid. For siRNA experiments, cells were transfected using lipofectamine RNAiMAX (Invitrogen) and 0.0125 µM of siRNA. We have previously established the efficacy of this siRNA targeting hnRNP A1 in Neuro-2a cells116. For experiments examining hnRNP A1 knockdown and nucleocytoplasmic transport, co-transfection of siRNA and the plasmid of interest was performed using lipofectamine 2000 (Invitrogen), 400 ng of plasmid, and 0.0125 µM of siRNA. Cells were seeded at 25,000 cells per well on 8-well plates for 24 hours prior to transfection. 16 hours post-transfection, media was changed to differentiation media as previously published116. 72 hours after transfection, cells were harvested or fixed for downstream experiments. Nuclear import or export inhibition were achieved using Importazole (Sigma-Aldrich SML0341) at a concentration of 40 µM and Leptomycin B (Sigma-Aldrich L2913) at a concentration of 10 ng/mL, respectively, suspended in DMSO and diluted in differentiation media.

2.2 siRNA Oligonucleotides and Plasmids

The siRNA sequence for scrambled/negative control siRNA (siNEG) was 5’-UGGUUUACGUUGACAAA-3’, and siRNA targeting A1 (siA1) was 5’-GUAUCCAUUAUCAUGUGUA-3’116, both synthesized by Integrated DNA Technologies (IDT). The N-Lentiviral-S-tdTomato-C plasmid was a gift from Jeffrey Rothstein (Addgene plasmid # 112579166; RRID: Addgene_112759). The N-pAAV-hSyn-2xNLS-tdTomato-C plasmid was generated using HiFi DNA assembly cloning (NEBuilder HiFi DNA Assembly Cloning Kit, New England BioLabs). Briefly, the 2xNLS-tdTomato sequence was PCR cloned out of N-pAAV-MBP-2xNLS-tdTomato-C a gift from Viviana Gradinaru (Addgene plasmid # 104054183; RRID: Addgene_104054) using the forward ctgagagcgctggagaagatgggaagcccaaag and reverse tatcgataagcttgatatcgtcacaccttcttcttctt PCprimers. The cloned product was inserted into the N-
pAAV-hSyn-mScarlet-C plasmid a gift from Karl Deisseroth, (Addgene plasmid # 131001\textsuperscript{184}; RRID: Addgene\_131001) between the BamHI and EcoRI restriction enzyme sites with two-fragment HiFi DNA assembly cloning. This cloning fully replaced the mScarlet gene cassette with the 2xNLS-tdTomato sequence.

For bacterial growth and plasmid expansion, sterile terrific broth (Sigma-Aldrich 71754) cultures were supplemented with Ampicillin (ThermoFisher BP176025) (final concentration 100µg/mL) and inoculated with -80°C frozen, plasmid transformed NEB\textsuperscript{®}5-alpha competent \textit{E. coli} (NEB C2987H) glycerol stocks. Cultures were grown overnight at 37°C with 300rpm shaking in an incubator shaker, and plasmids were isolated using a PureLink\textsuperscript{TM} HiPure Plasmid Midiprep kit (ThermoFisher K210005), following the manufacturer’s protocol. Plasmid concentrations were determined using a Nanodrop ND-1000 spectrophotometer (ThermoFischer).

2.3 Protein Isolation from Cells

Neuro-2a cells were cultured on 6-well dishes coated with poly-D-lysine. Cells were harvested via scraping into Dulbecco’s phosphate buffered saline (D-PBS) and centrifuged for 5 minutes at 500 x g. Pelleted cells were resuspended in 1 mL D-PBS. 800µL of the cell suspension was used for protein extraction and the remaining 200 uL was used for RNA extraction. For protein harvest, cells were lysed using CytoBuster (Millipore) containing protease inhibitors (Roche). Cells were rotated in lysis buffer and then centrifuged at 16 000 x g for 5 minutes. The resultant supernatant was isolated and frozen at -80°C until Western blot experiments.

2.4 Western Blotting

Protein lysates were precipitated in acetone at -20°C for at least 20 minutes followed by centrifugation at 18000 x g for 20 minutes. The supernatant was removed, and the pellet was resuspended in 1X sample buffer with β-mercaptoethanol. 40 µg of protein was loaded into each well and were separated via SDS-PAGE on a 10% acrylamide gel run at 120V. Proteins were transferred to PVDF membrane using overnight wet transfer at 4°C at 30V. Membranes were blocked with 10% normal goat serum for 1 hour at room temperature and then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: mouse anti-hnRNP A1 (1:1000; Millipore 05-1521), rat anti-Nup98 (1:1000; Abcam ab50610), rabbit anti-β-actin (1:1000; Cell Signaling Technology 4967), mouse anti-β-actin (1:2000; Cell Signaling Technology 3700), and rabbit anti-RanGAP1 (1:1000; Abcam ab92360). Membranes were washed
and incubated with secondary antibody. The following secondary antibodies were used: goat anti-mouse IgG (1:3000; Bio-Rad 1706516), goat anti-rabbit IgG (1:3000; Bio-Rad 1706515) and goat anti-rat IgG (1:9000; Jackson 112-035-003), all conjugated to horseradish peroxidase. Membranes were developed using Clarity Western ECL substrate (Bio-Rad) for 5 minutes and visualized using the Bio-Rad ChemiDoc system. Protein levels were quantified using ImageJ by densitometry and normalized to ß-actin.

2.5 Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) brain tissue from both sexes was obtained from The Netherlands Brain Bank (https://www.brainbank.nl/) and sectioned at 10 µm. Sections were deparaffinized through 5-minute sequential washes of xylene (2X), 50/50 xylene and 100% ethanol, 100% ethanol (2X), and 95% ethanol. Slides were incubated in 0.667% H₂O₂ in methanol for 30 minutes to block any endogenous peroxidases followed by 5-minute washes in 95% ethanol and 70% ethanol. For antigen retrieval, slides were incubated in Tris-EDTA buffer with pH 9 (10 mM Tris, 1 mM EDTA, 0.05% Tween-20) in a steamer for 45 minutes. Slides were washed in 0.1M PBS (3X 5 minutes) and blocked in 10% FBS in 0.1M PBS for 15 minutes. Rabbit anti-Lamin B1 (1:1000; Abcam ab16048) was incubated overnight at 4°C diluted in blocking solution. Slides were washed in 0.1M PBS and incubated with donkey anti-rabbit conjugated to biotin (1:200 Jackson 711-065-152) diluted in 10% FBS and 3% human serum in 0.1M PBS for 1 hour at room temperature. Slides were washed in 0.1M PBS 3 times and incubated with avidin peroxidase 10µg/mL (Sigma A3151) diluted in 10% FBS in 0.1M PBS for 1 hour and washed in 0.1M PBS. Slides were counterstained with hematoxylin for 10 minutes, rinsed in tap water, destained in 0.5% HCl in 70% ethanol, rinsed with tap water, and placed in Scott solution (0.2% KHCO₃ and 2% MgSO₄*7H₂O in distilled water). Slides were dehydrated in sequential 5-minute washes in 70% ethanol, 95% ethanol (2X), 100% ethanol (2X), 50/50 xylene and 100% ethanol, and 100% xylene (2X). Slides were coverslipped using vectamount (Vector Laboratories H-5700-60) and imaged using an Olympus BX61VS Scanning microscope under a 40X objective, with a numerical aperture of 0.95. Images were processed in Olympus VS-ASW FL 2.7 software, and analysis was performed using QuPath v0.4.3 (see abnormal staining quantification). Representative images were taken using a 100X oil objective, numerical aperture 1.3, on an Olympus BX53 microscope and processed using Olympus CellSens Standard 1.5 software.
2.6 Immunocytochemistry

Neuro-2a cells were cultured in 8-well chamber slides coated with poly-D-lysine (Sigma-Aldrich). Cells were fixed with 3.7% formaldehyde in D-PBS for 15 minutes at room temperature, washed three times in PBS and permeabilized with 0.1% Triton X-100 in PBS (0.1% PBS-T) for 10 minutes. Cells were blocked with 5% bovine serum albumin (BSA) in 0.1% PBS-T for 1 hour, followed by overnight incubation at 4°C with primary antibodies diluted in blocking solution. Primary antibodies used include rabbit anti-Lamin B1 (1:1000; Abcam ab16048), mouse anti-hnRNP A1 (1:500; Millipore 05-1521), rabbit anti-hnRNP A1 (1:500; Abcam ab4791), rabbit anti-POM121 (1:500; Novus NBP2-19890), mouse anti-RanGAP1 (1:250; Santa Cruz sc-28322), rat anti-Nup98 (1:500; Abcam ab50610), rabbit anti-Nup214 (1:250; Bethyl Laboratories Inc. A300-716A-M), chicken anti-β-tubulin (1:500; Aves Lab TUJ), and rabbit anti-β-tubulin (1:1000; Sigma-Aldrich T2200). Cells were washed thrice in 0.1% PBS-T and incubated with secondary antibodies for 30 minutes at room temperature. Secondary antibodies used include donkey anti-mouse Alexa Fluor 488 (1:1000; Jackson Immuno Research 715-546-151), goat anti-rabbit Alexa Fluor 488 (1:1000; Jackson Immuno Research 111-545-006), goat anti-mouse DyLight 594 (1:1000; Jackson Immuno Research 115-515-006), goat anti-rabbit DyLight 594 (1:1000; Jackson Immuno Research 111-586-006) donkey anti-rat Alexa Fluor 594 (1:1000; Jackson Immuno Research 712-586-153), and donkey anti-chicken Alexa Fluor 647 (1:1000; Jackson Immuno Research 703-606-155). Slides stained with the conjugated antibody rabbit anti-hnRNPA1 Alexa Fluor 647 (1:250; Abcam ab197854) were washed in 0.1% PBS-T three times and incubated overnight at 4°C overnight diluted in blocking solution. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and imaged with a 40X or 63X objective, with a 1.40 numerical aperture, on an Axio Observer 7, inverted fluorescent microscope (Carl Zeiss Canada Ltd.). The 3D images were created by imaging cells through the z-plane every 0.29µm for approximately 50 stacks per image. Images were processed using ZEN 3.1 Blue Edition software (Carl Zeiss Canada Ltd.).

2.7 Fluorescence Quantification

ImageJ was used to measure the fluorescence intensity of stained cells. Regions of interest were generated from DAPI (a nuclear stain) to prevent bias and outline the nuclei. The outlined nuclei were overlayed on the fluorescent channel for hnRNP A1 or tdTomato. The corrected mean
nuclear fluorescence was calculated by measuring the mean nuclear fluorescence intensity for hnRNP A1 or tdTomato and subtracting the mean background fluorescence intensity. Cells quantified per replicate ranged from 19-87 depending on the plasmid transfection efficacy. For 3D and abnormal staining quantification, cells were included in the treatment group if they had less than 50% corrected mean nuclear hnRNP A1 fluorescence compared to the control group (i.e., greater than 50% hnRNP A1 knockdown). Thus, only cells with greater than 50% hnRNP A1 knockdown were used for analysis. The corrected mean nuclear fluorescence was used to identify individual cells with hnRNP A1 knockdown as it did not rely on the size of the nucleus which can be variable in Neuro-2a cells. The corrected total nuclear fluorescence was calculated by taking the integrated density of the region of interest and subtracting the area of the nucleus multiplied by the background fluorescence to represent overall hnRNP A1 fluorescence in the siNEG and siA1 treatment groups.

2.8 Abnormal Staining Quantification

Human brain tissues were analyzed using QuPath v0.4.3\textsuperscript{185}. Tissue section images were loaded into QuPath in the .vsi format. The image type was set to Brightfield H-DAB, the brightness and contrast was altered appropriately to view the channels separately and to best identify Lamin B phenotypes. All samples were renamed using a random letter code to blind cases during analysis. After abnormal phenotypes were counted, samples were unblinded to statistically test the results. A square 2000 pixels by 2000 pixels, created using the Objects $\rightarrow$ Annotations $\rightarrow$ Specify Annotation tab, was randomly placed in approximately layer III of the cortical gray matter. Five squares were analyzed per tissue section. Within each square, glial cells were identified to avoid confusion when counting neurons. Neurons were classified as normal, internal, or incomplete based on Lamin B staining using the counting tool. MS tissue was analyzed indiscriminately due to blinding, meaning that likely normal appearing gray matter and plaques within MS samples were both included in the analysis. Over 100 neurons were quantified per case.

Cells treated with siNEG and siA1 were stained for five different markers of the NPC and nuclear envelope. For each protein, various phenotypes were defined based on staining phenotypes in both treatment groups and previous publications\textsuperscript{155,166,186,187}. Phenotypes were then binned into normal or abnormal based on the prevalence of the phenotypes in the siNEG group. Each protein of interest displayed different phenotypes and thus, phenotypes were defined as normal or abnormal separately for each marker of interest. The percent of abnormal phenotypes was
calculated by adding the counts of the different abnormal phenotypes and dividing by the total number of cells counted. The mean percentage of abnormal staining phenotypes between the two treatment groups was compared for all five protein markers using one-tailed, independent t-tests. There were 26-59 cells analyzed per replicate.

2.9 Three-Dimensional Quantification

Cells with hnRNP A1 knockdown or siNEG treated cells were stained for Lamin B. The 3D ImageJ suite plugin for ImageJ was used for 3D quantification. A Z-stack of Lamin B tiff images was combined into a stack in ImageJ with the distance between images and the scale in the images appropriately calibrated. Images were processed with ImageJ using the optimal auto threshold on the z-plane most in focus and applying the same threshold to all images in the stack. Images were processed again to be compatible with the 3D quantification tool using 3D fast filers in the plugin using the median method and default settings. The 3D manager window was used to segment the image. The Lamin B staining was outlined, and all individual measurements for a single nucleus were combined and then measured, returning all the values used for 3D quantification. Three replicates were quantified per group.

2.10 Gene Ontology Analysis

Differentially expressed genes in Neuro-2a cells with hnRNP A1 knockdown were previously identified through bulk RNA-sequencing analysis and analyzed in ShinyGO 0.77. Different pathway databases were analyzed with different parameters as the number of pathways returned varied greatly, and the analysis was optimized for each pathway. In general, the false discovery rate (FDR) cut-off was set at 0.05, the minimum pathway size was set at 2, the maximum was set at 2000, and approximately half of the pathways were shown and sorted. Redundancies were not removed. KEGG pathway analysis involved showing 20 pathways selected by FDR and sorting by fold enrichment in ShinyGO software. The top 100 pathways, selected by FDR and sorted by fold enrichment, were displayed for GO cellular component. The top 80 pathways sorted by fold enrichment were displayed for GO molecular function. GO biological process analysis included 500 pathways sorted by FDR.
2.11 Experimental Design and Statistical Analysis

Graphical representations and data analysis were completed using GraphPad Prism 10 software (GraphPad Software, San Diego, CA, USA). Paired t-tests were used to compare hnRNP A1 knockdown as replicates 1, 2 and 3 had slightly different exposure times (exposure time between treatments in the same replicate were identical), making the data on hnRNP A1 fluorescence dependent on the imaging parameters and, therefore, related (Figure 3.2). One-tailed independent t-tests were used to compare means when the treatment was expected to produce a specific directional effect. Two-tailed independent t-tests were used to compare means when the expected treatment effect was unknown. The data was assumed to be normally distributed and the variance between samples was assumed to be similar for all samples that had three or less replicates. The percent of abnormal phenotypes in human samples was normally distributed (Shapiro-Wilk W: Control W=0.9232, p=0.5505; MS W=0.9811, p=0.9877) and the variance between the two samples was statistically equal (F-test: F_{11,4}=1.863, p=0.5761). Therefore, an independent t-test was used to analyze the samples. G*Power 3.1 was used to complete a power analysis related to Fisher’s exact test to determine the sample size needed to achieve a power of 0.8 using the effect size found in the experiment in Figure 4\textsuperscript{190}. Values in graphs are plotted as the mean ± standard error of the mean (SEM) in all instances, with p<0.05 considered statistically significant.
CHAPTER 3: Results

3.1 Lamin B and the Nuclear Pore Complex Structure is Affected by hnRNP A1 Knockdown

The NPC can be altered by dysfunctional RBPs and is involved in neurodegenerative diseases. Therefore, I used knockdown of hnRNP A1 to model dysfunction and loss of nuclear staining, which is present in MS, and evaluated staining patterns of the nuclear and NPC structures\(^94,114\). Due to issues with the current method used in the literature, a novel method to assess Lamin B staining was also generated.

3.1.1 Knockdown of hnRNP A1 Significantly Alters the Nuclear Envelope

First, I treated Neuro 2A cells, a cell line with a neuronal phenotype, with siNEG and siA1 (Figure 3.1) and assessed the effects on the nuclear envelope and NPC through Lamin B staining. Cells treated with siNEG demonstrated two predominant, normal phenotypes, including a ring phenotype of Lamin B staining surrounding the edges of the nucleus or diffuse Lamin B staining throughout the nucleus (Figure 3.2A). Treatment of cells with siA1 knocked down hnRNP A1 expression (Figure 3.1) and increased the prevalence of abnormal Lamin B staining phenotypes by a factor greater than two (Figures 3.2B, C). Abnormal Lamin B staining phenotypes were defined based on previous publications and their low presence in the control group, siNEG treatment\(^155,166,187,191\). Abnormal phenotypes included punctate (Lamin B ‘spots’ in the center of the nucleus), incomplete (Lamin B ring did not entirely surround the nucleus), and internal (lines of Lamin B staining within the nucleus). When examining individual phenotypes, I found that the normal ring phenotype significantly decreased with siA1 treatment, while the diffuse phenotype was similar between treatments (Figure 3.2D). The proportion of cells with an incomplete phenotype significantly increased with hnRNP A1 knockdown. Likewise, the internal phenotype was also significantly increased (Figure 3.2E), suggesting that these two phenotypes largely contributed to the overall increase in abnormal Lamin B staining. The punctate phenotype was similar in number regardless of treatment (Figure 3.2E). Increased abnormal Lamin B phenotypes demonstrate that a loss of hnRNP A1 function alters nuclear envelope structure and, by extension, possibly NPC structure.
Figure 3.1 siA1 treatment significantly decreased hnRNPA1 expression compared to siNEG treatment in cells labelled with Lamin B. Cells treated with siA1 showed significant hnRNP A1 (green) knockdown. Scale bars=20 µm, n=3 replicates, one-tailed, paired, ratio t-test, *p<0.05. Data are plotted as mean of one replicate of the experiment.
A) Normal: Ring, Diffuse

B) Abnormal: Punctate, Internal, Incomplete

C) Lamin B

D) Lamin B Ring

E) Lamin B Punctate, Internal, Incomplete
Figure 3.2 Cells treated with siA1 demonstrate abnormal Lamin B staining. (A) Cells treated with siRNA targeting hnRNP A1 (siA1) were binned into distinct phenotypic categories based on the Lamin B staining pattern. Ring and diffuse phenotypes were considered normal based on the phenotypes present in non-targeting siRNA (siNEG) treated cells. (B) Punctate, internal, and incomplete phenotypes were considered abnormal with arrows pointing to the defining characteristic. (C) Quantification of the normal and abnormal staining phenotypes of Lamin B in siNEG and siA1 treated cells, with a significant increase in the number of abnormal phenotypes with hnRNP A1 knockdown. (D) Assessment of individual normal Lamin B phenotypes demonstrate a significant decrease in ring but not diffuse phenotypes between siNEG and siA1 treatment. (E) Assessment of individual abnormal Lamin B phenotypes identified a significant increase in the incomplete and internal phenotypes, but not the punctate phenotype, between treatment groups. Data are graphed as mean ± SEM. Scale bars=10 µm, n=3 replicates, one-tailed independent t-test, *p<0.05, ns=not significant.
3.1.2 Qualitative Phenotypes of Lamin B Staining Can Be Identified and Quantified Using Three-Dimensional Images

Next, I sought to define and measure NPC phenotypes quantitatively. As proof of principle, I developed a pipeline to build three-dimensional (3D) renderings from Z-stack images to quantify Lamin B staining phenotypes. 3D images revealed that the ring and diffuse phenotypes were qualitatively similar as both had a ring of staining around the nucleus, but diffuse cells had more staining on the dome-shaped area surrounding the edge of the nucleus, whereas the ring phenotype had less staining on the dome (Figure 3.3A). The abnormal phenotypes in Figure 3.3B were qualitatively distinct from the normal phenotypes in Figure 3.3A. After finding observable differences in the 3D images between normal and abnormal phenotypes, I collected 3D measurements to generate an automatic method to assign Lamin B phenotypes within cells to reduce potential bias, generate similar results regardless of conditions of the data quantification, and decrease the time associated with manually identifying the phenotype of the cells.

3D images of Lamin B staining enabled the collection of several measurements to define the quantitative characteristics of the manually assigned phenotypes. A mathematical approach was developed using the known phenotypes (ring, diffuse, folded, internal, punctate) to automatically assign phenotypes based on subtractive selection across quantitative measurements using siNEG cells (Figure 3.3C). The automatic phenotyping process is illustrated in Figure 3.3C, where cells were initially grouped and examined based on the number of objects within the cell (Figure 3.3Ci). More objects in a cell corresponded to cells with punctate phenotypes. A threshold was established wherein cells with an object count 3.3x the average of siNEG cells not currently assigned a phenotype were defined as having a punctate phenotype. This threshold was determined by taking the fold change in Figure 3.3Ci and making precise adjustments to determine the optimal value, 3.3, when the maximum number of punctate cells was correctly identified using the quantitative measurement. These cells, identified as punctate staining, were removed from the group based on the number of distinct objects stained for Lamin B. The remaining cells were then examined using moment 3, a 3D measurement of the 3D shape of the staining to identify cells with an internal Lamin B phenotype (Figure 3.3Cii). The remaining cells were assessed based on their flatness, which examines the depth of the nucleus (Figure 3.3Ciii). This allowed for the separation of diffuse phenotype cells as the diffuse phenotype has a hollow hemisphere of Lamin B staining, whereas the remaining cells had less depth of staining in the z-dimension. This left the ring and
incomplete phenotypes as the remaining cells to be defined. The moment 3 measurement was used again to separate the incomplete from the ring phenotype, which had a higher moment 3 measurement than the incomplete phenotype (Figure 3.3 Civ).
Figure 3.3 3D quantification and method of automatic Lamin B phenotyping of siNEG and siA1 treated cells. (A-B) 3D renderings of Lamin B staining (white) were generated from a Z-stack of images. The normal phenotypes, ring and diffuse (A) are visually distinct from the abnormal phenotypes: punctate, internal, and incomplete (B). These images were used to quantify each of the Lamin B phenotypes. (C) Schematic demonstrating how the five different phenotypes were separated and assigned to generate a novel automated phenotyping script. Cells were examined based on different 3D measurements (graphs) to separate normal (green) and abnormal (dark blue) phenotypes. After separating out an individual phenotype, the remaining cells (tan) were assessed based on another measurement until all phenotypes could be mathematically grouped. Initially, the number of objects, which is the count of distinct, disconnected Lamin B objects in the cell, was used to define the punctate phenotype (i). Next, moment 3, a quantification of the 3D shape of Lamin B staining, was used to identify the internal phenotype (ii). The remaining population was examined based on flatness, which is the depth of the staining in the third (z) dimension and defined the diffuse phenotype (iii). The last two phenotypes, ring and incomplete could be separated based on their moment 3 measurement (iv). Scale bars=10 µm, n=3 replicates, Moment 3 graphs are two-tailed independent t-tests; Number of Objects and Flatness graphs are one-tailed independent t-tests. Data is normalized to the values in the previous step (connecting node in C) within replicates and plotted as the mean ± SEM with *p<0.05, **p<0.01.

3.1.3 The Novel and Manual Methods of Lamin B Phenotype Identification Are Equivalent

To validate our automated system, I generated three new replicates of siNEG and siA1 treated cells stained for Lamin B to analyze using our novel phenotyping program. The first replicate was used to compare the manual method of separating cells into normal and abnormal staining phenotypes and the computer-based phenotyping program. Here, I found no differences in the number of cells classified as normal or abnormal between the two methods (Figure 3.4A, B). The same trend, no differences between the manual or computer-based methods, was found when examining the separate treatment groups, siNEG (Figure 3.5A, B) and siA1 treated cells alone (Figure 3.5C, D). After confirming there were no differences between the manual and computer-based phenotyping methods, I used the latter to quantify the percentage of cells with Lamin B abnormalities in siNEG and siA1 treated cells (Figure 3.4C). I observed that significantly more cells in the siA1 treatment group demonstrated abnormal Lamin B staining compared to siNEG treated cells, thus confirming our previous observations using an unbiased methodology.
Figure 3.4 Quantification of the manual and computer-based phenotyping approaches. (A) Contingency table containing the phenotypes of an experiment of Lamin B-stained cells using manual phenotyping and the computer script to automatically assign phenotypes in both siNEG and siA1 treatment groups. The statistical test demonstrates that there are no differences between the two methods. (B) Graphical representation of data in (A), there are no differences comparing the normal and abnormal phenotype counts between the two quantification methods. A power analysis found that a sample size of 5817 is needed to achieve a power of 0.8 due to the small effect size. With a sample size that large any detected differences would be biologically irrelevant. The small effect size also indicates the certainty that the two groups are not different. (C) Quantification of Lamin B phenotypes using the computer script demonstrating a significant increase in the percent of cells with abnormal staining in siA1 treated cells. Data are plotted as the mean with n=1 replicate in (B) and the mean ± SEM with n=3 replicates in (C), one-tailed independent t-test, **p<0.01.
Figure 3.5 Contingency tables and graphs reveal no difference between the automatic and manual Lamin B phenotyping methods. (A, C) Contingency tables with the normal and abnormal phenotype counts of siNEG (A) and siA1 (C) Lamin B-stained cells. The statistical test demonstrates a lack of differences between the two methods in both conditions. (B, D) Illustration of data in (A, C) comparing the normal and abnormal phenotype counts between the two quantification methods in siNEG (B) and siA1 (D) treated cells. n=1 replicate.
3.1.4 hnRNP A1 Knockdown Significantly Alters the Nuclear Pore Complex Structure

To further substantiate our finding that hnRNP A1 knockdown alters the NPC, I investigated the phenotypes of additional proteins in the NPC. The normal and abnormal phenotypes were defined based on the presence of the phenotypes in the control and experimental groups separately for each protein. Nup98 staining was predominantly ring-shaped or diffuse in the nucleus in the control condition, making up the two normal Nup98 phenotypes (Figure 3.6A). A ring with cytoplasmic staining, an incomplete ring, staining at the cell membrane, and diffuse staining throughout the cell were the abnormal phenotypes (Figure 3.6B). The occurrence of abnormal Nup98 phenotypes significantly increased with hnRNP A1 knockdown (Figure 3.6C). Normal RanGAP1 phenotypes were ring, punctate on the nucleus and diffuse in the nucleus (Figure 3.7A). Abnormal phenotypes of RanGAP1 were punctate staining through the whole cell or cytoplasm, an incomplete ring of staining, and internal staining within the nucleus (Figure 3.7B). hnRNP A1 knockdown resulted in a significant increase in the percentage of abnormal phenotypes (Figure 3.7C). POM121 staining demonstrated two normal phenotypes, ring and diffuse nuclear (Figure 3.8A). Cytoplasmic staining with or without a ring around the nucleus, staining throughout the whole cell, internal staining, and incomplete ring staining were abnormal POM121 phenotypes (Figure 3.8B). The incidence of abnormal phenotypes in cells was significantly elevated with siA1 treatment (Figure 3.8C). There were three normal and two abnormal phenotypes of Nup214 staining. The normal phenotypes included ring staining, diffuse nuclear staining, and diffuse whole cell staining (Figure 3.9A). The abnormal phenotypes were an incomplete ring and cytoplasmic staining (Figure 3.9B). The presence of the abnormal phenotypes was the same in the siNEG and siA1 treatment groups (Figure 3.9C). While multiple NPC markers were altered, the phenotypes of all NPC proteins were not affected by hnRNP A1 knockdown.

In addition to assessing the visual appearance of NPC protein staining to evaluate the structure and composition of the NPC, protein expression was examined via Western blotting (Figure 3.10A). Treatment with siA1 resulted in an approximately three-fold downregulation of hnRNP A1, confirming the efficacy of siRNA (Figure 3.10D). I also found significant downregulation of Nup98 and RanGAP1 protein expression levels with hnRNP A1 knockdown (Figures 3.10B-C). These results further implicate the involvement of changes in the NPC in cells with dysfunctional hnRNP A1.
Figure 3.6 Abnormal phenotypes of Nup98 staining significantly increased with siA1 treatment. (A) Ring and nuclear Nup98 were the normal phenotypes. (B) There were four abnormal Nup98 phenotypes including ring & cytoplasmic staining, incomplete ring staining around the nucleus, Nup98 cell membrane localization and diffuse staining throughout the whole cell. Arrows point to the defining feature of the phenotype in (A) and (B). (C) Quantification of the normal and abnormal phenotypes in siNEG and siA1 treated cells. Data is graphed as the mean ± SEM. Scale bars=10µm, n=3 replicates, one-tailed independent t-test, **p<0.01.
Figure 3.7 RanGAP1 staining phenotypes showed a significant increase in abnormal phenotypes with hnRNP A1 knockdown. (A) Normal RanGAP1 staining included a ring of staining around the nucleus, nuclear punctate staining, and diffuse staining throughout the nucleus. (B) Abnormal RanGAP1 phenotypes included punctate staining throughout the whole cell and in the cytoplasm only, an incomplete ring of staining, and internal staining of RanGAP1. Arrows point to the defining characteristic of each phenotype. (C) Quantification of the abnormal phenotypes in both treatment groups. Data is graphed as the mean ± SEM. Scale bars=10 µm, n=3 replicates, one-tailed independent t-test, **p<0.01.
Figure 3.8 The prevalence of abnormal POM121 phenotypes significantly increased with siA1 treatment. (A) The two normal phenotypes of POM121 staining included a ring of staining around the nucleus and diffuse staining throughout the nucleus. (B) The abnormal phenotypes of POM121 included a ring with cytoplasmic staining, staining in the cytoplasm only, diffuse staining throughout the cell, incomplete ring staining, and internal staining in the nucleus. Arrows point to the defining aspect of each phenotype. (C) Quantification of the prevalence of the phenotypes in the control and experimental group. Data is graphed as the mean ± SEM. Scale bars=10 µm, n=3 replicates, one-tailed independent t-test, **p<0.01.
Figure 3.9 The prevalence of abnormal Nup214 phenotypes was the same between siNEG and siA1 treated cells. (A) A ring of staining around the nucleus, diffuse staining throughout the nucleus or whole cells were normal phenotypes of Nup214. (B) Abnormal phenotypes were an incomplete ring of staining around the nucleus or diffuse staining in the cytoplasm. Arrows point to the defining characteristic of each phenotype. (C) Quantification of the abnormal phenotypes in cells with A1 knockdown compared to control cells. Data is graphed as the mean ± SEM. Scale bars=10 µm, n=3 replicates, one-tailed independent t-test, ns=not significant.
Figure 10 Nup98 and RanGAP1 protein levels were significantly decreased with hnRNP A1 knockdown. (A) Western blots for Nup98, RanGAP1, β-Actin, and hnRNP A1 in siNEG and siA1 treated cells. Three individual replicates are shown for each condition (Rep1, Rep2, Rep3) with the left sample treated with siNEG and the right band treated with siA1 per replicate. (B) Quantification of hnRNP A1 band density levels confirming knockdown with siA1 treatment (C-D) Quantification of band density levels for the NPC proteins, RanGAP1 (C) and Nup98 (D) from (A) normalized to β-actin. Data is plotted as the mean ± SEM, n=3 replicates, paired t-test *p<0.05, **p<0.01.
3.2 Structural Changes in the Nuclear Pore Complex Impact Nucleocytoplasmic Transport

Nucleocytoplasmic transport is the predominant and most important function of the NPC as it is responsible for RNA export to synthesize new proteins and protein import to maintain homeostasis. To investigate the function of the NPC, active nucleocytoplasmic transport was assessed.

3.2.1 hnRNP A1 Knockdown Alters Gene Expression Related to Nucleocytoplasmic Transport

hnRNP A1 dysfunction, a phenotypic characteristic of MS brain, altered the expression of over a thousand genes in differentiated Neuro-2a cells treated with siRNA targeting hnRNP A1 (siA1) in a previous study. Significantly altered transcripts included those involved in the NPC and nucleocytoplasmic transport (Appendix A). Differentially expressed genes were newly analyzed using GO analysis across four different databases: GO biological process, GO cellular component, GO molecular function, and KEGG, which identified hundreds of significantly enriched pathways. Specifically, I found that pathways related to the nuclear envelope, matrix, pore complex, and nucleocytoplasmic transport were highly prevalent in the top 20 pathways across the databases (Figure 3.11). In total, GO biological process analysis yielded 62 pathways related to the NPC and nucleocytoplasmic transport. Therefore, I further investigated nucleocytoplasmic transport in the context of MS using a protein transport assay across the nuclear envelope.

3.2.2 hnRNP A1 Knockdown Alters Active Nucleocytoplasmic Transport

Loss of hnRNP A1 function leads to alterations in the structure of the NPC, however, structural changes are not always indicative of functional changes. Therefore, I first examined functional changes associated with the NPC by investigating active nucleocytoplasmic transport, which was achieved by performing co-transfection of siRNA with a plasmid, Shuttle-tdTomato (S-tdTomato) containing an NLS, an NES, and a fluorescent marker (Figure 3.12A). The S-tdTomato construct is continuously shuttled between the nucleus and cytoplasm through active transport. The NLS is more potent than the NES and therefore, more plasmid was expected to be found in the nucleus under control conditions. Cells were treated with a nuclear import inhibitor (importazole) and nuclear export inhibitor (leptomycin B) to visualize both cytoplasmic and nuclear accumulation of the S-tdTomato plasmid to establish baseline cytoplasmic and nuclear accumulation of the plasmid (Figure 3.12B, C). Cells treated with siNEG demonstrated
predominantly nuclear localization of the S-tdTomato plasmid quantitated by a high nuclear-to-cytoplasmic fluorescence ratio (Figures 3.12D, E, G). Conversely, treatment with siA1 caused a significant increase in cytoplasmic localization of the plasmid, indicated by a decrease in the nuclear-to-cytoplasmic fluorescence ratio (Figures 3.12E-G). This indicates that hnRNP A1 knockdown driven structural alterations in the NPC also have a functional consequence in that active nucleocytoplasmic transport across the nuclear envelope is compromised.

Figure 3.11 hnRNP A1 knockdown in cells altered mRNA transcripts related to pathways of nucleocytoplasmic transport and nuclear structure. Bubble plot of significantly altered pathways related to the nuclear structure, NPC, and nucleocytoplasmic transport identified in the top 20 pathways in different databases. The size of the dots is the number differentially expressed genes in the pathway and colours correspond to different databases.
Figure 3.12 hnRNP A1 knockdown leads to deficits in active nucleocytoplasmic transport. 
(A) Overview of the S-tdTomato plasmid transfected into cells. (B) siNEG cells treated with a nuclear import inhibitor (Importazole, Imp) to visualize cytoplasmic accumulation of the plasmid. Arrowheads indicate cytoplasmic localization of the S-tdTomato plasmid. (C) siNEG cells treated with a nuclear export inhibitor (Leptomycin b, Lmb) to visualize nuclear accumulation of the plasmid. (D) High power view of the S-tdTomato plasmid in siNEG treated cells. Dotted lines outline the nucleus, created using DAPI staining. (E) Cells were co-transfected with siNEG or siA1 and the S-tdTomato plasmid. Quantification of tdTomato fluorescence was measured in the nucleus and cytoplasm using the corresponding DAPI and beta-tubulin images to outline the nucleus and cytoplasm, respectively. (F) High power view of the S-tdTomato plasmid in siA1 treated cells. Dotted lines outline the nucleus, which were created using the corresponding DAPI image. Arrowheads indicate cytoplasmic localization of the S-tdTomato plasmid. (G) Quantification of the nuclear to cytoplasmic ratio of tdTomato signal showing a significant decrease in the nuclear/cytoplasmic ratio in cells treated with siA1. Scale bars=20 µm, n=3 replicates. Data are plotted as mean ± SEM. One-tailed independent t-test, **p<0.01.

3.2.3 Nuclear Envelope Integrity Remains Intact With hnRNP A1 Knockdown

To assess the integrity of the nuclear envelope following hnRNP A1 knockdown, I used a different plasmid that contained two NLS sequences, 2xNLS-tdTomato, and performed co-transfection with siNEG or siA1 (Figure 3.13A). Because this plasmid contains two NLS sequences, it was expected that the plasmid would be nuclear under normal conditions where the nuclear envelope is intact. As a positive control, digitonin was used at both a low concentration (20µg for 4 minutes) that would not compromise the nuclear envelope and a high concentration (40µg for 10 minutes) to permeabilize the nuclear envelope. In control cells treated with a low level of digitonin, the plasmid was predominantly located within the nucleus, as expected (Figure 3.13B). Cells treated with the higher concentration of digitonin displayed cytoplasmic expression of the plasmid, demonstrating that large holes in the nuclear envelope cause the plasmid to leak out of the nucleus (Figure 3.13C). The plasmid appeared exclusively nuclear in cells treated with siNEG or siA1 despite hnRNP A1 knockdown (Figure 3.13D-F). The ratio of the plasmid in the nucleus to the cytoplasm was similar between the two treatment groups (Figure 3.13G), indicating that hnRNP A1 knockdown does not completely compromise the nuclear envelope integrity.
Figure 3.13 The nuclear envelope is not compromised by siA1 treatment. (A) Overview of the 2xNLS-tdTomato plasmid transfected into cells. (B) Cells treated with a low concentration of digitonin (20 µg for 4 minutes) to permeabilize the plasma membrane only. (C) Neuro-2a cells treated with high concentration of digitonin (40 µg for 10 minutes) to permeabilize the nuclear envelope and cause plasmid leakage into the cytoplasm. (D) Zoomed in section of 2xNLS-tdTomato in siNEG treated cells. Dotted lines outline the nucleus, created using DAPI staining. (E) Representative images of cells co-transfected with 2xNLS-tdTomato with two NLS sequences. Quantification of 2xNLS-tdTomato fluorescence was measured using DAPI to outline the nucleus and Beta-tubulin III to outline the cytoplasm. (F) Zoomed in section of 2xNLS-tdTomato in siNEG treated cells. Dotted lines outline the nucleus, created using DAPI staining (G) Quantification of the nuclear to cytoplasmic ratio of 2xNLS-tdTomato signal showing no difference between siNEG and siA1 treated cells. Scale bars (white) = 20 µm, n=3 replicates, one-tailed independent t-test, ns=not significant.
3.3 Nuclear Structure Alterations Are Present in MS Tissue Establishing Clinical Relevance

The *in vitro* findings establish the impact of hnRNP A1 dysfunction on the NPC and nucleocytoplasmic transport. Because hnRNP A1 dysfunction is a feature of neurons from MS, I sought to validate my findings of NPC alterations *in situ* using human brain samples. Therefore, Lamin B was assessed in postmortem control and MS brain tissue to determine whether NPC alterations are found in a disease with hnRNP A1 dysfunction.

3.3.1 Lamin B is Significantly Altered in Neurons from MS as Compared to Healthy Controls

Next, I examined whether there were NPC alterations in MS brains as previous publications have demonstrated neuronal hnRNP A1 dysfunction\textsuperscript{94,114}. Human brain tissue samples were age-matched, and there were no differences in sex between healthy control and MS samples (Table 3.1). In control tissue, Lamin B predominantly formed a ring of staining around the neuronal nuclei with diffuse staining within the nucleus (Figure 3.14A, B), similar to what was observed *in vitro*. This normal ring phenotype was also present in MS tissue (Figure 3.14A, B). A smaller portion of neurons from control tissue demonstrated abnormal Lamin B phenotypes, including an incomplete ring of staining around the nucleus and Lamin B staining internal to the nuclear ring. However, in MS cases, the occurrence of the two abnormal Lamin B staining phenotypes was significantly increased compared to controls (Figure 3.14C).

<table>
<thead>
<tr>
<th>Table 3.1 Summary of postmortem tissue characteristics</th>
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<tbody>
<tr>
<td>Age (mean ± SD)</td>
</tr>
<tr>
<td>Control: 53.25 ± 3.775</td>
</tr>
<tr>
<td>MS: 54.58 ± 7.489</td>
</tr>
<tr>
<td>P-value: 0.7415</td>
</tr>
<tr>
<td>Postmortem delay in hours (mean ± SD)</td>
</tr>
<tr>
<td>Control: 6.773 ± 2.192</td>
</tr>
<tr>
<td>MS: 7.820 ± 2.650</td>
</tr>
<tr>
<td>P-value: 0.4900</td>
</tr>
<tr>
<td>Sex (Male, female, unknown)</td>
</tr>
<tr>
<td>Control: 3,1,1</td>
</tr>
<tr>
<td>MS: 6,6,0</td>
</tr>
<tr>
<td>Fisher's Exact test P-value</td>
</tr>
<tr>
<td>Control: -</td>
</tr>
<tr>
<td>MS: 0.5846</td>
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Figure 3.14 Lamin B was significantly altered in post-mortem MS tissue compared to controls. (A) Control and MS gray matter stained for Lamin B (brown) and hematoxylin (blue). Arrows point to abnormal phenotypes. (B) Higher magnification illustrating normal Lamin B phenotypes from control and MS tissue and abnormal Lamin B phenotypes from MS tissue in neurons. Arrows point to the defining feature of the phenotypes. (C) Quantification of abnormal
Lamin B phenotypes in neurons. Data is the mean ± SEM. Scale bars=10 µm, n=5 controls and n=12 MS cases, two-tailed independent t-test, *p<0.05.
CHAPTER 4: Discussion

Alterations in the NPC and nucleocytoplasmic transport have been shown to contribute to the pathogenesis of multiple neurodegenerative diseases, including ALS, FTD, HD and Alzheimer’s disease\textsuperscript{172,173,179,192,193}. Dysfunctional RBPs are also present in these diseases and MS, where it is implicated in neuronal damage. Alterations in the NPC and nucleocytoplasmic transport and dysfunctional RBPs are closely related, can exacerbate each other, and negatively affect cell viability. Data presented in this thesis shows for the first time that NPC and nucleocytoplasmic transport alterations are present in MS and an \textit{in vitro} model of disease, which can potentially contribute to dysfunctional RBPs, perturbed RNA metabolism, and neuronal damage.

4.1 Loss of hnRNP A1 Function is Sufficient to Perturb the NPC and Nuclear Envelope

Here, I used a previously validated siRNA sequence to knockdown hnRNP A1 and examined the effects on the NPC and nuclear envelope\textsuperscript{116}. For each experiment, I confirmed decreased hnRNP A1 expression via Western blotting or immunofluorescence and then quantified the number of cells with abnormal staining of specific NPC markers and Lamin B, as in previous studies\textsuperscript{155,166,186,187,191}. Lamin B, an ancillary marker of the nuclear envelope, normally presented with a ring or diffuse phenotype. However, with siA1 treatment, there was a significant increase in abnormal phenotypes, which could largely be attributed to an increase in the presence of internal and incomplete phenotypes. Interestingly, the punctate phenotype did not increase with hnRNP A1 knockdown, suggesting that it may demonstrate nuclear envelope budding or cell division\textsuperscript{194–197}. All five phenotypes (ring, diffuse, internal, incomplete, punctate) were then used to generate 3D images of Lamin B staining.

In 3D, the normal Lamin B phenotypes, diffuse and ring, showed the following characteristics. The diffuse phenotype revealed a dome of staining around the nucleus, which was flat on the side where the cell rests on the slide. The ring phenotype showed defined Lamin B staining around the cell without internal staining. The abnormal phenotypes were similar in two dimensions as in 3D. The 3D images enabled the creation of a novel method to automatically identify Lamin B phenotypes to reduce bias, save time, and provide uniformity across the research field. A computer script was created to process the data to simplify and accelerate data processing for large experimental datasets. The script is available open-source on GitHub (https://github.com/tes465/LaminB-ID) to allow other researchers to utilize the new method. The
output of the script included identifying phenotypes for each individual cell, total counts for each phenotype, and the percent of abnormal phenotypes in each treatment group. The program first analyzes and defines phenotypes from the control group. Then, based on measurements from the control group, the program analyzes and phenotypes an experimental group. Individual phenotypes are identified using specific measurements that show significant differences between individual phenotypes thus ensuring that the measurement reliably identifies the correct phenotype. There are several caveats to the program script. For example, one assumption of the program is that there is a large population of normal cells in the control group. If approximately greater than 75% of the cells in the control group are abnormal, the script would inaccurately assign phenotypes. However, this issue can be avoided by using a proper negative control where the nuclear envelope and Lamin B staining are unaltered. Further, this method and computer script were optimized to analyze Lamin B staining. Similar optimization steps would need to be performed in order to apply this approach to automatically phenotype other protein markers of the NPC.

After establishing the computer-based phenotyping method, I compared it to the manual method and found no significant difference between the two methods in either the siNEG or siA1 groups, demonstrating the accuracy of the method in both conditions. This novel method might be slightly biased towards identifying cells as normal, even when they are abnormal, which is supported by the slightly higher count of normal cells with the novel method compared to the manual method. (Figure 3.4A-B, Figure 3.5). After confirming that there were no differences in quantification between the manual and novel methods, the latter was applied to three new independent experiments. The novel method identified a significant increase in the presence of abnormal Lamin B in cells treated with siA1 compared to siNEG, paralleling the manual quantification, and demonstrating the reliability of the method.

In addition to examining changes in Lamin B phenotypes, I investigated several other NPC proteins to determine the effect of hnRNP A1 knockdown. I selected markers from different substructures within the NPC to achieve an overview of the NPC structure. Nup98, found on the outer rings and central channel, faces the pore and is responsible for forming the permeability barrier\textsuperscript{135}. POM121 is a structural NPC protein located in the outer portion of the transmembrane ring in the nuclear envelope\textsuperscript{127,128,138}. RanGAP1 is located in the cytoplasm and the cytoplasmic ring and filaments of the NPC, along with Nup214\textsuperscript{127,128,138}. RanGAP1 plays a major role in active nucleocytoplasmic transport, while Nup214 provides structure to the cytoplasmic filaments\textsuperscript{139}. 

56
While the specific phenotypes of all the markers analyzed differed slightly, the normal and abnormal phenotypes can be generalized. The normal phenotypes were ring shaped or had exclusive nuclear staining. The abnormal phenotypes belonged to three main groups: cytoplasmic staining, an incomplete ring, or internal nuclear staining. The abnormal phenotypes of POM121, Nup98, and RanGAP1 were all significantly increased with hnRNP A1 knockdown.

There are several possible explanations for the appearance of abnormal phenotypes. For example, incomplete phenotypes may represent an uneven distribution of the NPC on the nuclear envelope. Uneven distribution and clustering of NPCs have been observed in cells with decreased Lamin expression\textsuperscript{198}. Cells with loss of Lamin B staining in a specific area of the nuclear envelope may indicate a loss of the NPC within the same area of the nuclear envelope. Further, incomplete phenotypes might demonstrate a rupture of the nuclear envelope. Abnormal internal staining phenotypes could represent the removal and translocation or mislocalization of specific NPC proteins or the entire defective NPC from the nuclear envelope. The identified cytoplasmic phenotypes may represent mislocalization of NPC proteins, which can occur with RBP dysfunction, indicative of potential nucleocytoplasmic transport alterations.

The NPC proteins which showed significant alterations with hnRNP A1 knockdown (POM121, Nup98, and RanGAP1) represent the three main functions of proteins in the NPC – providing scaffold support for NPC structure, establishing the permeability barrier, and allowing active nucleocytoplasmic transport. Interestingly, Nup98 and RanGAP1 protein levels were also significantly decreased demonstrating a relationship between NPC phenotype abnormalities and overall NPC protein abundance. This further supports that hnRNP A1 loss of function alters the overall structure of the majority of NPCs both through phenotype and levels of necessary proteins. However, the lack of increased abnormal Nup214 staining demonstrates that not all proteins of the NPC are affected by siA1 treatment. Thus, while the specific composition of some of the NPC proteins is altered, the NPC likely retains some of its overall structure and function as a complete loss of nuclear import or export leads to immediate cell death\textsuperscript{199}.

The nuclear envelope was significantly altered by hnRNP A1 knockdown, as demonstrated by Lamin B staining. The Lamin B phenotypes provided evidence that the NPC could also be affected by siA1 treatment. However, it did not provide direct, concrete evidence of NPC alterations. Specific proteins that constitute part of the NPC were assessed to examine the structure of the NPC. The levels of the NPC proteins assessed were decreased, indicating degradation of the
NPC and the specific protein components. The visual appearance of the NPC proteins was also disrupted. Together, these findings provide evidence that the structure of the NPC is altered due to the loss of hnRNP A1. However, the mechanism of the structural changes to the NPC is unclear. Structural changes in the NPC alone are not important if they do not impact cellular functions. Therefore, the next steps of this project were to investigate a potential mechanism of hnRNP A1 dysfunction-induced changes in the NPC and assess active nucleocytoplasmic transport.

4.2 Active Nucleocytoplasmic Transport is Altered Affecting Protein Localization

The model system used in this thesis, siRNA mediated knockdown of hnRNP A1 in vitro, was previously established in our lab\textsuperscript{116}. Loss of hnRNP A1 function was found to alter RNA abundance in cells leading to neuronal damage and cell death, but the exact mechanism of how hnRNP A1 knockdown leads to death was not fully elucidated. RNA sequencing data from cells with hnRNP A1 knockdown revealed thousands of differentially expressed genes between cells treated with siNEG and siA1\textsuperscript{116}. I utilized the differentially expressed gene list to perform GO analysis to identify enriched pathways significantly affected by hnRNP A1 knockdown. Using the top 20 pathways identified in four databases, I found numerous pathways related to the NPC and nucleocytoplasmic transport, emphasizing the importance of hnRNP A1 in these processes. For example, the GO biological process database analysis identified more than 60 pathways related to nucleocytoplasmic transport and six of these pathways were included in the top 20, demonstrating that the NPC function was significantly and highly affected by hnRNP A1 knockdown.

Given the GO analyses and observed structural changes in the NPC, I then investigated the main function of the NPC, which is active nucleocytoplasmic transport. To study active nucleocytoplasmic transport, I used a plasmid containing a fluorescent protein with a NLS and NES, called S-tdTomato. The protein undergoes continuous transport between the nucleus and cytoplasm and has previously been used to assess active nucleocytoplasmic transport\textsuperscript{153,161,166,200}. Here, I found that hnRNP A1 knockdown significantly altered nucleocytoplasmic transport, as there was an increase in tdTomato fluorescence in the cytoplasm with siA1 treatment compared to control. This is indicative of mislocalization of the plasmid, which contains both a NLS and NES, like RBPs, due to hnRNP A1 dysfunction. Therefore, it is likely that RBPs that undergo the same mechanism of nuclear import and export can mislocalize. TDP-43, which is mislocalized in MS, undergoes a similar form of nucleocytoplasmic transport\textsuperscript{114,201}. It is plausible that hnRNP A1 knockdown-induced alterations in nucleocytoplasmic transport leads to the mislocalization of
additional proteins, including TDP-43, causing RBP dysfunction and subsequent cell death and neurodegeneration.

There are a few explanations that could account for altered nucleocytoplasmic transport and cytoplasmic accumulation of the tdTomato construct following hnRNP A1 knockdown. The permeability of the NPC may be increased, allowing larger molecules to passively cross the nuclear envelope. This would cause the tdTomato plasmid to leak out of the nucleus and into the cytoplasm, resulting in its cytoplasmic accumulation. Increases in the passive permeability of the NPC have been associated with abnormal Lamin B staining, damage to the NPC and neurodegenerative diseases\textsuperscript{193,202–204}. Another potential justification for the cytoplasmic accumulation of tdTomato plasmid is that nuclear import is decreased due to a blockage in the NPC. However, an obstruction in the NPC is unlikely as the NPC is resistant to clogging even in the presence of increased transport proteins\textsuperscript{205}. Active nucleocytoplasmic transport itself may be directly affected through alterations to the Ran gradient or transport molecules. Upregulation of nuclear import proteins or decreasing nuclear export has been observed to reduce the toxicity associated with dysfunctional RBPs, supporting the potential that hnRNP A1 knockdown directly affects nucleocytoplasmic transport\textsuperscript{166,206,207}. Another potential reason for changes in nucleocytoplasmic transport is that the integrity of the nuclear envelope is compromised. Interestingly, nuclear envelope ruptures occur in neurodegenerative diseases that also exhibit alterations in the nuclear lamina and the incomplete Lamin B and NPC staining phenotypes observed here\textsuperscript{123,208}.

The integrity of the nuclear envelope was assessed in this study due to the likelihood that it could be disrupted due to hnRNP A1 knockdown. The 2xNLS-tdTomato protein was used as it should be exclusively located in the nucleus. A low digitonin control was used to permeabilize the cytoplasmic membrane and confirmed that the location of the 2xNLS-tdTomato was exclusively nuclear. The high digitonin treatment permeabilized the nuclear envelope and was used as a positive control to visualize leakage of the plasmid to the cytoplasm with a compromised nuclear envelope. In both the siNEG and siA1 conditions, the plasmid was nuclear, demonstrating that the nuclear envelope remained intact. Although Lamin B staining demonstrated that the nuclear envelope structure was altered by hnRNP A1 knockdown, it remained intact even in cells with incomplete nuclear staining phenotypes of Lamin B and the other NPC proteins assessed. As the nuclear envelope is not ruptured, the incomplete Lamin B and NPC phenotypes likely demonstrate that the distribution of the NPC becomes uneven and non-homogenous. Uneven NPC distribution
is associated with loss of Lamins, specifically Lamin B, but the consequences and impact of uneven distribution are unknown$^{198,209}$.

The exact process of how hnRNP A1 loss of function leads to alterations in the NPC and nucleocytoplasmic transport is unclear. While the RNA sequencing data indicates that NPC and nucleocytoplasmic transport are affected by hnRNP A1 loss of function, it also suggests a potential mechanism. The RNA targets of many RBPs have been established by ultraviolet crosslinking and immunoprecipitation followed by RNA sequencing (CLIPseq) experiments. Much of this data has been collected and compiled into a CLIPseq experiment database known as CLIPdb$^{210}$. According to CLIPdb, hnRNP A1 binds and regulates many RNA transcripts that code for proteins of the NPC and nucleocytoplasmic transport$^{210}$. Knockdown of hnRNP A1 changed the abundance of transcripts that code for the NPC and proteins involved in nucleocytoplasmic transport (Appendix A)$^{116}$. Therefore, a rational explanation is that hnRNP A1 knockdown affects the synthesis of new proteins involved in the NPC and transport by modulating the underlying RNA transcripts. Changes in NPC protein levels then lead to changes in the structure and function of the NPC. Several notable changes in the function of the NPC could include increases in the permeability barrier due to decreased synthesis of proteins in the central channel of the NPC responsible for forming the barrier, changes in active nucleocytoplasmic transport through altered synthesis of nuclear transport proteins, or both. This study proves that loss of hnRNP A1 function affects the NPC and nucleocytoplasmic transport, which is known to negatively affect cells and may correlate with the pathogenesis of neurodegenerative diseases.

Alternatively, hnRNP A1 is an essential protein to cellular functioning. Loss of hnRNP A1 is known to negatively impact the health of neuronal cells and increase cytotoxicity$^{116}$. However, that study did not delve into the mechanism of how the loss of hnRNP A1 function mechanistically leads to cellular toxicity consequences on the cell. This study provides evidence that hnRNP A1 knockdown alters the structure and function of the NPC, which is involved in neurodegenerative diseases and has harmful effects. Altered nucleocytoplasmic transport observed caused a predominantly nuclear protein (tdTomato), which undergoes continuous active nucleocytoplasmic transport, to become mislocalized to the cytoplasm. It is likely that other proteins that undergo nucleocytoplasmic transport, including other RBPs, become mislocalized and cannot function properly. The mislocalization of RBPs to the cytoplasm can induce the formation of cytoplasmic protein inclusions, which can lead to the mislocalization of Nups and transport proteins into the
inclusions, causing damage to the NPC\textsuperscript{146,153,154}. Together, dysfunction of the NPC, nucleocytoplasmic transport and multiple RBPs would severely alter RNA metabolism, including RNA processing and stability, having a widespread impact on cells.

RNA metabolism could also be affected if RNA transcripts become trapped in the nucleus because of changes in nucleocytoplasmic transport. Nuclear accumulation of RNA is a consequence of alterations in the nuclear envelope, nucleocytoplasmic transport, and cytoplasmic protein aggregation and contributes to neurodegeneration\textsuperscript{156,165,211,212}. Furthermore, bulk accumulation of mRNA in the nucleus further affects RNA metabolism. For instance, hnRNP A1 is known to bind and regulate thousands of RNA transcripts. Approximately 10\% of the altered RNA transcripts affected by hnRNP A1 knockdown in the RNA sequencing dataset were not known to bind hnRNP A1\textsuperscript{116,210}. Dysfunction of additional RBPs and nuclear RNA accumulation could explain how hnRNP A1 knockdown affects these additional genes in that they are indirectly dysregulated following hnRNP A1 loss. Altered RNA metabolism likely also affects protein synthesis. Widespread changes in protein expression due to issues with target RNA transcripts are a potential mechanism of how hnRNP A1 knockdown eventually results in cell death. Therefore, changes in the nuclear envelope, NPC, and nucleocytoplasmic transport caused by hnRNP A1 knockdown are a possible mechanism of how hnRNP A1 loss leads to neuronal damage and cytotoxicity.

4.3 Changes in the Nuclear Envelope Are Recapitulated in Multiple Sclerosis

Although hnRNP A1 is dysfunctional in MS and other neurodegenerative diseases, the model of hnRNP A1 dysfunction used in this thesis may not completely recapitulate human disease. Therefore, I analyzed Lamin B phenotypes in human MS tissue to determine whether my \textit{in vitro} findings could be validated in humans. Lamin B staining was used as it provides a readout of overall nuclear structure, binds to the NPC and was indicative of NPC and nucleocytoplasmic transport alterations \textit{in vitro}. Control tissue exhibited normal staining phenotypes of Lamin B, which were similar to those observed in the \textit{in vitro} experiments. For example, Lamin B was primarily nuclear and provided a clear outline of the nuclear envelope thus recapitulating the ring and diffuse phenotypes observed \textit{in vitro}. Internal and incomplete phenotypes of Lamin B were also present in human tissues, reflecting two of the abnormal Lamin B phenotypes that were significantly increased in cells with hnRNP A1 knockdown. While abnormal Lamin B staining was present in control tissue, this was expected since the nuclear envelope and NPC can be affected by
aging\textsuperscript{145,146,213}. However, we still observed a significant increase in abnormal Lamin B phenotypes between MS and control tissue in age-matched samples ensuring that the significant difference in abnormal phenotypes was not due to age. This result supports the validity of the \textit{in vitro} model used, demonstrates the relevance of the findings, and signifies that the nuclear envelope, the NPC, and nucleocytoplasmic transport are altered in MS. As the analysis was blinded, the MS tissue was analyzed indiscriminately, leading to inflammatory lesions and normal appearing gray matter both being analyzed. However, current research in our lab has shown that hnRNP A1 dysfunction is present throughout the MS cortex and therefore we expected abnormal Lamin B staining throughout the cortex as well.

In this study, hnRNP A1 knockdown was used to model the loss of nuclear hnRNP A1 and its dysfunction, which is observed in MS and other neurodegenerative diseases. Knockdown of hnRNP A1 altered RNA metabolism and abundance, and GO analysis identified pathways related to the NPC and nucleocytoplasmic transport. Interestingly, RNA sequencing of MS tissue and an animal model of MS, which both exhibit hnRNP A1 dysfunction, has also identified significantly altered pathways related to molecular transport\textsuperscript{82}. This provides evidence that similar mechanisms underlie hnRNP A1 dysfunction across multiple model systems, including the \textit{in vitro} system used here. Evidence in this thesis also pointed towards potential changes in nucleocytoplasmic transport, which has never been shown in MS. Therefore, these findings are important to MS research as processes uncovered here likely affect MS patients. Changes in nucleocytoplasmic transport, present in other neurodegenerative diseases, are believed to be involved in the pathogenesis of disease.

In neurodegenerative diseases, nucleocytoplasmic transport becomes altered, causing RBP mislocalization, nuclear RNA accumulation, altered protein synthesis, cellular damage, and neurodegeneration. Some studies have investigated the potential of therapeutics to target this mechanism to decrease cell death in neurodegenerative diseases. Upregulating a single NPC protein rescues other abnormal NPC proteins and reestablishes active nucleocytoplasmic transport in a model of ALS\textsuperscript{152}. Modulating the expression of nuclear transport receptors can also remedy mislocalization associated with altered nucleocytoplasmic transport and reduce toxicity\textsuperscript{166,206,207}. As similar processes are occurring in MS, these treatment methods have the potential to repair the NPC and nucleocytoplasmic transport in MS and represent a novel therapeutic avenue to treat MS and reduce neurodegeneration.
CHAPTER 5: Conclusions and Future Directions

MS is an autoimmune disease of the CNS in which demyelination and neurodegeneration contribute to disease pathogenesis. Current MS treatments modulate the immune system, effectively reduce relapses, and delay the progression from RRMS to SPMS. However, neurodegeneration still occurs, increasing disability in MS patients, and the treatment options for progressive forms of MS are limited. Identifying mechanisms of neuronal death in MS is essential to understanding the processes occurring, identifying new treatment methods, and designing new therapeutics to reduce and prevent neurodegeneration.

Dysfunctional RBPs and NPC and nucleocytoplasmic transport alterations are involved in multiple neurodegenerative diseases, including HD, ALS, and FTD\textsuperscript{165,166,168,172,173,177}. Dysfunctional RBPs have also been recently discovered in MS and relevant models, where dysfunction correlates with markers of neurodegeneration and neuronal loss\textsuperscript{60,114,115}. The hallmarks of dysfunctional RBPs include mislocalization to the cytoplasm, the formation of protein inclusions, and loss of nuclear staining. Loss of nuclear staining leads to a loss of hnRNP A1 function, which can be modelled by its knockdown\textsuperscript{116}. Here, I used knockdown of hnRNP A1 to model its dysfunction in MS and other neurodegenerative diseases and investigated the structure of the nuclear envelope and the NPC as well as functional nucleocytoplasmic transport.

First, I assessed Lamin B staining in cells with hnRNP A1 knockdown as an overview of nuclear envelope structure and the NPC. The nuclear envelope was significantly perturbed, which could be visually observed in both two and three dimensions (Figure 5.1). I then created a novel method to automatically phenotype Lamin B staining patterns \textit{in vitro}. This computer script can improve the research field by reducing bias and time and creating uniform classifications of Lamin B staining patterns. Future studies can tailor the method so that it may be applied to \textit{in vivo} animal models to further understand changes in the NPC in more disease-relevant systems. Additional studies can also adopt the method to phenotype other markers of the NPC in addition to Lamin B, making the method more widely applicable to different research studies.

Next, I investigated specific markers of the NPC to confirm whether the structure of the NPC itself was altered by hnRNP A1 knockdown. Abnormal phenotypes of POM121, RanGAP1, and Nup98 were significantly increased with loss of hnRNP A1. The abnormal phenotypes demonstrated the potential of mislocalization to the cytoplasm and uneven distribution of the NPC on the nuclear envelope (Figure 5.1). The protein levels of RanGAP1 and Nup98 were also
significantly decreased with siA1 treatment, supporting the possibility that the function of the NPC is altered as Nup98 plays a role in the permeability barrier and RanGAP1 plays a role in active nucleocytoplasmic transport. Future experiments should investigate whether upregulating one or multiple of the NPC proteins is sufficient to rescue the function of the NPC and reduce the cytotoxic effects of hnRNP A1 knockdown.

Multiple pathways related to the NPC and nucleocytoplasmic transport were identified by GO analysis of differentially expressed genes from cells with hnRNP A1 knockdown. This finding supported the conclusion that loss of hnRNP A1 affects the structure of the NPC and led to further investigation into the function of the NPC following hnRNP A1 knockdown. The active nucleocytoplasmic transport assay demonstrated mislocalization of the target fluorescent protein to the cytoplasm. This highlights the role that hnRNP A1 knockdown plays in facilitating functional changes in the NPC by altering active nucleocytoplasmic transport (Figure 5.1). We then examined whether hnRNP A1 knockdown caused nuclear envelope rupture as suggested by the incomplete staining phenotypes. Despite observable phenotypic changes in the nuclear envelope, this assay revealed that the nuclear envelope was still intact following loss of hnRNP A1. Together, the results suggest a mechanism whereby hnRNP A1 knockdown leads to altered RNA abundance of NPC and nuclear transport targets, affecting protein levels and distribution, and subsequent NPC functions. Future research should further explore this mechanism to determine whether nuclear transport proteins affect RBP localization, leading to the formation of protein inclusions and NPC damage, whether the NPC permeability is affected by decreased protein synthesis leading to leakage of proteins into the cytoplasm, or both. This can be achieved by Western blotting for nuclear transport proteins to determine whether hnRNP A1 knockdown leads to decreased protein expression or by differential centrifugation to confirm the localization of target proteins. Another model of hnRNP A1 dysfunction, in which transfection of hnRNP A1 with mutations contained within the M9 transport region or prion like domain could be used in the future to determine whether cytoplasmic mislocalization of hnRNP A1 also leads to a defective NPC and nucleocytoplasmic transport.

Lastly, the clinical relevance of the findings was established by staining MS and control brain tissue for Lamin B. Abnormal Lamin B phenotypes were significantly increased in MS tissue, supporting the validity of the *in vitro* model and the possibility that the NPC and active
nucleocytoplasmic transport are altered in MS. Future research is needed to confirm these possibilities by assessing additional markers of the NPC and transport in MS tissue.

This research is the first to specifically investigate the NPC and nucleocytoplasmic transport in the context of MS. Alterations in the NPC and nucleocytoplasmic transport are known to be involved in other neurodegenerative diseases where they contribute to cell death. Identifying how altered nucleocytoplasmic transport contributes to MS pathogenesis can uncover new mechanisms of neurodegeneration and disease progression. Further elucidating the cause of NPC and nucleocytoplasmic transport alterations may lead to the discovery of new treatment targets and therapeutics to reduce and prevent neurodegeneration in MS and other neurologic diseases.
Figure 5.1 Summary of the main conclusions of the thesis. hnRNP A1 knockdown resulted in an abnormal nuclear envelope structure, which was also observed in neurons in the brains of MS patients. The distribution of the NPC became uneven on the nuclear envelope, where the NPC was absent in specific areas. Nucleocytoplasmic transport was dysfunctional, shown by the cytoplasmic mislocalization of a protein that underwent continuous transport between the nucleus and cytoplasm. Made in BioRender.
CHAPTER 6: References


## Appendix A

### Table A.1
Select Differentially expressed genes identified by RNA sequencing in cells with hnRNP A1 knockdown.

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