# Mutations in Kinesin 5A effect on Mitochondrial Mobility in Amyotrophic Lateral Sclerosis & Hereditary Spastic Paraplegia

**A MAJOR QUALIFYING PROJECT**
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**Abstract**
Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease with no cure. Kinesin 5A (KIF5A), a molecular motor, has been identified as a novel gene associated with ALS, specifically a mutation in the C-terminal cargo binding domain. Mutations in the N- terminus motor head domain of KIF5A cause hereditary spastic paraplegia (HSP). Mitochondria is a common cargo of KIF5A. Mutations involving mitochondria transport are often correlated with neurodegenerative diseases. This project aimed to investigate the effects of different KIF5A mutations on mitochondrial mobility. We found increased motility in ALS mutant motor neurons and reduced motility in HSP mutants compared to $KIF5A^{WT}$cells, suggesting opposite mechanisms of action for these diseases.

**Acknowledgments**

I would like to thank Claudia Fallini for her all the guidance and support throughout this project as well as all the members of the Landers Lab for helping me throughout my time at UMMS. Thank you to Professor Manning for guidance throughout the duration of the MQP project. Thank you to the Biology and Biotechnology Department at WPI for funding transportation to and from UMMS.

## Introduction

*The Neuromuscular System*

The neuromuscular system is essential for movement of the body, breathing, posture, and many additional functions. This system is composed of cortical (or upper) and spinal (or lower) motor neurons, supportive cells such as glial cells, and muscle fibers. A motor neuron and the skeletal muscle fibers innervated by that motor neuron axonal terminals make up a motor unit which coordinate the contraction of a single muscle. Signals are sent throughout the body by electrical impulses triggered from the motor neuron propagated along the motor axon. This results in the release of the neurotransmitter, acetylcholine, from the motor nerve terminal, through the synaptic cleft and end at the synaptic contact, called the neuromuscular junction. Acetylcholine binds to receptors on the muscle fiber resulting in the opening of ion channels. This changes the muscle membrane potential causing a release of internal calcium ions leading to a cascade of events with the end result of muscle contraction (Keynes, 2010).

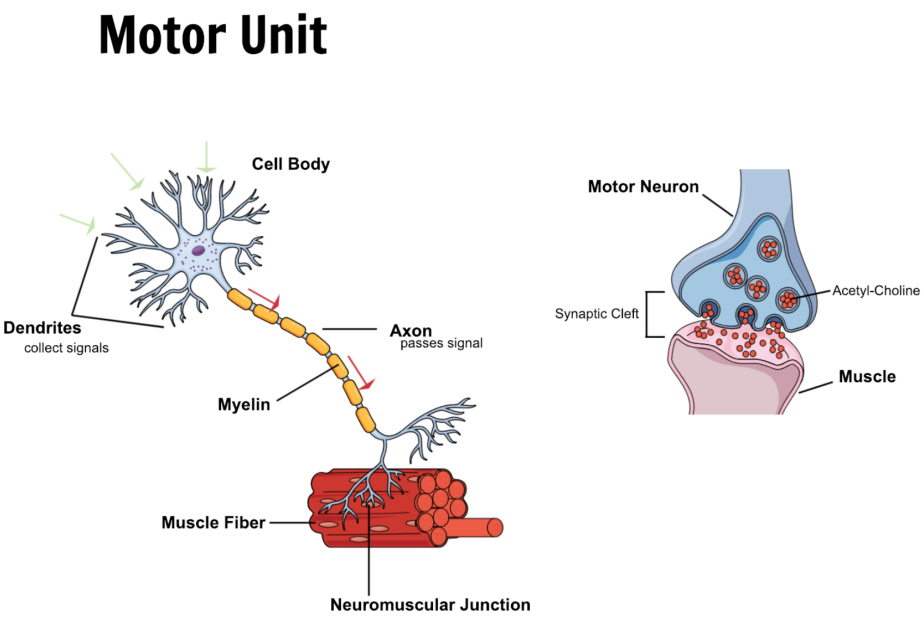


***Figure 1.*** *Groups of motor units often work together to coordinate the contractions of a single muscle. Image adapted from Location, Structure, and Function of Motor Neurons, In Bodytomy, n.d., Retrieved October 28, 2018, from https://bodytomy.com/motor-neurons-location-structure-function*

Neuromuscular disorders affect any component of the neuromuscular system and their supporting cells. Signs of dysfunction tend to vary based on the specific area affected. When damage occurs to the upper motor neurons primary lateral sclerosis occurs and symptoms such as muscle weakness without atrophy, hyperreflexia, and babinski sing occurs. In contrast, when lower motor neurons are damaged, progressive and spinal muscle atrophies occur with phenotypes of severe muscle weakness and paralysis, hypoactive reflexes (Tawil, 2010).

*Amyotrophic Lateral Sclerosis: Clinical Description*

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that is characterized by motor neuron death in the brain and spinal cord, affecting both the upper and lower neuromuscular system (Wim, 2015). ALS is diagnosed based solely on symptoms, there is currently no biomarker that can be used for diagnosis and/or prognosis. The onset of ALS can be so subtle that the symptoms are often overlooked and mistaken for less severe muscle atrophies or dystrophies. Early symptoms include muscle twitches, tightness and weakness in extremities, slurred and nasal speech, and difficulty chewing or swallowing. This affects daily life by making simple tasks like buttoning shirts, writing, or turning a key difficult (Brown, 2017). Early symptoms are often seen in different parts of the body varying with the patient. However, regardless of where the symptoms first appear, muscle weakness and atrophy spread to other parts of the body as the disease progresses (Chio, 2012). Eventually individuals are no longer able to stand or walk, get in or out of bed on their own, or use their hands, arms, and legs. Individuals with advanced ALS have difficulty swallowing and chewing food, making eating more difficult and increasing the risk of choking. In addition to ALS patients burning calories at a faster rate, individuals tend to lose weight rapidly and often become malnourished. People with ALS usually retain their ability to perform higher mental processes such as understanding, problem solving, reasoning, and remembering. Because of this they are aware of their progressive loss of function and may become anxious and depressed as the disease progresses. Patients with ALS have difficulty breathing as the muscles of the respiratory system weaken due to the death of motor neurons. Eventually individuals lose the ability to breathe on their own and must depend on a ventilator. Most people with ALS die from respiratory failure within 3 to 5 years from when their symptoms first appeared. Currently, there is no cure for ALS (Siddique, 2007).

*Genetics of ALS*

Amyotrophic lateral sclerosis is common world-wide and affects people of all races and ethnic backgrounds. In 2016 the CDC estimated that 14,000-15,000 Americans have ALS. Potential risk factors for ALS have been identified and include age and gender. Although the disease can occur at any age, symptoms most commonly develop between the ages of 55 and 75. Men are slightly more likely than women to develop ALS when they are younger. As age increases, there is no difference between men and women (Peters, 2015).

About 10% of ALS cases are familial, usually inherited as dominant traits and the remaining 90% of cases of ALS are sporadic meaning they occur without a family history. However, these numbers may be inaccurate because of misdiagnosis in past family members, or due to the genetic complexity of ALS. As research tools and understanding of ALS continue to grow, the amount of cases classified as familial may increase. (Brown, 2017)

There are a number of known genes that play a role in ALS such as C9ORF72, SOD1, NEK1, TDP43, FUS, and UBQLN2. In most cases, only one mutated copy is needed to cause disease. It is also known that these genes cluster in three categories: protein homeostasis, RNA

homeostasis and trafficking, and cytoskeletal dynamics. Mutants in any category can lead to deposition of cytoplasmic proteins and RNA aggregates, disturbances of protein degradation mechanisms, mitochondrial dysfunction, endoplasmic reticulum stress, defective nucleocytoplasmic trafficking, altered neuronal excitability and altered axon transport (Lee, 2017). It is essential to identify novel genes associated with ALS as these will help our understanding of the mechanisms associated with pathology of ALS to develop therapeutic treatments.

*Kinesin Family Member 5A (KIF5A)*

Intracellular organelle transport is essential for morphogenesis and functioning of the cell. Kinesins make up a large family of molecular motors that transport cargoes such as organelles (e.g. mitochondria, peroxisomes, and lysosomes), protein complexes (e.g. components of the cytoskeleton, virus particles), and mRNAs. (Brenner, 2018)

Kinesin family member 5A (KIF5A) is mainly expressed in neurons and has been identified as a novel gene associated with familial ALS. KIF5A acts as a microtubule motor in intracellular protein and organelle transport (Ebbing, 2008). Previous research has shown that mutations in a singular KIF5A gene can lead to abnormalities in both mice and humans. Knockout mice die during embryonic development indicating that KIF5A is essential for normal function and survival (Wang, 2010). KIF5A is on chromosome 12 and can structurally be divided into three domains. The N-terminal domain binds microtubules and is responsible for ATP hydrolysis and the kinesin motor activity. (Brenner, 2018)

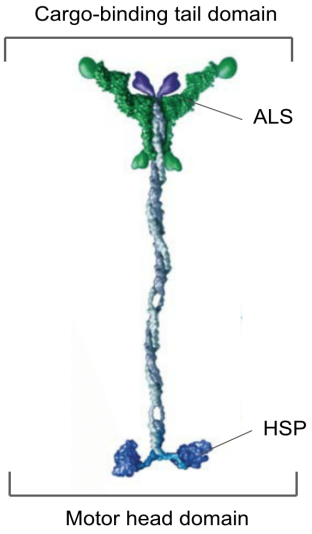


***Figure 2.*** *Three dimensional representation of KIF5A. The cargo-binding tail domain shown in green is the location of mutations associated with ALS. The motor head domain is shown in blue. Mutations associated with HSP are in this area. Image adapted from How Fast Do Molecular Motors Move On Cytoskeletal Filaments, In Cell Biology by the Numbers, n.d., Retreived Feburary 20, 2019, from http://book.bionumbers.org/how-fast-do-molecular-motors-move-on-cytoskeletal-filaments/*

The central coiled-coil or stalk domain mediates heavy chain dimerization. The C-terminal domain interacts with cargo molecules. The central role of kinesins in cellular transport leads us to speculate that mutations in KIF5A cause disease by disrupting axonal transport.

There are known mutations in the neuronal specific KIF5A gene that have been shown to be causative for a dominant form of hereditary spastic paraplegia (HSP). HSPs are a heterogeneous group of genetic neurodegenerative diseases and are characterized by slowly progressive spasticity and weakness in the lower limbs due to progressive distal degeneration of the longest ascending and descending axons in the spinal cord. Unlike ALS, the lifespan of individuals with HSP is normal, although with a decreased quality of life. Similarly to ALS, there is currently no cure for HSP. (Reid, 2002)

It is known that mutations contributing to HSP are missense mutations located in the N-terminal motor domain of KIF5A. These mutations are also known as the third most frequent cause of autosomal dominant hereditary spastic paraplegia. Cellular phenotypes associated with HSP include impairment of microtubule dynamics along axons, axon swelling, and cargo stalling (Tarrade, 2006). Interestingly, mutations in the C-terminal cargo binding region of KIF5A have been identified as causative for ALS and are loss of function alterations (Reid, 2002). This shows that mutations in different regions of KIF5A result in different but overlapping neurodegenerative diseases and for this reason we focused on these mutations for our project.

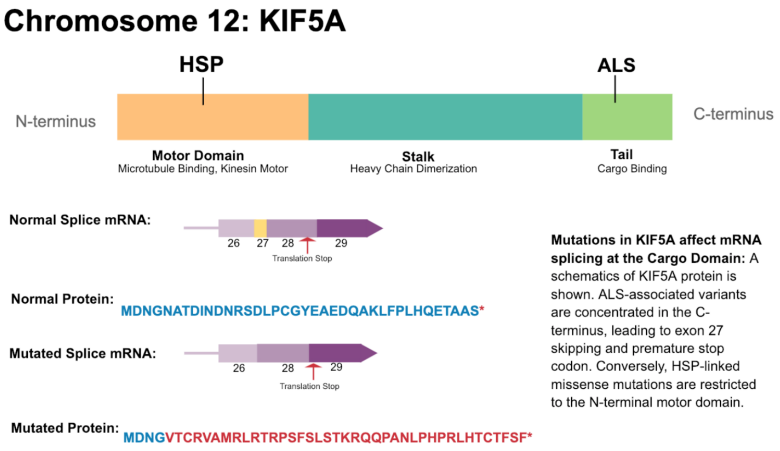


***Figure 3***. *Schematic of KIF5A and mRNA splicing as a result of mutations in KIF5A*

*Research Plan: Overlapping Neurodegenerative Diseases*

The objective of this project is to compare the effects of missense HSP mutations and the loss of function ALS mutation in KIF5A on axonal transport. For the purposes of this project we will be focusing primarily on mitochondria. Mitochondrial movement throughout axons is

integral for neuronal function. Mitochondrial dysfunction has been linked to a host of developmental and degenerative neurological disorders.

Our hypothesis is that the N- and C- terminal mutations in KIF5A will cause different phenotypes in the axonal transport and neuron survival. Comparing HSP and ALS KIF5A phenotypes may allow for a greater understanding of the mechanisms linked with the differing genotypes and mutations and function of KIF5a. Currently, it is unknown how KIF5a mutations lead to motor neuron degeneration. This information can add to the understanding of molecular mechanisms which lead to this motor neuron degeneration. Primary motor neurons expressing wild-type, ALS mutant, or HSP mutant forms of KIF5A will be used to measure the effects on the axonal transport (i.e. rate/speed, distance, and the ratio of anterograde versus retrograde transport) of known KIF5A cargoes, specifically mitochondria. These experiments will establish whether ALS/HSP mutations act through a dominant negative, loss of function or gain of function mechanism. This work will be a stepping stone to investigation of how the movement and distribution of KIF5A throughout an axons lead to different but overlapping neurodegenerative diseases.

## Materials and Methods

### *Cloning of GFP KIF5A Short*

*Gibson Assembly*

Gibson assembly is a molecular cloning method that allows the integration of DNA fragments into a plasmid. This method requires around 30 base pair overlap between DNA fragments and the adjacent DNA fragments. Three main enzymes are needed for the reaction: exonuclease, DNA polymerase, and DNA ligase. Exonuclease chews back DNA from the 5' end, so to not inhibit polymerase activity and allows the reaction to occur in one single process. The resulting single-stranded regions on adjacent DNA fragments can anneal and DNA polymerase incorporates nucleotides to fill in any gaps. DNA ligase covalently joins the DNA of adjacent segments, thereby repairing any nicks in the DNA. The resulting product is the fragments joined into one in a closed circular assembly. This process is shown in Figure 4.

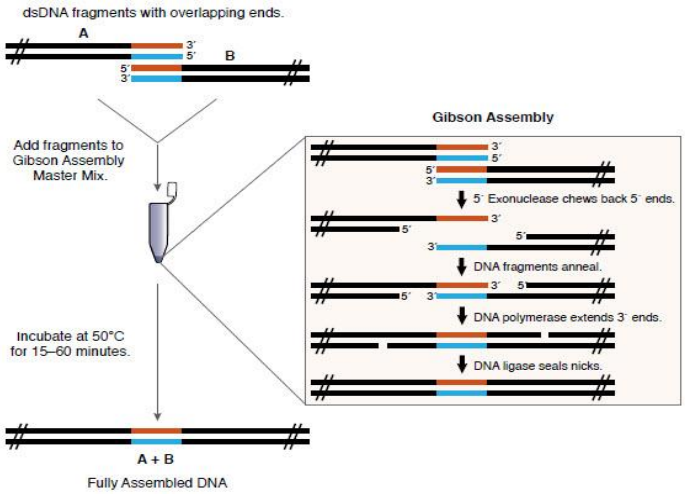


***Figure 4.*** *Overview of Gibson Assembly method*
*From Gibson Assembly Master Mix, In New England BioLabs, n.d., Retrieved March 23, 2019, from https://www.neb.com/products/e2611-gibson-assembly-master-mix#Product%20Information*

This method was used to clone KIF5A short into the pEGFP-C1 (Clonetech) backbone. pEGFP-C1 vector was cut using BamHI (Thermo Scientific) restriction enzyme. A reaction of 2µg vector, 5uL of 10x Cutsmart Buffer (Thermo Scientific), 0.5uL BamHI, and $H_2O$ to 50uL was made and placed at 37°C for 30 minutes.

KIF5A Short was PCR amplified from full length KIF5A. The following primers were designed to be complementary to BamHI areas for the KIF5A short segment. This will allow for the areas to anneal to one another.

Primers for inserting KIF5A into GFP vector

Fragment 1

KIF5A Fw frag1 use: CTGCAGTCGACGGTACCGCGGGCCCGGATGGCGGAGACCAACAAC

KIF5Av5 rev frag 1: GACTCCAGGGACAGCATGGTG

Fragment 2
KIF5A v5 fw frag 2: GCTGTCCCTGGAGTCTGAGTTG
KIF5a short Rev frag2 use: GATTATGATCAGTTATCTAGATCCGGTGTCCATTGTCCATGTTGGCCT

Green: Region that overlaps in pEGFP_C1 vector
Yellow: Region that is in KIF5A short sequence

Fragments from the KIF5A vector were amplified using primers above in a reaction of 10ng/1uL and 10 uL of Taq 2X Master Mix (NE BioLabs). The reaction was run with the following conditions: Initial denaturation 95°C for 30 seconds, 95°C for 50 seconds, 45°C for 60 seconds, 68°C for 1 minute, for 30 cycles, and a final extension of 68°C for 5 minutes. The products of the two fragments and the cut GFP vector were run on a 1% agarose gel to confirm cutting and amplification. The linearized pEGFP-C1 vector was extracted using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's recommendations and the yield quantified by absorbance at λ260nm using Nanodrop Spectrophotometer.

Gibson assembly was performed with a 1:2 vector to insert DNA molecular ratio. The previous absorbance levels were used to calculate the total amount of fragment and vector needed using NEBiocalculator. 1.1uL of vector, 0.8 uL of Fragment 1, and 0.4 uL of Fragment 2 were added to 10 uL of HiFi DNA Assembly Master Mix (NE BioLabs) and 7.7 uL of $H_2O$. The assembly was incubated at 50 °C for 15 minutes.

*Transformation*

Transformation is genetic alteration of a bacteria resulting from the direct uptake, incorporation and expression of exogenous genetic material from its surroundings. This method was utilized to amplify and select individual cloned plasmids. The pEGFP-C1 vector contains a kanamycin resistance gene, therefore if the bacteria cells successful take up the vector, they will be able to grow when plated acting as a confirmation of vector incorporation.

Chemically competent *E. Coli* cells were thawed on ice, and 2µl or 10µl of DNA was added and incubated on ice for 30 minutes. The tubes were then placed at 42°C for 30 seconds to allow the DNA to be taken up by the cells. After 30' at 37°C incubation in LB medium, cells were plated on LB agar plates containing 50 mg/L kanamycin and grown overnight. Isolated colonies were picked using a pipet tip and placed in 2 mL of LB and 0.2uL of kanamycin. The tip and mixture was incubated in a shaker overnight at 37°C. The bacteria were pelleted and a QIAprep Spin Miniprep Kit (Qiagen) was used to extract the plasmid according to manufacturer's recommendations.

***Cloning of V5-KIF5A Short***

*GFP KIF5A Short Vector Digest*

The pEGFP-KIF5A Short vector was digested using Kpn2I and BshTI (Thermo Scientific) as outlined above to remove the GFP coding sequence. 1uL of rSAP (NE BioLabs) was added and placed back in 37°C for 30 minutes to dephosphorylate the vector and prevent re-

ligation. The vectors were run with electrophoresis on a 1% agarose gel. The linearized digested vector was extracted from the gel using QIAquick Gel Extraction Kit and quantified.

*V5 Oligos Annealing*

2µg of both forward and reverse v5 oligos (V5-for: CCG GTA TGG GTA AGC CTA TCC CTA ACC CTC TCC TCG GTC TCG ATT CTA CGG AAA ACC TGT ATT TTC AGG GCT; V5-rev: CCG GAG CCC TGA AAA TAC AGG TTT TCC GTA GAA TCG AGA CCG AGG AGA GGG TTA GGG ATA GGC TTA CCC ATA) were added to Annealing Buffer (10mM Tris, pH7.5-8.0, 50mM NaCL, 1mM EDTA) to a total volume of 50uL and placed at 98°C for 5 minutes and removed and slowly cooled at room temperature. The oligos were phosphorylated using T4 PNK, 2uL ATP, 10X PNK Buffer (NE BioLabs), 1uL of the oligo mixture, and $H_2O$ to 20uL. The mixture was placed in 37°C for 30 minutes, 65°C for 20 minutes, and quantified using a spectrophotometer.

*V5-KIF5A Short Ligation and Transformation*

A 1:10 dilution of V5 oligos were added to 4uL T4 ligase buffer (BioLabs), and 1uL of T4 ligase (NE BioLabs), and 2.5 ul of KIF5A short linearized vector. This mixture sat for 10 minutes at room temperature and placed on ice for 30 minutes. 5uL of ligation reaction was added to a tube of chemically competent *E. Coli* cells and placed on ice for 30 minutes and then heat shocked for 30 seconds. 1mL of SOC was added and placed in 37°C for 1 hour. 200 uL was plated on LB-kanamycin agar plates. Plasmids that have integrated into the vector will grow and confirm insertion.

*Colonies Picking and Testing*

Isolated colonies were picked using a pipet tip and placed in 2 mL of LB and 0.2uL of kanamycin. The tip and mixture was incubated in a shaker overnight. The bacteria was pelted and the QIAprep Spin Miniprep Kit (Qiagen) was used to extract the plasmid. The isolated DNA was used for PCR to verify the insertion of the V5-tag. Twenty uL reaction was made for each colony using 10uL of Amplitaq Gold 360 Master Mix 2X (Applied Biosystems), the V5-for and KIF5A-reverse oligos (GAC TCC AGG GAC AGC ATG GTG) for the V5 tag were added and 0.2uL of DNA. The remainder of the volume was $H_2O$. The samples were run in the following conditions: 98°C for 5 seconds, 59°C for 1 minute and 30 seconds, 72°C for 1 minute repeated for 30 cycles. These samples were run using gel electrophoresis on a 1% gel to determine if the insert was integrated into the vector.

## *Cell Culture and Transfection*

*Primary Motor Neuron Culture*

Spinal motor neuron culture is an incredible model system for studying motor neuron diseases such as ALS. Motor neuron survival *in vitro* requires a specific combination of multiple

growth factors and supplemental reagents. This process was completed by a trained member of the lab.

Primary motor neurons (MNs) were isolated from E12.5 mouse embryonic spinal cords dissociated in 0.1% trypsin (Worthington) at 37°C for 12 minutes. MNs were purified using a 6% Optiprep (Sigma-Aldrich) density gradient and plated on glass coverslips coated with 0.5g/L poly-ornithine (Sigma-Aldrich) and laminin (Thermo Fisher). Cells were grown at 37°C and 5% $CO_2$ in Neurobasal medium (Thermo Fisher) supplemented with 0.25% Glutamax, 2% B27, 2% horse serum, and 10ng/ml BDNF, GDNF, and CNTF.

*Transfection*

Constructs designed to express mutant KIF5A into primary motor neurons (MNs) are shown in Figure 5. The tagless KIF5A in pGW1 backbone was used to analyze mitochondrial movement. The V5 tagged KIF5A in pEGFP-C1 backbone was used to determine the distribution of KIF5A throughout primary motor neurons. To visualize mitochondria for live cell imaging, MNs were transfected with the dsRED-mito construct, a red fluorescent protein that specifically labels mitochondria in living cells.

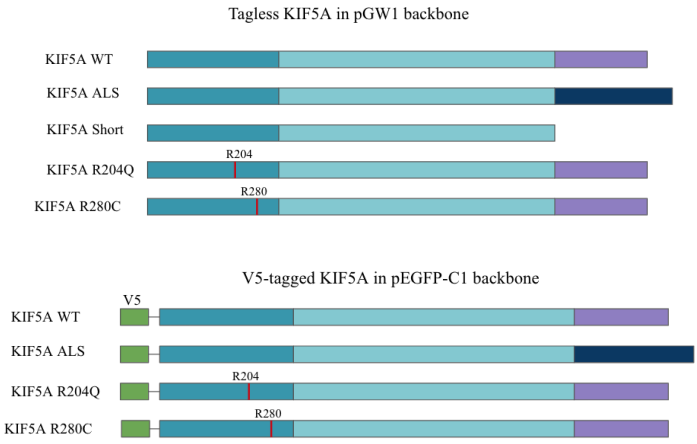


***Figure 5**. Schematic representation of the constructs used in these project.*

Transfection is the process of inserting genetic material, in this case plasmid DNA, into mammalian cells. The insertion of DNA into a cell allows the expression, or production, of exogenous proteins using the cells own machinery. It is critical that DNA is transported to the nucleus for effective transfection. There, DNA can be transiently expressed or become incorporated into the genomic DNA. In motor neurons, transfection is performed using magnetic beads which the plasmid attaches to and the beads act as scaffolds to direct transfection to individuals cells shown in Figure 6.

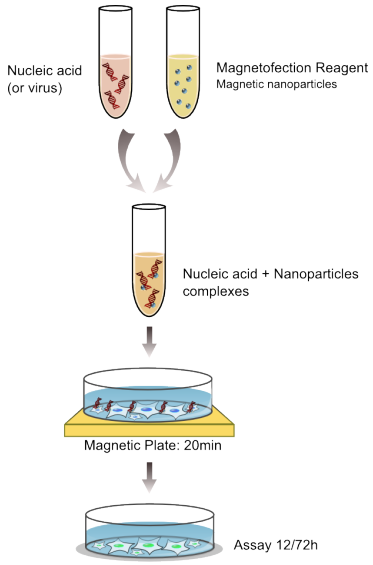


***Figure 6.*** *Localized transfection with magnetic beads coated nucleic acids From Magnetofection In OZBiosciences, n.d., Retrieved March 13, 2019, from https://www.ozbiosciences.com/content/13-Magnet-assisted_transfection*

MNs at 2 days *in vitro* (DIV) were transfected using 1.75µl NeuroMag reagent (OZ Biosciences) + 0.5µg DNA. Complete growth medium was replaced with serum free neurobasal medium 1 hour prior and after transfection. The NeuroMag reagent was incubated with DNA for 15 minutes at room temperature and added to cells. The cells were placed on a magnetic plate for 15 minute. Complete growth medium was replaced 1 hour after transfection.

*Immunohistochemistry*

Immunohistochemistry is used to show the distribution and localization of specific proteins in cells by exploiting the principle of antibodies binding specifically to antigens in biological tissues.

Cells were washed for 5 minutes in PBS, fixed with a 4% PFA in PBS for 15 minutes and washed with PBS in two 5 minute periods. Antigen retrieval was completed by heating 10 mM Citrate Buffer (2mM citric acid + 8mM sodium citrate) pH 6 to 80°C and added to the cells for 20 minutes. Coverslips were washed for 5 minutes two times in PBS. Cells were permeabilized in PBS-Triton 0.2% for 5 minutes and blocked with PBS-BSA 5% for 45 minutes. Primary antibodies (mouse anti-V5, 1:10,000; rabbit anti-βtubulin, 1:1000) were diluted in 5% BSA overnight at 4°C. After incubation cells were washed with PBS for 15 minutes three times. Fluorescent-conjugated secondary antibodies (donkey anti-mouse Alexa488, 1:500; donkey anti-rabbit Alexa546) were diluted in PBS-BSA 5% and added to coverslips and incubated in the dark at room temperature. After an hour cells were washed with PBS for 15 minutes three times. Slides were rinsed in $H_2O$ and mounted in mounting medium, Prolong Gold with DAPI in the dark overnight at room temperature.

***Microscopy and Analysis***

*Microscopy of Primary Motor Neurons*

Live cell imaging is utilized to study living cells using time lapse microscopy and allows for a better understanding of biological functions over a period of time after transfection. Conditions that mimic those of a cells normal environment must be obtained to keep cells alive during analysis. Cells were imaged at 3 days after transfection using a Nikon TiE widefield microscope equipped with temperature- and $CO_2$-controlled environmental chamber. Movies were acquired with a 20x lens at a rate of 1 frame every 2 seconds for 2 minutes.

Fixed cells were imaged using the same Nikon TiE widefield microscope at 20X. Images were acquired using three different filters to observe (DAPI, □-Tubulin, and the V5-KIF5A tagged constructs).

*Video Analysis*

Videos captured were analyzed using the ImageJ plugin MTrackJ. For tracking speed and direction of mitochondria movement, reference points were established at the cell body. Individual mitochondria were tracked throughout the length of the axon, marking each individual movement over the 2 minute period. Length (microns), mean velocity (microns/second), and distance to reference were all collected for each primary axon as shown in Figure 7.

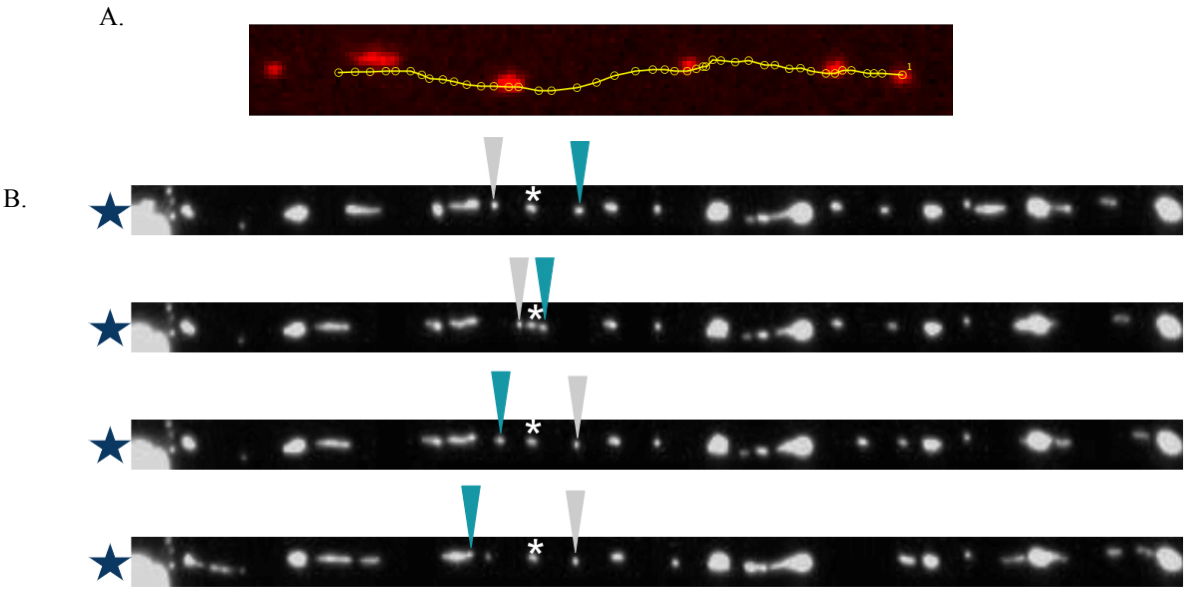


***Figure 7.*** *(A) Tracking of individual mitochondria. (B) An individual motor axon with several labeled mitochondria. The dark blue star indicates the location of the cell body. The gray arrow indicates a mitochondria moving in the anterograde direction (i.e. toward the growth cone) and the blue arrow the mitochondria moving in the retrograde direction (i.e. toward the cell body). The white star indicates a static mitochondria.*

To determine the percent of moving mitochondria, the number of immobile mitochondria was established via the minimum intensity projection, which only highlights

immobile objects as depicted in Figure 8. Total number of mitochondria was calculated using ImageJ particle analysis tool and a ratio was calculated to determine the total percent of moving mitochondria.

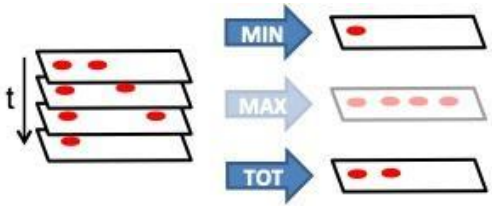


***Figure 8***. *Schematics of minimum intensity projection. Red dots represent mitochondria.*

*Statistics*

Statistical analyses were performed using Prism 7 software package (GraphPad). D'Agostino-Pearson normality tests were run on data sets. According to results, parametric (One-way ANOVA) or nonparametric (Kruskal-Wallis test) statistical tests were performed. These tests allowed for statistical significance to be tested. If P values were  less than 0.05 the results were considered significant.

**Results and Discussion**

*1. ALS and HSP-linked Mutations in KIF5A Alters the Percentage of Moving Mitochondria*

To compare the effects of different mutations in KIF5A on cellular transport, we performed live cell imaging experiments to visualize and quantify the axonal trafficking of mitochondria, a primary cargo of kinesins. Primary motor neurons were transfected with either wild type or mutant KIF5A, and the percentage of mitochondria moving for each construct was assessed. Time lapse images were acquired every second for a total of 2 minutes, and kymographs, a 2D plot of time over distance, were created. This was useful to demonstrate and visualize the amount of moving mitochondria throughout individual motor axons.

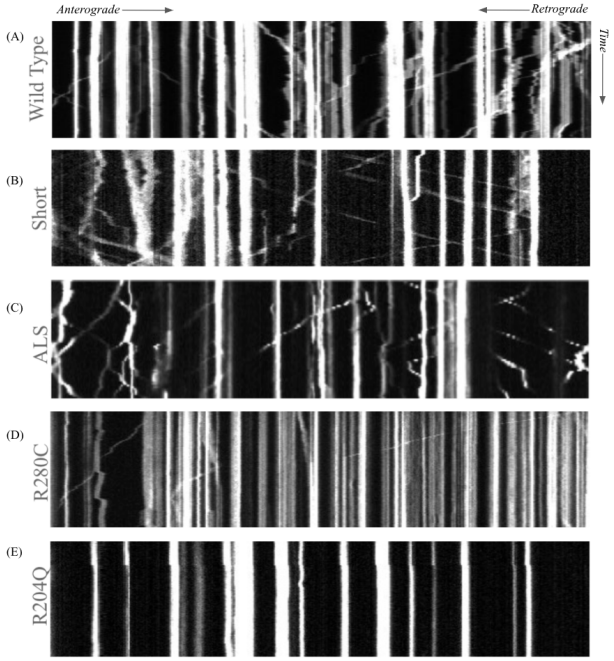


***Figure 9.*** *Kymographs of representative neurons transfected with the different KIF5A constructs. Cell bodies are localized to the left, while the axon tip is on the right.*

The KIF5A-ALS mutant (Figure 5) has a slightly greater number of total moving mitochondria compared to the wild type control. On the contrary, the HSP-linked mutation R280C exhibits low levels of movement predominantly in the retrograde direction, while HSP mutation R204Q shows no movement in either direction. Since we wanted to determine if the mutation in the ALS linked mutation in the C terminus is a gain or loss of function, we designed a construct that removed the mutated amino acid sequence (i.e. KIF5A-Short). Interestingly, these preliminary and qualitative images demonstrate the Short construct has mitochondrial mobility similar to the WT, indicating no immediate differences in mitochondrial movement.

To further our observations and quantify the percentage of mitochondria moving in either direction, we scored the number of immobile mitochondria compared to the total number of

mitochondria present in individual proximal axon. The values in white in Figure 10 represent the number of axons assessed.

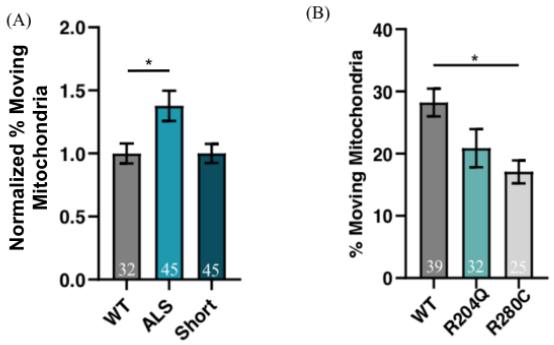


***Figure 10.*** *Quantification of the percentage of moving mitochondria. Bars are mean and SEM. N as indicated in the bars represent the number of cells analyzed obtained from 4 independent experiments. Statistical analysis was performed using one way-ANOVA (* p= 0.0116).*

This analysis shows a 37.7% increase of mitochondria moving in the KIF5A mutant in comparison to the WT. The Short construct shows no difference to the WT, consistently with the kymograph analysis. Interestingly, this indicated that deletion of the extra C-terminal sequence present in the ALS mutant had no effect on mitochondria movement. A significant decrease in the HSP- linked mutations can be seen in percent of moving mitochondria particularly in the R280C construct with a 44% decrease. The R204Q construct had a decrease of 31% in mitochondrial mobility.

Utilizing kymographs and percent moving mitochondria, we observed the effects of mutations in KIF5a on the number of moving mitochondria within axons. Interestingly, the HSP linked mutations had the inverse effect on mitochondria mobility than that of ALS linked mutations.

*2. Mutations in KIF5A Disrupt Anterograde Mitochondria Transport*

To further test the effects of mutations in KIF5A on mitochondria transport, the velocity of each mitochondria was determined for each motor neuron. Time lapse images allowed for individual mitochondria to be tracked. The white arrows in Figure 11 indicate an individual mitochondria moving in the anterograde direction.

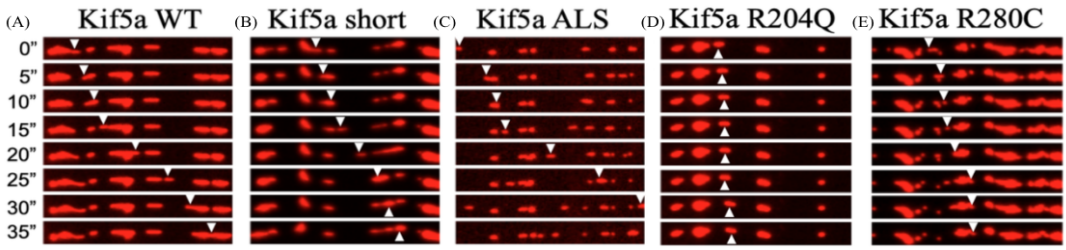


***Figure 11***. *Time lapse images of representative neurons expressing WT or mutant KIF5A. A mitochondria (red) moving in the anterograde direction is highlight by the arrowhead.*

Initial qualitative tracking results obtained from Figure 11 show individual moving mitochondria through individual axons. KIF5A ALS mutant was found to be moving at a higher velocity and the HSP linked mutants moving much slower. Similarly to the results in Figure 10, the KIF5A Short does not differ much from the KIF5A WT.

Further quantification was completed with each motor neuron to compare the velocity of mitochondria from each cell expressing specific constructs (Figure 12). To enable comparison across multiple experiments, mitochondrial velocities in cells expressing the ALS and short construct versions of Kinesin 5A were normalized to mitochondrial velocities in cells with wild-type Kinesin 5A.

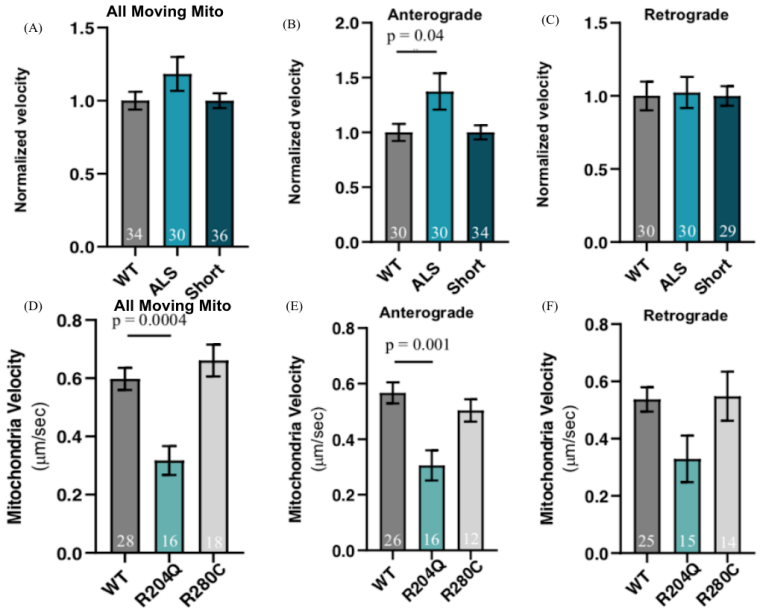


***Figure 12.*** *Analysis of mitochondria velocities. Quantification of anterograde, retrograde, or total mitochondria velocity. Bars are mean and SEM. N as indicated in the bars represent the number of cells analyzed obtained from 4 independent experiments. Statistical analysis was performed using one way-ANOVA. All values are normalized to the mean of WT.*

These analyses showed an increase in mitochondria velocity in ALS mutants. In particular, when mitochondria moving in anterograde and retrograde direction were analyzed separately, we found a 40% increase in speed in the anterograde direction. No changes in mitochondrial velocity were seen in the retrograde direction for the ALS mutant as also seen in the slopes of kymographs in Figure 8. The KIF5A-Short construct had no effects on mitochondria movement in either direction, suggesting that the extra C-terminal sequence in the

ALS mutant has a toxic gain of function. The HSP mutants, particularly the R204Q mutant shows significant decrease of mitochondria velocity with a 50% decrease in velocity was seen in overall and anterograde directions in comparison to the WT. A trend of lowered velocity was seen in the retrograde direction but did not achieve significance. The HSP mutation R280C did not have an observed decrease or increase in any direction in comparison to the WT. This analysis shows mutations in the motor head N terminus, particularly at R204C cause a decrease in mitochondria velocity and or movement, while mutations in the cargo binding C terminus cause increased velocity.

Again, these results support that mutation in KIF5A have an effect on not only the amount of moving mitochondria but at what rate they are being transported demonstrating the potential differences in the overlapping diseases of HSP and ALS.

*3. ALS-linked Mutations Affect KIF5A Cellular Distribution*

Since we saw that HSP and ALS-linked mutations in KIF5A altered mitochondria velocity in opposite ways, we wondered whether this could also impact the cellular distribution of KIF5A itself. Thus, we performed immunocytochemistry to visualize and quantify the localization of KIF5A along the motor axons. V5-tagged KIF5A was visualized using a specific antibody, while the whole neuron was labeled with an antibody against the microtubule protein β-Tubulin.

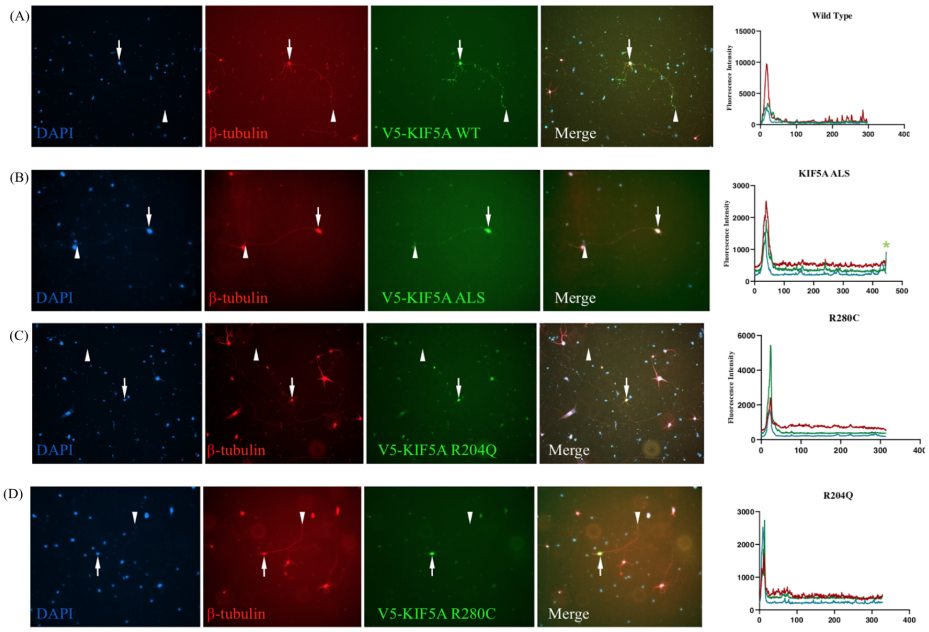


***Figure 13.*** *Analysis of KIF5A distribution in primary motor neurons.* ***A-D.*** *low magnification representative images of motor neurons transfected with V5-tagged KIF5A (green). β-tubulin (red) was used as a marker of the whole neuron, highlighting the cell body (arrows), axon, and growth cone (arrowhead). DAPI (blue) staining indicate the cell nucleus. Line plots show the fluorescence intensity of V5-KIF5A) along the whole neuron in comparison to β-tubulin and DAPI.*

Our results show that while wild type KIF5A localizes in the cell body as well as along the length of the axon, the ALS and HSP MN present differences in KIF5A localization. HSP mutant MNs have very little V5-KIF5A in the axon and the growth cone and similar amounts in the cell body. On the contrary, ALS mutant KIF5A accumulates at high concentrations in the cell growth cone, in line with our previous data showing increased anterograde movement of mitochondria. Further quantification using line plots as shown in Figure X shows the spike of V5-KIF5A at the growth cone in the ALS linked mutant (asterisk). This spike is not observed in any other construct including the WT.

From the immunohistochemistry images, the level of V5-tagged KIF5A was taken from the cell body and from the growth cone. Ratios were taken from these values by dividing the cell body intensity by the axonal one.

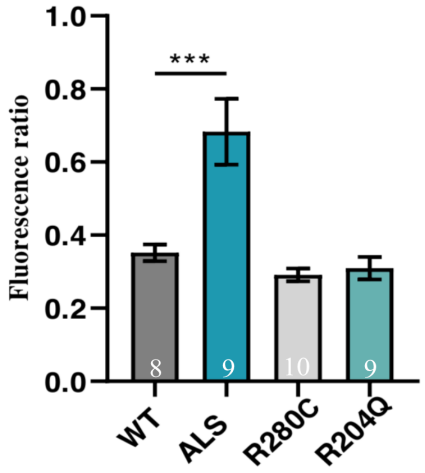


***Figure 14***. *Quantification of growth cone to cell body ratio of KIF5A intensity. Bars are mean and SEM. N as indicated in the bars represent the number of cells analyzed obtained from 1 experiment. Statistical analysis was performed using one way-ANOVA (***p = 0.0004).*

This analysis revealed a 48% increase in the KIF5A ALS mutant cell body/growth cone ratio in comparison to the WT demonstrating an incredibly high amount of KIF5A in the growth cone. No significant difference was seen in either of the HSP mutations. Although preliminary, these results further demonstrate the effects of mutations in KIF5A leading to KIF5A distribution and supporting the previous work completed in this study.

**Conclusions and Recommendations**

ALS and HSP are complex diseases that are even challenging to study on the cellular level. The increasing prevalence of adults over the age of 65 in the United States and around the globe means that the both hereditary spastic paraplegia and amyotrophic lateral sclerosis will only become more common in the coming years, making research such as this even more imperative.

Previous work has demonstrated that mutations in KIF5A are linked to ALS and HSP, suggesting missense mutations in the N terminus play a role in HSP and that gain/loss of function mutations in the C- terminus could be linked to ALS. This study demonstrates that mutations in KIF5A have an impact on mitochondrial transportation throughout primary motor neurons.

Our work shows that ALS-linked mutation in the cargo binding region of KIF5A results in mitochondria moving at an increased velocity, particularly in the anterograde direction. We also demonstrated a larger percent of total moving mitochondria in KIF5A-ALS. Finally, increased concentration of KIF5A protein in the growth cone of motor axons in comparison to WT KIF5A was observed when completing immunohistochemistry with V5 tagged constructs, suggesting that ALS-linked mutation in KIF5A increases its anterograde transport along the motor axon.

Mutations in the motor head domain, associated with HSP, resulted in significantly less mobility of mitochondria shown in the decreased velocity, particularly in the R204C construct. A significantly lowered percent of moving mitochondria was seen in both constructs. V5 tagged HSP constructs showed little distribution of mitochondria throughout motor axons in comparison to WT.

Potentially the most interesting discovery was that the deletion of the extra C-terminal sequence present in the KIF5A ALS mutant (i.e. KIF5A-short) caused no difference in mitochondria mobility compared to WT. This suggests a gain of function in the ALS mutant. Due to decrease in mitochondrial movement, HSP mutations support a loss of function, supporting the previous work.

The results of the study will help provide ideas for future research. Further work will include creating a Short V5 tagged construct to visualize the distribution of KIF5A throughout the motor neuron and a more detailed analysis of KIF5A distribution to help further solidify that the mutation in the C-terminus associated with ALS is a gain of function. Completely further quantitative analysis on more cells would also allow for stronger results.

It would be beneficial to further expand this work by investigating the molecular mechanisms linking KIF5A C-terminal mutations with increased mitochondria mobility. Further experimentation could be completed to determine if the mutation occurring in the cargo binding region of KIF5A is mutated in a way creating a higher affinity for mitochondria or if the mutation in the C-terminus is making it so the motor protein is unable to become inactive by binding to the N-terminus.

## References


Brenner, D., Yilmaz, R., Müller, K., Grehl, T., Petri, S., Meyer, T., ... & Weber, M. (2018). Hot-spot KIF5A mutations cause familial ALS. *Brain*, *141*(3), 688-697.

Brown, R., & Al-Chalabi, A. (2017). Amyotrophic Lateral Sclerosis. *New England Journal Of Medicine*, *377*(2), 162–172.

Chio, A., Logroscino, G., Traynor, B., Collins, J., Simeone, J., Nalysnyk, L., & White, L. (2012). Global epidemiology of amyotrophic lateral sclerosis: a systematic review of the published literature. *Journal Of Neurology*, *259*, S51–S52.

Ebbing, B., Mann, K., Starosta, A., Jaud, J., Schöls, L., Schüle, R., & Woehlke, G. (2008). Effect of spastic paraplegia mutations in KIF5A kinesin on transport activity. *Human molecular genetics*, *17*(9), 1245-1252.

Keynes, R., Aidley, D., & Huang, C. (2010). *Nerve and muscle* (4th ed.). Cambridge;: Cambridge University Press.

Lee, H., Kim, K., Jeon, G., & Sung, J. (2017). ALS. *Journal Of The Neurological Sciences*, *381*(sS), 33–33.

Peters, O., Ghasemi, M., & Brown, R. (2015). Emerging mechanisms of molecular pathology in ALS. *Journal Of Clinical Investigation*, *125*(5), 1767–1779.

Reid, E., Kloos, M., Ashley-Koch, A., Hughes, L., Bevan, S., Svenson, I. K., ... & Rubinsztein, D. C. (2002). A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10). *The American Journal of Human Genetics*, *71*(5), 1189-1194.

Siddique, N., Sufit, R. L., & Siddique, T. (2007). Degenerative motor, sensory, and autonomic disorders. In *Textbook of Clinical Neurology: Third Edition* (pp. 781-811). Elsevier Inc.

Tarrade, A., Fassier, C., Courageot, S., Charvin, D., Vitte, J., Peris, L., ... & Seilhean, D. (2006). A mutation of spastin is responsible for swellings and impairment of transport in a region of axon characterized by changes in microtubule composition. *Human molecular genetics*, *15*(24), 3544-3558.

Tawil, R., & Venance, S. (2011). *Neuromuscular disorders*. Chichester, West Sussex, UK: Wiley-Blackwell.

Wang, L., & Brown, A. (2010). A hereditary spastic paraplegia mutation in kinesin-1A/KIF5A disrupts neurofilament transport. *Molecular neurodegeneration*, *5*(1), 52.

Wim Robberecht, & Thomas Philips. (2013). The changing scene of amyotrophic lateral sclerosis. *Nature Reviews Neuroscience*, *14*(4), 248–264.