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Neuromuscular Junction Defects in a Mouse Model of Charcot-Marie-Tooth Disease Type 2O

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NEUROMUSCULAR JUNCTION DEFECTS IN A MOUSE MODEL OF CHARCOT-MARIE-TOOTH DISEASE TYPE 2O

by

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ABSTRACT

Charcot Marie Tooth disease (CMT) represents the most common inheritable peripheral group of motor and sensory disorders; affecting 1 in 2500 people worldwide. Individuals with CMT experience slow progressing weakness of the muscle, atrophy, mild loss of motor coordination and in some cases loss of sensory function in the hands and feet which could ultimately affect mobility. Dynein is an essential molecular motor that functions to transport cargos in all cells. A point mutation in the dynein heavy chain was discovered to cause CMT disease in humans, specifically CMT type 2O. We generated a knock-in mouse model bearing the same mutation(H304R) in the dynein heavy chain to study the disease. We utilized behavioral assays to determine whether our mutant mice had a phenotype linked to CMT disease. The mutant mice had motor coordination defects and reduced muscle strength compared to normal mice. To better understand the disease pathway, we obtained homozygous mutants from a heterozygous cross, and the homozygotes show even more severe deficits compared to heterozygotes. They also developed an abnormal gait which separates them from heterozygous mice. In view of the locomotor deficits observed in mutants, we examined the neuromuscular junction (NMJ) for possible impairments. We identified defects in innervation at the later stages of the study and abnormal NMJ architecture in the muscle as well. The dysmorphology of the NMJ was again worse in the homozygous mutants with reduced complexity and denervation at all the timepoints assessed. Our homozygous dynein mutants can live up to two years and therefore make the design of longitudinal studies possible.

Altogether, this mouse model provides dynein researchers an opportunity to work towards establishing the link between dynein mutations, dynein dysfunction and the onset and progression of disease.

I would like to dedicate this work to my parents; the late James Kwaku Sabblah and Esther Hilary Ashiagbor. Thank you for your sacrifice and for all you taught me in my formative years, I wouldn't have made it this far without you guys.

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ATP	ADENOSINE TRIPHOSPHATE
BicD	BICAUDAL D
CMT	CHARCOT MARIE TOOTH DISEASE
Lis1	LISENCEPHALY 1
PSC	PERISYNAPTIC SCHWANN CELLS
µm	MICROMETER
NMJ	NEUROMUSCULAR JUNCTION
MDa	MEGADALTON
kD	KILODALTON
ZW10	ZESTE WHITE 10 (KINETOCHORE PROTEIN)
Lis1	LISSENCEPHALY 1
NudE	NUCLEAR DISTRIBUTION ELEMENT

CHAPTER 1:INTRODUCTION

1.1 Cytoplasmic Dynein

Intracellular transport is at the heart of the maintenance of function and structure in the eukaryotic cell. Cytoskeletal based molecular motors therefore play essential roles in the cell since they transport various cargoes from one part of the cell to the other. Cytoplasmic dynein is a 1.6 Mega Dalton motor protein that transports cargo retrogradely on microtubule tracks. Dynein is important in mitosis and the transport of intracellular cargoes such as mRNA-protein complexes, viruses and other organelles towards the center of the cell *(Bhabha et al, 2016).*

Dynein belongs to the AAA+ (ATPases Associated with various cellular Activities) family of proteins *(Neuwald, 1999).* This sets it apart from the other molecular motors kinesin and myosin which are part of the G-protein family *(Vale and Milligan, 2000).* Myosin functions in the muscle and moves on actin tracks, but dynein and kinesin move along microtubules transporting cargo. There are over 15 kinesins which function as plus and minus-ended motors, whereas cytoplasmic dynein is solely a minus-end directed cargo. Dynein consists of 4 types of subunits namely the heavy chains, intermediate chains, light intermediate chains and light chains *(Figure1A).* The heavy chain is the largest subunit with a molecular weight of approximately 500kDa. There are two heavy chains per molecule, and each contains the ATPase domains at the C-terminal end responsible for mechanical force for movement.

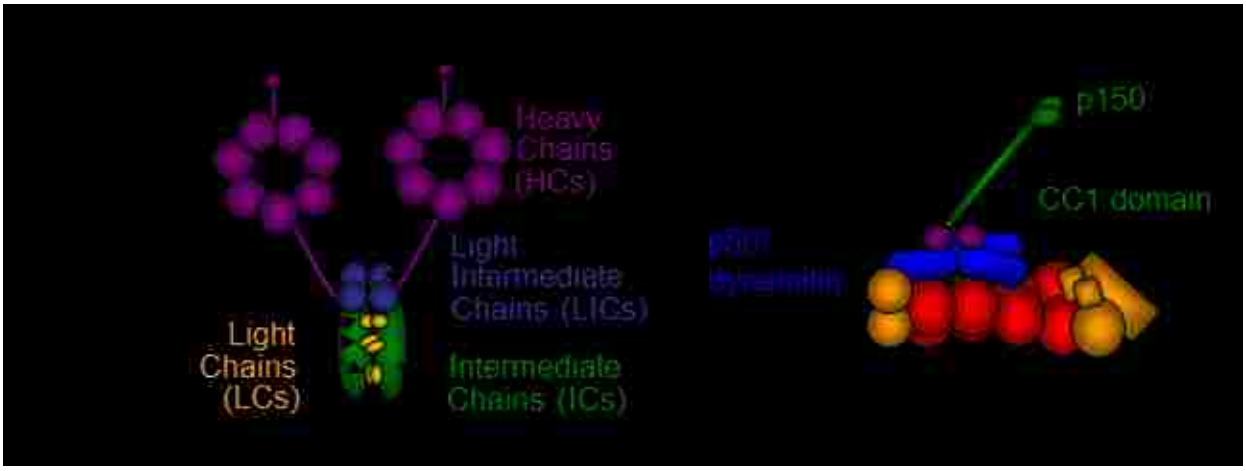


Figure 1 The Structure of Dynein and Dynactin

A) The 4 subunits of dynein as well as the functional sites required for normal activity in cells B) The major activator of dynein, with its subunits and some of the functional domains

There are 6 AAA+ domains in the dynein heavy chain and AAA1 is believed to be where most of the ATP hydrolysis occurs *(Gibbons et al,1987).* AAA3 and AA4 are also able to cleave ATP and further experiments show that when nucleotide binding is interrupted in these domains there is a corresponding change in dynein motility *(Cho et al, 2008; DeWittt et al, 2015; Silvanovich et al, 2003).* AAA5 and AAA6 do not bind nucleotides but help the motor detach from microtubules via a coupled rotational movement *(Schmidt et al, 2014).* Cytoplasmic dynein binds microtubules with a 15nm coiled coil stalk projecting from AAA4. The stalk has a specialized α-helical domain for microtubule binding *(Carter et al, 2008; Carter et al, 2011).* The tail segment or N-terminal region of the dynein heavy chain dimerizes and provides the sites for attachment of the other dynein subunits namely the intermediate chain, light intermediate chain and light chains. The intermediate chain is the site of attachment of important dynein regulators such as Nudel/NudE, Lis1 and dynactin *(McKenney et al, 2011; Nyarko et al, 2012)*. The light chains are the sites of cargo attachment and are also the smallest of the dynein subunits. Rp3 (13kD), Tctex-1 (12.4kD), LC8 (10.3kD) and LC7/robl (11kD) *(King et al, 1998. King et al,2002)*. siRNA depletion experiments involving some light chains points to a role in endosome trafficking and mitosis *(Palmer et al, 2009.*)

Dynein is ubiquitously expressed in all cells and is critical during development. For instance, during mitosis it generates the force that is required to organize the microtubules and position the spindles. Dynein transports checkpoint proteins that play a role in regulating mitosis *(Howell et al, 2001 & Varma et al, 2008).* Dynein function requires dynactin, an adaptor found to make the motor protein processive *(King and*

Schroer,2000). Dynactin has a size of about 1MDa and is made up of 11 subunits with the largest being p150 *(Figure 1B)*. Dynactin interacts with dynein via its p150 subunit, which bears a CAP-Gly (cytoskeleton-associated protein glycine-rich) domain to attach to microtubules thereby ensuring that dynein does not fall off the microtubules. Dynactin is needed in most of the dynein cellular functions; there is some evidence for the attachment of cargos to both dynein and dynactin *(Allan, 2011).* The intermediate chain of dynein and p150 of dynactin interact directly to ensure the proper microtubule arrangement within cells and architecture of the centrosome *(King et al, 2003)*. The Arp1 filament of dynactin associates with βIII spectrin, a surface protein on Golgi and some membranes which might implicate a role in the movement of vesicles. Interactions have also been reported with COPII vesicle protein SEC23 and a Rab7 GTPase effector, RILP (Rab-interacting lysosomal protein) *(Kardon & Vale, 2009)*.

Apart from dynactin, there are other accessory proteins that are necessary for proper dynein function, these include LIS1, NudE/Nudel, BicD and ZW10. Mutations in LIS1 have been implicated in Lissencephaly or smooth brain; a neurological disorder affecting development with the hallmark features of a smooth cerebrum, intellectual defects and seizures. The disorder arises from impairments in proliferation of progenitor cells, nucleokinesis and neuronal migration *(Reiner, 2013).* Lis1 is the only known dynein regulator which is able to interact directly with the motor domain, specifically AAA3 and AAA4, suggesting a more significant effect on dynein's motion and function as well *(Huang et al, 2012. Toropova et al, 2014).* More evidence of Lis1 involvement in neurodevelopment is observed in its association with Nudel and NudE. These

homologous proteins are essential for mitosis and neurodevelopment. *(Bradshaw et al, 2013).* NudE recruits Lis1 to dynein and their added effect allows dynein to generate the drag force to move heavy cargo like the nucleus. Nudel on the other hand may work conversely by preventing the motor protein from attaching to microtubules *(McKenney et al, 2010).* The Axon initial segment (AIS) is the location where nerve impulses are initiated, it also serves as a neuronal barrier that helps differentiate dendritic identity from axonal identity during neuronal development *(Palay et al,1968; Rasband, 2010)*. The maintenance of a peculiar identity by either the axon or dendrite is found to be dependent partly on the activity of motor proteins. Nudel has been shown to associate with an essential organizer of the AIS, Ankyrin G. A Lis1- mediated pathway leads to the activation of dynein and transport of cargo out of the AIS *(Kuijpers et al,2016)*.

ZW10 is a part of a complex that attaches to the kinetochore and binds it to microtubules. ZW10 is responsible for targeting dynactin and dynein to the kinetochore *(Stehman et al, 2007, Starr et al, 1998).* Although it is directly bound to the p50 protein of dynactin, ablation of ZW10 results in the loss of dynein at kinetochores, indicating that ZW10 perhaps indirectly stabilizes dynein at the kinetochores. BicD was discovered in *Drosophila* as a factor that links dynein to some of its cargoes. Structural studies showed that the CC1 and CC2 domains at the N-terminal of BicD bind to dynein and dynactin, whereas the CC3 domain might specifically bind to cargoes such as Rab6 and some pathogen-linked proteins *(Liu et al, 2013)*. BicD like some other linker proteins are being appreciated more and more as more than just cargo adapters but regulators of dynein

motility. For instance, there are suggestions that BicD could influence the processivity of dynein *(Schlager et al, 2014)*.

1.2 Dynein Heavy Chain Mutations & Disease Models

Axonal transport is particularly vital to the functions of neurons given the distance that separates the soma from the synaptic region at the tip of the axon and dendritic compartments. Transport and timely delivery of cellular cargo to the right targets prevents the disruption of neuronal functions that could possibly result in disease.

Our initial understanding of dynein structure and functions have come from experiments in cell culture targeting dynein transport mechanisms and their effect on the so-called housekeeping roles of dynein in the cell. Previous researchers showed that dynein null mice die at the embryonic stage suggesting the need for dynein in development *(Harada et al, 1998)*. This broadly established dynein as an essential factor that supports life making it even more worth studying. Other studies brought insights into more of the roles of dynein, such as the trafficking of endosomes and lysosomes, organelles and mitosis *(Moore and Cooper, 2010).* Genetic manipulations in yeast and fungi have been invaluable in shedding more light on how dynein mutations might result in disease *(Sivagurunathan, S. et al. 2012, Qiu et al, 2013).* Some of the mutations disrupt axonal transport, which is a well-known mechanism in the occurrence of neurodegenerative diseases like Parkinson's, Alzheimer's and Huntingtin's (*Liu et al, 2012; Lloyd, 2012; Vicario Orri et al, 2015; Hoang et al,2017)*. Some of these mutations affect not only the

motor proteins themselves and but accessory proteins that aid them perform multiple roles. Additionally, the cytoskeleton, which constitute the tracks on which the motors travel, can also be targets of mutations that in turn hinder molecular motors and eventually result in disease *(Poirier et al,2013; Kumar et al, 2010)*. This makes the case for the need to the understand all the complex interactions associated with molecular motors as it is crucial to the pathology of neurodegenerative disease.

The heavy chain of cytoplasmic dynein, in particular, has been the protein of interest with over 20 mutations recorded to date and known to cause various neurological disorders in humans *(Figure 2)*. The dynein heavy chain is a 532 kDa protein encoded by a single gene, DYNC1H1 *(Banks and Fisher, 2008).* Interest in the heavy chain of dynein was further heightened with the generation of animal models by ENU mutagenesis named 'legs at odd angles' or *Loa , (Rogers et al, 2001)* 'cramping' *or Cra (Hrabe de Angelis et al,2000)* and the 'sprawling' mutation or Swl produced by irradiation *(Duchen, 1975).* There are some phenotypes that these early dynein mouse models have in common. The mice showed abnormal hind limb posture when suspended by the tail as well as abnormal gait and general muscle weakness. These mutations also disrupt dynein-driven retrograde transport involving various cargoes in one way or the other. As much as there are common features with the various mutations there are also unique the ways the function of the protein is altered based on where the mutation is in the heavy chain. *(Hafezparast et al, 2003)* reported the loss of motor neurons in both *Loa* and *Cra* heterozygote mice, *(Dupuis et al, 2009)* on the other hand reported sensory neuron degeneration in *Cra* heterozygous mice, *(Chen et al, 2007)* showed that *Swl* heterozygotes also lose

proprioceptive sensory neurons and muscle spindles. Experiments with homozygous mice from all 3 genotypes revealed severe defects in development, which explains why they are lethal at the embryonic stage. *Loa* homozygotes showed delayed migration of neurons on the hippocampus and defects in the layers of the cortex *(Ori-McKenney and Vallee, 2011).* Homozygotes with the *Cra* mutation were found to have defects in morphology and arborization of dendrites culture from the striatum *(Braunstein et al, 2010).* Embryos from *Swl* homozygotes were found to be abnormal and had developmental defects and died by E8.5. *(Zhao et al, 2016)*.

Actual human mutations in the dynein heavy chain documented over the years are characterized by developmental deficits and a dysfunction of parts of the neurological system *(Schiavo et al, 2013).* The incidence of malformations of cortical development (MCD) in 11 patients was traced to mutations in the dynein heavy chain *(Poirier et al,2013)*. Some of the mutations possibly affect specifically the motor and sensory neurons and are characterized by weakness in the muscle and atrophy *(Weedon et al, 2011).* In some cases, progressive muscle degeneration is observed classically referred to as spinal muscular atrophy with lower extremity predominance or SMA-*LED (Harms et al, 2012, Tsurusaki et al, 2012)*. Some human mutations in the dynein heavy chain were found to cause severe intellectual disability *(Willemsen et al, 2012).* Another study employing exome sequencing of the dynein heavy chain uncovered 8 *de novo* mutations and 1 familial mutation from a screen of patients with various brain malformations such as microcephaly and abnormalities on the cortex *(Poirier et al, 2013)*.

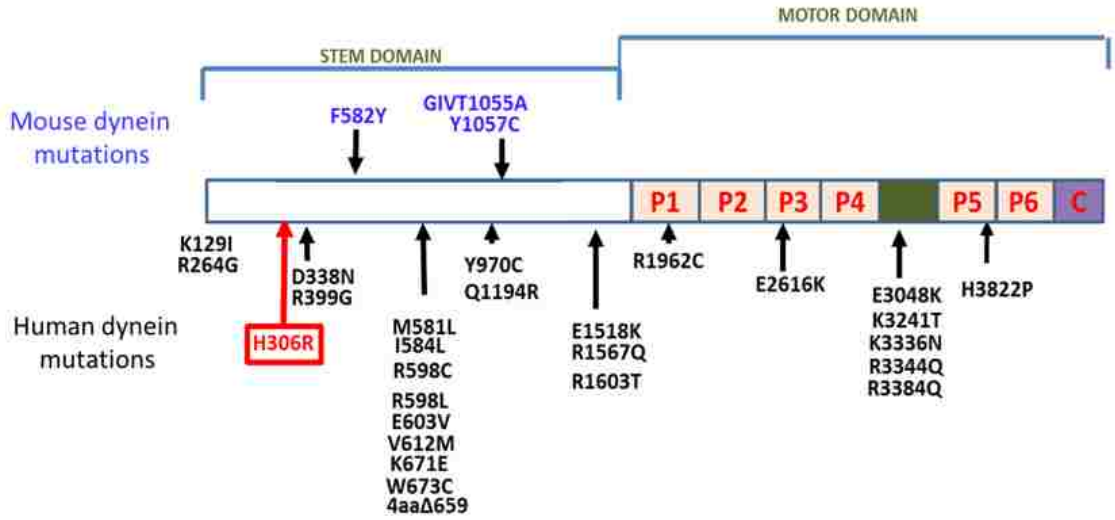


Figure 2 Schematic representation of mutations in the dynein heavy chain.

The diagram shows the different mutations that have been reported to date in the dynein heavy chain (about 4644 amino acids). Human mutations are differentiated from the mouse mutations, P1-P5 represent the ATPase domains, and C represents the C-terminal end.

The wide phenotypic spectrum which has clearly become the hallmark of dynein –related disorders could be due to the multifarious roles of dynein inside the organism and in the case of neurological disorders, the transport of different cargos that impact different processes. These factors could determine the sensitivity of a group of neurons over the other and therefore predict which neurological function will be compromised.

Different phenotypes are observed in human subjects with dynein heavy chain mutations, however the question of how and why still remains. It is possible that it is a result of the myriad of roles that dynein performs in cells. While there is no simple straightforward answer, we can learn a lot of useful information by relating the structure of dynein to its functions. It is important to investigate how mutations alter the dynein molecule and whether or not it's functions are altered as a result.

Evidence of the heterogeneity in phenotypes were initially observed in the animal models. For example, sensory deficits were seen in *Swl* and *Loa* mice but not in mice with the *Cramping* mutation. There was a reduction in the number of alpha motor neurons in the spinal cord of *Loa* and *Cra* mice but no motor neuron loss was recorded in *Swl* mice *(Banks and Fisher, 2008)*. Heterogeneity in the dynein heavy chain mutations was reiterated when 34 mutations were generated in *Neurospora crassa* (*Sivagurunathan et al, 2012,*) The length-dependent theory of dynein heavy chain mutations based on disrupted transport over long distances in axons would presume a bias towards the peripheral nerves instead of nerves in the brain or spinal cord. Yet, there are patients with deformities in the cerebral cortex and cognitive impairments all arising from mutations in

the dynein heavy chain. The effects of dynein heavy chain mutations on the CNS have previously been observed alongside peripheral neuropathies. For instance, in CMT2O, which predominantly manifests in muscle weakness and atrophy, at least 2 patients were reported with severe intellectual disability as a result of dynein heavy chain mutations *(Weedon et al, 2011).*

1.3 Importance of Axonal Transport

The distance that separates the soma from the synapse in neurons makes timely communication between these compartments very crucial. This issue of distance is accentuated by the fact that protein synthesis does not occur in some axons, hence the need to ferry the needed proteins from the soma. *(Morfini et al,2009)*. The unique functions of dynein in neurons unlike the 'so-called' general housekeeping functions have been studied extensively as a result of phenotypes observed from many heavy chain mutations.

Defects in axonal transport have been highlighted in relation to neurodegenerative diseases in amyotrophic lateral sclerosis (ALS) *(LaMonte et al, 2002 & Strom et al, 2008),* spinal muscular atrophy (SMA), *(Puls et al, 2008),* Huntingtin disease *(Trushina et al, 2004)* and tauopahies *(Ishihara et al, 1999, Ebneth et al, 1999)*. The cargos involved and the extent to which they facilitate disease progression varies. The transport of synucleins may play a role in the pathogenesis of Parkinson's disease *(Jenssen et al, 1999),* presenilin-1 and the amyloid precursor protein transport are important in

Alzheimer's *(Papp et al, 2002),* and problems related to the transport of mitochondria accounts for some of the dynein associated mutations *(Eschbach et al, 2013).*

Axonal transport is dependent not only on the motors but on other conditions, for instance the cytoskeleton. The neuronal cytoskeleton provides the structural integrity and due to its dynamic properties, the neuron is also able to change morphology and grow over time *(Chevalier-Larsen and Holzbaur, 2006).* The microtubules in the axon serve as the tracks for transport by molecular motors; dynein and kinesins transporting various cargo in the retrograde and anterograde direction respectively *(Figure 3).* The rapid polymerization and depolymerization in microtubules is required for the axon and growth cone earlier in the developmental cycle. This however is significantly reduced in the mature neuron as the microtubules are stabilized more in part by microtubule associated proteins (MAPs). These features of MAPs are vital in regulating transport in the axon through the interaction of the molecular motors with the microtubules *(Dixit et al, 2008).*

Although these motors have significant differences structurally as well as in their kinetics, experiments show that both kinesins and dynein's are transported bidirectionally suggesting a mechanism for the regulation of their functions *(Hendricks et al, 2010).* Additionally, apart from cargo transported predominantly in one direction a number of organelles are transported by both dynein and kinesin in both directions. Bidirectional transport of cargo may involve the attachment of either dynein or kinesin molecules. This ensures that each motor is available for respective movement and prevents the situation where one motor will be stuck in one compartment of the cell and be unable to

move cargo when required. Kinesin–driven transport towards the synapse may translocate mitochondria, synaptic vesicles, axolemma precursors *(Elluru et al, 1995, Leopold et al, 1992)*. Dynein is known to transport microtubules in neurons as experiments interrupting dynein transport revealed that the microtubules are stuck at the centrosome *(Ahmad et al, 1998)*.

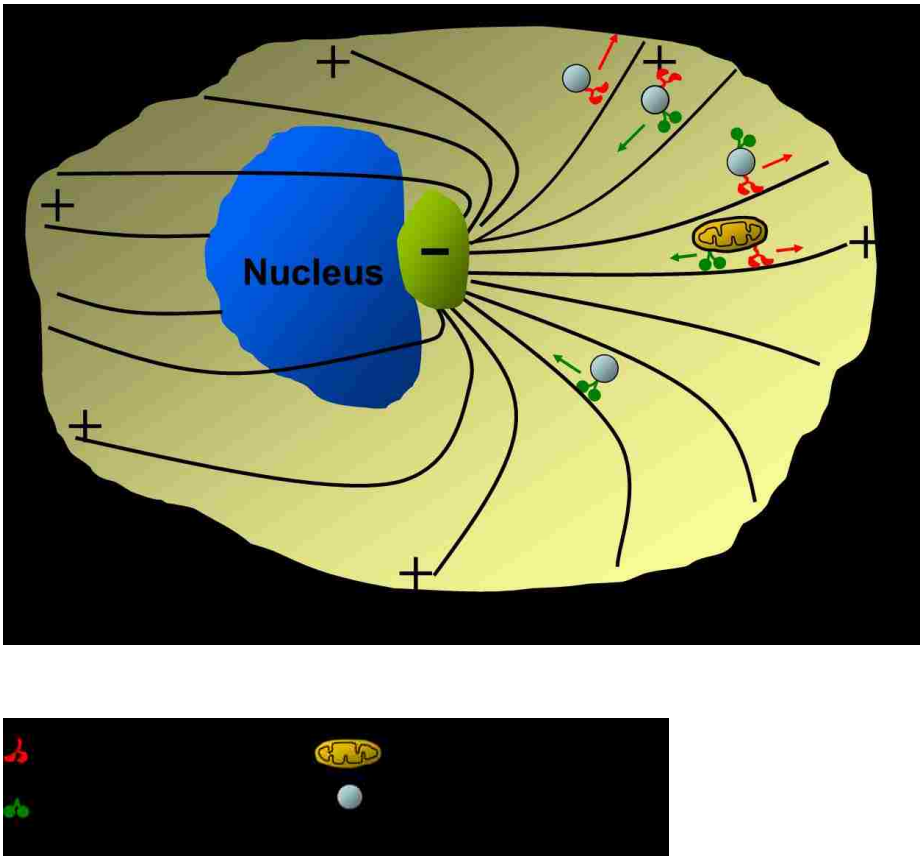


Figure 3 Microtubule based intracellular transport

The molecular motors dynein and kinesin generate force within cells to transport various cargo such as vesicles, signaling molecules, RNA and mitochondria utilizing microtubules as tracks. The majority of kinesins move cargo away from the nucleus to the minus end, whereas cytoplasmic dynein is responsible for retrograde transport towards the nucleus.

Dynein and kinesin transport neurofilaments which provide structural support to the axon and dendrite and aids in the transmission of nerve impulses *(Roy et al, 2000, Yuan et al, 2015)*. It has also been suggested that some neurofilament proteins offer stability by interacting with synaptic proteins from the brain and peripheral synapses *(Ralph and Nixon, 2016).* The movement of cytoskeletal proteins ie. microtubules, actin, neurofilament is believed to be proceed at a much lower rate than organelle transport *(Baas and Buster, 2004).* This is probably needed to allow remodeling of the cytoskeleton at the growth cone, so that axons can be withdrawn if need be and reorganization of microtubule polarity in dendrites.

In the developing neuron, dynein plays a role in neuronal migration via its interaction with other binding partners. *(Sasaki et al, 2000)* showed that the Lis1, which is the mutant protein in the neurological condition Lissencephaly or smooth brain, marked by abnormal cortical layering in the brain and large ventricles interacts with dynein and could possibly be a regulator of retrograde axonal transport. Neuronal migration defects were also confirmed in a dynein mutant mouse model by *Ori-McKenney and Vallee, 2011*. Dynein was identified as part of the postsynaptic density (PSD) in the dendritic spines of rats *(Cheng et al, 2006).* This finding in addition to what is already known about the transport of synaptic components to the soma for recycling suggests a role for cytoplasmic dynein in the set up and maintenance of synaptic connections. Furthermore, synaptic plasticity is affected by the activity of dynein and kinesin motors via the transport of specific components (*Yagensky et al, 2016).* Cargoes transported by dynein aid in maintaining neuronal health and survival, for instance signaling molecules,

proteins to be recycled *(Delcroix et al 2004, Susalka and Pfister, 2000).* The clearing of misfolded proteins helps prevent their eventual accumulation and/ or aggregation which could have deleterious effects on the neuron. Signaling molecules could also be transported as receptor –ligand complexes and are vital in the signaling pathway that ensures survivals of neuronal cells *(Harrington and Ginty, 2013).*

(Yano et al, 2001) reemphasized the important role of dynein in the neurotrophic signaling cascade when they reported the binding of dynein to the neurotrophic receptors TrkA, TrkB and TrkC. Brain –derived neurotrophic factor (BDNF) is one such neurotrophic factor known to control dendritic growth and morphology in cortical neurons *(McAllister et al, 1997).* When BDNF mediated transport is perturbed by dissociating dynein from the adaptor snapin, dendritic growth is reduced *(Zhou et al, 2012).* Dynein is also instrumental in the lysosome degradation pathway. The transport of autophagosomes to the cell body by dynein allows them to mature as they acquire other components en route. *(Maday et al, 2012).* Jip-1 and huntingtin, which are major players in the transport of autophagosomes have been shown to associate with dynein and kinesin *(Fu et al, 2014).*

There is growing evidence that supports the possibility of local protein synthesis in the axon, which largely stemmed from the identification of components of the protein synthesis apparatus *(Steward and Levy, 1982, Giuditta et al,2002, Fumy et al, 2010).* Axonal protein synthesis can be typically observed when there is an injury and there is the need to turn on biochemical and molecular switches to reprogram a growth cone for

axonal regeneration *(Yoo et al,2010).* All of this means that mRNA and indeed components of the translation machinery must be transported to the site of translation, this is accomplished by dynein and kinesin activity *(Zhang et al, 2001).* Further involvement of dynein in mRNA transport is seen with RNA granules, which are complexes of mRNA plus other proteins that help target it to a specific location *(Bassell and Kelic, 2004)*. Stress granules contain ribonucleoproteins and enable the cell mount up the appropriate response under harsh conditions like toxin exposure, elevated temperature, and factor generally repress translation in one way or the other *(Kedersha and Andersen,2002).* In spinal cord neurons subjected to arsenite stress, dynein was discovered to be key not only in the composition of stress granules but equally in the disintegration of the complexes as well. *(Tsai et al, 2009. Loschi et al, 2009).* Axonal transport is central to a lot of the processes in the neuron that promotes its survival, and it is also important in the pathology of neurodegenerative diseases as a whole; during both the subtle and overt stages. Exploring how these diseases influence the intricate interactions of motor proteins like dynein with different cargoes and accessory proteins will be key to the development of effective treatment and management solutions.

1.4 Charcot Marie Tooth Disease

Charcot-Marie-Tooth disease (CMT) is named after 3 neurologists, Jean-Martin Charcot, Pierre Marie and Howard Tooth, who discovered it in 1886 *(Daroff et al, 2012)*. The disease also referred to as hereditary motor and sensory neuropathy (HMSN) describes multiple inheritable motor and sensory neuropathies affecting various proteins *(Figure 4. Pareyson and Piscosquito, 2014)*. 1 in 2500 individuals are believed to be affected by CMT disease *(Nelis et al,1996)*, making it the most common inheritable peripheral nerve disorder worldwide *(Magy and Vallat, 2015)*. To date over seventy genes have been implicated in the different types of CMT disease. Electrophysiological readings led to the broad classification of the forms of the disease as either demyelinating (CMT1) and non-demyelinating (CMT2) or Axonal *(El-Abassi and Carter, 2013)*. CMT1 involves mutations in genes encoding myelin proteins or transcription factors for example PMP222, MPZ, and CX32. When proteins that function in the maintenance of the axon are affected, it is classified as CMT2. Some examples are CMT2B (mutation in the KIF1B kinesin gene), CMT2B (mutation in RAB7) and CMT2E (mutation in neurofilament gene NEFL) *(Zhao et al, 2001, Meggouh et al, 2007, Mersiyanova et al, 2000)*. CMT disease reduces the quality of life of its sufferers since they have to deal with limited mobility arising from distal limb weakness, deformity and pain. Some of the common deformities include pes cavus, pes planus and hammer toes *(Figure 5, Pareyson and Marchesi, 2009, Tazir et al, 2014).* Less common features like gait problems and loss of sensation in the hands and feet can also impose further difficulty on patients. CMT disease has a variable onset based on patient data and usually begins with muscle

weakness and atrophy of the feet and then slowly advances to the upper limbs (*McCorquodale et al, 2016)*.

1.4.1 Diagnosis

Due to the heterogeneity of the phenotypes and variable onset in some cases, physicians cannot rely heavily on family history. As a result, genetic testing has proved invaluable in the diagnosis of CMT disease. Clinical evaluation may also involve investigating sensory and motor symptoms, muscle strength in the arm and leg and nerve conduction velocities. Advances in sequencing have proved useful with the use of DNA arrays, exome sequencing and next generation sequencing in genetic tests.

Nerve conduction velocities (NCVs) above 38 m/s are classified as CMT2 whereas demyelinating CMT1 NCVs are usually less than 38m/s. Mid-point values from 36-45 m/s are categorized as Intermediate CMT or X-linked CMT *(Hoyle et al, 2015)*. Thus, the electrophysiological test is needed to distinguish the demyelinating form from the non-demyelinating form to help inform the most appropriate treatment regimen. Research into therapeutic options for the various forms of CMT diseases to a very large extent depends on the genes or proteins involved and the cellular processes that are altered *(Figure 4)*. For instance, in animal models of CMT1A, vitamin C and progesterone antagonists have been found to limit the expression of PMP22. These results however were not replicated in human subjects *(Sereda et al, 2003, Gess et al, 2015)*. Other strategies involve targeting ligands, inhibitors or receptors involved in transducing signals downstream of the affected genes. Targeting the genes of interest poses a challenge in subtypes of the

disease with multiple mutations, like CMTD, where there are about 15 reported mutations in the enzyme glycyltRNA synthetase (GlyRS) *(He et al, 2011*). Gene therapies comprising the use of induced pluripotent stem cells (IPSCs), RNA antisense technologies aimed at modifying gene expression could provide more promising outcomes. More extensive research is required regardless of the strategy, to move these remedies successfully from the pre-clinical to the clinical phase.

1.4.2 Management

Clinically, physical therapy and exercise are commonly employed in managing the associated symptoms of CMT disease. Gait, balance and posture can be corrected after consistent application so that the risk of injury or fall is greatly reduced. These exercises are helpful in reducing the stiffness in affected muscles and can be important in preventing deformities in the limbs later on in life. *(Mathis et al, 2015).* Some ankle and foot deformities such as pes cavus and hammertoes require surgical interventions due to their severity. Physical and occupational therapists offer assistive or orthotic devices such as special shoes, braces, crutches and canes to ease mobility in patients *(Bird, 2016).* Orthoses can help provide extra support to the local site affected and compared to surgical procedures, serve as simpler, non-invasive procedures to manage the cognate symptoms of CMT disease. In designing physical activities for neuromuscular disease patients, it is highly recommended to start with low-intensity exercises rather than high intensity regimens *(Abresch et al, 2012)*. This allows relief and/or recovery to occur at a steady pace over a period of time without compounding the symptoms.

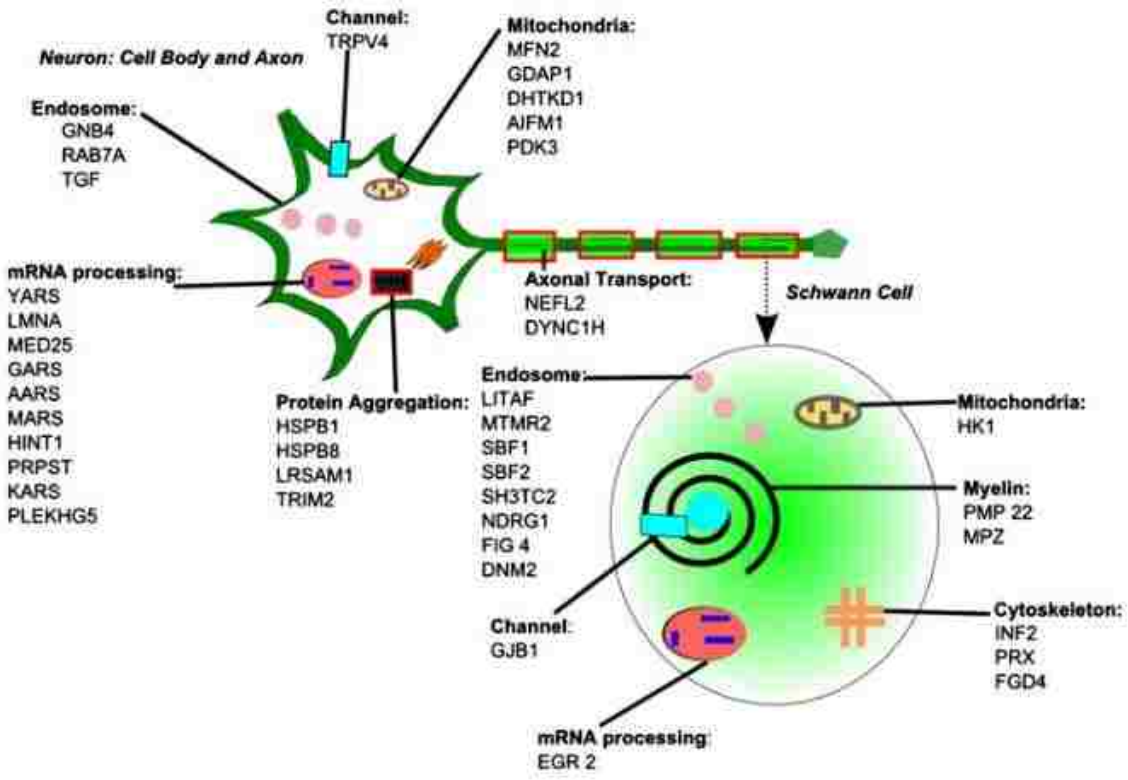


Figure 4 CMT target genes affected and cellular processes involved

Many genes account for CMT disease phenotype. Mutations affecting proteins in the Schwann cell are classified as the demyelinating form of CMT, while those affecting axon and cell body proteins fall under the non-demyelinating category of CMT disease (*Jerath and Shy, 2015)*.

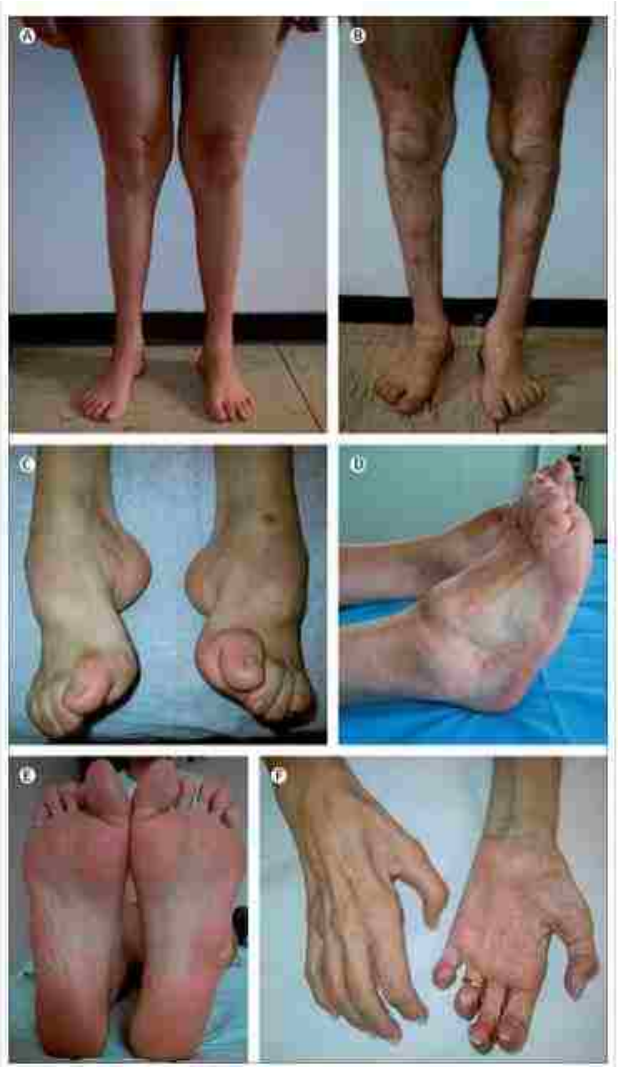


Figure 5 Classical features of Charcot Marie Tooth Disease

CMT disease impairs ion of motor and sensory nerves resulting in a range of phenotypes that mainly affect the lower limbs and the hands in some cases. (a,b)Atrophy of the muscle in the lower limbs, (c) pes cavus (d) hammertoes, (e) callosities of the foot and (f) claw hands *(Pareyson et al, 2009)*

Efforts directed towards pharmacological interventions have not been so successful, for example initial findings about ascorbic acid for the treatment of CMT1A looked promising but actual data from patients did not show much improvement in their health *(Visioli et al, 2013)*. The plant –derived curcumin compound that is known to have multiple therapeutic effects was found to boost the production of Schwann cells by reducing myelin protein zero protein misfolding *(Patzko et al, 2012).* Mice bearing mutations in the heat shock protein HSPB1 recorded modest improvements in some symptoms when histone deacetylase 6 (HDAC6) inhibitors were administered. HDAC inhibitors alter the transport defects through tubulin deacetylation *(d'Ydewalle et al, 2011)*. In CMT1A rats, there was an observed amelioration of the disease phenotype when a progesterone antagonist, onapristone was administered. Onapristone reduces the levels of the Pmp22 gene which is normally overexpressed in CMT1A disease *(Sereda et al, 2003)*. However, onapristone could not be used in humans due to its toxicity, prompting the need for further studies into alternative progesterone antagonists *(Shy,2006)*. Going beyond just CMT1A, scientists are looking into different strategies and approaches to find remedies that can work in humans. One example of such is a massive drug screen involving 3,000 drugs in a high throughput screen that led to a shortlist of 9 with significant activity against different targets. More toxicity studies excluded 5 of them, leaving just 4 suitable compounds *(Jang et al, 2012)*. Although this screen was specifically targeting modulators of myelin genes it is worth looking into for other forms of CMT disease.

Dietary supplements like some essential fatty acids, vitamin E as well as the drug 3, 4-diaminopyridine have been experimented as treatment options, but once again they are not backed by convincing data in patients *(Williams et al, 1986 & Russell et al, 1955).* One major challenge that has hampered the search for remedies in the past has been the absence of adequate historical records on the disease. This is because sometimes symptoms are observed late, as the disease progresses slowly. Also,it is possible patients were previously misdiagnosed when parameters were not clearly defined. This has been addressed to some extent with the emergence of focused groups like the INC (International Neuropathies Consortium) which are working hard to collate data on patients *(Gutman and Shy, 2015).* Another initiative to ensure success in dealing with CMT disease is to encourage collaboration among health experts; neurologists, geneticists, physiatrists and orthopedic surgeons to provide more holistic care and to possibly give patients a better quality of life. *(McCorquodale et al, 2016)*. Such collaborative efforts facilitate data sharing and enables providers monitor disease progression and patient recovery more closely.

1.5 Skeletal Muscle Function

Voluntary movement in the body is achieved through striated cardiac and skeletal muscles *(Kho et al, 2012)*. Skeletal muscles attach to the bone via connective tissues and are responsible for the movements of bones at a joint. Skeletal muscles therefore possess tensile and contractile properties that enable them to support movement. These are made possible by a myriad of cytoskeletal and structural proteins that are well regulated with

coordinated functions to obtain the proper assembly. Sheets of connective tissue known as fascicles, cover multiple muscle fibers organizing them into bundles. Skeletal muscles have numerous nuclei, distinguishing them from other cell types. However, they possess organelles such as mitochondria, endoplasmic reticulum, and glycogen granules just like other cells in the body *(Fox, 2011)*.

1.5.1 Muscle Sarcomeres

Sarcomeres are the units of contraction in skeletal and cardiac muscle *(Goulding et al, 1997)*. The highly ordered patterns account for the striations in these muscle types. The proper assembly of various proteins into the final ordered structure is indispensable in the maintenance of proper muscle structure and function. The components of the sarcomere appear as bands or lines under the microscope. There are 4 main components of the sarcomere; Z-line, I-band, A-band and M-line *(Figure 6)*. The Z-line delineates the sarcomere and consists of proteins such as alpha-actinin, capZ, desmin and myotilin that serve as attachments of the thin filaments. The I-band is made up of mainly actin and other associated proteins including troponin, tropomodulin and nebulin. The region consisting of mainly thick myosin filaments and thin filaments is referred to as the A-band. Contraction of the muscle occurs when the sarcomeres shorten due to the sliding of the myosin filaments over actin. *(Cadot and Gomes, 2016).* At the center of the A-band, are a group of specialized proteins belonging to the myomesin family that make up the M-line or M-bridge *(Henderson et al, 2017)*. These function to provide stability to the myosin heavy chain and in sensing and transducing intracellular signals.

The highly ordered sarcomere lattice would suggest a fixed, rigid structure, however, it can undergo reorganizations in response to different cues like stress or injury.

1.5.2 Myonuclei and Muscle Development

Skeletal muscle fibers are characterized by numerous nuclei and units of contraction known as sarcomeres in tandem which account for the striations. The muscle nuclei are maintained over a range of specific internuclear distances possibly to mark out domains for more efficient transcriptional control *(Bruusgard et al, 2003, Pavlath et al,1989)*.The debate as to whether nuclei number is related to the size of the muscle fiber is still pretty much unresolved, especially when it has been observed that increase in muscle size is not always accompanied by a large presence of myonuclei *(Verheul et al,2004; Aravamudan et al, 2006)*. Progenitor cells in the muscle known as satellite cells are positioned between within the basement membrane and sarcolemma. Their location within the vicinity of myonuclei at the periphery, likely enhances the replacement of damaged nuclei in the event of an injury *(White et al, 2010)*. The maintenance of a maximum number of nuclei marks the attainment of maturity in the muscle and is necessary for muscle functioning *(Davis and Fiorotto, 2009)*. This makes satellite cells therapeutic targets for the treatment various neuromuscular disorders if their regenerative capacity can be harnessed. There are several factors that influence proliferation in satellite cells; muscle type, age and chemical modulators *(Manzano et al, 2011)*. Testosterone for instance, enhances the proliferation of satellite cells and increases muscle nuclei abundance in rats *(Joubert &Tobin, 1989)*. The satellite cells maintain an actively dividing population during development, and a smaller population that stays in a quiescent mode. The larger proliferating group is high in numerosity and most active during early postnatal days accounting for the high cell number, after which both number and activity decline in adulthood *(Neal et al, 2012)*.

Nuclei positioning is very important for the normal functions of the muscle fiber, conversely, the misalignment of the nucleus is linked to disorders of the muscle *(Gueneau et al, 2009, Romero,2010)*. Molecular motors, microtubules and a host of adaptors and regulators are responsible for the placement of nuclei in different cell systems. Work done in the muscles of *Drosophila* points to joint roles by both dynein and kinesin in positioning nuclei *(Folker et al, 2014)*. This study proposed a mechanism with segregated roles for the motors where their ability to generate force is harnessed at leading and trailing ends of the migrating nuclei. In mutant *Drosophila* embryos with impaired dynein and kinesin function, changes occurred in the shape of the nuclei and the pace of myonuclei transport was much slower.

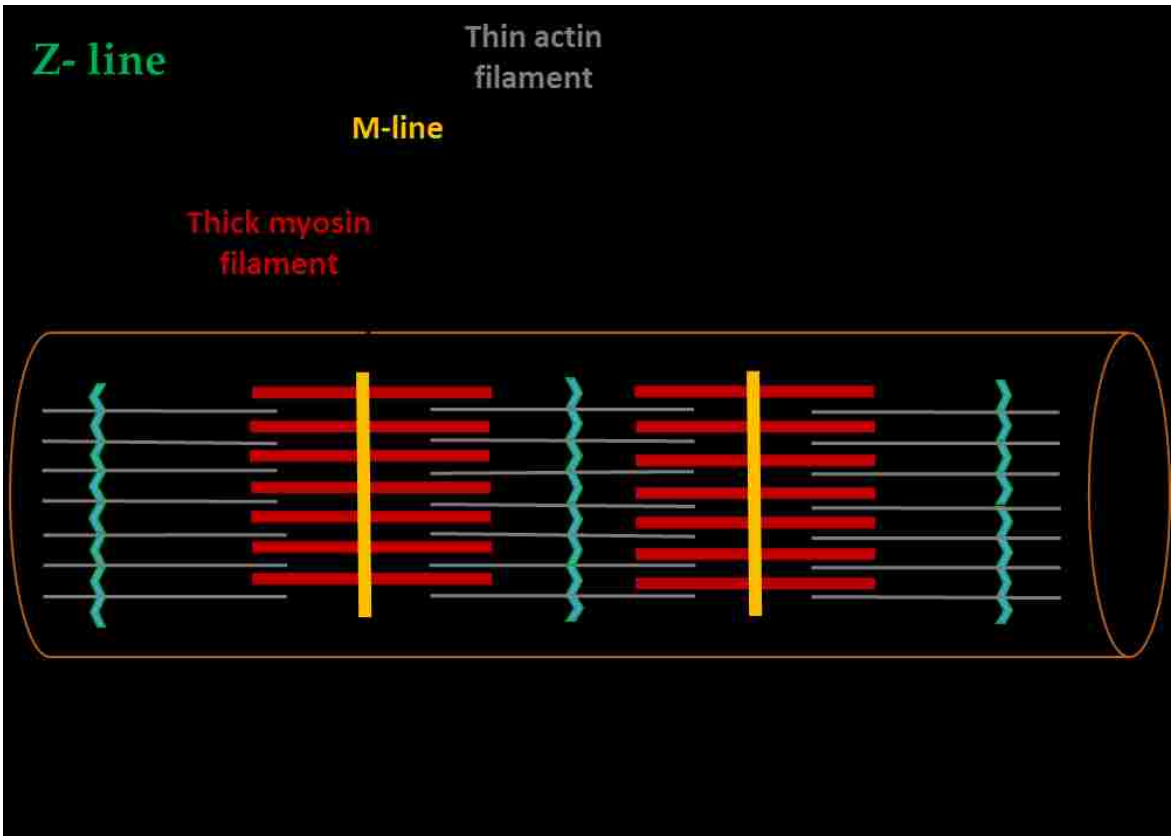


Figure 6 Illustration of the contractile unit of the muscle

Long tubular structures called myofibrils make up each muscle fiber, and these myofibrils possess repeating sarcomeres. The distance from one Z-line to the other represents the length of one sarcomere. Thick and thin filaments overlap on the A-band. The I-band has only thin filaments and the H-zone only has thick filaments whose center is marked by a group of proteins constituting the M-line.

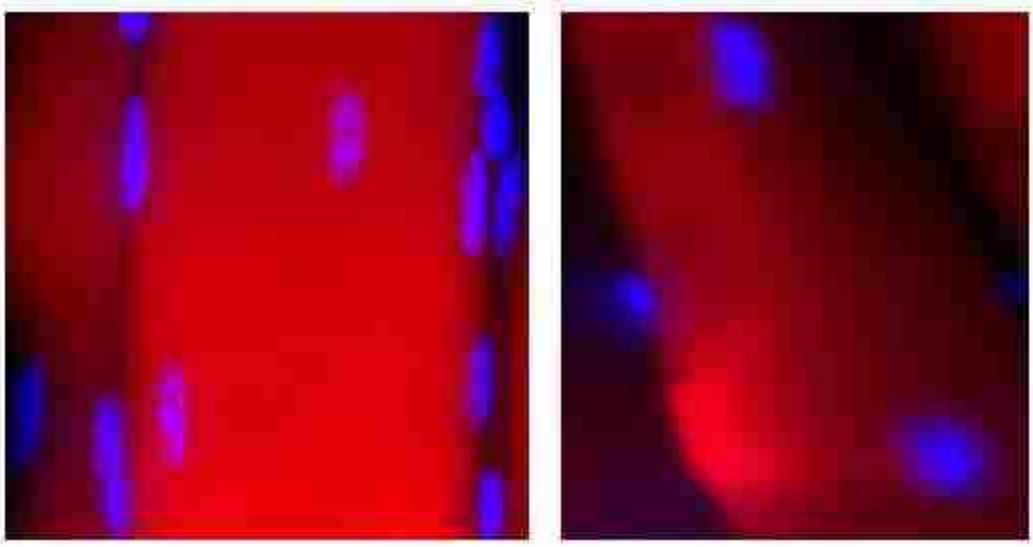


Figure 7 Nuclei placement in muscle fibers

Muscle fibers stained in red and DAPI-stained nuclei (blue) from a wild-type mouse muscle tissue. Nuclei are normally positioned at the periphery of the muscle nuclei based on the need to maintain transcriptionally controlled sections within the fiber. When there is some muscle pathology, the arrangement could be disrupted leading to the presence of more centrally-positioned nuclei within the muscle fiber.

1.5.3 The Neuromuscular Junction

The neuromuscular junction (NMJ) is a peripheral synapse that maintains the connection between the motor neurons and muscle fibers. This connection ensures that action potential initiated by the nerves are carried across to ensure contraction of the muscle *(Tintignac et al, 2015).* The formation and maturation of the NMJ consists of a myriad of molecular events that are well regulated and coordinated by key players. This multitiered set up also makes it vulnerable to disease at diverse levels when the normal functions are disrupted. Disorders of the peripheral nerves such as Charcot Marie Tooth Disease, SMAs, myasthenias and muscular dystrophies usually affect the neuromuscular junction, making it important in understanding the pathology and progression of disease. Mutations that affect signaling pathways that control the maturation and stability of the synapse or the withdrawal of nerve inputs can all interfere with the normal functioning of NMJs and lead to muscle weakness, fatigue, atrophy and paralysis in very severe cases *(Ferrraro et al, 2012).* The setup of the peripheral synapse begins with motor axons making contact with their muscular target once the myotubes fuse to form the myoblasts. *(Lin et al, 2001)* observed that majority of the AChRs are innervated by E13.5, with complete innervation of all AChRs achieved by E16.5. Transmission at the synapse occurs once the growth cones of the motor axons reach the myotubes. The NMJ at this point is functional and is characterized by having multiple inputs from a number of axons at the time of birth.

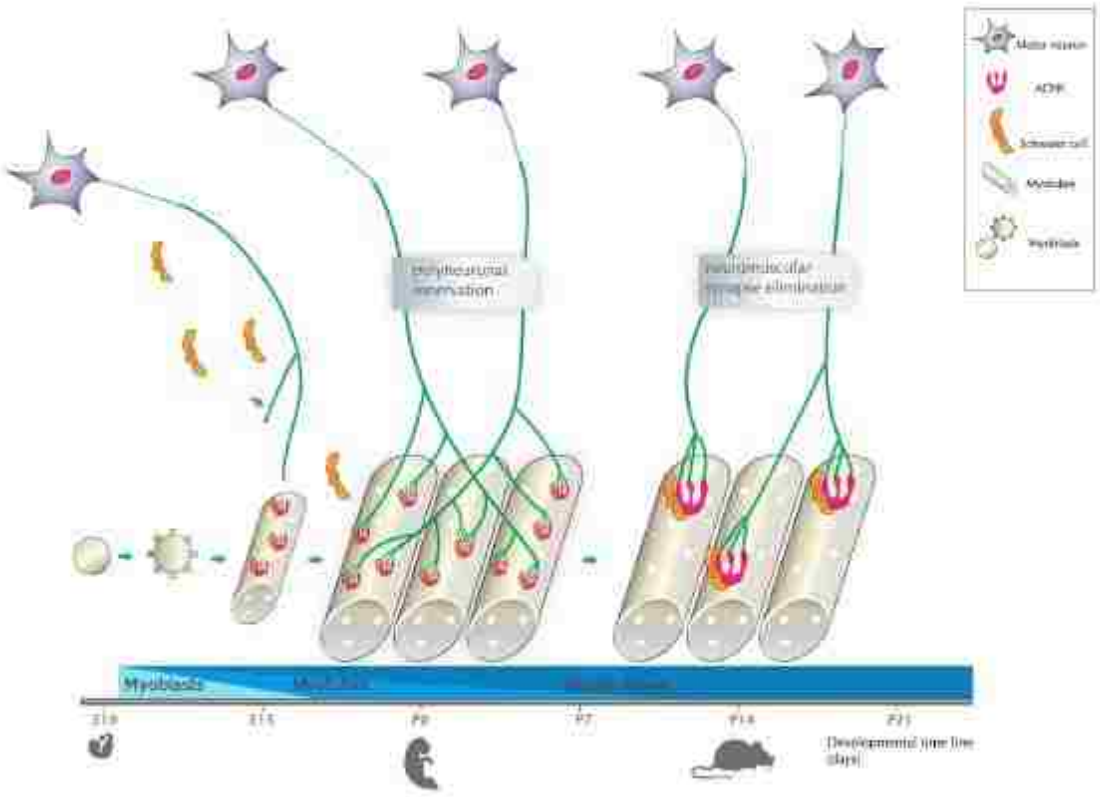


Figure 8 The developmental timeline of neuromuscular junctions in mice

Motor neurons contact muscles by E12.5 after the myoblasts fuse to form myotubes. Terminal Schwan cells start move towards the end-plate some time before E15. Acetyl choline receptors also cluster as the axons approach the myotubes. One of the most significant changes that occurs afterwards is the shift from poly-innervated muscle fibers to single innervated fibers beginning from birth to P14. *(Bloch-Gallego, 2015)*

1.5.3.1 Synapse Maturation

The most noticeable feature is the change in shape or morphology of the post-synaptic apparatus, from a solid plaque to a highly-branched structure commonly referred to as the 'cookie to pretzel transformation'. The end plate, which was previously flat is elevated to form gutters or troughs. There is also an increase in other cellular components at the end plate which help make it more stable, some of these include the basal lamina and various cytoskeletal proteins. The AChRs undergo a change in Ca permeability that favors the initiation and generation of action potential. Similarly, the ion and ligand gated channels are also reorganized to achieve the same purpose. Structural changes occur as the AChRs mature; the most significant is the replacement of the γ – subunit to an ε- subunit. The morphology of AChR clusters at the endplate also changes during maturation. Nerve inputs are withdrawn till the end plate has just one input. The transition from an immature polyinnervated apparatus into the fully-developed form in the adult is responsible for the rearrangement of AChR structure *(Figure 8. Sanes and Lichtman, 1999).* Further changes occur as the muscle fiber increases in surface area due to growth. The invaginations that develop are typically about 1um deep and are spaced at 3um from one another. The AChRs located on the crests of the folds are as a result easily accessible to the nerves. Another major change in the mature post-synaptic apparatus is the subunit change that occurs in the AChR. One subunit out of the pentamer ($\alpha_2\beta\delta\gamma$), specifically the γ-subunit is traded for an ε-subunit ($\alpha_2\beta\delta\varepsilon$) to make the receptor more stable *(Missias et al, 1996, Sanes and Lichtman, 2001)*.

1.5.3.2 Transmission of Nerve Impulses

Innervation of the muscle by motor neurons is critical for normal functioning and structural development of the peripheral synapse. The innervation precedes the cascade of molecular events that occur to ensure the proper development and maturation of the NMJ. In a functional neuromuscular synapse, the transmission of signals in the post-synaptic compartment is facilitated by a surge in the number and density of acetyl choline receptors at the end plate. The acetylcholine receptors (AChR) can accumulate up to over 10,000μm^2 in density in the synaptic zones. This number is vastly different from regions farther away where the density is approximately 10μm^2 *(Sanes and Lichtman, 1999)*. Subsynaptic nuclei located beneath the receptors are partly responsible for this phenomenon. These nuclei positioned at the NMJ in clusters of between 3- 8 nuclei maintain a transcriptional profile distinct from nuclei outside the synaptic region and transcribe genes needed for the developing NMJ *(Simon et al,1992, Briguet and Ruegg,2000).* The reorganization and specification that occurs in and around the synaptic region is achieved via three pathways. Agrin is released by the nerves and it binds to the muscle specific receptor tyrosine kinase (MuSK) present in the muscle. This leads to other downstream events that result in the clustering of acetyl choline receptors. Preferential expression of AChR genes in the synaptic nuclei also occurs and this is mediated by neuregulin and erbB kinases. The upregulation in transcription of AChR genes requires the suppression of gene expression in regions outside of the synaptic region. Acetyl choline binds to AChRs to trigger the influx of calcium and the generation of a voltage –dependent signal to down regulate the expression of genes. The nerves were initially thought to be required for clustering of the acetyl choline receptors in the initial stages of development. *(Lin et al, 2001)* examined neuromuscular junction development

early at E14.5 and identified a concentration of AChRs, suggesting that neural cues may not be needed to initiate NMJ development. This observation confirmed what was previously reported by other researchers *(Lupa and Hall, 1989 & Dahm and Landmesser, 1991)*. Experiments with mutations in the MuSk show it is a major player in the neural-independent post synaptic organization seen early in development. That notwithstanding, the nerve input is required for further development and maintenance of the neuromuscular junction. The involvement of the MuSK pathway in initiating synaptic differentiation help explains why mice lacking the receptor die at birth, and why mutations lead to congenital myasthenic syndrome in humans *(Dechiara et al, 1996 and Chevessier et al, 2004).* MuSK depends on agrin binding to dimerize and activate its kinase activity. Further downstream, other kinases responsible for the phosphorylation of the AChR subunits are also activated. A membrane protein which associates with the AChRs known as Rapsyn or Receptor-associated protein at the synapse is vital in AChR clustering. Studies into the precise function of Rapsyn indicate that it is required for phosphorylation of AChRs. The cytoskeleton is another crucial factor in triggering AChR clustering. The MuSK-agrin path way mediates the positioning of the receptors on actin filaments after they have undergone some rearrangements. This process may be important to provide additional stability to entire post-synaptic apparatus *(Strochlic et al, 2005).*

1.5.4 The Role of Glial Cells at the Neuromuscular Junction

The peripheral synapse is a tripartite system consisting of the motor neuron, acetylcholine receptor and perisynaptic Schwann cells (PSCs) also known as terminal Schwann cells. Details about the exact functions and mechanisms of these specialized Schwann cells located at the synapse are still being uncovered. The glial cells associated with the peripheral synapse differ from myelinating Schwann cells in many ways: the most obvious being location with the PSCs covering the nerve terminal and the myelinating Schwan cells wrapping around the axon further upstream, accounting for saltatory transmission of nerve impulses. Both myelinating and non-myelinating Schwann cells share similar proteins or markers such as myelin-associated glycoprotein, myelin protein zero (MPZ), galactocerebroside and 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase) *(Feng & Ko, 2008)*. Mature PSCs however uniquely express genes not found in myelinating glia such as LNX1, an E3 ubiquitin ligase *(Young et al, 2005)* and Nav 1.6 *(Musarella et al, 2006)*. These biomarkers have proved very useful in the investigation of PSCs and their effects on NMJ development. PSCs possess more neurotransmitter receptors and ion channels in addition to receptors for ATP and acetylcholine. They also produce chemicals like nitric oxide, prostaglandins and glutamate, which regulate synaptic communication *(Auld et al, 2003)*. The density of PSCs at the NMJ significantly increases as the animal progresses to adulthood, possibly in response to roles in the maintenance and proper functioning of the synapse *(Love and Thompson, 1998*).

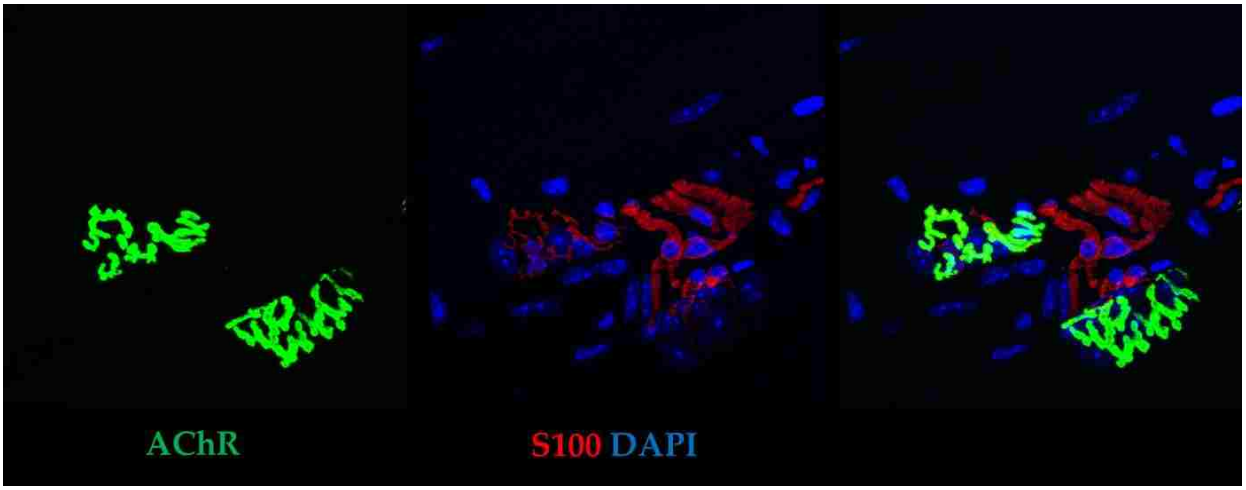


Figure 9 Glial cells at the neuromuscular synapse

Non-myelinating glial cells, also known as terminal Schwann cells (stained in red) are a part of the tripartite apparatus of the peripheral synapse. Terminal Schwann cells play a role in the maintenance of NMJs and guide resprouting axons to their target.

1.5.4.1.1 Roles of PSCs in the developing NMJ

PSCs and axons make their way early to the muscles at about the same time in the developmental timeline. There is evidence to show that although the nerves still successfully associate with muscles, they are significantly defasciculated when PSCs are ablated *(Suguira and Lin, 2011)*. Whilst the axons may not need the PSCs to locate their targets, they start to retract when the PSCs are depleted from the NMJ. Thus, PSCs may rather be important in maintaining the neuromuscular synapses through various means. PSCs were found tightly associated with the AChR dense areas in developing NMJs up to 14 days after birth. More probing revealed how they assist in input removal through the phagocytosis of nerve terminals *(Smith et al, 2013)*. This is explained by the ability of the PSCs to sense the strength of synaptic inputs from incoming nerves at polyinnervated NMJs *(Jahromi et al, 1992; Darabid et al, 2013)*. Researchers have been interested in the molecular switches that regulate the PSCs in the course of development and how that affects neuromuscular function as a whole. ErbB2 is a tyrosine kinase receptor which acts downstream of neuregulin and is normally found in Schwann cells and muscles during NMJ development. Studies with erbB2 deficient mice revealed not only aberrations in NMJ development but the loss of Schwann cells at nerve terminals of developing embryos. The mice also die at birth most likely because of breathing difficulties caused by neuromuscular anomalies in the diaphragm *(Lin et al, 2000)*. The removal of PSCs adversely affects neuromuscular junction development as the nerves of the muscle retract and the motor neurons degenerate *(Wolpowitz et al,2000)*.

1.5.4.1.2 Roles of PSCs in the mature NMJ

A lot of what is now known about PSCs came from studies with frogs (*Rana pipiens)*, although they differ from rodents in many ways. In frogs, the PSCs extend long projections into the synaptic area and the amphibians PSC is maintained in a dynamic state and are actively involved in transforming the entire NMJ structure, this contrasts with the relatively more stable set up in mammals *(Ko and Robitaille, 2015)*. Injury or damage to the nerve also triggers the phagocytic activity of the PSCs. This process clears debris and guides axons to reinnervate the muscles. Through a similar mechanism, PSCs isolates surrounding connective tissue and basal lamina when there is an injury, or even when the axon is severed. The axon is unable to initiate its own regeneration and depends on the PSCs and other key players to make contact again with target cells. In the Schwann cells of both adult frogs and rodents, there are receptors for the binding of Ca^{2+} and ATP *(Parinello et al, 2010)*. The presence of muscarinic and purinergic receptors in PSCs makes it possible to modify Ca levels and ultimately the expression of specific genes. PSCs generally influence NMJ structure and function often with the input of upstream signals. Neuregulins, for instance via the erbB kinases trigger changes in the Schwann cells that maintain efficient synaptic transmission and stabilize motor terminals (*Trachtenberg and Thompson, 1997)*. PSCs produce neuronal factors that stimulate a cascade of downstream events. Neuregulin-2, required for transcription by acetyl choline receptors and agrin, an inducer of receptor aggregation, are expressed by PSCs *(Yang et al, 2001, Rimer et al, 2004)*. It is safe to speculate that expression of these factors allows the maintenance of the NMJ to continue even after maturity, or it may be a backup mechanism during denervation or neuronal injury.

1.6 Animal Behavioral Assays

The use of standardized protocols to examine the phenotype of animals with mutations has become valuable in establishing disease model systems and in translational research. These protocols allow the mutant animal to be pathologically characterized to either confirm or rule out the existence of disease. The benefits of standardized phenotypic assessment of animals include the possibility of differentiating spontaneous and induced mutations, strains of animals and analyzing the impact of transgenic or genetically manipulated models of disease *(Rogers et al, 1997)*. There are certain conditions that must be generally satisfied regardless of the specific application of behavioral assessment. It is vital to avoid partiality by keeping benchmarks for all groups, and these include but are not limited to, initial time point and endpoint, features or characteristics to be examined, a scoring system if needed and an accurate record keeping system maintained for all tests carried out. Behavioral assays also cover observations on the morphological characteristics of the cohort; body mass, body length and notes on the functional activities as well. An often-used well-designed system for behavioral testing is the SHIRPA analysis *(Rogers et al,2001)*. This comprises a protocol involving three stages of screening to assess neurological impairments in an animal. This can be very useful in cases where it is not possible to predict exactly which system will be affected for instance, whether there is more of a sensory deficit or a cognitive deficit in the case of neurological disease models. The initial screen of SHIRPA is based on observation of the general health of animals for features like hair color, gait, posture, excitability, aggression, defecation, salivation etc. It is a general analysis that also requires a scoring system to properly document findings. The next level of screening is more detailed and

targets an underlying pathological condition. Motor performance, balance and coordination and nociception are some of the measurements that can be taken and these can be done with the appropriate measuring device or equipment. Histopathological procedures, biochemical analysis of plasma or serum for metabolic investigations are done at this level of analysis. The third of level of screening is custom-made for a known condition that does not necessarily require a battery of assays to establish the presence of a phenotype. At this stage investigators will be interested in defects of a cognitive or psychiatric nature. The assays are usually for more specific conditions, anxiety for instance can be measured with the open-field activity test, elevated maze or light-dark box; learning and memory defects can be examined with the Morris water maze. At this final stage of screening some other techniques like nerve conduction, electromyography, electroencephalography and magnetic resonance imaging could be utilized.

The reproducibility of behavioral data is important like in any other assay for that matter, for that reason it is important that the environment in which these tests are carried out are carefully controlled to limit sources of variation. Concerns about a valid environment are validated by data suggesting interactions between genes and external factors in the environment *(Gottlieb, 1998)*. The environmental conditions are within the power of the investigator as well as associated parameters as standard equipment, protocol, husbandry conditions etc. These as potential sources of variability can be eliminated by standardizing experimental parameters for all test groups. The situation is quite different from more intrinsic factors that have to do with the neighboring genes where the mutation is found and the effects that could arise from using a strain of the organism or animal from a particular genetic background *(Wahlsten, 2001)*.

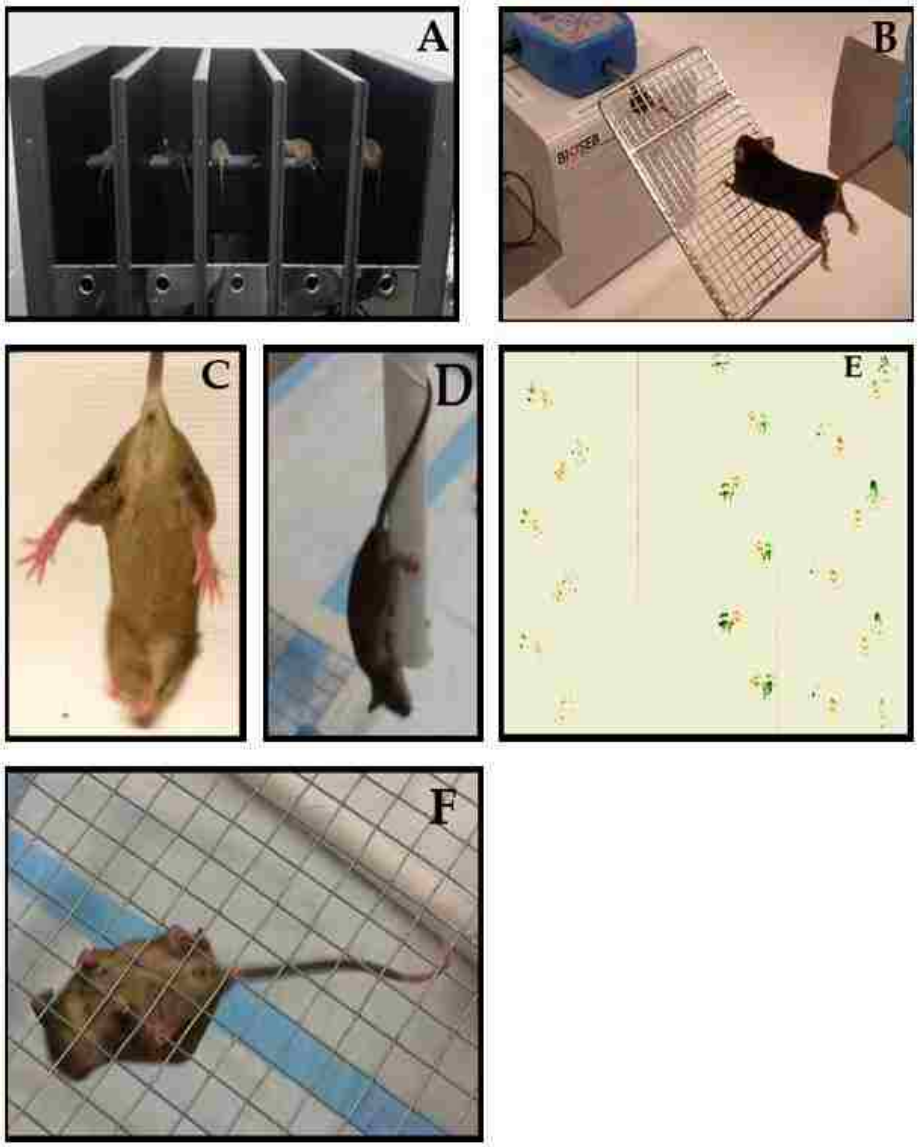


Figure 10 Examples of behavioral assays used in assessing motor phenotype in mice

A) The rotarod is useful for determining motor coordination B) The grip strength meter records the muscle strength C) The tail suspension assesses hind limb posture as a reflex response when mice are suspended by the tail D) The suspended pole tests balance and muscle strength E) foot print assay is for gait analysis F) the wire mesh tests balance and muscle strength in the limbs of mice

Records of past experimental procedures from literature are a good starting point in other to pick a particular background for genetic manipulation. It might be just easier to maintain the same genetic background for a related mutation. A lot of pointers can be obtained from research geared at ascertaining the effects of multiple factors on the accuracy of behavior data. They can help inform an investigator on the experimental design or choice of assays. (*McIlwain et al, 2001)* in one such study, looked at the effects of serial testing (using different assays) on mice and whether or not the sequence of the assay also had any effects on the output. There were both similarities and differences in mice that performed single assays versus those that did multiple assays. There were however fewer differences resulting from the order of assays when two groups of mice were compared. Deductions from studies of this nature always require caution, after the factors are meticulously examined. In making decision regarding an experiment, every investigator must pay attention to the results obtained making use of relevant statistical tools and analysis to scrutinize the data obtained in several ways to erase doubt.

The choice of a behavioral test is obviously made based on the expected phenotype of the animal, and as such the right tool or equipment for the assay has to be picked as well. Regarding the equipment, it is possible to design an apparatus that achieves the same goal with certain tests thereby save cost from not purchasing an automated device *(Figure 10)*. There are number of advantages with an automated system compared to non-automated ones. Scores can be done more accurately, and this enhances the chances of data reproducibility, the investigator or tester interferes very little on the process, it can be more efficient and drastically reduces the expense of energy *(Hanell and Marklund, 2014)*. The caveat however with an automated procedure is to find ways of verifying the

data, making sure it makes sense and satisfies metrics like trends and normal distribution. It may be therefore helpful to consider the level of automation, in such circumstances a process that is almost completely automated would necessarily analysis of the output data to ensure that it makes scientific sense.

CHAPTER 2:MATERIALS AND METHODS

2.1 Generation of DYNC1H1 H304R Knock-in Mouse Model

An 8.04 kb region used to construct the targeting vector was subcloned from a positively identified C57BL/6 BAC clone (RP23:60K23). The BAC was subcloned into a 2.45 kb backbone vector (pSP72; Promega) containing an ampicillin selection cassette for retransformation of the construct prior to electroporation. The region was designed such that the long homology arm extended 5.3 kb 5′ to the point mutation (A to G) in exon 5 A pGK-gb2 FRT NeoR cassette was inserted into the gene in intron 5–6. The short homology arm extended 2.05 kb 3′ of the FRT-flanked Neo cassette.

The CMT2O mutation CAC to CGC (mouse aa304: His to Arg) within exon 5 was generated by 3-step PCR mutagenesis. Four primers, PT1 – 3 and LUNI, were designed and used to amplify a ∼2.5 kb fragment that incorporated the mutation at the desired position. The point mutation was engineered into primers PT2 and PT3. The final PCR fragment carrying the point mutation (middle arm) was then used to replace the wild-type sequence using conventional sub cloning methods at an endogenous enzyme site, MfeI, and at a MluI site in the Neo cassette. The targeting vector was confirmed by restriction analysis after each modification step, and by sequencing using primers designed to read from the selection cassette into the 3′ end of the middle arm and the 5′ end of the SA. Sequencing showed the presence of the mutation and that no errors were introduced in the PCR amplified region *(Sabblah et al, 2018).*

The targeting vector was linearized and electroporated into C57BL/6 × 129/SvEv hybrid embryonic stem cells. After selection by G418 resistance, surviving colonies were expanded and screened for recombinant clones by PCR using primers LAN1 and A1. Positive clones had the presence of the point mutation confirmed by a second round of PCR screening using primers SQ1 and LUNI followed by sequencing of that PCR product. Integration into the correct genomic location was confirmed by southern blot analysis. Three clones were confirmed as integrated into a parental chromosome at the targeted site and were then used for implantation *(Sabblah et al, 2018)*.

Positive stem cells were microinjected into C57BL/6 blastocysts. The blastocysts were transferred to pseudopregnant foster mothers and chimeras were obtained. Those chimeric mice were crossed to mice constitutively expressing Flp recombinase to produce F1 heterozygous knock-in targeted, NeoR cassette deleted mice. PCR was used to confirm the deletion of the Neo cassette and sequencing of the four founder mice confirmed the presence of the point mutation *(Sabblah et al, 2018)*.

2.2 Mouse Breeding and Colony Management

Four founder animals (F1) with the confirmed H304R/+ mutation from InGenious Technologies were crossed to Jackson Laboratory mouse hybrid strain, C57Bl6/129 (Stock # 101045) generating wildtype and H304R+/R mice. The animals were out crossed to the C57Bl6/129 strain up to the F7 generation. The progeny of these mice were used to breed the behavioral group for this study. Animals were bred in pairs and/or trios for behavior and tissue harvest. Founder females were bred with one male whereas founder male was bred with either one or two Jackson wildtype females. Females were

checked for pregnancy twice/ week. Males were housed singly for one week prior to breeding. Females were housed in pairs or groups until paired with a male. Females that did not produce a litter after three matings were removed from the breeding pool. Both males and females were bred from 6-8 weeks old. Males were retired from breeding after 1 year and females were retired from breeding after 6 litters or 5 months whichever came first. The animals were housed in microisolation cages with ad libitum access to food and water under controlled temperature (22±2 °C) and humidity (50±10%) and maintained on a 12-hour light/dark cycle. All mice were given red housing for environmental enrichment *(Sabblah et al, 2018)*.

All females were examined for plug positivity and the males were removed. The pups were weaned on post-natal day 21. The animals were uniquely identified by tail tattoo using SoMark Tail Tattoo system (SoMark Innovations, San Diego, CA). Tail snips were taken at the time of tattoo. The tail snips were genotyped using PCR to determine the genotype of the animals.

Western blot analyses were performed to determine if the H304R mutation altered the amount or stability of dynein molecules in brain tissue. Brain tissue from wild-type and H304R/+ male mice was homogenized in 35 mM PIPES pH 6.96, 5 mM MgSO4, 1 mM EGTA, 0.5 mM EDTA supplemented with 1 mM dithiothreitol, 0.2 mM ATP, and a protease cocktail. A high-speed supernatant of each sample was generated by spinning the samples in an SW50.1 rotor for 30 minutes at 40,000 rpm. The samples were run on SDS-PAGE gels, blotted and probed using standard techniques, utilizing antibodies against dynein intermediate chain (clone 74.1, BioLegend) and glyceraldehyde-3-

phosphate dehydrogenase (clone O411, Santa Cruz Biotechnology) as a loading control *(Sabblah et al, 2018)*. Signal was detected using goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Invitrogen) and the ECL Prime Western Blot Detection Reagent (Amersham). Signal was captured on X-ray film and quantified using NIH ImageJ software *(Sabblah et al, 2018)*.

2.3 Motor behavioral assays

Mice were given motor skills behavior tests every two weeks beginning at 1 month of age. The researchers performing the behavioral tests were blinded to the genotype of the mice they were examining, and the mice were only identifiable by tail tattoo codes. The tail suspension test was performed by gently lifting a mouse by its tail for 15 seconds. The test mouse was recorded from both ventral and lateral angles to fully capture the tail suspension reflex. The recorded tail suspension videos were then analyzed, and each test was scored into one of two categories: typical or atypical response. Mice exhibiting a typical response had their hind limbs splayed apart from each other and held away from the body *(Chen et al, 2007)*. In an atypical response, mice hind limbs were clenched together and held either away from or near the body. The categorical tail suspension data for mice of different genotypes were binned in three-month intervals and statistically compared using the Fisher's exact test (two-tailed distribution).

The accelerating rotarod test utilized an instrument (IITC Life Science, model# 755) programmed to start at 5 RPM, ramp to 40 RPM over 60 seconds, and then maintain 40 RPM speed for a total time of three minutes *(Sabblah et al, 2018)*. The elapsed time until

the mouse fell off the rotating rod was automatically collected with the Series 8 software provided with the instrument *(Hennis et al, 2013)*. Each mouse performed three successive runs and these 3 readings were averaged for each test day. Each test mouse's averaged rotarod reading from test days was averaged again for 3-month bins. The wild-type and H304R/+ binned rotarod datasets were statistically compared for 3-month bin using the two-tailed Student's t-test *(Sabblah et al, 2018)*.

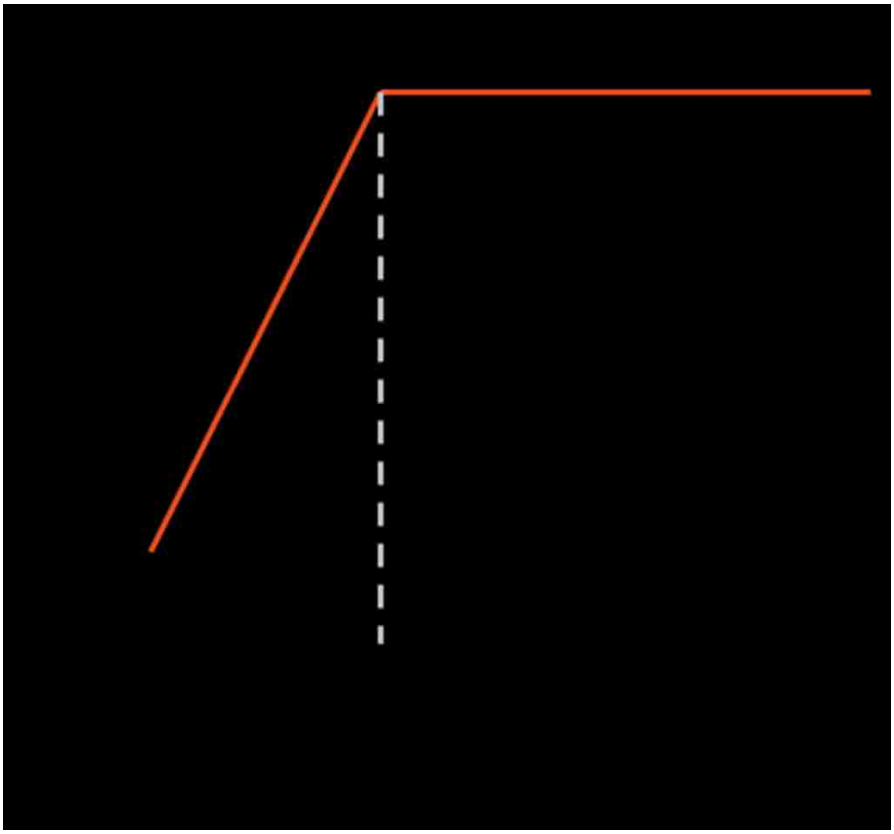


Figure 11 Parameters for the motor coordination assay

After a series of trials with different parameters, the optimized conditions for our motor coordination assay on the rotarod were as follows: a speed of 5 to 40 rpm with a ramp time of 60 seconds over a total duration of 180 seconds.

The limb muscular strength of mice was measured with a digital grip strength instrument (BIOSEB Research Instruments, model# BIO-GS3). Each test mouse was placed on the steel grid attached to the instrument and the grip strength reading was collected as per the manufacturer's protocol. The manufacturer's RSIC software recorded the maximum force (in grams) exerted by the mouse on the steel grid. The grip strength in the front two limbs and all four limbs were measured for each test mouse *(Mandillo et al, 2008)*. Mice could not grip the instrument solely with hind limbs, preventing the direct assessment of hind limb strength. All the grip strength measurements were taken in quadruplicates and the 4 reading were averaged on each test day. Each test mouse's averaged grip data from test days was averaged for 3-month bins. The wild-type and H304R/+ binned grip datasets were statistically compared using the two-tailed Student's t-test.

2.4 Tissue Preparation

Animals were anesthetized via intraperitoneal injection with SomnaSol ™ (7.5 mg/kg, Henry Schein, Dublin, OH, USA). Cardiac perfusion was performed using 25 mls of 0.9% Saline (Hospira, Lake Forest, IL, USA) and then 4% paraformaldehyde (Sigma-Aldrich, ST Louis MO, USA). Gastrocnemius muscles were carefully dissected from animals and kept in 4% paraformaldehyde *(Dequen et al,2008).* The muscles were transferred into 30% sucrose for cryopreservation overnight at 4^{o}C. Muscles were embedded in OCT Compound in plastic cryomolds (Sakura Fine Tek, Torrance, CA, USA) and stored at -80 ^{o}C until ready for sectioning. 20 µm sections were obtained from muscles using a cryostat (Leica CM1850) and attached to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Fixation was done by immersing slides in acetone for 20

mins at room temperature. The slides were then stored at -20 ^{o}C until ready for staining and imaging *(Sabblah et al, 2018)*.

2.5 Immunohistochemistry and Imaging

For neuromuscular junction analysis cryosections were first washed with PBS for 10 minutes each to clear out the OCT. The edges of the sections were marked with a PAP pen to restrict the staining solution. Sections were then permeabilized for 10 mins with 0.5% Triton X100 and blocked for up to three hours in 5% bovine serum albumin and goat serum with 0.1% TritonX100 in PBS at room temperature. Staining was done in a humidified chamber with antibodies against neurofilament (2H3, DSHB and chicken NF-H, EMD Millipore). The sections were washed three times with block solution for a duration of 10 mins each. Alpha subunits of Acetylcholine receptors (AChRs) and neurofilament were stained with the appropriate Alexa Fluor secondary antibodies (goat anti-mouse 546, goat anti-chicken, 546, 488 alpha-bungarotoxin, Life Technologies, Eugene, OR, USA). Two washes were first done with block solution for 10 minutes each and then two final washes with PBS for 10 minutes each time. The sections were mounted in antifade reagent containing DAPI (Life Technologies, Eugene, OR, USA) to stain the nuclei. Images of neuromuscular junctions were obtained using a Zeiss LSM 710 Confocal microscope *(Sabblah et al, 2018)*.

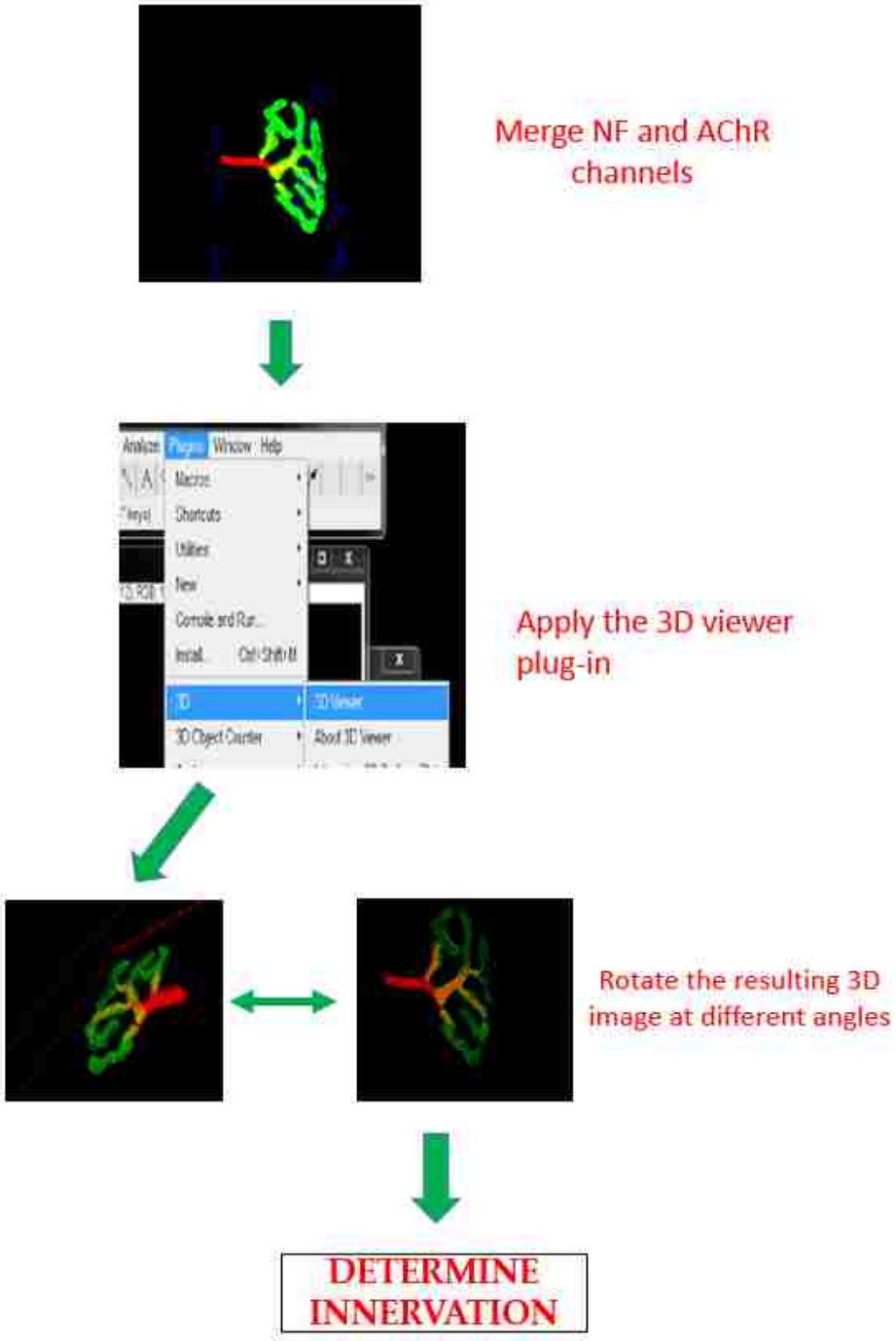


Figure 12 Determination of Innervation using Image J Software

A schematic representation of the steps involved in determining innervation at the NMJ as well as the appropriate plug-in to use

2.6 Analysis of NMJ Architecture

Stacks obtained from confocal imaging were processed further using ImageJ for analysis of innervation patterns and acetylcholine receptor (AChR) architecture *(Figure 12)*.

The *3D Viewer* plug-in was used in determining innervation at the NMJs *(Schmid et al, 2010)*. The respective channels corresponding to neurofilament and AChR staining are merged prior to applying the plug-in. The plugin enables rotation of the reconstructed confocal image to examine the degree of innervation from different angles, this is useful to find out whether the nerve occupies the post-synaptic region or not.

The 3D object counter was used to measure the volume and surface area of the AChRs *(Figure 14)*. The image is prepared by *'smoothening'* to reduce noise and applying a binary mask so that the images appear black against a white background. The threshold is then be set to exclude objects that are outside the region of interest.

We utilized another set of plug-ins; *Skeletonize 2D/3D* and *Analyze Skeleton 2D/3D* to generate a single line version of the image and evaluate different indicators of the resulting structure *(Figure 13, Arganda-Carreras et al, 2010)*. These indicators constitute the complexity parameters used in characterizing the AChR's *(Table 1)*. We obtained counts of branches, junctions, triple points, end-point voxels and slab voxels. The program also calculates the average branch length, maximum branch length and longest shortest path. The student's T-test was used to determine the statistical significance of the data obtained.

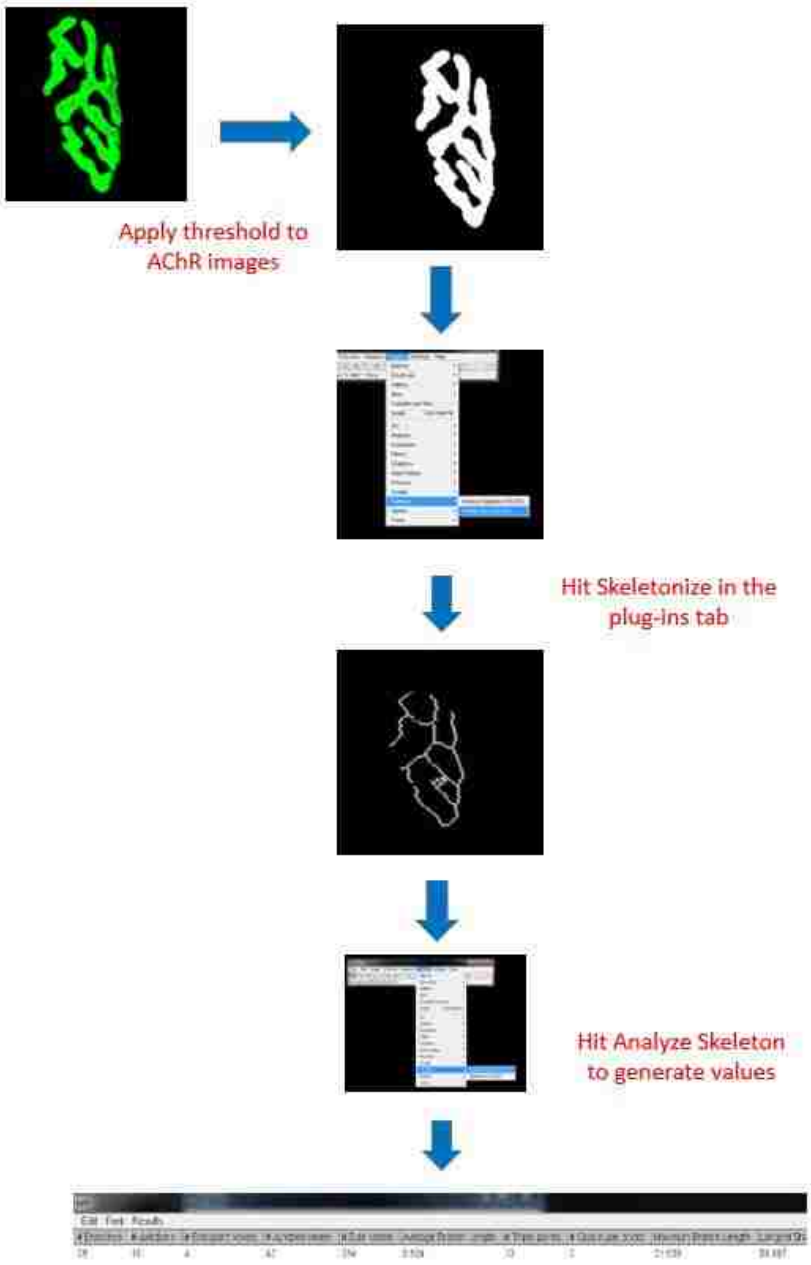


Figure 13 How to analyze skeletons of the NMJ structure

Diagram showing how to process confocal images into skeletonized images and obtain analyses that characterize the complexity and morphology of the NMJ

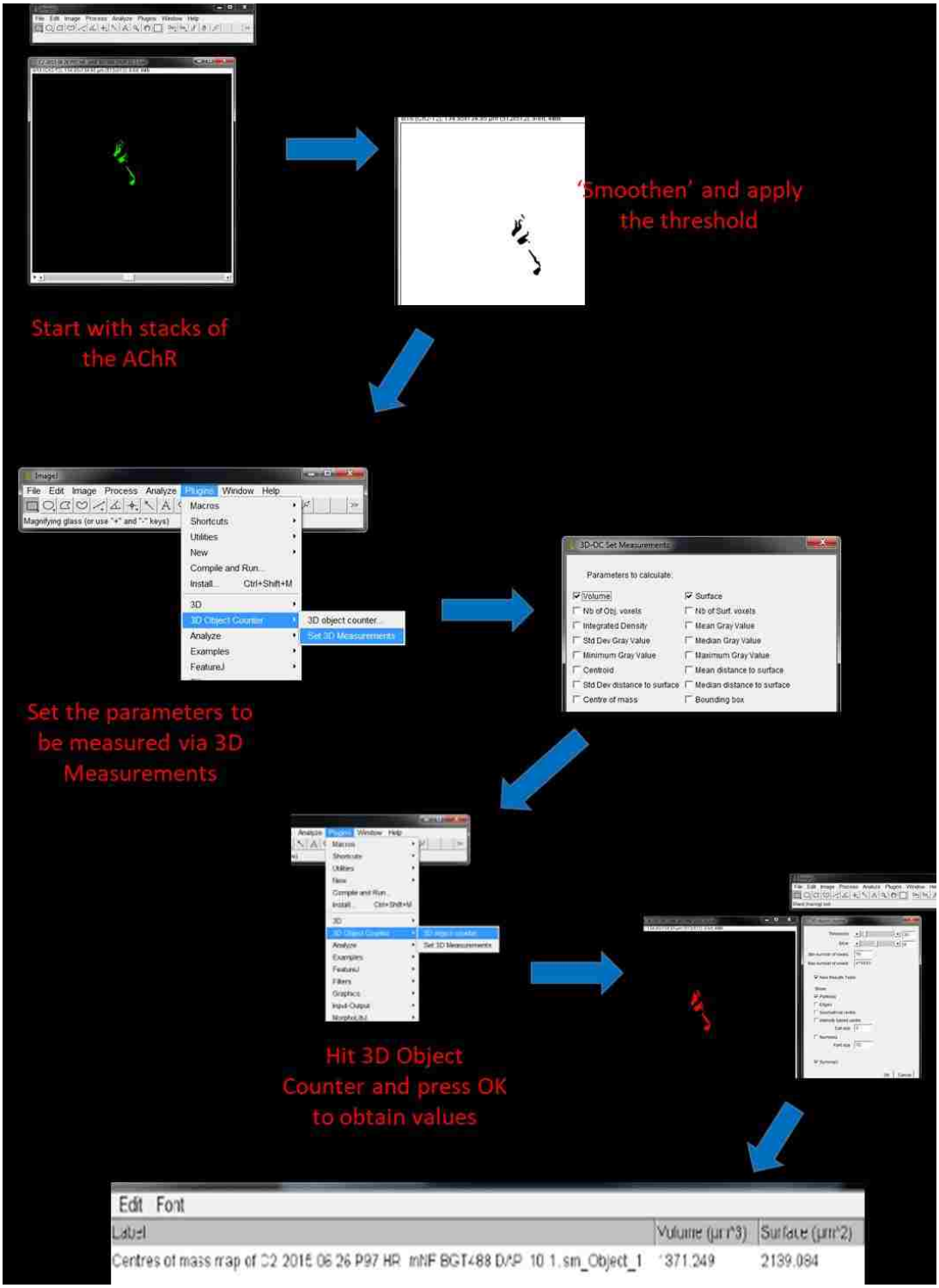


Figure 14 How to measure surface area and volume of AChRs

The schematic outlines how to process image stacks from the confocal image to obtain a calculation of the surface area and volume.

Table 1 List of Complexity Parameters Used in Characterizing Neuromuscular Junction Architecture

Parameter	Definition
Branches	The number of elongated segments in the skeletonized NMJ that link a junction point to the nearest junction or end point.
Junctions	The number of vertices in the skeletonized NMJ that connect two or more branches.
Triple points	The number of vertices in the skeletonized NMJ that connect three branches.
Voxels	The three-dimensional building blocks (“pixels”) in the 3D skeletonized NMJ.
Endpoint voxels	The number of voxels with only one neighbor voxel.
Slab voxels	The number of voxels with two neighbor voxels.
Junction voxels	The number of voxels with more than two neighbor voxels.
Average branch length	The mean branch length of all branches in the skeletonized NMJ.
Maximum branch length	The length of the longest branch in the skeletonized NMJ.
Longest shortest path	The shortest path distances between all possible endpoint pairs in the skeletonized NMJ are determined. The longest of these shortest path distances is then identified.
Area to Volume Ratio	The surface area to volume ratio of the NMJ.

2.7 Determination of Nuclear Distribution

To analyze nuclei morphology at the NMJ, we processed confocal images with Image J software. Confocal images in the *'Maximum Intensity Projection'* format were utilized for length and width measurements of the nuclei. First the area around the NMJ was delineated to help identify nuclei associated with the synapse for analysis. This was done with the help of the *MorphoLibJ* plug-in *(Legland et al, 2016). 'Morphological filters'* are applied to images of the AChRs using a disk to set a 38-pixel dilation for all images to capture associated nuclei, any nuclei that has more than 50% of its area within this region is counted. This is further confirmed by rotating the image in 3D. Nuclei that do not meet this criterion and that fall outside the dilated region are excluded from the analysis. After the background is adjusted on the image, the wand tool is used to mark out the boundaries of the dilated region *(Figure 15)*. This region is saved using the ROI manager, and later applied to images of the nuclei to obtain the relevant nucleus for analysis. The line drawing tool was used to mark out the length and width of the nucleus which were perpendicular to each other and drawn from the farthest points of the nuclei in each direction. The longest distance was considered the length and the shortest the width, the ellipticity value is obtained by dividing the width by the length.

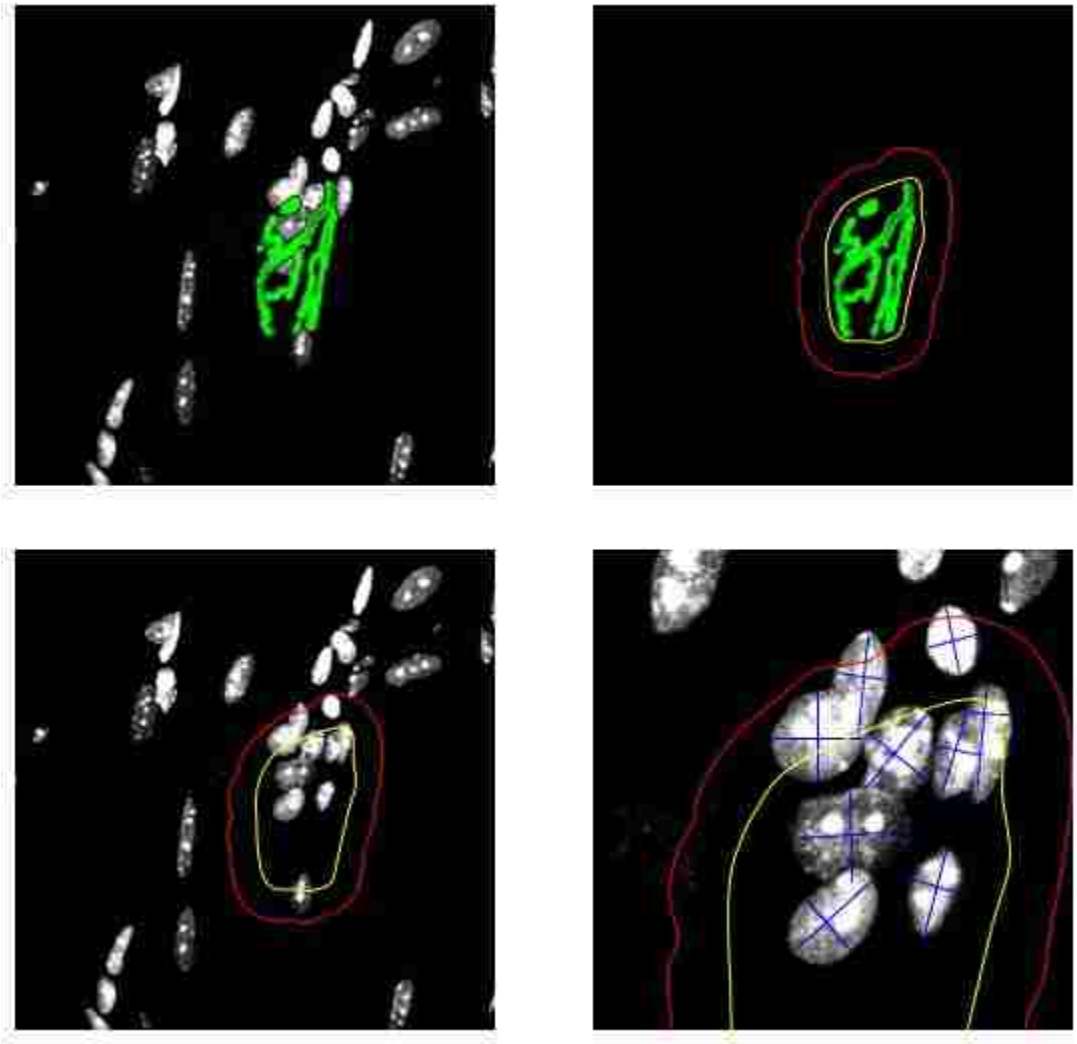


Figure 15 Illustration of analysis on sub-synaptic nuclei

A 10µm radius is marked on confocal images of the NMJ to include relevant sub-synaptic nuclei for the analysis. The length and width of all nuclei that fall within this radius are determined and the ellipticity calculated.

CHAPTER 3:BEHAVIORAL CHARACTERIZATION OF CMT2O MOUSE MODELS

3.1 Introduction

A lot of what is known about dynein function has come from studies in different systems, such as yeast, fungi, fruit flies and mice *(Wickstead and Gull, 2007, Moore et al, 2009, Egan et al, 2012)*. Knockdown studies in mice provided strong evidence showing that dynein is imperative in supporting life *(Harada et al, 1998)*. The use of animal models of disease however requires first, the investigation and identification of phenotypes related to the disease. The dynein mouse models *Loa, Cra,* and *Swl* have been well characterized with motor and sensory abnormalities *(Schiavo et al, 2013)*.

The first characterized human mutation in the dynein heavy chain was found to cause CMT disease, specifically type 2O. *(Weedon et al, 2011)*. We therefore made a knock-in mouse with the corresponding H304R mutation that causes CMT2O to study the disease and obtain further insight into how dynein function is altered. CMT2O patients usually develop muscle weakness, gait problems and delayed milestones *(Weedon et al,2011)*. Based on the presentation of the disease in humans, a good mouse model should have similar motor phenotypes. To find out if our CMT2O mouse models had a phenotype reminiscent of CMT disease, we conducted a longitudinal assessment of the locomotor skills of wild-type and mutant mice bearing the H304R mutation. We employed 3 assays namely; the tail suspension assay, grip strength measurements and motor coordination assessment. These assays have been widely used to determine neuromuscular impairments in various animal models of disease *(Brooks and Dunnett, 2009)*. Dynein mouse models (*loa, cra, swl*) and some CMT2 mouse models (CMT2D, CMT2E) have

been assessed for their locomotor properties via the tail suspension and motor coordination on the rotarod *(Rogers et al, 2001, Dequen et al, 2010, Shen et al, 2011).* The choice of these motor behavioral assays gives us a basis for comparison of results with studies involving other CMT2 and dynein mouse models.

3.2 Results

3.2.1 Investigation of defects in the heterozygous mouse

We generated a heterozygous knock-in mouse line that carries the corresponding H304R mutation in the mouse cytoplasmic dynein gene. We characterized potential loco-motor phenotypes by examining both male and female littermate wild-type and H304R/+ mice in a 12-month longitudinal study. Over the time points analyzed, H304R/+ mice had no obvious physical or cage behavioral phenotypes that could be distinguished by eye from littermate wild-type animals. We tracked the general health of those animals and saw no obvious health differences in the animals outside of a modest weight gain in 3-month old female H304R/+ mice that disappeared as the mice aged

3.2.1.1 General observations and cage behavior

All mice regardless of genotype were bred using the same scheme and housed under standard conditions with unlimited access to food, water and cage enrichment. For purposes of our behavioral study, male and female mice were separated into different cages after weaning. All mice were periodically observed to assess their general well-being or to pick out signs of ill-health. Some of the signs we looked out for were; the level of activity, condition of the fur, skin and eyes, locomotion, posture and examination of cage litter for signs of fecal and urine elimination. This exercise is particularly critical for a novel mutation, because we do not know how the introduction of the transgene

affects the behavior of the litter. We recorded all health concerns encountered and the response to treatment as well. After comparing the genotypes of animals with health concerns, we ruled out the contribution of the H304R dynein heavy chain mutation. We tracked the weights of the mice and found no significant genotype –specific effect on weight change over the duration of the study.

3.2.1.2 Picking out the right tools to determine phenotype

We started out by analyzing behavioral phenotypes making use of some well-known assays, namely; wire mesh, the hanging rod both of which test for locomotion and balance *(Figure 16)*. For gait analysis we painted the feet of the animals; utilizing two different colors to differentiate front paws from hind paws *(Crawley,2008)*. Although these assays were suitable for other phenotypes, with the exception of the tail suspension assay, we obtained very little reproducible results with our mice. We resorted to the use testing methods that required minimal tester involvement in our bid to find out whether the H304R mutation had any phenotype based on published work on dynein and CMT2 mouse models (*Rogers et al, 2001 Bogdanik et al,2013, Zhang et al,2014)*. We therefore settled on the rotarod and grip strength meter which came with software packages to automatically record data for analysis. The use of these equipment resulted in more efficient work as it eliminated a lot of optimization and troubleshooting time. Testers were blind to the genotype of the mice, and they carried out initial trials with the mice before actual performance measurements. The rotarod required testing out different parameters to determine the most suitable testing condition.

Figure 16 Mouse footprints assay

A sample of the output from the footprint assay with fore and hind paws distinguished by different colors and showing 2 patterns of footsteps. A) Regular footprints from a mouse that took normal steps. B). Irregular pattern of footprints obtained from a mouse that could be jogging or running.

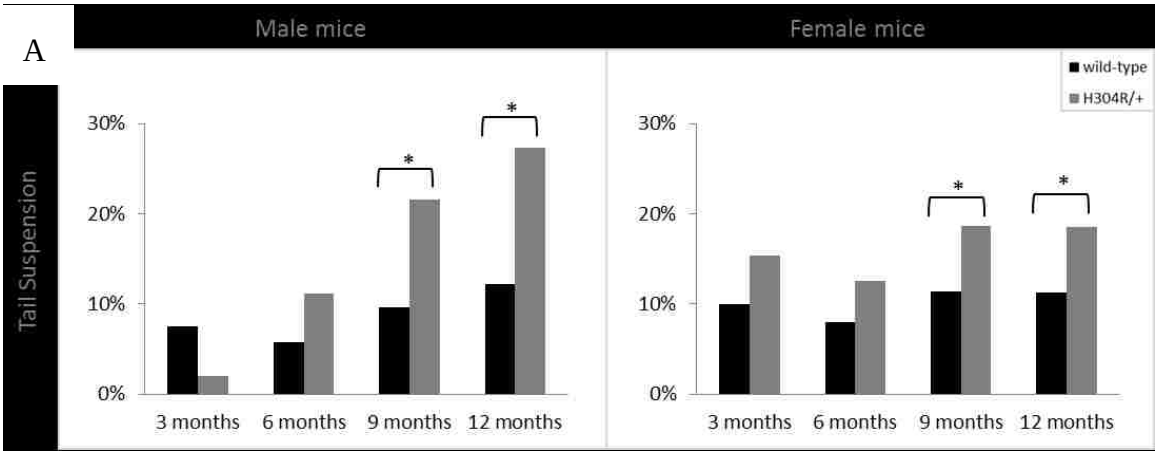
3.2.1.3 H304R/+ mutant mice show an altered tail suspension reflex

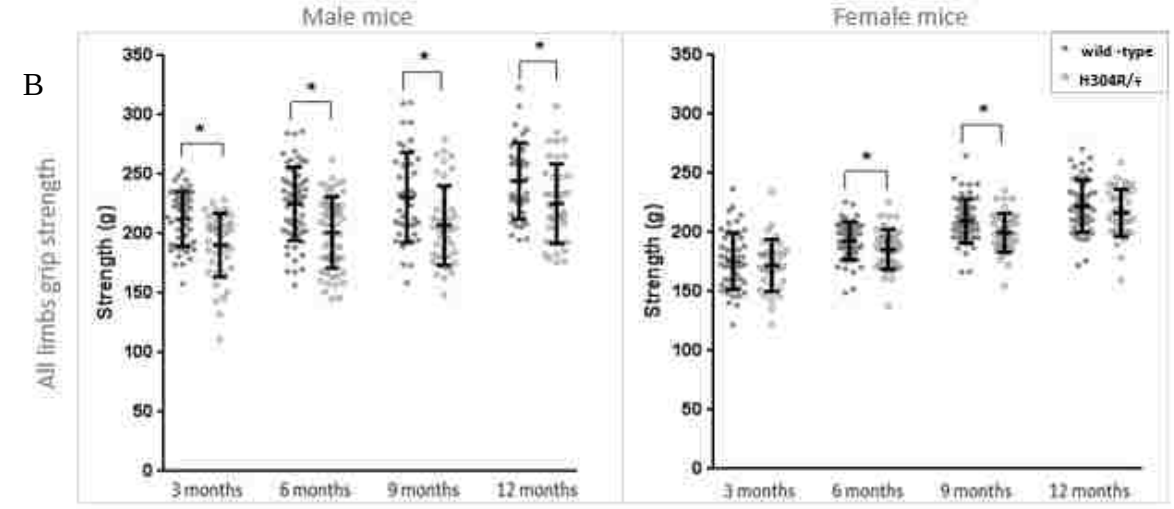
Wild-type mice generally display a characteristic tail suspension reflex of splayed hind limbs held away from their body when suspended by their tails. Several studies of *Loa/+*, *Cra/+* and *Swl/+* mice pointed out that those mice display an atypical phenotype of clenching their hind limbs when suspended by their tails. We performed a similar tail suspension assay at successive 3-month time points and found that the majority of male and female wild-type and H304R/+ mice displayed the normal splayed tail suspension reflex phenotype at each time. However, both male and female H304R/+ mice showed a significant increase (p <0.05) in atypical tail suspension responses relative to wild-type at 9 and 12 months *(Figure 17A)*. These data suggest that the H304R/+mice have a hind limb defect but that the defect is less pronounced than the phenotypes in Loa/+, Cra/+, and Swl/+ mice as only a subset of H304R/+ mice display the atypical response.

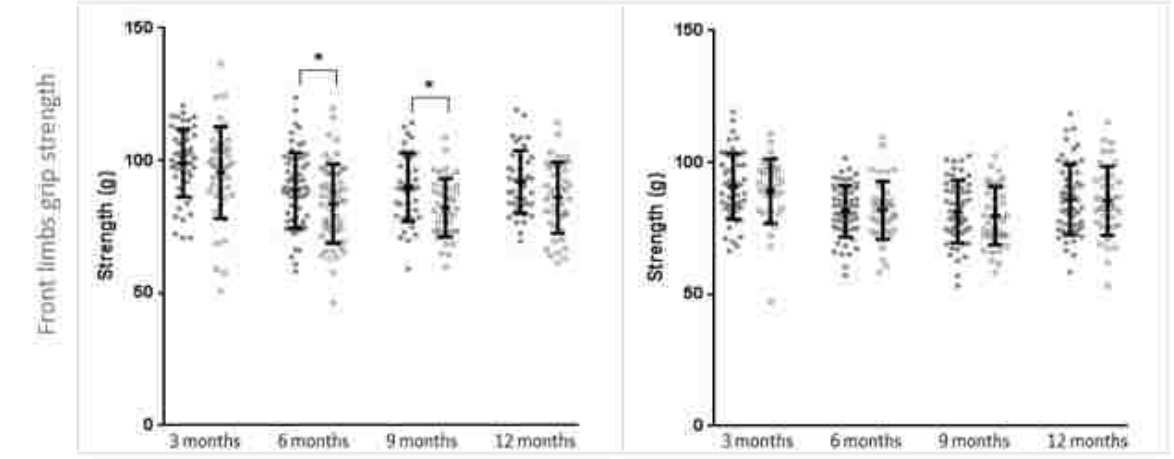
3.2.1.4 H304R/+ mutant mice have reduced muscular strength

We next analyzed the limb muscular strength in our mice using a standard grip strength assay. Male H304R/+ mice exhibited significant weakness in all limb grip strength relative to wild-type male mice at all ages examined. To further tease apart the contribution of front and hind limbs to this phenotype, we examined the strength of the front limbs solely. We found that there was a significant, progressive weakness in front limb grip strength of male H304R/+ mice relative to wild-type mice that occurred in the later 9 and 12-month time points ($p < 0.05$) *(Figure 17B)*. These data suggest that the

mice have reduced function in the hind limbs at an early age, and that front limb weakness progresses as the mice age.


A
Male mice
Female mice
wild-type
H304R/+
Tail Suspension
30%
20%
10%
0%
*
3 months
6 months
9 months
12 months


B
Male mice
Female mice
wild-type
H304R/+
All limbs grip strength
Strength (g)
350
300
250
200
150
100
50
0
*
3 months
6 months
9 months
12 months


Front limbs grip strength
Strength (g)
150
100
50
0
*
3 months
6 months
9 months
12 months

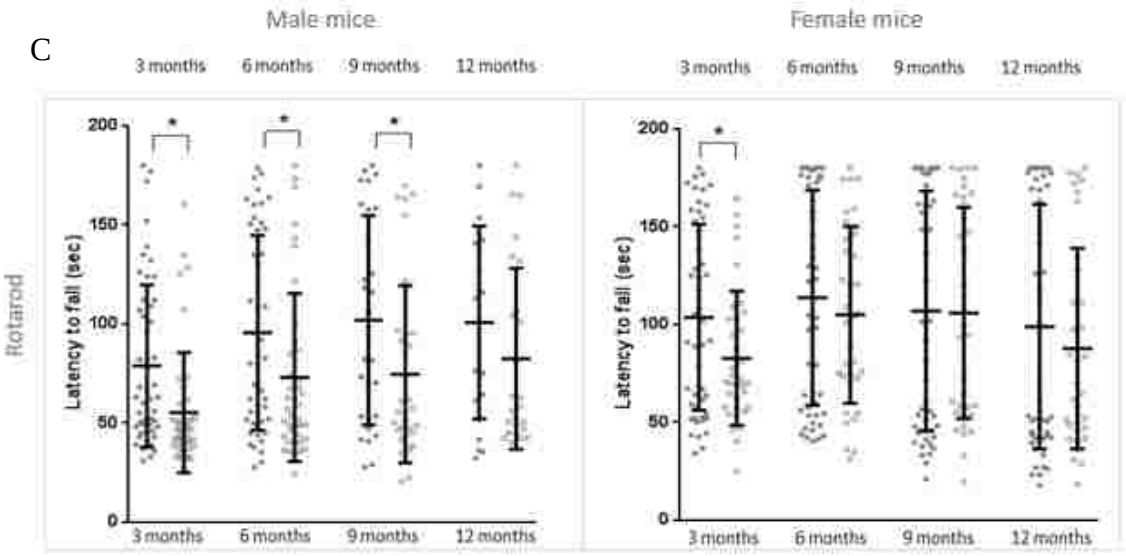


Figure 17 Motor performance test results

Behavioral phenotypes of H304R/+ mice. **(A)**The atypical tail suspension reflex was scored and counted for each test mouse per 3-month long time intervals and the scores were statistically compared between the wild-type and H304R/+ mice groups using Fisher's Exact Test (two-tailed distribution, $p<0.05$). **(B)**The grip strength was measured in the all limbs of the male and female mice, and in the front limbs of the male and female mice. The data was statistically compared between wild-type (filled circles) and the H304R/+ (empty circles) mice groups using the *t-test* (two-tailed distribution, *= $p<0.05$). **(C)**The rotarod performance was measured for both the male and female mice. Grip and rotarod readings from each test mouse was then averaged per 3-month long time intervals respectively. The data was statistically compared between wild-type (filled circles) and the H304R/+ (empty circles) mice groups using the *t-test* (two-tailed distribution, *= $p<0.05$).

When we examined female mice, we found subtle defects in the combined grip strength at 6 and 9 months of age for H304R/+ females, but no defect in front limb grip strength at any age. The grip strength data is consistent with our tail suspension data, showing that there is a general trend of reduced neuromuscular function in H304R/+ animals as they age.

3.2.1.5 H304R/+ mutant mice have motor coordination defects

The rotarod test is a performance-based test for the evaluation of muscular endurance, motor coordination and balance in mice. We utilized an accelerating profile where the mice were tested for their ability to adjust to increasing revolutions during a ramp time followed by a consistent speed for the remainder of the assay. When we examined the ability of wild-type and H304R/+ littermates to perform this assay, we found that individual animals of a particular genotype could exhibit a wide range of abilities. However, as a group, H304R/+ males had reduced rotarod performance at 3, 6, and 9-month time points *(Figure 17C)*. Female H304R/+ mice only displayed a significant difference in rotarod performance at the earliest time point examined. This difference disappeared as the female mice aged. *Loa/+* mice had been previously characterized with the rotarod test and showed similar defects.

3.2.2 Characterization of the homozygous mouse model

The H306R mutation originally discovered in the cytoplasmic dynein heavy chain in humans occurs as an autosomal dominant heterozygous genetic defect *(Weedon et al,2011)*. Heterozygous mutations in *loa, cra, swl* mice have provided some clues about how a mutation in dynein can lead to neurological dysfunction. Homozygous mice with the *loa, cra, swl* mutation result in early lethality and therefore a study of the progression of the disease and the effects on the motor in these disease models hasn't been possible. A homozygous dynein mouse model that is able survive long enough for a longitudinal assessment will fill that gap. Our H304R/R homozygous mouse fortunately addresses that, with homozygous animals able to survive beyond the first year of life. Of particular interest was the discovery of homozygous dynein in an individual from a genetic screen involving 30 subjects *(Scoto et al,2015).* This individual with the homozygous mutation had arthrogryposis from birth, cognitive impairment and ADHD (attention deficit hyperactive disorder). These features were more severe than what the heterozygous parents had. One parent had muscular weakness and denervation in the lower limbs whereas the other showed no obvious symptoms.

Generally, the homozygous mice had deficits at all time points looked at. Furthermore, the gender differences that were a feature of the heterozygous mice, (ie. females having a weaker phenotype) appear to have diminished in the homozygous mice. A study in Germany looked at gender differences among CMT patients and gender was found to play a role in the incidence of muscle force in the lower limbs, with men being affected more than women *(Wozniak et al, 2015).* This finding agrees with the pattern observed in

our behavioral assays in heterozygotes, where female mice generally had a weaker phenotype compared to males.

3.2.2.1 H304R/R mice survive after birth

After obtaining the approval to breed homozygous mutants from two heterozygous parents, we were initially curious in finding out whether the homozygous mice will survive after birth and if so, how long their life span will be. The homozygous mutation is lethal in other dynein mutants, with mice surviving only a few days after birth. H304R/R homozygous mice survive up to 2 years after birth and as such can undergo a longitudinal assessment of phenotypes similar to what was done in heterozygous mice *(Sabblah et al, 2018)*. H304R/R homozygous mice can be differentiated from wild-type and even heterozygotes by the 30th day after birth. Some of the mice are often smaller and develop a characteristic high steppage gait.

3.2.2.2 H304R/R homozygous mice have severe neurological impairments

We carried out motor performance assays, namely tail suspension, muscle strength and motor coordination in homozygous mice to characterize their phenotype. We tested both male and female mice at 5 timepoints altogether:1 month, 3 months, 6 months, 9 months and 12 months. We were anticipating a harsher effect of the mutation on neuromuscular performance, however, we didn't know what to expect in terms of progression and how the general activity of the mice will be affected and whether gender differences will be seen, as was the case with heterozygotes.

Our neurological assessment of mice based on hind limb clasping in the tail suspension assay confirmed our expectations as homozygous mice presented a more robust phenotype *(Figure 18)*. About 60% of homozygous animals clenched their hind limbs and often writhed in an attempt to assume a more comfortable posture *(Figure 19A)*. This peculiar behavior often made the homozygous distinguishable from the other genotypes.

3.2.2.3 Homozygous mice show a pronounced motor phenotype

Atrophy of the muscle, weakness and gait problems are well-documented in CMT2 and peripheral neuropathies in general. Other dynein mouse models also presented with muscular issues as well, therefore it was only a question of how severely the H304R/R homozygous model will be affected. We first evaluated muscle strength in all limbs of the mice on the grip meter. Lower values were consistently recorded for homozygous mice throughout the study. In female homozygous mice the deficiency in the muscle was seen earlier at 3-months whereas in heterozygotes differences occurred at 6 months *(Figure 25)*. To tease out the contribution of each set of limbs to the phenotype, we took grip strength readings in the fore limbs. We found reduced muscular strength in forelimbs of mice meaning that in the homozygous mice both fore and hind limbs are targets of the peripheral neuropathy. Both males and females suffered in the fore and hind limbs.

Motor coordination discrepancies were also observed consistently at all time points in both male and female mice. Homozygous mice on the average, fell of the rotarod before the 60 second ramp time set on the instrument *(Figure 11)*. Although the motor phenotypes didn't progressively worsen in homozygous mice over time, the performance of mice on the assays reveals the severity of the homozygous mutation.

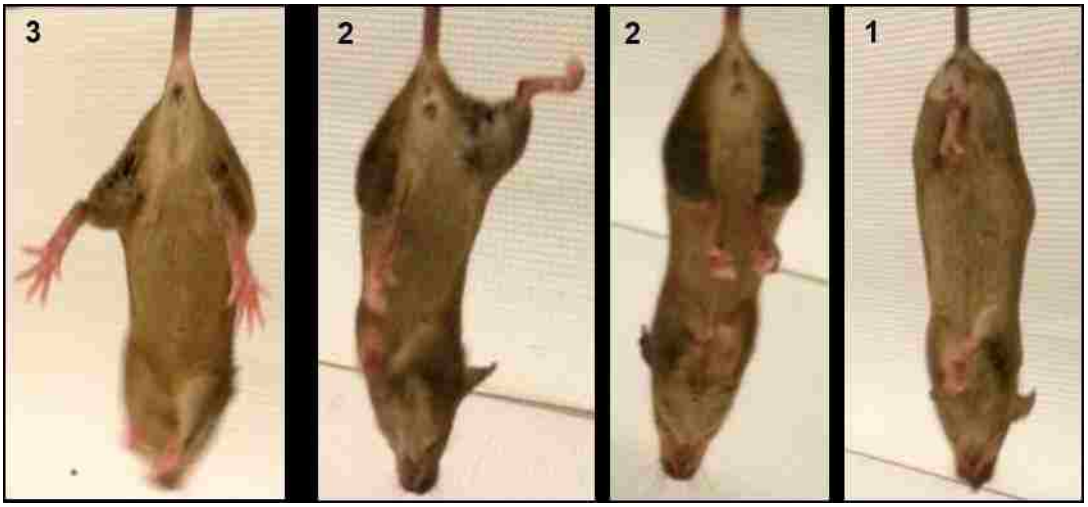
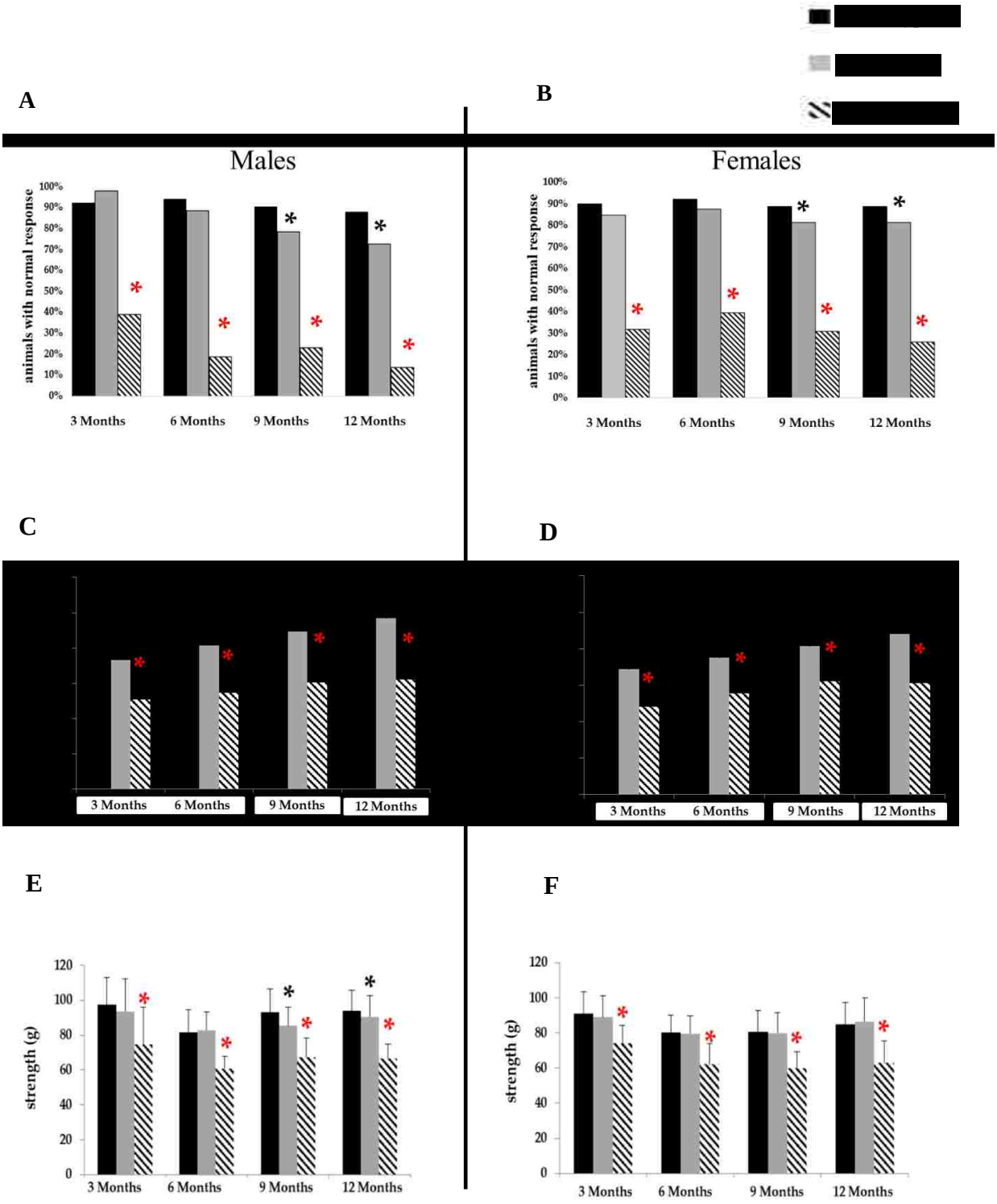


Figure 18 Hind limb posture in mice on the tail suspension assay

Scores are given based on widely apart animal hold their hind limbs apart. Wild-type mice are typically awarded a score of 3 the highest score, majority of the heterozygous mice score 2 displaying mostly 2 forms of partial limb clasping. Homozygotes largely obtain a score of 1 as they are unable to keep their feet apart and have them clutched


A
B
Males
Females
animals with normal response
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
3 Months
6 Months
9 Months
12 Months
C
D
3 Months
6 Months
9 Months
12 Months
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9 Months
12 Months

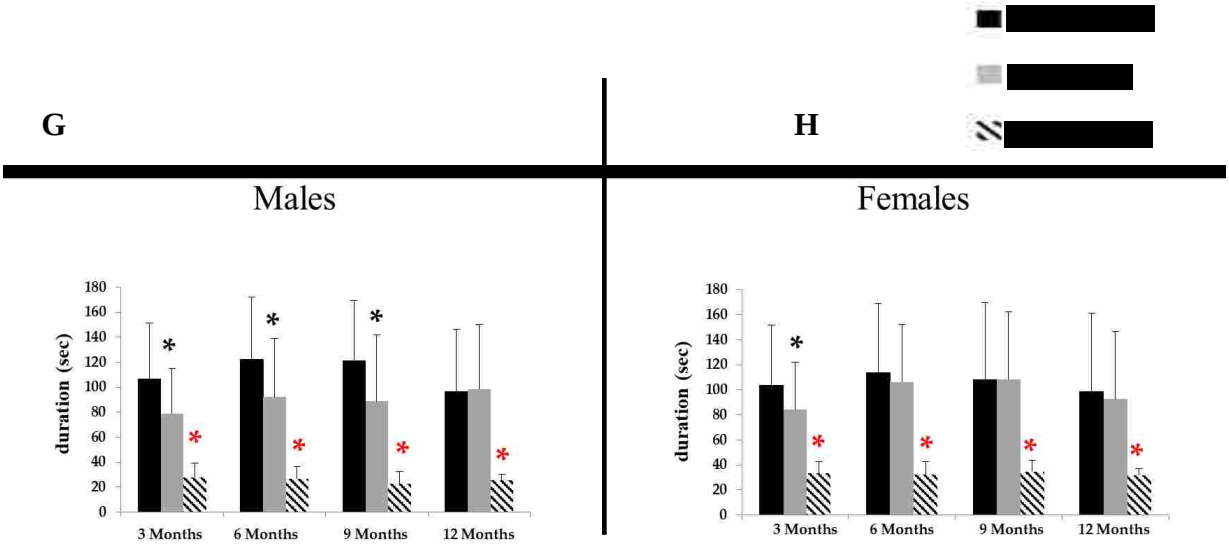


Figure 19 Phenotypic assessment of homozygous mice

Hind limb posture assessed in mice (A, B). Muscle strength in all limbs was measured with the help of a grip meter (C, D). Fore limb strength alone was also measured (E, F). Motor coordination based on the latency of the animal to fall was determined on the rotarod (G, H)

3.3 Discussion

3.3.1 Assessment of H304R Phenotype

To gain understanding of the onset and progression of axonal CMT as well as the role of cytoplasmic dynein in cellular neuropathy, we have generated and initially characterized a knock-in mouse carrying the H304R mutation in the cytoplasmic dynein heavy chain. This mutation corresponds exactly to the human H306R DHC mutation that results in CMT2O *(Weedon et al,2011)*. We examined both male and female H304R/+ mouse cohorts in a 12-month longitudinal assessment study to determine if the mutant DHC allele causes dominant locomotor deficits similar to those exhibited by individuals with CMT2; we also assessed whether H304R/+ male mice have neuromuscular pathologies.

H304R/+ male mice exhibit phenotypes that would be expected for a mouse model of CMT disease. The mice displayed muscle weakness, loss of motor coordination and atypical tail suspension reflexes compared to littermate controls. In addition, although our assay could not distinguish hind limb muscle strength directly, the progression of limb weakness from early onset "all limb" only to significant weakness in the forelimbs specifically at the later time points strongly suggests that the mouse disease pathology is following the progression of symptoms in humans from difficulty walking to progressive inability to use hands effectively.

Interestingly, we found differences in severity of phenotypes based on the sex of the animal, with female animals having weaker phenotypes overall compared to male counterparts when measuring grip strength and motor coordination but similar atypical tail suspension reflexes. We believe that our female H304R/+ mice exhibit milder CMT characteristics that will require more sensitive locomotor assays to distinguish. The initial

study of human CMT Type 2O characterized 8 males and 5 females in detail, with no obvious differences between the sexes being noted *(Weedon et al,2011)*. It is possible that our observed difference in expressivity of H304R/+ between males and females is due to specific sexual differences in the characteristics of mice; it will be extremely interesting to determine if the variation in expressivity occurs at the cellular level. Especially when sex differences have been observed among CMT patients in a study looking into the severity of the disease *(Wozniack et al, 2015)*.

3.3.2 Phenotypic features of the H304R/R homozygous mouse

The phenotypes observed in our CMT2O homozygous mice were expectedly more severe than heterozygous animals, but there is also a difference in the way the homozygotes are affected. Homozygous mice are distinguishable from heterozygotes in appearance, they generally weigh less and are smaller and develop a high steppage gait at about 14 days after birth. Our behavior data shows that the predominant motor phenotype due to the H304R mutation in the dynein heavy chain is more pronounced in the homozygotes as it develops earlier than in the heterozygotes in some cases. Significantly the sex differences in the phenotype were eliminated as the both males and females recorded comparable behavioral deficits. For instance, there were no deficits recorded in the heterozygous female fore limb strength, however the homozygotes displayed fore limb weakness at all the time points looked at compared to male mice *(Figure 19)*. Although the reason for the minimized effect of the CMT-related phenotype is not known in females, it is obvious the homozygous mutant protein and therefore dynein is affected strongly enough to produce a robust phenotype in males. Another factor that is worth noting is in terms of the progression of the disease is that the ability of the animals to recover is lost with the

homozygous mutation. Motor coordination is restored in male mice by the 12th month and in female mice by the 6th month in heterozygotes, in the homozygous however, all time points are affected. Simultaneously there appears to be the early development of a motor phenotype in the homozygous animals, this is also corroborated in the data on NMJ analysis.

CHAPTER 4:HISTOLOGICAL ANALYSIS OF MUSCLE FUNCTION

4.1 Introduction

Cytoplasmic dynein has specialized roles in specific cell types in as much as there are general functions in many cells. This is perhaps one of the factors underlying the wide range of symptoms observed in living organisms when there is mutation in the cytoplasmic dynein gene. A typical investigation into a dynein therefore should include the isolation and investigation of different subcellular components for further analysis. Different studies have shown how dynein mutations can affect some cellular targets and not others *(Dupuis et al, 2009, Braunstein et al, 2010, Eschbach et al, 2011)*. The *loa, cra* and *swl* dynein mutations have been examined at the tissue level for neuromuscular abnormalities. The neuromuscular junction was found to be affected by perturbations in dynein function in the *cra* heterozygous mice *(Courchesne et al, 2011)*. The link between dynein and neuromuscular junction dysmorphology could be due to dynein activity in motor nerves *(LaMonte et al, 2002, Levy and Holzbaur, 2006)*.

The results of our initial loco-motor studies, altered behavior in tail suspension, grip strength, and rotarod assays, suggested to us that there might be a physical defect in the neuromuscular system in the mutant H304R/+ mice. To examine this possibility directly, we characterized the sarcomere and neuromuscular organization of the gastrocnemius lower limb muscle from male wild-type and mutant mice. We chose to examine specifically male mice, as they exhibited more significant motor behavior skills defects than female mice. Fixed gastrocnemius muscle tissue slices were labeled with antibodies for key components of those structures and imaged using standard confocal microscopy.

Our survey of 9 and 12-month tissue showed no defect in the number or overall organization of sarcomeres between wild-type and H304R/+ mutant mice

4.2 Results

4.2.1 Investigation of Defects in the heterozygous mouse

We tested the possibility of the defects in mice arising from an improperly assembled motor protein by comparing the levels of dynein in the brains of wild-type to mutant (H304R/+) mice. Quantification done on the immunoblot showed no difference in the levels of the intermediate chain of dynein *(Figure 18)*. We were unable to specifically examine dynein heavy chain protein levels in the brain samples due to incomplete transfer of the approximately 4700 amino acid protein from the gel to the membrane.

The results of our initial loco-motor studies, altered behavior in tail suspension, grip strength, and rotarod assays, suggested to us that there might be a physical defect in the neuromuscular system in the mutant H304R/+ mice. To examine this possibility directly, we characterized the sarcomere and neuromuscular organization of the gastrocnemius lower limb muscle from male wild-type and mutant mice. We chose to examine specifically male mice, as they exhibited more significant motor behavior skills defects than female mice. Fixed gastrocnemius muscle tissue slices were labeled with antibodies for key components of those structures and imaged using standard confocal microscopy. We examined sarcomere organization by staining the Z-line with alpha-actinin *(Figure 19)*. We saw no qualitative difference between images of wild-type and H304R/+ mutant mice. We also measured the mean sarcomere distance in the muscles and saw no

significant difference (p= 0.08) between those of wild-type (2.22 µm sarcomere length from 11 muscle fibers and 689 individual sarcomeres) and H304R/+ (2.20 µm sarcomere length from 11 muscle fibers and 702 individual sarcomeres).

4.2.1.1 Determining complexity in the NMJ architecture

Complexity is a classical feature of the mature neuromuscular junction and resulting changes in architecture could also be valuable in predicting the involvement of some kind of neuromuscular pathology. It is therefore important to be able to capture and quantify the various changes that could occur in the event of an underlying disease in the course of development. NMJ complexity in simple terms is descried as a 'pretzel-like' structure after a series of transformations have occurred from the initial 'cookie' structure or plaque early in development. In situations where the changes are subtle, it is important to have analytical tools that are both sensitive enough and at the same time reproducible. Complexity has been previously defined based on different ways of studying neuromuscular junctions *(Courchesne et al, 2011, Sleigh et al,2014)*. We used plug-ins from ImageJ software to determine NMJ complexity based on 10 parameters *(Table1)*. The advantage is that minor changes over time can be noticed as was the case for early time points in our longitudinal study of the H304R mutation in the dynein heavy chain.

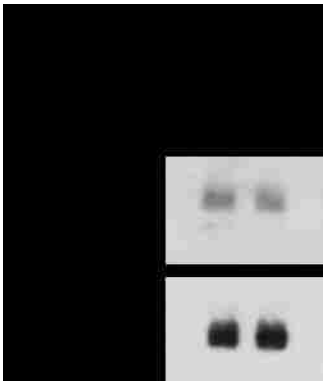


Figure 20 Dynein expression in brain of mice

Protein levels of the dynein intermediate chain from brain tissue high speed supernatant of both wild-type and heterozygous mice. Statistical analysis using the student's t-test, two-tailed distribution, showed no significant difference in protein expression ($p=0.23$)

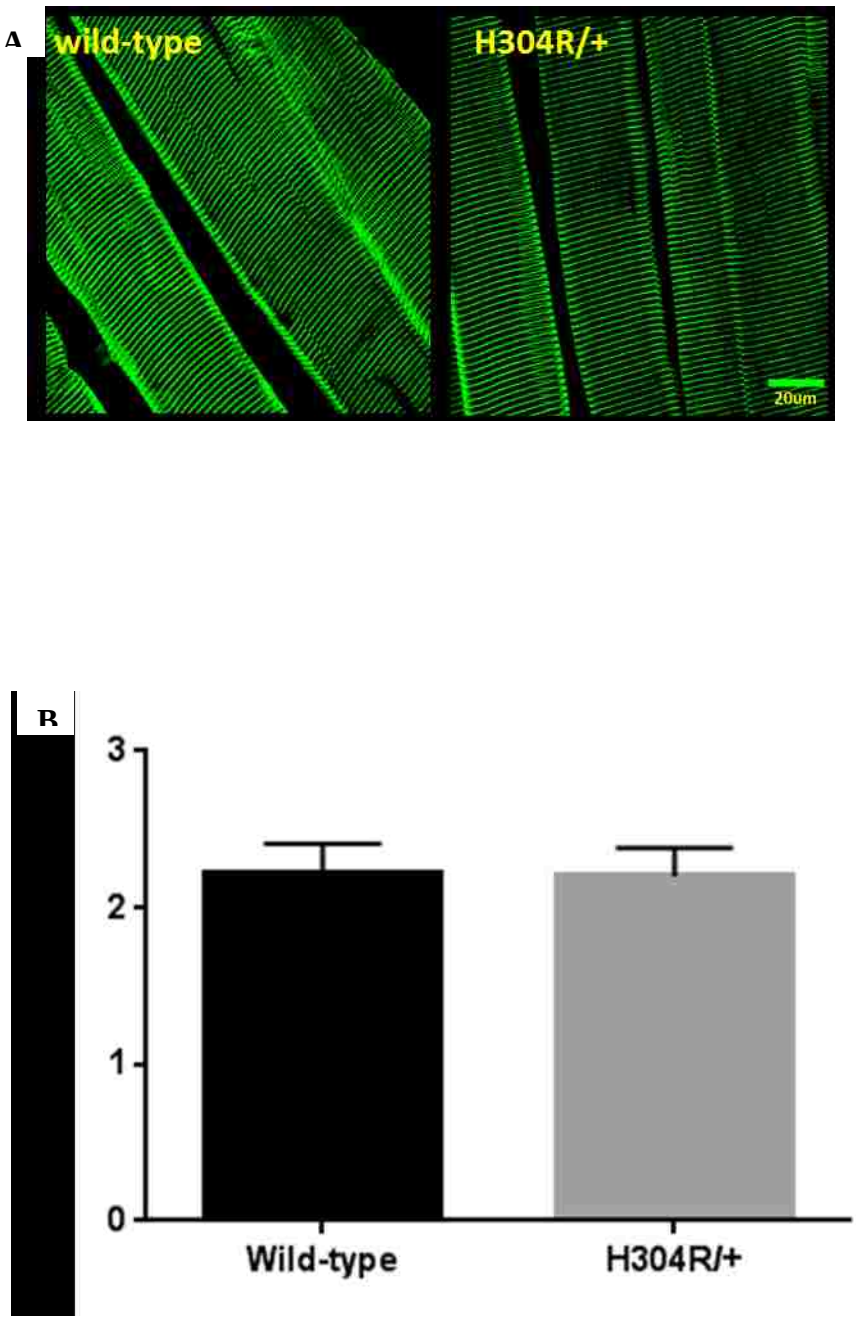


Figure 21 Sarcomere organization in heterozygous mice

A) Gastrocnemius muscles were stained with α-actinin to show the Z-line (in green) in the sarcomeres of wild type and mutant H304R/+ mice. B) No differences were observed in the length of the sarcomere in wild type and H304R/+ muscle tissue

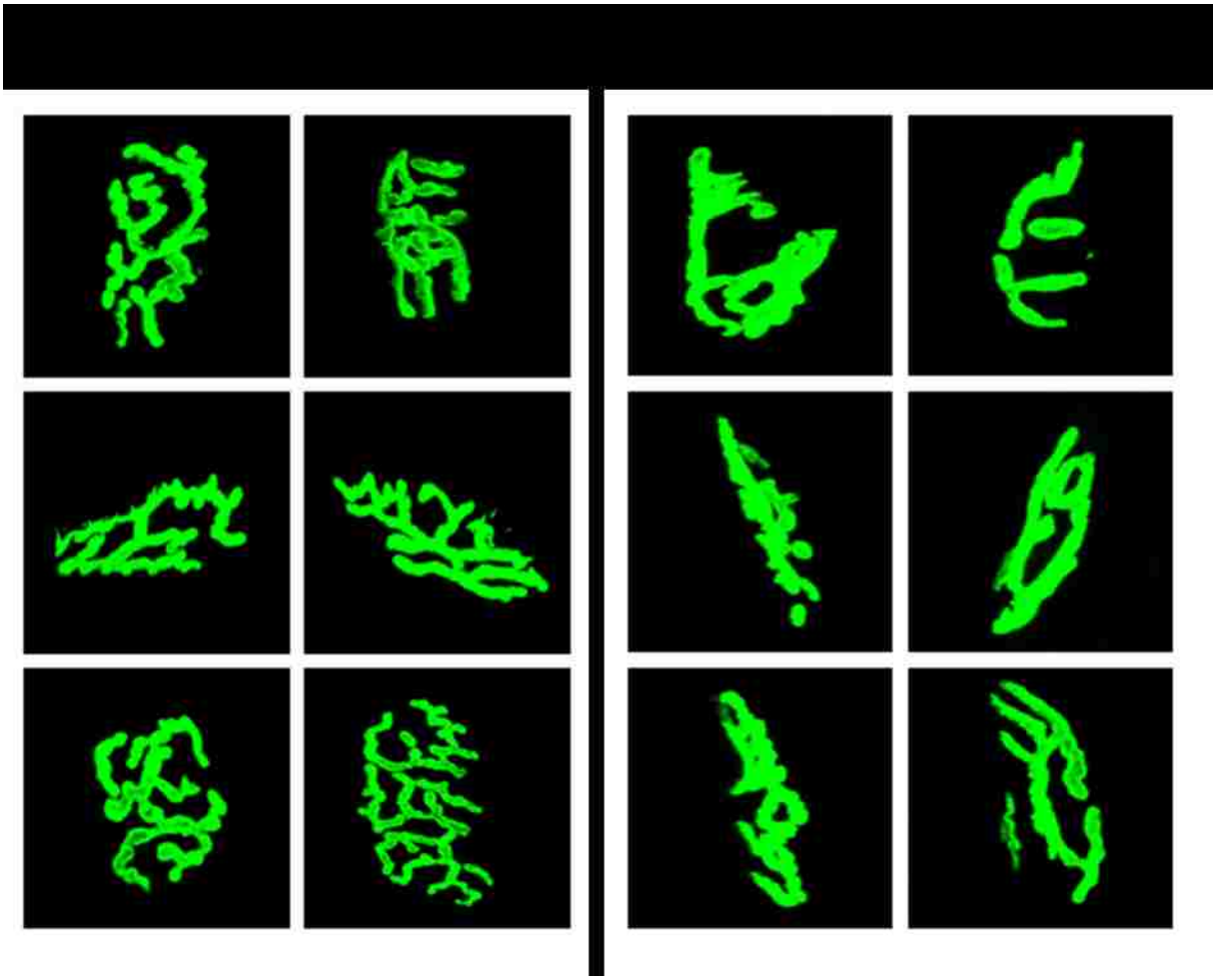


Figure 22 General morphology of NMJs in mice

AChRs from wild-type and heterozygous (H304R/+) mice at 9 and 12 months were stained to assess the morphology of the NMJ. Wild-type muscles had more of the typical complex morphology compared to mutant heterozygous mice.

4.2.1.2 Neuromuscular junction dysmorphology

We labeled 9 and 12-month tissue slices with fluorescent alpha-bungarotoxin to examine the NMJs of wild-type and H304R/+ mice. NMJs from wild-type mice displayed the classic pretzel shaped morphology with a highly branched complex appearance *(Sanes and Lichtman, 2001)*. However, NMJs from H304R/+ animals presented a mixture of NMJ morphologies ranging from the classic morphology to those with obvious defects in size, branches, and complexity.

To more thoroughly characterize the NMJ defect, gastrocnemius tissue samples were analyzed from both wild-type and mutant H304R/+ mice from 1, 3, 6, 9, and 12-month old male animals. Qualitatively, we saw abnormal NMJ complexity/morphology in 1-month H304R/+ tissue and no discernable phenotype in 3-month H304R/+ tissue. This was followed by an increasing percentage of NMJs with more obvious defective morphologies in the 6, 9, and 12-month time-points. However, not all NMJs were abnormal in the H304R/+ mice: there was a range of NMJ morphologies present at all time points. We found that an individual mouse could display a mixture of normal and abnormal NMJ morphologies and that there was a range of abnormalities present in the NMJs

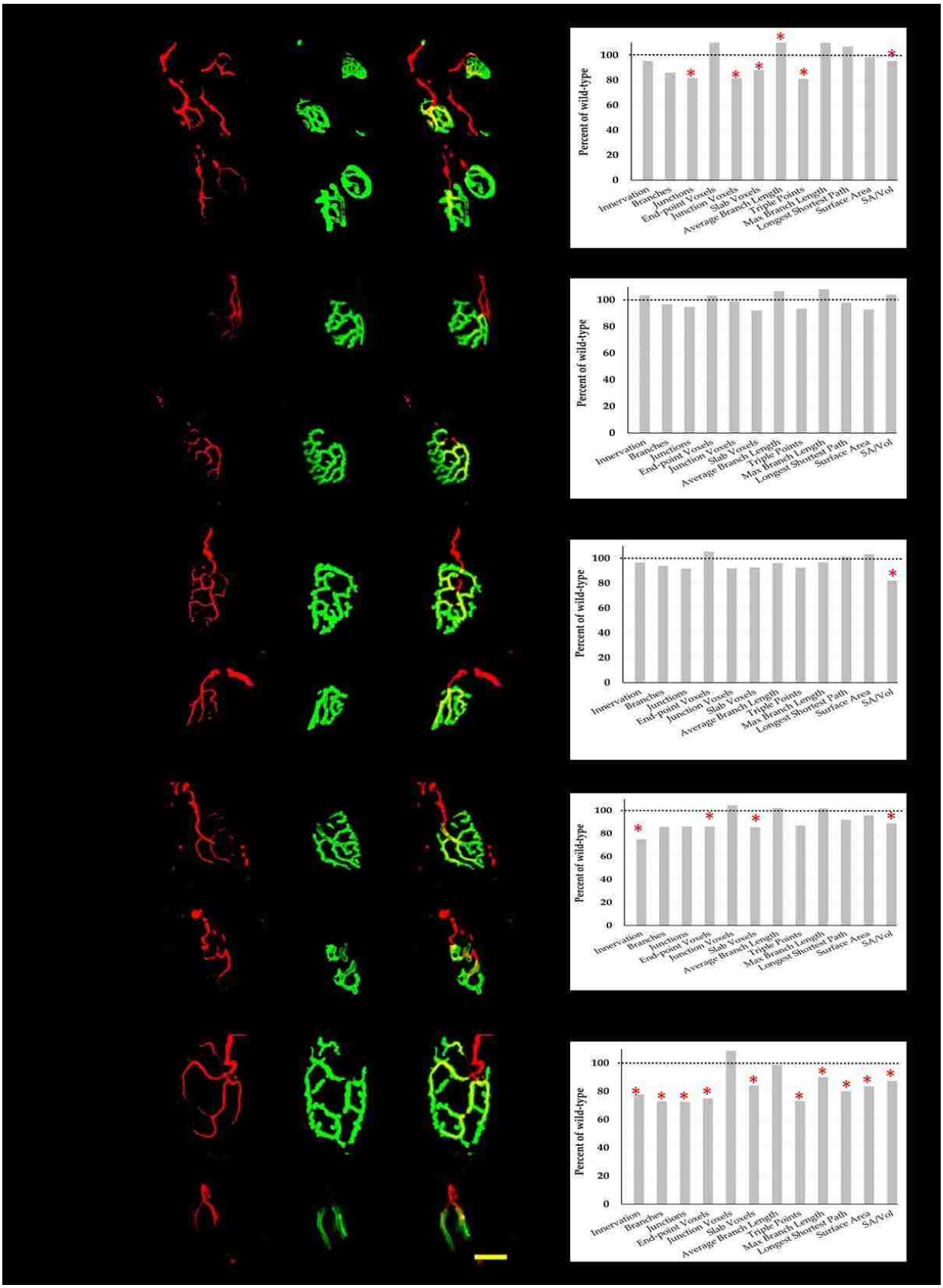

Percent of wild-type
100
80
60
40
20
0
Innervation
Branches
Junctions
End-point Voxels
Junction Voxels
Slab Voxels
Average Branch Length
Triple Points
Max Branch Length
Longest Shortest Path
Surface Area
SA/Vol

Figure 23 Altered neuromuscular junction morphology

Confocal images of representative NMJs stained with neurofilament (red) and alpha-bungarotoxin (green) at different time points. A: 1 month, B: 3 months, C: 6 months, D: 9 months, E: 12 months. Graphs show twelve parameters that were examined for the NMJ architecture in wild-type and H304R/+ animals, displayed as the H304R/+ percentage of the wild type value, grey bars represent H304R/+, black dotted line is wild-type. Statistically significant differences between wild type and H304R/+ values are indicated by * (two-tailed distribution, $p<0.05$). Scale bar =20μm

To quantitatively characterize the defects in NMJ morphology we processed image stacks of AChR fluorescence as described in Materials and Methods. Our analysis identified several interesting features that occur as the mice reach and pass through adulthood. At one month of age, H304R/+ NMJs have statistically significant defects in six of the parameters examined *(Figure 21A)*. Notably, the one-month H304R/+ NMJs have fewer branches as seen by the branch, junction voxel, and triple point parameters. Furthermore, the branches present are longer than those in wild-type animals. Finally, there is a reduction in the surface area to volume ratio of the entire NMJ. These characteristics can be seen in *Figure 21A* as these representative H304R/+ NMJs show fewer longer branches present. At three months of age the overall morphology of H304R/+ and wild-type NMJs were indistinguishable and the quantitative analysis was unable to identify any parameters with statistically significant differences.

At six months of age we observed a small subset of H304R/+ NMJs that appeared less complex than the typical wild-type NMJ. However, these were only a fraction of the overall NMJs and the surface area/volume ratio was only parameter with a statistically significant difference between genotypes *(Figure 21C)*. At the 9-month time point, there were a sizeable percentage of H304R/+ NMJs with obvious morphological defects *(Figure 21D)*. Our analysis showed that there was a reduction in most of the quantitative parameters we measured, and that reduction was statistically significant for four of the parameters in H304R/+ mice compared to wild-type littermate control mice *(Table 1)*.We found the decrease in the percentage of NMJs that were innervated to be especially interesting as it may indicate that some form of degeneration may have occurred in these older NMJs.

The NMJs of 12-month old H304R/+ mice had severe defects from both qualitative and quantitative assessments. Many of the H304R/+ NMJs had a wider junction diameter with a notable reduction in the number of branches present *(Figure 21E)*. Our quantitative analysis of H304R/+ NMJs identified ten parameters with statistically significant decreases relative to age matched wild-type NMJs *(Table 1)*. These differences are indicative of the gross abnormalities present in many of the H304R/+ NMJs. The percentage of NMJs that were innervated correctly was dramatically reduced in the H304R/+ NMJs at 12 months similar to the 9-month time point.

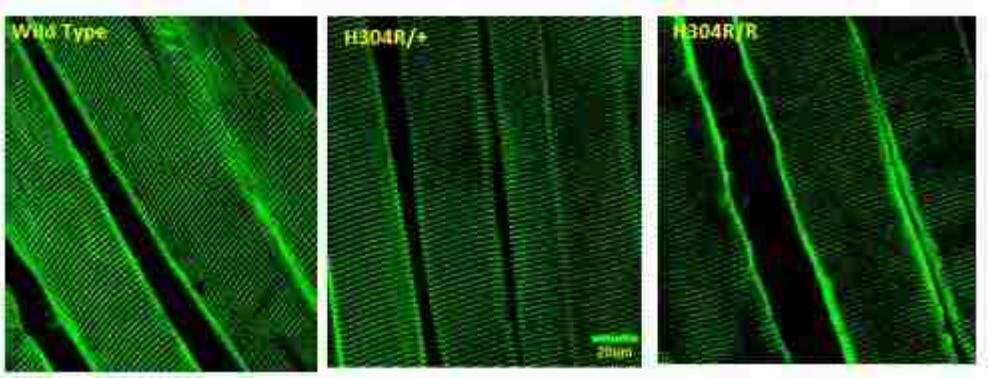
4.2.2 Analysis of Defects in the CMT2O Homozygous Mouse Model

To delve deeper into how a point mutation (H304R) in the dynein heavy chain leads to dynein dysfunction and result in CMT disease-related phenotypes in our mouse model, we bred two heterozygotes to generate homozygous mice with the mutation in both dynein heavy chain genes. We assessed the phenotype of the homozygous mice through our behavioral assays and discovered stronger phenotypes than previously noticed in heterozygous mice and therefore a wider difference when compared to wild-type versus heterozygotes compared to wild-type. We were interested in whether neuromuscular abnormalities in the tissue of homozygous mice would similarly be worse than heterozygotes. Firstly, we examined sarcomere organization in 12-month-old male homozygous mice and compared the results to both age-matched wild-type and heterozygous mice. We chose 12-month old animals because they represent the stage at which mice show with the most drastic effect from the H304R dynein mutation. Sarcomere measurements were taken based on alpha-actinin staining of Z-lines in muscle

fibers of gastrocnemius muscles. There were no obvious genotype-specific differences judging from the arrangement of Z-lines in muscle fibers from confocal images *(Figure 24A)*.

However, we noticed a slight reduction in sarcomere lengths in homozygous mice with a mean sarcomere length of 2.11µm *(Figure 24B)*. This value represents a 5% decrease compared to wild-type (2.22µm) and a 4% decrease compared to heterozygote mean sarcomere length (2.20µm). This difference in sarcomere length between wild-type mice and mutants was statistically significant ($p<0.05$). More experiments are needed to determine the reason for the change in sarcomere length, and if it is a direct or indirect result of dynein dysfunction. Sarcomere organization in the fruit fly, was found to occur after nuclear placement in the muscle fibers *(Auld and Folker, 2016)*. Should this be true in mice as well, it could partly explain why sarcomere lengths in homozygous mice may be slightly altered. This is because our preliminary assessment of myonuclear positioning of subsynaptic nuclei suggests some discrepancies in homozygous mice at the 12-month time point *(Figure 29)*.

A



B

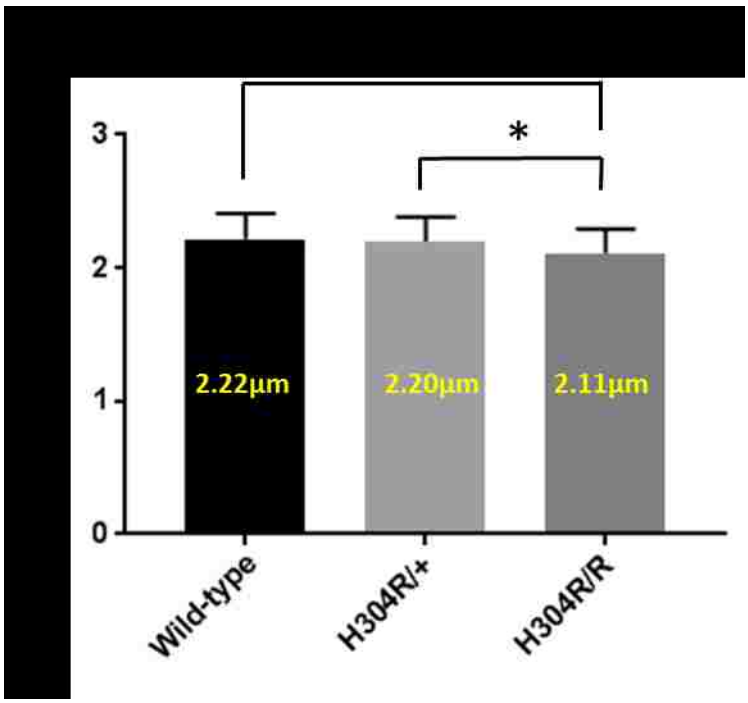


Figure 24 Sarcomere organization in homozygous mice

a) Representative images of sarcomeres in gastrocnemius muscles of 12-month-old mice for each genotype b) graph showing mean sarcomere lengths in muscle fibers for each genotype. The mean values were obtained from 682 wild-type,702 H304R/+ and 744 H304R/R individual sarcomeres

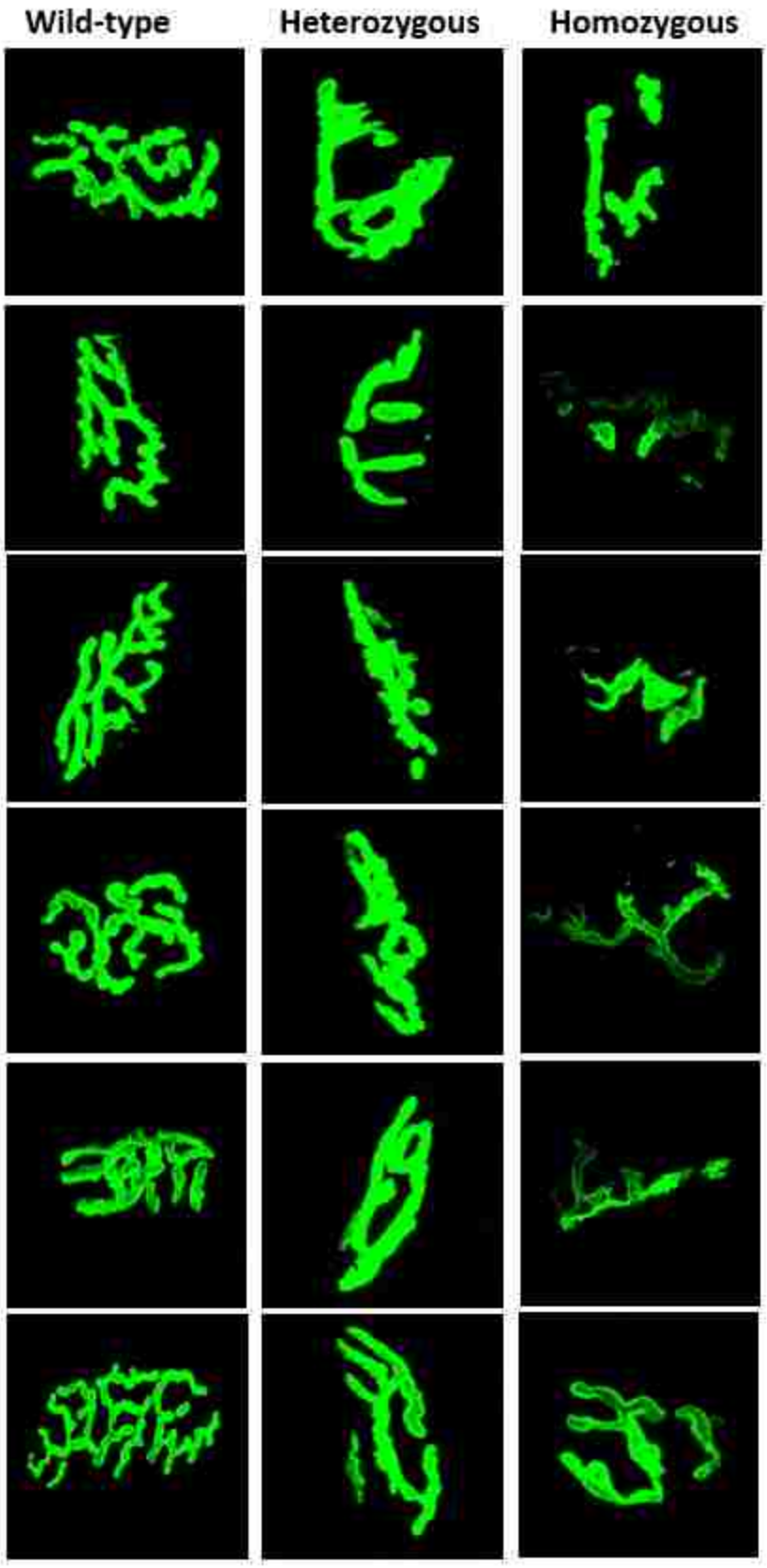
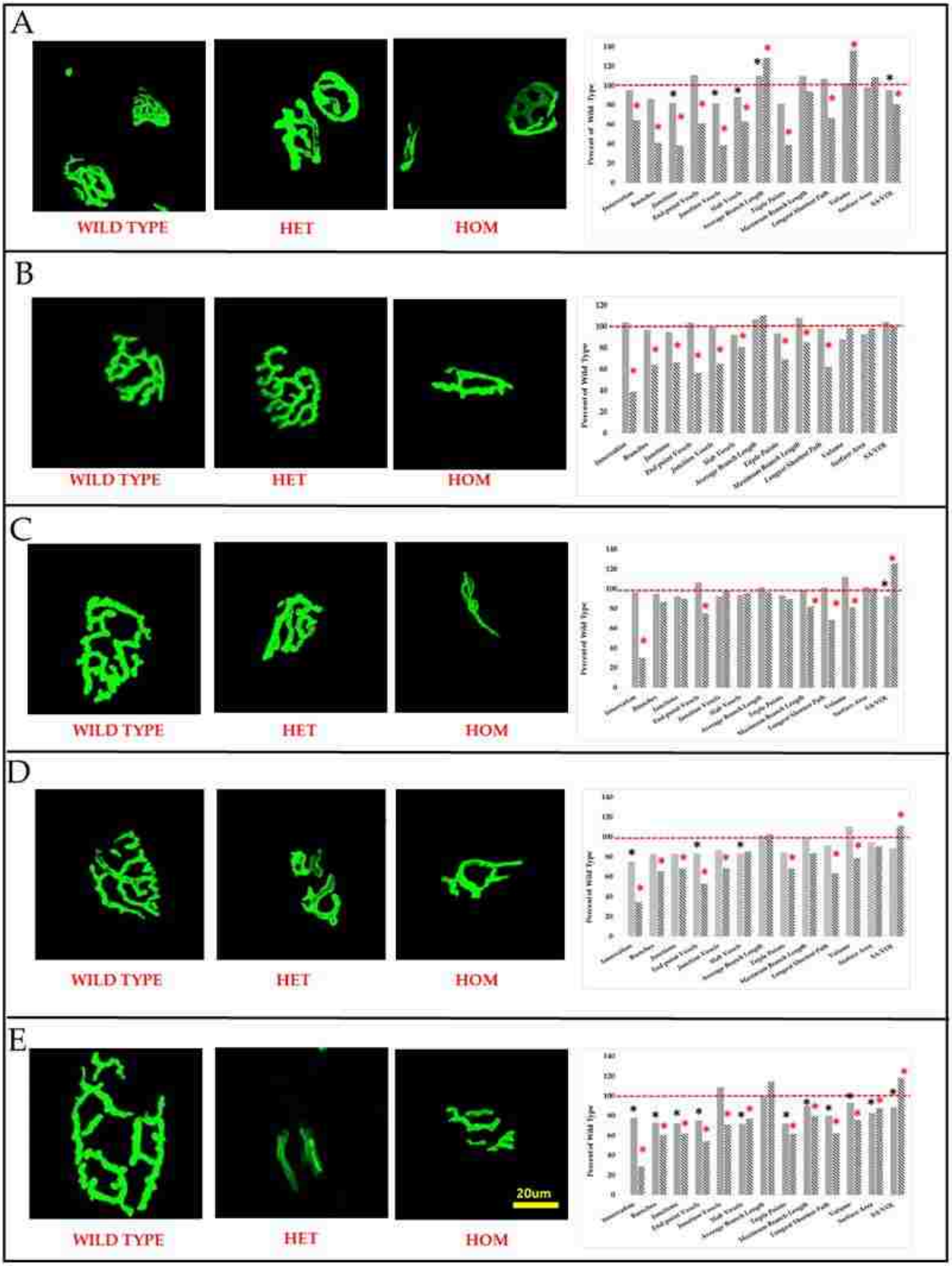

Wild-type
Heterozygous
Homozygous

Figure 25 Comparison of NMJ morphology across genotypes

Representative images of AChRs for each genotype based on alpha bungarotoxin staining at 12 months. Homozygous NMJs were clearly distinguishable from wild-type and even heterozygotes by being less branched and less connected branches. They also displayed fragmentation and had more regions of low fluorescent intensity. Heterozygous NMJs compared to wild-type were generally less complex.

4.2.2.1 H304R/R homozygous mice display more defects in motor endplate architecture

Heterozygous mice showed aberrations in the organization of the NMJ as well as innervation defects at 9 and 12 months compared to wild-type mice. We compared the general morphology of NMJs from gastrocnemius muscles of homozygous mice to wild type and heterozygotes and we saw more severe abnormalities *(Figure 23)*. The NMJs were generally disconnected and unlike the heterozygote muscles, there were more fragmented NMJs. Homozygous mice displayed very low NMJ complexity and in some instances had regions of low receptor density. They appeared smaller in size and quite a number of them had no nerves at all. We proceeded with a longitudinal assessment of NMJ innervation and architecture at 1 month. 3 months, 6 months, 9 months and 12 months. In terms of innervation, H304R/R mice were notably different from H304R/+ mice by showing consistent deficits at all time points from 1 month to 12 months. The number of NMJs that were innervated was higher at 9 months and 12 months compared to wild type but also lower than that of heterozygous mice.


A
WILD TYPE
HET
HOM
B
WILD TYPE
HET
HOM
C
WILD TYPE
HET
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D
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E
20um
WILD TYPE
HET
HOM

Figure 26 Abnormal NMJ architecture in gastrocnemius muscles of H304R/R mice

Confocal images of representative NMJs stained with neurofilament (red) and alpha-bungarotoxin (green) at different time points. A: 1 month, B: 3 months, C: 6 months, D: 9 months, E: 12 months. Graphs show twelve parameters that were examined for the NMJ architecture in wild-type and H304R/+ and H304R/R animals as the percentage of the wild type value. Grey bars represent H304R/+, striped bars are H304R/R, red dotted line is wild-type. Statistically significant differences between wild type and H304R/+ shown by * and differences between wild type and H304R/R values are indicated by * (two-tailed distribution, $p<0.05$). Scale bar =20µm

Table 2 Morphological assessment of wild-type and mutant NMJs

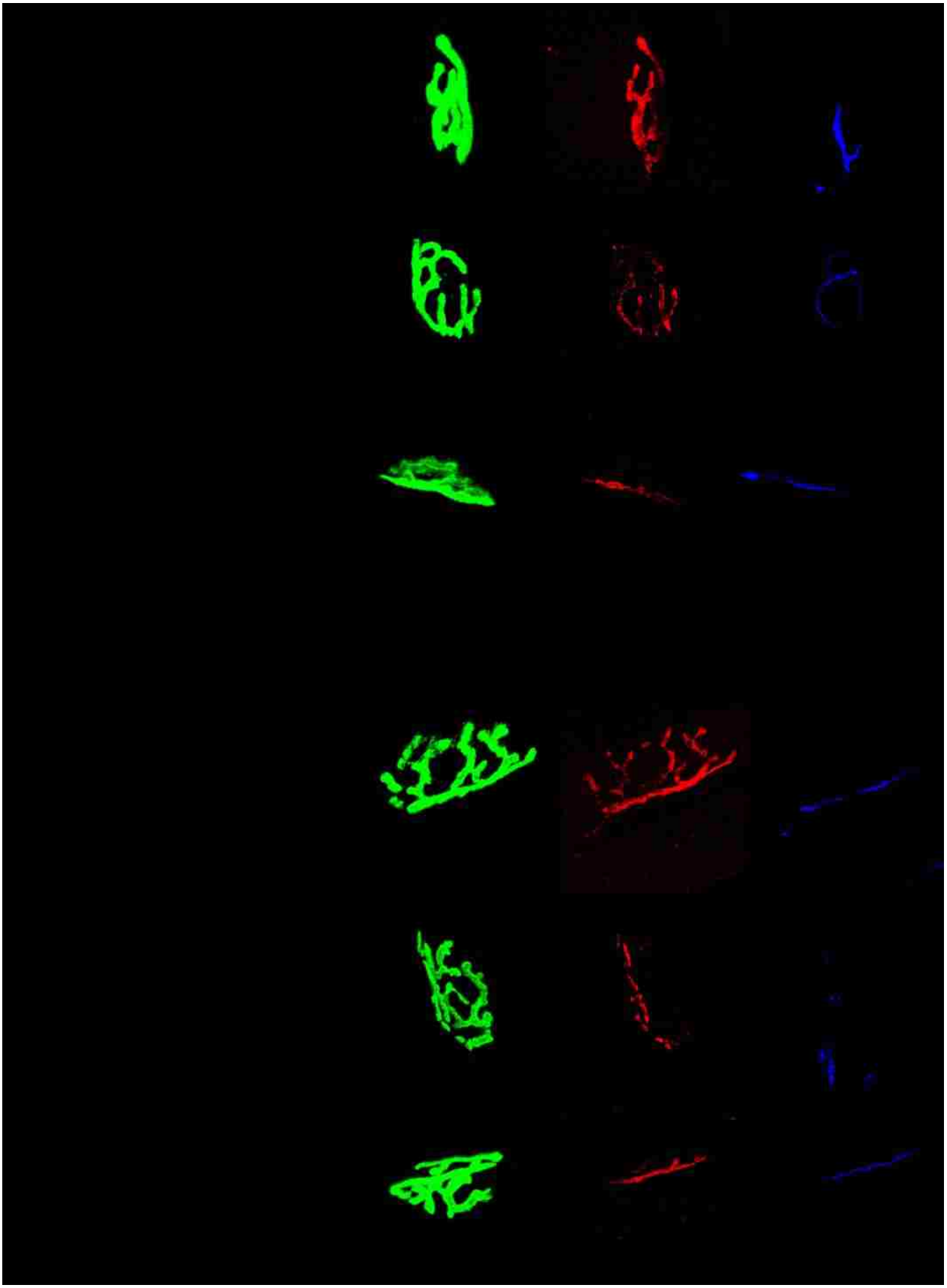
Timepoint	Genotype	*n*	*N*	% of NMJ Innervated	Branches	Junctions	End-point Voxels	Junction Voxels	Slab Voxels	Average Branch Length	Triple Points	Maximum Branch Length	Longest Shortest Path	Volume	Surface Area	SA / VOL
1 month	wild-type	67	5	95.4%	27.0 ± 11.9	15.3 ± 7.1	7.1 ± 2.8	23.2 ± 14.6	353.4 ± 140.5	6.1 ± 1.4	14.4 ± 5.8	19.6 ± 5.8	56.6 ± 15.8	687.4 ± 253.3	1523.5 ± 476.6	2.3 ± 0.3
	H304R/+	88	5	91.0%	23.3 ± 11.7	12.6 ± 7.1	7.9 ± 3.3	23.8 ± 13.9	311.6 ± 116.8	6.8 ± 2.1	11.7 ± 6.9	21.5 ± 7.9	60.6 ± 18.3	704.1 ± 247.0	1500.0 ± 485.9	2.2 ± 0.3
	H304R/R	56	4	64.3%	11.0 ± 6.7	5.8 ± 4.1	4.3 ± 2.0	11.2 ± 7.6	222.1 ± 101.0	7.9 ± 4.8	5.6 ± 4.0	18.4 ± 6.6	37.7 ± 11.9	933.2 ± 293.2	1659.1 ± 428.4	1.8 ± 0.3
3 month	wild-type	67	6	93.3%	21.9 ± 9.1	11.6 ± 5.2	8.8 ± 3.1	21.8 ± 10.4	359.5 ± 111.6	7.8 ± 2.0	11.1 ± 4.9	22.3 ± 6.3	70.6 ± 20.3	1018.5 ± 400.3	1872.0 ± 561.38	1.9 ± 0.3
	H304R/+	72	6	97.2%	21.2 ± 11.9	11.0 ± 6.7	8.9 ± 3.9	21.5 ± 14.1	331.4 ± 145.9	8.1 ± 3.1	10.3 ± 6.2	24.0 ± 9.7	69.1 ± 21.7	896.0 ± 393.4	1738.2 ± 680.4	2.0 ± 0.3
	H304R/R	95	6	38.8%	14.0 ± 8.6	7.7 ± 5.3	4.8 ± 2.2	14.1 ± 10.5	269.4 ± 134.4	8.3 ± 5.4	7.6 ± 5.3	19.0 ± 6.3	44.1 ± 12.2	1004.8 ± 419.0	1841.3 ± 622.6	1.9 ± 0.4
6 month	wild-type	98	5	88.8%	19.3 ± 9.5	10.1 ± 5.4	7.6 ± 3.4	20.1 ± 13.5	335.0 ± 137.0	8.2 ± 2.9	9.5 ± 4.9	25.1 ± 8.3	68.3 ± 23.3	1278.0 ± 630.4	2020.7 ± 790.6	1.7 ± 0.3
	H304R/+	82	5	85.3%	18.3 ± 10.0	9.4 ± 5.7	8.1 ± 3.4	18.6 ± 13.3	313.7 ± 142.5	8.4 ± 2.9	8.3 ± 5.5	24.4 ± 9.4	69.3 ± 22.5	1430.1 ± 766.7	2059.8 ± 807.3	1.6 ± 0.3
	H304R/R	87	5	30.5%	16.7 ± 11.2	9.1 ± 6.7	5.7 ± 2.7	19.8 ± 27.8	321.0 ± 150.0	8.0 ± 5.2	8.6 ± 6.3	20.4 ± 7.2	47.4 ± 15.1	1042.3 ± 468.4	2035.2 ± 728.5	2.1 ± 0.5
9 month	wild-type	63	5	86.2%	22.0 ± 11.5	11.4 ± 6.6	9.1 ± 3.6	22.7 ± 17.6	362.8 ± 363.3	8.2 ± 3.2	10.8 ± 6.2	25.3 ± 9.0	74.7 ± 24.6	1051.7 ± 670.3	1925.0 ± 922.0	2.1 ± 0.5
	H304R/+	65	5	64.7%	18.2 ± 11.7	9.5 ± 7.0	7.6 ± 3.3	19.7 ± 17.8	300.9 ± 142.2	8.3 ± 3.1	9.08 ± 6.5	25.1 ± 9.5	68.2 ± 24.2	1159.3 ± 879.3	1813.4 ± 969.6	1.8 ± 0.6
	H304R/R	75	5	34.5%	14.3 ± 7.7	7.8 ± 4.6	4.9 ± 2.2	15.5 ± 10.0	309.3 ± 130.1	8.4 ± 4.5	7.4 ± 4.3	21.1 ± 7.5	47.5 ± 13.8	828.4 ± 399.5	1739.5 ± 641.0	2.3 ± 0.6
12 month	wild-type	118	6	94.9%	22.8 ± 11.3	11.7 ± 6.4	9.7 ± 4.0	19.9 ± 12.8	387.7 ± 155.7	8.0 ± 2.5	11.2 ± 6.0	25.9 ± 9.5	78.5 ± 24.4	1438.3 ± 718.6	2281.7 ± 907.3	1.7 ± 0.3
	H304R/+	97	6	73.9%	16.6 ± 8.9	8.5 ± 5.0	7.3 ± 3.3	21.7 ± 48.3	278.4 ± 143.5	7.9 ± 2.5	8.1 ± 4.7	23.2 ± 8.5	62.7 ± 26.4	1337.18 ± 742.3	1890.6 ± 894.6	1.5 ± 0.3
	H304R/R	88	6	28.7%	13.78 ± 8.3	7.3 ± 5.0	5.3 ± 2.4	14.1 ± 9.6	298.8 ± 140.2	9.1 ± 7.5	6.9 ± 4.8	20.6 ± 8.8	48.9 ± 14.5	1091.2 ± 546.7	2004.9 ± 811.0	2.0 ± 0.4

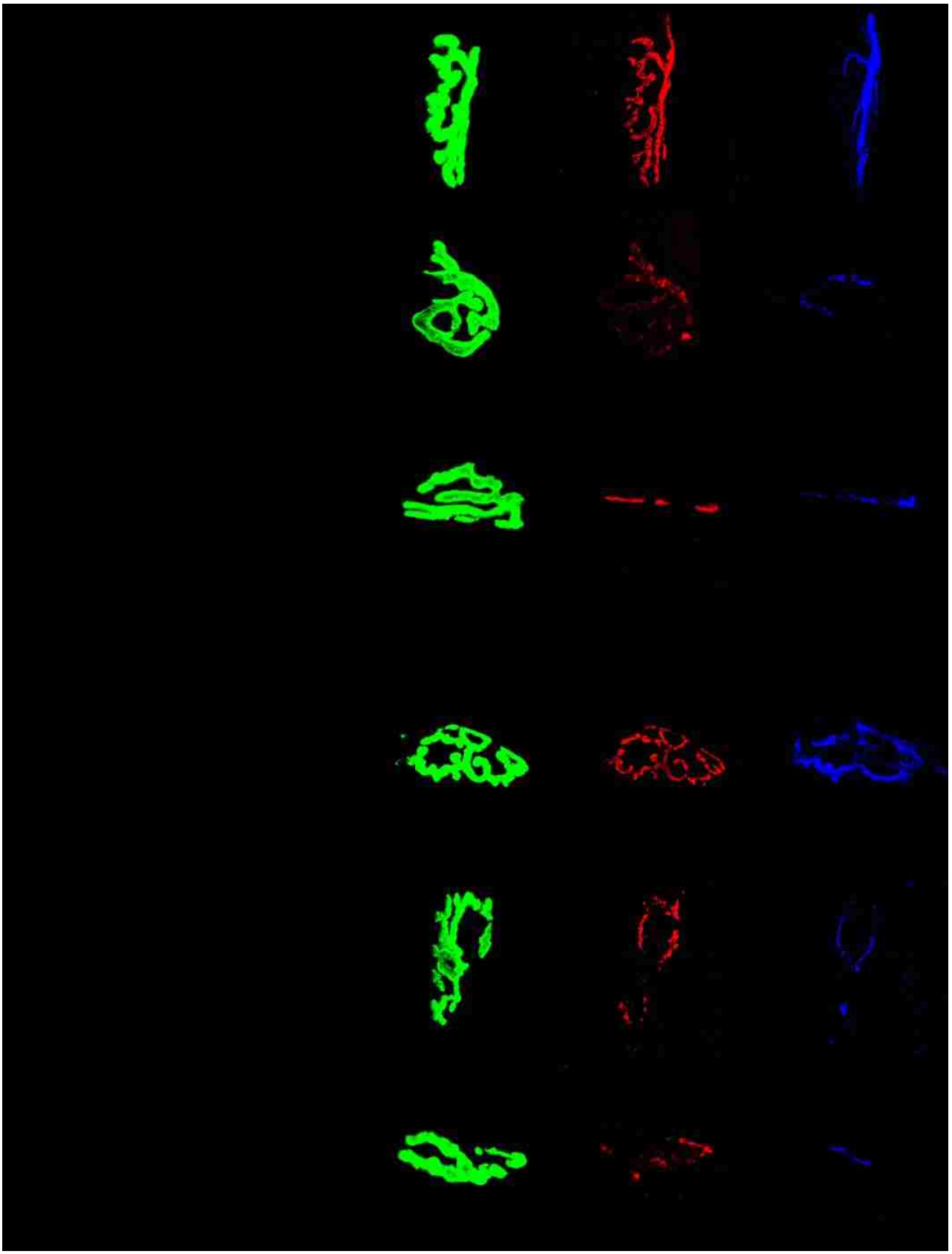
The number of AChRs analyzed (*n*), number of animals per genotype (*N*) and mean values (± standard deviation) of different parameters assessing NMJ complexity. Innervation is shown as the percent of examined NMJs with nerves that occupy more than half of the receptor volume. SA / VOL: surface area to volume ratio.

The dysmorphology of NMJ architecture is seen more strongly at 1 month than in H304R/+ with differences observed in more of the parameters. Whereas there were no abnormalities observed in H304R/+ mice at 3 months, homozygous mice continue to show differences in complexity when compared to wild-type and heterozygotes *(Figure 26B)*. However, at 6 months the effect of the H304R mutation on NMJ complexity seems to be slightly reduced compared to earlier time points. There were significant differences in 6 of the complexity parameters compared to 9 parameters at 3 months. Previously in H304R/+ mice only surface area to volume ratio was different from normal mice. At 9 months, the NMJ abnormalities in the homozygous mice are more pronounced, an obvious deterioration from the 6-month time point. The defects in NMJ organization are most severe at 12 months compared with wild type, with lower values than heterozygous *(Figure 26E)*.

4.2.2.2 H304R/R homozygous mice show abnormalities in innervation

Innervation defects were consistently recorded in homozygous mice from 1 month to 6 months, worsening by 9 months and 12 months. Our analysis pointed to denervation in a number of homozygous NMJs with negative staining for neurofilament. Even though less NMJs are innervated in homozygous mice, we focused on the NMJs with nerves present and asked if synaptic activity was normal in these nerves. To ascertain the functionality of the neuromuscular synapse beyond the mere presence of neurofilament positive staining, we assessed synaptophysin occupancy at the NMJ as a measure of a functioning synapse.





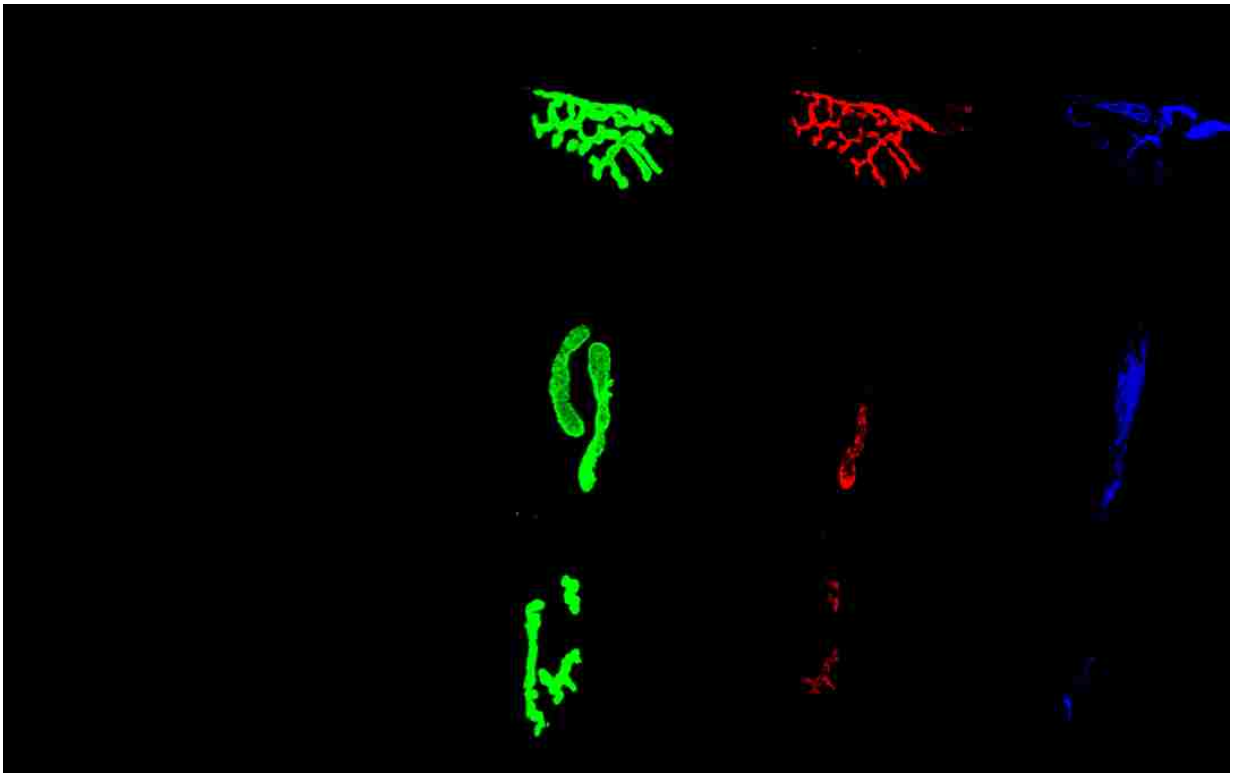
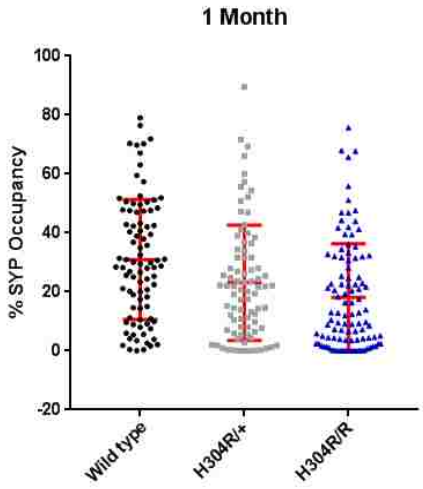
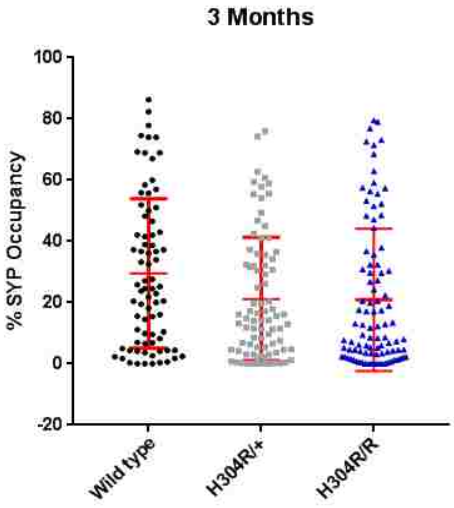


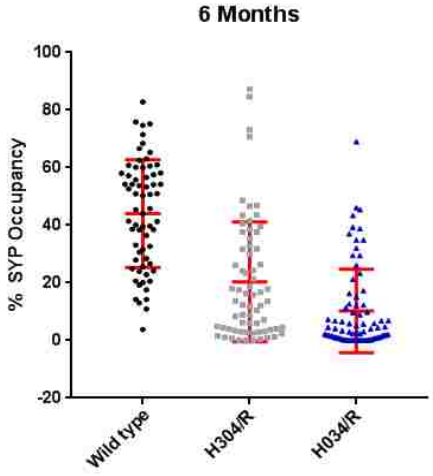
Figure 27 Synaptic dysfunction in H304R/R homozygous mice

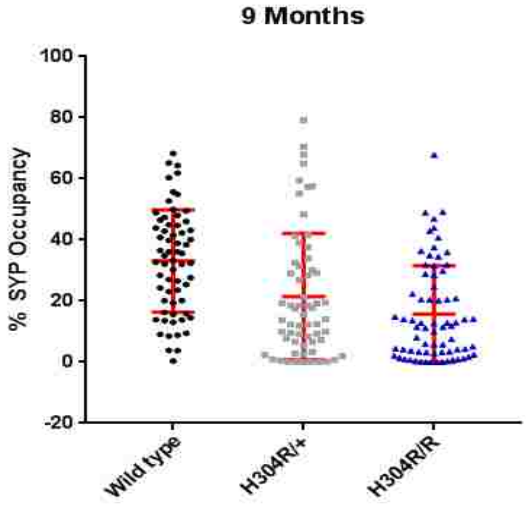
Representative images of synaptophysin density (red) at each time point in pre-synaptic nerves (blue) at the neuromuscular junction of mice. The post-synaptic apparatus is stained with alpha–bungarotoxin (green).

Synaptophysin is a membrane protein of synaptic vesicles which is widely used as a marker for synaptic vesicles *(Valtorta et al, 2004)*. The synaptophysin occupancy is obtained from the area of synaptophysin staining divided over the area of AChR staining. In heterozygotes, there were no statistically significant differences in synaptic vesicle density at early time points, significant differences were observed beginning from 6 months till 12 months *(Figure 25)*. Although a significant reduction in the number of innervated NMJs is not observed in heterozygotes till 9 months, a reduction in synaptic vesicle density occurs earlier at 6 months. The density of synaptic vesicles does not drastically reduce over time but remains almost constant from 6 to 12 months. In H304R/R mice, synaptic vesicle density was consistently lower than wild-type and H304R/+ mice, however not all these differences were statistically significant. Differences between wild –type and homozygous were significant at all time points examined.


1 Month
% SYP Occupancy
100
80
60
40
20
0
-20
Wild type
H304R/+
H304R/R


3 Months
% SYP Occupancy
100
80
60
40
20
0
-20
Wild type
H304R/+
H304R/R


6 Months
% SYP Occupancy
100
80
60
40
20
0
-20
Wild type
H304/R
H034/R


9 Months
% SYP Occupancy
100
80
60
40
20
0
-20
Wild type
H304R/+
H304R/R

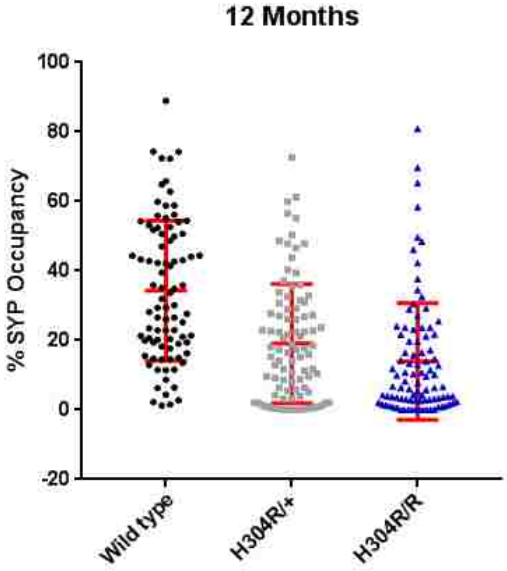

12 Months
% SYP Occupancy
100
80
60
40
20
0
-20
Wild type
H304R/+
H304R/R

Figure 28 Synaptic vesicle density at the presynaptic apparatus

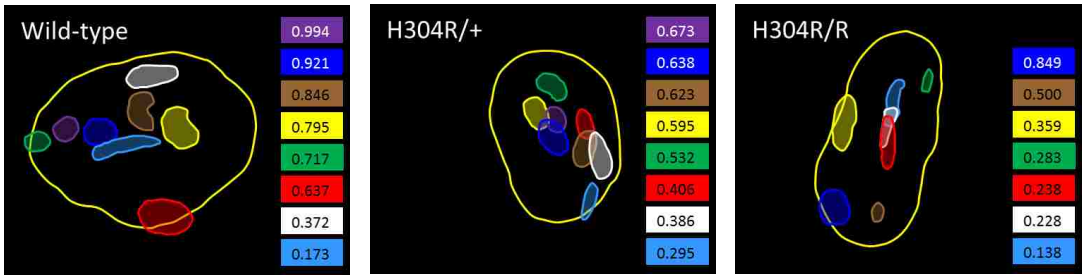
Gastrocnemius muscle sections were stained for synaptophysin and the AChR and images acquired via confocal microscopy. Heterozygous mice display deficits in synaptophysin density beginning from 6 months, whereas homozygotes show defects from the first month up to 12 months. Synaptic vesicle density is expressed as a percentage of AChR area and was computed as follows: (synaptophysin area/ AChR area) x100. Statistical analysis was carried out using Graph pad and by employing the one-way-ANOVA, $p<0.05$

4.2.2.3 Homozygous mice show differences in sub-synaptic nuclei distribution

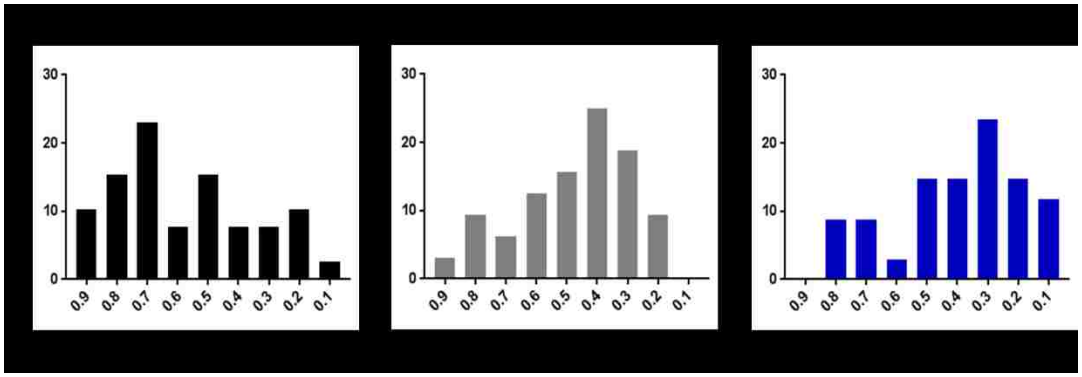
Molecular motors are required for the positioning of nuclei in different cell types; and mispositioning of nuclei is a hallmark of some muscular disorders. We noticed differences in the appearance of nuclei associated with the NMJs between wild-type and heterozygous mice. These nuclei looked different in terms of their morphology, and arrangement and at times, the number of nuclei also varied between genotypes. After we generated homozygous mice with the dynein H304R mutation we continued to notice these differences. The nuclei at the neuromuscular junction or sub-synaptic nuclei play some distinct roles that sets them apart from the other nuclei in the muscle *(Moscoso et al, 1995)*, it is therefore possible they would be targeted as the H304R mutation leads to disruptions in NMJ architecture. We proceeded to investigate the differences observed in sub-synaptic nuclear arrangement at the NMJ by focusing on the morphology of the nuclei and the number present at the synapse. We chose to look first at mice that were 12 months of age, since we saw a more pronounced neuromuscular dysfunction at this time point. We determined the ellipticity of the nuclei to differentiate more oval elongated nuclei from more rounded nuclei. The neuromuscular synapse is also infiltrated by nuclei from the glial cells which help stabilize the entire apparatus also. Our initial study of nuclear morphology and distribution in 12-month-old mice revealed the abundance of more elongated nuclei in the muscles of homozygous mice compared to wild type and even heterozygotes. The heterozygotes seemed to have more mid-point ellipticity scores as seen also in the histogram that quantifies the distribution of these nuclei *(Figure 29)*.

Further studies into establishing the identity of nuclei either as myonuclei or glial cell nuclei as well as results from other timepoints are ongoing.

A


Wild-type
0.994
0.921
0.846
0.795
0.717
0.637
0.372
0.173
H304R/+
0.673
0.638
0.623
0.595
0.532
0.406
0.386
0.295
H304R/R
0.849
0.500
0.359
0.283
0.238
0.228
0.138

B


30
20
10
0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

C

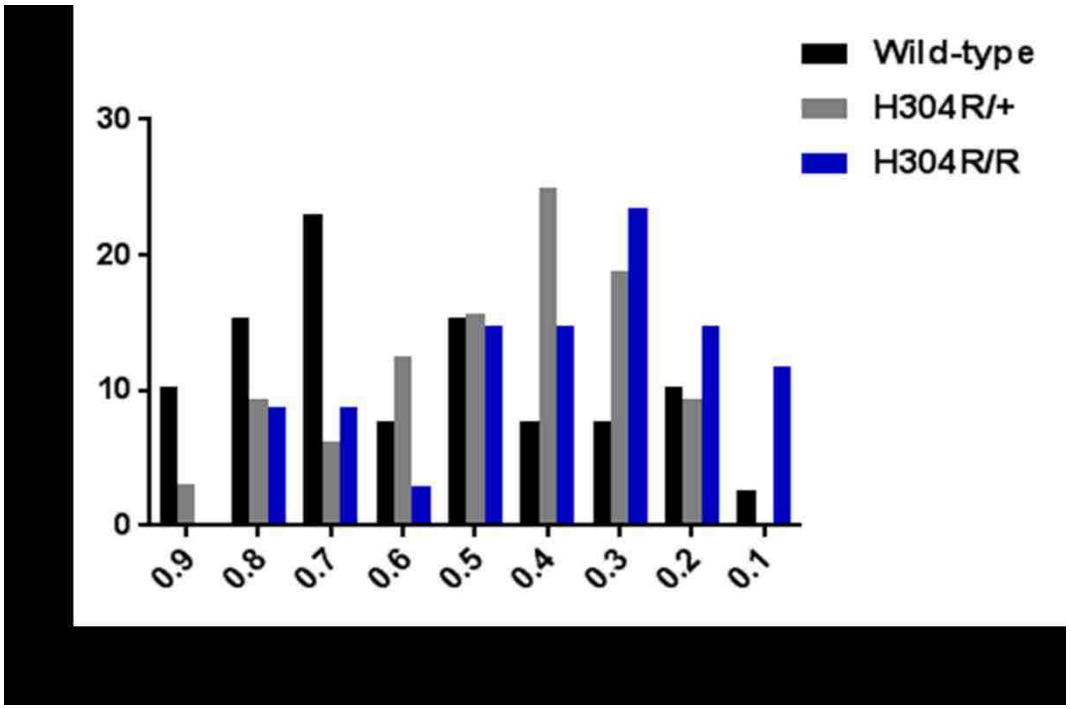

Wild-type
H304R/+
H304R/R
30
20
10
0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

Figure 29 Distribution of nuclei at the neuromuscular junction

A) Schematic depicting nuclear morphology at the NMJ from three genotypes (wild-type, heterozygous and homozygous) based on confocal images from12 month old mice. The area enclosed by the NMJ is marked in yellow as well as the nuclei analyzed. The ellipticity scores are shown in boxes and have been color-coded to match the respective nuclei. Ellipticity is calculated by dividing the width by the length of the nuclei. B) Histogram showing nuclear ellipticity in each genotype as a percent of all nuclei. (Number of nuclei analyzed: wild-type=39, H304R/+ =32, H304R/+=34) C) Distribution of nuclear ellipticity at the NMJ compared across genotypes. Black bars are wild-type, grey bars heterozygotes and dark blue bars homozygotes

4.3 Discussion

4.3.1.1 Changes in NMJ architecture in H304R heterozygous mice

Based on the motor behavior phenotypes we observed, we decided to examine NMJ morphologies in male H304R/+ mice and saw significant differences in NMJ morphologies over time *(Table 2)*. We found that NMJs appeared under-developed at one-month of age, with fewer branches, and with those branches having significantly longer lengths. However, this phenotype disappeared at 3 months, as the NMJs were indistinguishable from control animals at that age. At later time points the NMJs lost complexity, and many times appeared to have a larger synapse diameter. One of the most interesting features of the H304R/+ phenotype was the apparent recovery of NMJ morphology in young adult mice followed by degeneration as the mice aged. The defects seen at the earliest time point seem to represent a problem in development of the NMJs, as less complexity was present. In contrast, the defects seen in the later time points suggests that there may be a separate mechanism in the older animals that is causing possible retraction or degeneration at the NMJ during aging. Future work will be needed to test those possibilities.

4.3.1.2 Altered neuromuscular junction morphology in H304R/R animals

Although CMT disease occurs in humans as an autosomal dominant heterozygous mutation, there are some benefits in generating homozygous individuals. From a biochemical standpoint isolating components of the mutant dynein complex for further analysis is more feasible in a homozygous animal than a heterozygous. This is because the population of wild-type or normal dynein that would be present in every isolated

fraction is eliminated. Another reason why a homozygous mouse model is exciting is the possibility of studying understanding the function or dysfunction of dynein more clearly as the protein function is more severely affected compared to the heterozygous model. *loa/loa* homozygous mice die within a day of birth, however studies on the embryos have shed more light on the essential functions of dynein in neurons during development (*Hafezparast et al, 2003, Ori-McKenney and Vallee, 2011)*.

Homozygous mice displayed dysmorphology at the NMJ at all time points analyzed, a more careful scrutiny of the results point to a lessening of the effects on the NMJ at 6 months until it increases again from 9 months till the animals are 12 months. Unlike heterozygous mice where there is no difference in NMJ complexity at 3 months, the H304R/R mice have less severe abnormalities at 6 months. It is possible that the heterozygotes are able to overcome some of the relatively milder developmental problems by 3 months, but the homozygotes are never able to fully do so since they have more severe effects. Even at 6 months when the where there is a diminished effect compared to other time points, abnormalities at the NMJ still remain when compared with wild type mice. The H304R/R homozygous mouse model is not the first attempt at generating a homozygous dynein mouse model, but it is the first to survive up to about 24 months. The other dynein homozygous mouse models were either embryonic lethal or the mice died soon after birth. Therefore, only data from mouse embryos have been studied, there is no data on the progression of disease in the adults. This means that with the H304R/R homozygous dynein mouse model there is the opportunity to systematically investigate disease pathways and obtain purified mutant protein that can be used in

various cellular assays to better understand how dynein structure and function is altered in a diseased mouse.

4.3.1.3 Nuclear morphology at the Neuromuscular Junction

Nuclei that are found localized specifically at the NMJ continue to be the subject of much interests because of how they contribute to the maintenance of the NMJ and the transmission of nerve impulses. An understanding of how they could be affected in disease conditions will be important in finding out more about how the entire neuromuscular synapse is altered. We conducted an analysis of nuclear morphology in wild-type and mutant mice. We observed severe neuromuscular deficits in 12-month-old male mice, and so we started by looking at these cohorts. Differences exist in the morphology of nuclei in the different genotypes with the wild-type mice having more rounded nuclei and less elongated nuclei. There is a shift from a mixture of both rounded and oval morphs in heterozygous mice to a predominantly elongated oval nuclei in homozygous tissue. This observation could be due to a number of possibilities; the presence of more glial cells in the wild type mice compared to the other 2 mutants (heterozygotes and homozygotes). It is also possible that the defects in dynein function affect the placement of glial cell nuclei during development resulting in a reduction in the number of myonuclei. Researchers in an ALS study examined the link between denervation and expression of the glial marker, S100 *(Carasco et al,2016)*. They found no expression of S100 under conditions of denervation, mice with an ALS mutation (SOD1-G93A) are more susceptible to losing the expression of S100 compared to normal

mice. This scenario is entirely possible in our study, since we have shown that homozygous mice have more denervated NMJs. Further studies would help us establish whether a correlation exists between the denervation of the NMJ and reduced expression of S100 in a dynein disease model. It will also be important to ascertain whether the differences in nuclear distribution and morphology between wild-type and H304R mutants (both heterozygous and homozygous) occur at other time points too.

CHAPTER 5:CONCLUSION

5.1 The generation of a novel mouse model to study dynein function

Dynein function has been widely studied for several years in various systems and that has contributed immensely to our understanding of how the motor protein functions within cells. The ability to genetically manipulate portions of the dynein-dynactin complex have been invaluable in deciphering how its functions are needed in cells. The introduction of the three dynein heavy chain mouse models, *loa, cra, swl* drew the attention of the entire field to the critical role dynein plays in neuronal cells. Various studies into these mouse models brought to the fore how dynein-mediated retrograde transport may be essential for the survival of neuronal cells *(Hafezparast et al, 2003)*. There is therefore a two-fold limitation with these mouse models. First, the exact mechanistic pathway through which the mutation in the dynein heavy chain gene results in disease is not clear. The other limitation is that the *loa, cra, swl* mutations are not related to a disease in humans, there is therefore a bit of a difficulty in relating the findings to humans. We successfully generated a knock-in mouse model of CMT2O disease bearing a point mutation (H304R) in the dynein heavy chain which corresponds to H306R in humans. Our characterization of this mutant mouse revealed a predominant motor phenotype as seen in human CMT2 disease. This was confirmed through our assays by weakness in the muscle and motor coordination defects. The phenotypic study conducted in *loa, cra* and *swl* mice was not done in a longitudinal manner and so did not cover all the timepoints in the H304R study. However, phenotypic defects in H304R heterozygous mice are milder in comparison to

loa, cra, swl mice. The H304R/R homozygous mouse model on the other hand, appears to have more robust behavioral characteristics compared to *loa,cra, swl* heterozygotes. The H304R mouse model is therefore a novel and distinct system that can be used to study dynein function. The fact that it is linked to a disease in humans addresses the issue of relevance in the search for therapeutics and makes it suitable for translational research.

5.2 Connecting the dots between dynein mutations and neurological disorders

The ultimate goal of our study is to understand dynein function better, and in the specific case of our H304R mouse model, we hope to achieve this aim by following the onset and progression of CMT2O disease, caused by a mutation in the dynein heavy chain gene. We have shown that the neuromuscular junction is a pathological target in CMT2O disease *(Sabblah et al, 2018)*. The abnormalities in the NMJ are obvious in both heterozygous and homozygous models, with the deformities worsening in the homozygotes. There is still more work required to arrive at a mechanism for CMT disease in our H304R mouse model. A legitimate question that arises is how does dynein function affect NMJ architecture? It is known that dynein transport of essential cargoes in axons towards the nucleus is important in many neuronal processes *(Levy and Holzbaur, 2006)*. We can therefore hypothesize that NMJ defects in H304R mouse models are partly due to dynein dysfunction in the motor neurons that innervate the axon. There is evidence of that in our study where we saw reduced innervation and a concomitant increase in NMJ dysmorphology at later stages in heterozygous mice. Dynein activity in the muscle could also be a contributory factor. It was discovered that disrupting dynein function alters the components of the neuromuscular synapse in primary myoblasts from mice *(Vilmont et al, 2016)*. Our initial analysis of subsynaptic

nuclei at the NMJ suggests a difference in the distribution and morphology of nuclei in mutants compared to wild-type. This could be another pathway that disrupts the NMJ organization in H304R mutant mice since dynein is an essential player in myonuclear positioning *(Folker et al, 2014)*.

We cannot rule out the possibility of a structural defect in the dynein protein itself or a change in interaction with its binding partners. The dynein motor protein is a massive 1.6 MDa complex made up of four kinds of subunits that need to be present in the right stoichiometric proportions to be assembled into a functional unit. In addition to that, the dynein complex interacts with other key adaptors and regulators like dynactin, BicD, Lis1 that are required for complete function in cells. A mutation in any of the dynein subunits could possibly affect how the subunits interact or how other accessory proteins interact with dynein and ultimately alter dynein function to cause disease. Interestingly, a study involving 14 DYNC1H1 heavy chain mutations, did not find any change in the association of dynein with dynactin-BICDN complexes in majority of the human mutations, including the H306R mutation *(Hoang et al, 2017)*. With that being said, it will be interesting to know what the dynein subunit levels are in the H304R homozygous mutant as well, and what kind of outcomes will be obtained from single-molecule assays using only a homogenous population of mutant protein.

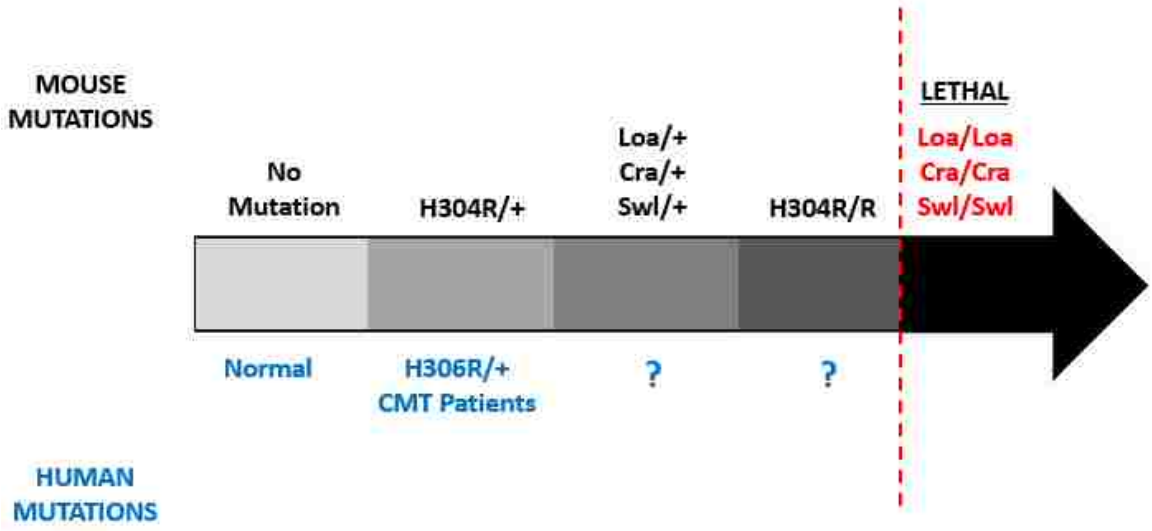


Figure 30 Severity of phenotypes continuum

Comparison of the severity of mouse DHC mutations with the CMT2O DHC phenotypes observed in human patients. Heterozygous mouse H304R/+ phenotypes are similar to those of H306R/+ CMT2O patients. Heterozygous mouse *loa/+, cra/+, swl/+* phenotypes are more severe than those of H304R/+ mice. Homozygous H304R/R phenotypes are more severe than *loa/+, cra/+, swl/+* phenotypes. Homozygous *loa/loa, cra/cra, swl/swl* phenotypes are the most severe resulting in death.

5.3 So how does the H304R mutation cause disease?

Studies in *N. crassa* involving the expression of various dynein heavy chain mutations resulted in 5 different classes of phenotypes based on the localization of cytoplasmic dynein in the hyphae *(Sivagurunathan et al, 2012)*. What this study taught us is that phenotypes generated from dynein heavy chain mutations are heterogeneous. Pleiotropic manifestations are observed in the different human mutations as well. The majority of the dynein heavy chain (DHC) mutations are described as SMA-LED (spinal muscular atrophy with a lower extremity predominance based on their features in patients. The SMA-LED phenotype is mainly characterized by lower muscle atrophy and weakness. There were other neurological defects in humans with mutations in the dynein heavy chain gene. Some patients with the H306R mutation which causes CMT2O disease had *pes cavus* and an abnormal gait. Less common manifestations of the disease include impaired speech and learning difficulties *(Weedon et al, 2011)*. Intellectual disorders have also been reported in other dynein heavy chain mutations *(Strickland et al, 2015, Scoto et al 2015).* Malformations of cortical defects were observed in some dynein heavy chain mutations as well *(Poirier et al,2013)*. Even though there are over 20 dynein heavy chain mutations discovered in humans, there isn't a lot known about the prevalence for any one individual mutation or all of the DHC mutations. The first publication about dynein heavy chain mutations in humans came out about 7 years ago, which makes it quite a short period of time to have volumes of data to compute prevalence rates. Perhaps epidemiological data will become available after a while when more cases are reported.

The point to note is that the heterogeneity of the disease manifestations could be strongly linked to the mechanism of the disease. In simple terms, multiple manifestations could be due to multiple disease pathways. Another significant observation regarding the phenotypes of DHC mutations is the variation in severity. Differences in the extent of the disease manifestation could occur within a cohort, varying from individual to individual, as previously reported in human patients *(Weedon et al, 2011, Scoto et al, 2015)*. We however wanted to capture any differences that might exist among different DHC mutations. By comparing our H304R mouse model with other dynein mouse models namely *loa, cra and swl,* we noticed differences in how the mice are affected by mutations in the DHC gene *(Figure 30)*. We are unable to determine which of the other DHC human mutations would correspond to those of the mouse DHC genes since there are no mouse models for those other human mutations.

Beyond the motor phenotype observed in the behavioral assays, we were interested in uncovering dynein related functions that could be contributors to CMT disease in the H304R mutant mice. Neuromuscular junction defects have already been reported in dynein and CMT2 mouse models *(d'Ydewalle et al, 2011, Sleigh et al, 2014, Spaulding et al, 2016, Courchesne et al, 2011)*. NMJ analysis in the dynein H304R mutant mice also unveiled dysmorphology and reduced complexity which were even worse in the homozygous mice.

To probe dynein dysfunction even further, it is important to consider the tripartite components of the neuromuscular apparatus, namely the motor nerves, the muscle fiber and the terminal Schwann cells. Dynein function has been studied in the motor and

neuron and in muscles but not much is known about dynein function in Schwann cells especially the terminal Schwann cells that cap the neuromuscular junction. We observed innervation defects in H304R mice, pointing to possible defects in dynein-mediated transport in the axons. The dynein complex transports cargo from the synaptic end of the axon to the soma, some of these include, molecules marked for degradation, recycling endosomes and ribonucleoproteins. Dynein dysfunction could lead to a reduction in transport efficiency and may even impact kinesin-mediated anterograde transport. This could be a very important factor in the development of disease because some cargo are transported in both anterograde and retrograde directions *(Brady et al, 1990, Hendricks et al, 2010).*

This could partially explain why the perturbation of dynein function via a point mutation in the heavy chain gene for instance, will result in defects in neuromuscular abnormalities in an organism. Knock out studies done in mice have shown that without dynein, mice die art the embryonic stage *(Harada et al,1998)*. As such we expect that mice that survive with a dynein mutation would not have a complete loss of dynein function. Additionally, the fact that H304R mutant mice are ambulatory means there may not be a grave malfunction such as the inability to transport cargo but rather an alteration of dynein function leading to a delay in the delivery of some kind of cargo sounds more likely. The slight change in dynein function is probably the reason why CMT2O patients have a slowly progressing motor and sensory neuropathy. In these patients also, the onset is variable and so is the phenotype of the disease. Motor neurons may be vulnerable to dynein mutations by their length, which represents a large distance over which cargo has to be transported. Most of the proteins required by neurons are synthesized in the soma

and must therefore be delivered to the various compartments where they are needed. This makes axonal transport indispensable to neuronal survival. The defective axonal transport model is a likely mechanism in the H304R dynein heavy chain gene mutation that causes CMT2O disease.

Work done in cultured muscle cells showed a direct involvement of dynein in the organization of the post-synaptic apparatus. The inhibition of dynein function led to aberrations in the trafficking of the muscle specific kinase (MuSK), which helps cluster the receptors and maintain the structural cohesion of post-synaptic components *(Vilmont et al, 2016)*. The possibility of MuSK transport being a key driver of NMJ dysmorphology in H304R mutant mice would require further studies using cells from the muscles of mice. Such an investigation at the cellular level will enable us to assess other dynein functions like the maintenance of Golgi and microtubule integrity. Dynein functions in muscle development although myosin is the molecular motor required for contraction in the muscle. Dynein is involved in the movement and placement of myonuclei, which is very critical in muscle function. As muscle cells are syncytial, the positioning of nuclei occurs at defined intervals to create sectors of transcriptional control for each nucleus *(Bruusgard et al, 2003)*. Live cell experiments in the fruit fly show dynein is needed to properly position myonuclei *(Folker et al, 2014)*. A similar mechanism may be needed to cluster nuclei at the synaptic region that are in close proximity to the AChR in the muscle. Preliminary results from our lab suggest a difference in nuclear distribution in the synaptic region *(Figure 29)*. The synaptic nuclei also contribute to the AChR pool that is clustered to ensure efficient communication from the nerves *(Ferraro et al, 2012)*. This means that changes in the organization of sub-

synaptic nuclei arising from the H304R mutation could be a factor in the reduced complexity observed in mutant NMJs.

There isn't much information about dynein function in glial cells. Dynein is known to play a role in myelination in zebrafish via its involvement in the expression of some vital genes *(Yang et al, 2015).* Work done in oligodendrocytes of zebra fish specifically identified myelin basic protein (MBP) as a cargo transported by the dynein/dynactin complex, and disrupting this complex reduces the expression of MBP, which is required to maintain myelin stability *(Herbert et al, 2017)*. Without much evidence specifically linking cytoplasmic dynein function to terminal Schwann cells, the scenario where dynein influences the expression of vital genes in the terminal Schwann cells still remains a possibility that has to be adequately tested. The ubiquity of dynein and its multifarious roles in living organisms reflects in the mechanisms of diseases that result from DHC mutations. In H304R mutant mice it is likely that a combination of altered dynein transport defects in the motor neuron, defective trafficking of one or more synaptic components and dysfunction in glial cells at the neuromuscular junction contribute to the predominant motor defects observed in mutant mice.

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APPENDIX B
DEFENSE ANNOUNCEMENT

Announcing the Final Examination of Mr. Thywill Tsatsu Sabblah for the degree of Doctor of Philosophy in Biomedical Sciences

Dissertation Title: "NEUROMUSCULAR JUNCTION DEFECTS IN A MOUSE MODEL OF CHARCOT-MARIE-TOOTH DISEASE TYPE 2O"

Date: April 2, 2018 **Time:**2:00pm **Location: Lake Nona BBS, Rm103 (Live) / BMS 136 (Simulcast)**

ABSTRACT

Charcot Marie Tooth disease (CMT) represents the most common inheritable peripheral group of motor and sensory disorders; affecting 1 in 2500 people worldwide. Individuals with CMT experience slow progressing weakness of the muscle, atrophy, mild loss of motor coordination and in some cases loss of sensory function in the hands and feet which could ultimately affect mobility. Dynein is an essential molecular motor that functions to transport cargos in all cells. A point mutation in the dynein heavy chain was discovered to cause CMT disease in humans, specifically CMT type 2O. We generated a knock-in mouse model bearing the same mutation(H304R) in the dynein heavy chain to study the disease. We utilized behavioral assays to determine whether our mutant mice had a phenotype linked to CMT disease. The mutant mice had motor coordination defects and reduced muscle strength compared to normal mice. To better understand the disease pathway, we obtained homozygous mutants from a heterozygous cross, and the homozygotes show even more severe deficits compared to heterozygotes. They also developed an abnormal gait which separates them from heterozygous mice. In view of the locomotor deficits observed in mutants, we examined the neuromuscular junction (NMJ) for possible impairments. We identified defects in innervation at the later stages of the study and abnormal NMJ architecture in the muscle as well. The dysmorphology of the NMJ was again worse in the homozygous mutants with reduced complexity and denervation at all the timepoints assessed. Our homozygous dynein mutants can live up to two years and therefore make the design of longitudinal studies possible. Altogether, this mouse model provides dynein researchers an opportunity to work towards establishing the link between dynein mutations, dynein dysfunction and the onset and progression of disease.

Committee members:
Stephen J. King, Ph.D (**Chair)**
Yoon-Seong Kim, Ph.D
Ella R. Bossy-Wetzel, Ph.D
Deborah Altomare, Ph.D

Publications:

- **Sabblah TT*,** Nandini S*, Ledray AP, Pasos J, Conley Calderon JL, Love R, King LE, King SJ., A novel mouse model carrying a human cytoplasmic dynein mutation shows motor behavior deficits consistent with Charcot-Marie-Tooth type 2O disease, Sci Rep. 2018;8(1):1739
- **Sabblah TT**, Nandini S, Conley Calderon JL, Ordway, B, Love R, King LE, King SJ., Homozygous Charcot-Marie-Tooth disease mutations in the dynein heavy chain cause severe neurological dysfunction in mice, *Manuscript in preparation*

Approved for distribution by Dr. Stephen J. King, Committee Chair

The public is welcome to attend.

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