Characterizing the Onset and Progression of Charcot-Marie-Tooth Neuropathy in H304R Mutant Mice

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CHARACTERIZING THE ONSET AND PROGRESSION OF CHARCOT-MARIE-TOOTH NEUROPATHY IN H304R MUTANT MICE

by

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ABSTRACT

Dynein is a motor protein complex that transports various types of intracellular cargos from the cell periphery towards the cell center. Dynein mutations are linked to several neurodegenerative diseases, including Charcot-Marie-Tooth disease (CMT). A mouse model of CMT was generated with a knock-in H304R dynein allele. This mutation at position 304 corresponds to the H306R mutation found in humans that can cause CMT. Here, a behavioral test was developed to study the onset and progression of CMT symptoms in these mice. In the tail suspension test, mice were suspended briefly by their tails and the posture of their hind limbs was scored. Wildtype mice spread their hind limbs outwards in a characteristic splayed posture, whereas heterozygous and homozygous mutants display abnormal phenotypes.

In further investigation, the neuromuscular junctions of these mice were analyzed in order to understand the histological effects of the mutation and how the potential differences could result in the behavioral effects observed. The extent of neuromuscular junction innervation was examined along with the size and complexity of the neuromuscular junctions themselves through multiple criteria. This, when combined with the effects observed during the tail suspension behavioral test, seeks to establish the H304R mutant mouse as a successful model for CMT.

ACKNOWLEDGEMENTS

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INTRODUCTION

Charcot-Marie-Tooth (CMT) disease is one of the most common neuromuscular disorders, encompassing several different hereditary motor and sensory neuropathies. CMT is characterized by chronic motor and sensory polyneuropathy, where affected individuals experience distal muscle weakness, atrophy associated with sensory loss, and high-arched feet (*pes cavus*). CMT is the most common genetic cause of peripheral neuropathy with a prevalence of about 1 in 1214 (Braathen, 2012). Patients affected by CMT experience persistent weakness in the hands or feet, often requiring special shoes with proper ankle support, forearm crutches for gait stability or wheelchairs. Surgical solutions are often required to treat pes cavus. Fatigue can also be severely disabling in patients, contributed by weakness in skeletal muscles. This often leads to career and employment problems for the patient. (Bird, 1993; El-Abassi et al., 2014).

CMT type 2O was recently discovered in a large-pedigree family, in which the DYNC1H1 gene was mutated at position 306, changing a histidine to an arginine in the polypeptide (El-Abassi et al., 2014; Weedon et al., 2011). This gene encodes for cytoplasmic dynein 1 heavy chain 1, a subunit in cytoplasmic dynein. Cytoplasmic dynein is an intracellular motor protein that shuttles various cargos from the cell periphery towards the cell center by walking along microtubules within cells, moving from the plus end of the microtubule and towards the minus end (Schroer et al., 1989). Other mutations in DYNC1H1 have been linked to neurodegenerative diseases like SMA-LED (spinal muscular atrophy with lower extremity predominance) with very

similar phenotypes to the patients with the H306R mutation causing CMT 2O (Harms et al., 2012; Tsurusaki et al., 2012).

Dr. Stephen J. King's laboratory generated a mutant mouse model that contains a targeted knock-in mutation H304R in the DYNC1H1 gene, analogous to the human H306R mutation causing CMT 2O. There are other DYNC1H1 mutant mouse models that have been produced, most notably the *Loa* and *Cra1* mice. These other mice containing DYNC1H1 mutations exhibit defects in retrograde transport. The mutant mouse model generated by Dr. King's laboratory, however, is the first mutation in DYNC1H1 analogous to a known disease-causing mutation in humans, whereas *Loa* and *Cra1* are not analogous to human mutations. Homozygous mutant *Loa* and *Cra1* mice die within 24 hours of birth (Hafezparast et al., 2003) where H304R homozygous mutant mice survive.

It should be noted that compared to other neurodegenerative diseases, CMT is mild. While investigating the more pronounced and obvious effects of the *Loa* and *Cra1* mutations provide answers to the functionality of the dynein heavy chain, it does not link back to human disease. This suggests that the H304R mouse may be the best disease model to study CMT, because other similar mutations are too drastic to properly represent the human disease. Behavioral tests were designed in this study to detect the slight changes in fatigue and skeletal muscle weakness in H304R mice and the neuromuscular junctions were examined across various mouse ages in order to understand what is happening as the mice age.

PURPOSE AND SIGNIFICANCE

This research aims to characterize a novel mouse model for CMT2O and determine if it can be a successful model for human disease for further research into disease mechanics or therapies. This work aims to study the onset and progression of CMT2O symptoms in this mouse model by using the developed tail suspension test for fatigue and weakness in the muscles of the hind legs. This establishes a timeline for the symptoms observed which can be compared to the human disease. Further, studying the neuromuscular junctions of these mice begins to answer the question of what is happening in the tissues of these animals and what causes their symptomatic behavioral phenotypes. This research seeks to determine if there are characteristic signs in this mouse model that point to either a neurodegenerative or neurodevelopmental pathogenesis. After establishing a timescale for the onset of disease symptoms through behavioral testing, differences observed in the neuromuscular junctions can be compared to the behavioral data to investigate the correlation between any changes observed in the neuromuscular junctions and disease progression. By understanding what in the tissue is causing the skeletal muscle fatigue and weakness, treatments can begin to be developed and tested.

BACKGROUND

Cytoplasmic dynein 1 is a multisubunit molecular motor complex of approximately 1.5 MDa in size that moves various cargos towards the minus end of microtubules. Cytoplasmic dynein's multiple subunits assemble around a homodimer of heavy chains, which binds to microtubules and is responsible for the ATP-driven motor functionality. Dynein moves by taking steps and walking along a microtubule using microtubule-binding domains located within the dimerized heavy chain. This heavy chain is encoded by a single gene, DYNC1H1. Dynein contains two of these identical heavy chain subunits, each of which has a size of approximately 530 kDa (Pfister et al., 2006). Each heavy chain contains a stem domain at the N-terminus for interacting with various cargo and six ATPase domains. Within the stem domain is a region responsible for the heavy chain homodimerization (Schiavo et al., 2013). The H306R mutation that causes CMT2O is located within the homodimerization region, spanning amino acids 300 – 1140 (Tynan et al., 2000).

The housekeeping roles of functional dynein are vital and complete deletions of the dynein heavy chain results in embryonic lethality (Harada et al., 1998). The *Loa* mutant mice contain a mutation at position 580 in DYNC1H1, changing a phenylalanine to a tyrosine. The *Cra1* mice contain a tyrosine to cysteine mutation at position 1055 on the opposite side of the homodimerization region. Both the *Loa* and *Cra1* mutations are so damaging to the organism that homozygous mutants die within 24 hours of birth. Because the H306R human mutation has been linked to CMT2O, it is known that the mutation has an effect in humans. Given the

survivability of H304R homozygous mutants however, compared to lethal Loa and Cra1 mice, the H304R mutation can be concluded as less disastrous to the organism. This corresponds to the human mutation which, while disabling and debilitating, is mild compared to other neuropathies. This also points to a new system to study which potentially could be better representative of human disease.

RESULTS

Aim 1. Characterize the behavioral effects of H304R mutation through hind limb posture

The other mouse models containing mutations in the DYNC1H1 gene were named for the unusual phenotypes animals expressed upon suspension by their tails, *Loa* (legs at odd angles) and *Cra1* (Cramping 1) (Hafezparast et al., 2003). Mice in the H304R colony were briefly suspended by their tails and heterozygous animals appeared to be more likely to position their hind limbs in a manner similar to that observed with the *Loa* and *Cra1* mice. We have accordingly designed a behavioral test that examines the different phenotypes observed between wildtype, heterozygous and homozygous mutant mice.

In the tail suspension test we are using, H304R colony wildtype (+/+), heterozygous mutant (+/R) and homozygous mutant (R/R) mice are lifted by their tails from a resting position on the experimenter's hand for ten seconds before being set down again with all four limbs settled on the experimenter's hand. After five seconds of rest, mice are again lifted by their tails and suspended for another ten seconds. This process is repeated until the animal has been suspended for a total of three times. We believe that this lifting and setting-down of the mice tires the animals out faster than simply suspending them for a single thirty-second period and exposes a fundamental phenotypic difference in mice of different genotypes. Using two cameras connected to a computer, videos are made of all three suspensions from both the ventral and lateral angle of the mouse, allowing leg posture to be best assessed. With only one point-of-view,

we previously determined that it was difficult to say with any certainty how the animal was holding their legs while suspended. For example, while legs may be in close proximity to each other from the ventral angle, when observing at the lateral angle they may be held far away from the body. These video pairs are then analyzed and each mouse is assigned a score during each of the three individual suspensions, depending on how their legs are positioned. Analysis is performed without knowledge of the animal's genotype. Figure 1 shows images from the ventral camera showing the three distinct phenotypes observed. A score of 3 indicates that the animal has their legs spread and held away from the body in what is the normal response of mice A score of 1 indicates that the mouse's legs are held very tightly to their body, often times touching each other. Animals with clasped hind limbs are always assigned a score of 1 because in the observed clasped behavior, their limbs are always held tightly to their body. A score of 2 means that the animal's hind limbs are held closer, but the feet are not touching each other and the limbs are not tightly held to the mouse's body. A score of 2 corresponds to an intermediate stage.

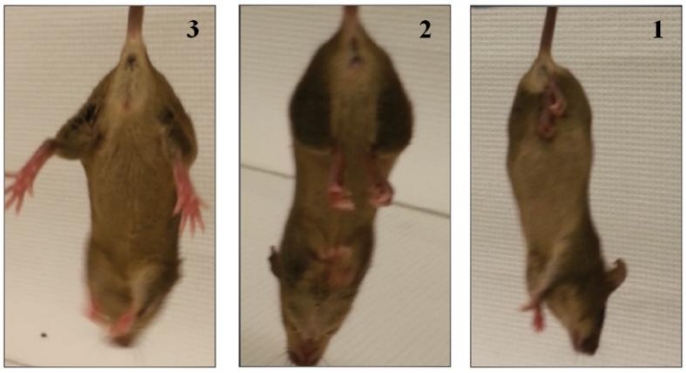

Figure 1: Tail Suspension Phenotypes

This project seeks to answer two questions. First, using leg posture as an indicator for muscle weakness, do the animals exhibit different phenotypes at different rates across the genotypes? This would indicate muscle weakness due to the H304R mutation and would make

sense given the prevalence of fatigue in skeletal muscles observed with CMT patients. Secondly, as the mice age, how do their scores change? This serves to study the onset and progression of the disease and examine the course of neurodegeneration over the animal's lifetime.

The scores given during the tail suspension behavioral test are not quantitative, rather being categorical. Because of this, tests such as the Student's t-test are not appropriate statistical tools for this data. However, because the categories are numbers, averages can be taken in order to qualitatively observe changes in the data. Analysis of Figure 2A points out several interesting observations. The average first suspension scores and third suspension scores were taken from animals of each genotype within each four-week grouping of animals. The number of tail suspensions in each pool ranged from 10 at minimum to a maximum of 147 (male +/R 8 to 11 weeks) with a tail suspension event in each pool performed by a single animal, with no other events in the pool coming from the same animal. The test averages serve to answer the question of how animals of each group perform as a whole at each point of the test and how this changes over time. For male mice, the distance between +/+ First Score and +/+ Third Score appears consistent and smaller than the distance between +/R First Score and +/R Third Score. This indicates that the difference in average scores is smaller and over the course of the test and that the phenotypes observed changed to more 2's and 1's in the +/R male population than the +/+ mice. Furthermore, as the animals age, there are more 2's and 1's in the +/R male animals during the first suspension. Comparing the Third Score of the +/+ male animals and the +/R male animals shows a larger difference than comparing the First Scores. This indicates that male +/R third suspension scores differ from those of the +/+ males more than the first suspension scores.

The difference between the first and third tail suspensions is only that of the time the animal is in the third suspension, they are likely to have been more fatigued by the prior two suspensions. This means that, with the addition of fatigue as a testing factor, +/R and +/+ animals differ. The R/R male animals consistently have lower average scores at all age groups. For both the +/R male animals and the R/R male animals, the average score decreases with age.

The female animals appear to respond to the test in a slightly different manner. In Figure 2 B, the downward slope of the +/R female mice first and second suspension scores is not as steep as it does with the males. While the +/R female scores do not seem to worsen quite to the same extent as the +/R male scores, the difference between the +/+ and +/R female scores appears similar to that between the +/+ and +/R male scores. The scoring differences with the R/R females is obvious and they do appear to have lower scores as the test progresses from the first suspension to the third, however the average scores for R/R females does not seem to decrease at the same steep rate that does happen with the R/R males. This suggests that there is a difference with the progression of disease between the genders, where males have lower average scores as they age compared to females.

The average scores from both the first and third tail suspensions can be seen in Table 1 for males and Table 2 for females. Here it is easier to see that the female heterozygous mutants have consistently lower scores on the third suspension than the wildtype littermates, though not to the same extent that the male heterozygotes differ from wildtype males. Homozygous mutant animals of both genders have drastically lower scores than wildtype and heterozygous mutants.

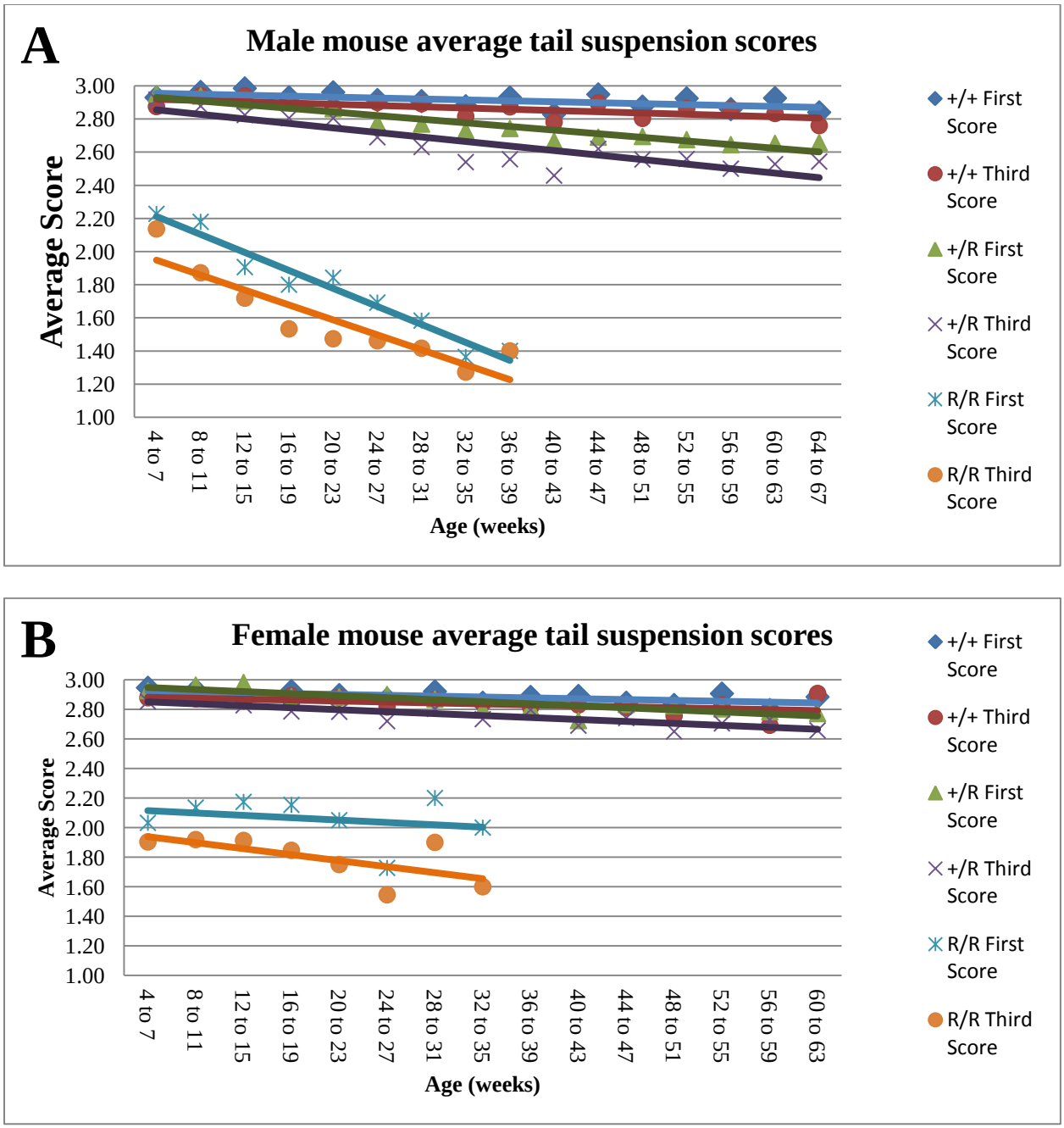

Figure 2: Average tail suspension scores for male and female mice

Table 1: Average tail suspension scores for male mice

Male average scores						
age (weeks)	+/+		+/R		R/R	
	First Score	Third Score	First Score	Third Score	First Score	Third Score
4 to 7	2.93	2.88	2.95	2.92	2.23	2.14
8 to 11	2.96	2.94	2.94	2.88	2.18	1.87
12 to 15	2.98	2.94	2.91	2.82	1.91	1.72
16 to 19	2.93	2.90	2.90	2.80	1.80	1.53
20 to 23	2.96	2.87	2.86	2.80	1.84	1.47
24 to 27	2.91	2.90	2.78	2.69	1.69	1.46
28 to 31	2.91	2.89	2.77	2.63	1.58	1.42
32 to 35	2.88	2.82	2.73	2.54	1.36	1.27
36 to 39	2.93	2.87	2.74	2.56	1.40	1.40
40 to 43	2.83	2.78	2.67	2.46		
44 to 47	2.95	2.90	2.69	2.62		
48 to 51	2.88	2.80	2.69	2.56		
52 to 55	2.92	2.86	2.68	2.56		
56 to 59	2.86	2.86	2.64	2.50		
60 to 63	2.93	2.83	2.65	2.53		
64 to 67	2.84	2.76	2.66	2.54		

Table 2: Average tail suspension scores for female mice

Female average scores						
age (weeks)	+/+		+/R		R/R	
	First Score	Third Score	First Score	Third Score	First Score	Third Score
4 to 7	2.95	2.88	2.93	2.85	2.03	1.90
8 to 11	2.93	2.87	2.96	2.89	2.14	1.92
12 to 15	2.88	2.86	2.98	2.83	2.17	1.91
16 to 19	2.92	2.89	2.89	2.79	2.15	1.85
20 to 23	2.90	2.88	2.88	2.78	2.05	1.75
24 to 27	2.87	2.82	2.90	2.72	1.73	1.55
28 to 31	2.92	2.86	2.87	2.80	2.20	1.90
32 to 35	2.84	2.84	2.80	2.73	2.00	1.60
36 to 39	2.88	2.81	2.81	2.80		
40 to 43	2.89	2.83	2.72	2.69		
44 to 47	2.84	2.81	2.83	2.74		
48 to 51	2.83	2.76	2.83	2.65		
52 to 55	2.91	2.83	2.80	2.70		
56 to 59	2.79	2.69	2.78	2.73		
60 to 63	2.88	2.91	2.77	2.66		

Figure 3 addresses the question of how much the scores change from the first suspension to the third suspension. Here, the average first score subtracted by the average third score for each group and this difference is graphed out. In Figure 3 A, +/+ male animals appear to have a consistent difference between their first and third tail suspension scores as they age, while +/R male animals have an increase in difference at 20 to 23 weeks (n = 87) that appears to maintain. Figure 2 A also shows that after 20 to 23 weeks the +/R male first suspension average score becomes lower, though the difference between the scores still remains two to three times more than that seen with the +/+ male animals, indicating that more +/R males are starting the test with lower scores and even more have lower scores after being fatigued. With the R/R male animals though, the score difference rapidly increases at 8 to 11 weeks of age (n = 39) meaning that with the addition of fatigue, animals had much lower scores. This trend decreases after 20 to 23 weeks, in which the average R/R male first suspension score drops but does not get much worse by the third suspension. Figure 3 B shows that the +/+ female animals remain consistent in their score differences and, for the most part, so do the +/R female animals, although they exhibit a larger drop in average score. The drop in average score increases in the R/R female animals, but upon examining Figure 2 B it is evident that this is because of an increasingly lower third suspension score and not because the first suspension scores are lower. Curiously, the phenotypic differences do not appear to become more prevalent as the animals age as seems to be the case with the males.

Figure 4 shows the individual scoring makeup of each group for the third tail suspension. In Figure 4 A, it is clear that the heterozygous male animals have fewer score of 3 as they age,

and the score of 1 appears consistently at age 24 to 27 weeks, distinct from similarly aged wildtype animals. The drastic scoring difference is also apparent with the homozygous mutant animals. In Figure 4 B, the difference between the heterozygous and wildtype female animals is far less prominent and does not change as the animals age in the same way the male animals do. Although the female homozygous mutant mice have a distinct scoring difference from the other genotypes, the scores themselves do not seem to drastically change as the animal ages, as is seen with male homozygous mutants. As a whole, female scoring patterns appear to be more consistent as the mice age, while male mutants are more likely to have lower scores as they age.

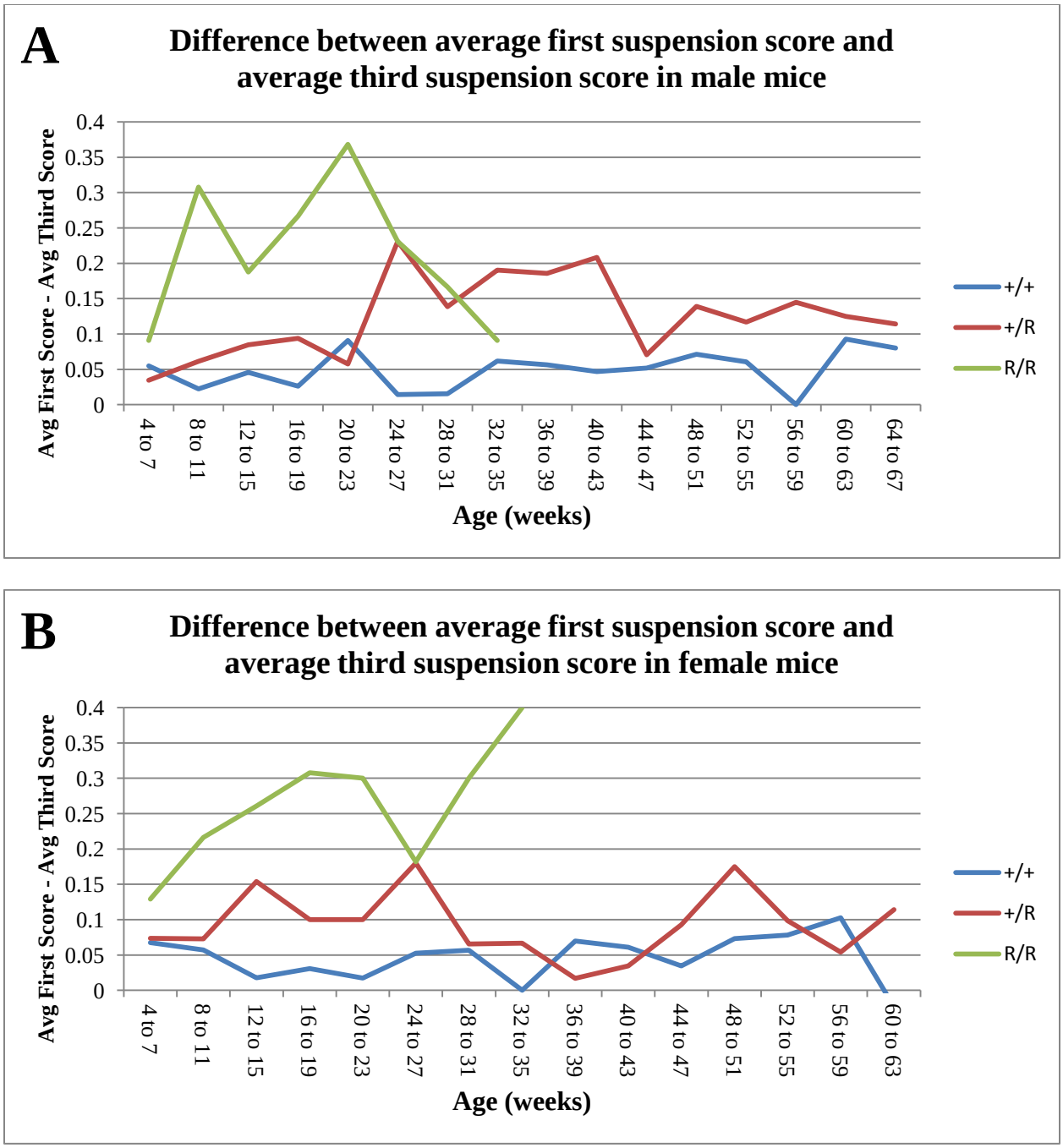

Figure 3: Differences between first and third tail suspension scores

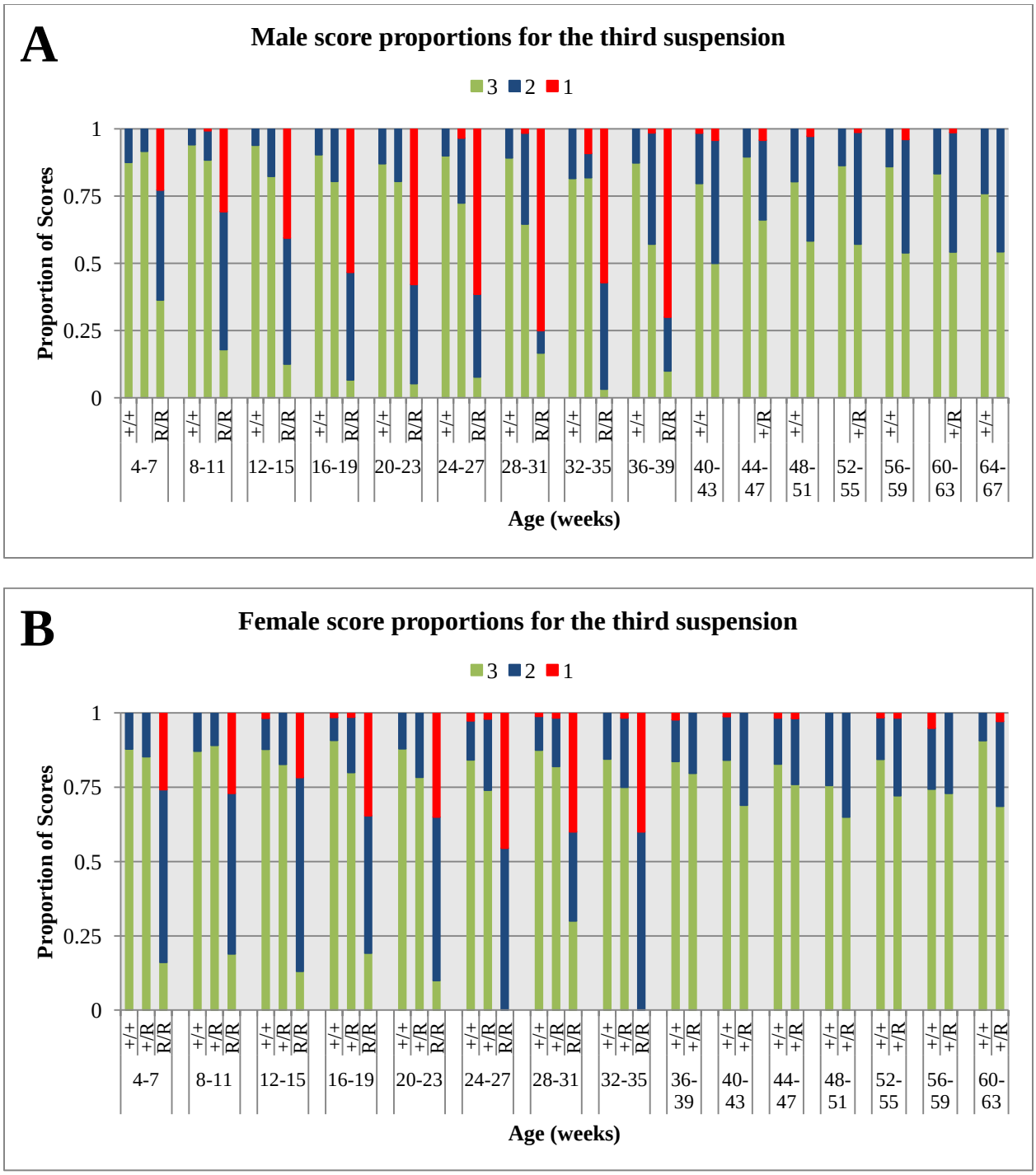

Figure 4: Proportional score makeup of each group in the tail suspension test

Because the scoring system was categorical (1, 2 or 3), statistical testing for significance was carried out utilizing Pearson's chi-squared test with the scores from the third tail suspension because it shows the greatest difference between heterozygous and wildtype animals. +/+ animals were compared to +/R animals to see if this difference observed during the third suspension was statistically significant, and R/R animals were compared to both +/+ and +/R groups. Results for these statistical tests can be found in Tables 3 and 4. All groups used for statistical testing had a minimum *n* of 10, with no individual animal tested twice within each group.

At first, the +/R males did not have any significant scoring difference from the wildtype littermates. At 12 to 15 weeks age however (*n* = 131 +/+, 142 +/R) the difference becomes significant (P = 0.000549). This trend continues as the heterozygous animals age, with the exception of 20 to 23 weeks of age (*n* = 77 +/+, 87 +/R) where the difference is not significant (P = 0.146986). R/R male animals perform significantly differently at all age groups when compared to +/+ and +/R male animals. Similarly, R/R female animals perform significantly differently than all +/+ and +/R female groups. Interestingly, the female +/R animals only have significantly different scores during ages 40 to 43 weeks and 60 to 63 weeks. Figure 4 B shows that for those two age groups, +/R animals had only a small proportional increase in the score of 2.

Scores of 3 are the most common among wildtype and heterozygous animals of both genders, though heterozygous animals have slightly more scores of 2 and vary rarely scores of 1. Score of 1 make up the majority of the scores for homozygous mutant mice. Upon investigation

into the frequency of each score, it was found that female wildtype mice tend to have lower third suspension scores than their male counterparts; however it is also the higher heterozygous female scores that contribute most to the lack of significance when comparing wildtype and heterozygous female populations. Figure 2 B illustrates this similarity.

In summary, the third tail suspension provides the biggest difference in phenotypes amongst any compared group of mice. With males, heterozygous animals performed significantly differently from the wildtype animals beginning at age 12 to 15 weeks, with the first score of a 1 appearing between 24 and 27 weeks. Homozygous mutants performed significantly differently than the other genotypes at all ages. Both male heterozygous and homozygous mutant mice populations had lower scores as they aged, while wildtype animals did not change in the same manner. In the female mice observed, homozygous mutants had significantly different scores than the other genotypes. The heterozygous mutants however did not perform significantly differently than the wildtype littermates until 40 to 43 weeks age. No further significant difference was observed until 60 to 63 weeks age. Unlike the mutant male mice, female mutants do not get lower scores as they age and wildtype animals occasionally have a score of 1 during their third suspension. While there does appear to be a consistent different between female wildtype and heterozygous average scores, chi-squared analysis does not consider it to be significant.

Table 3: Statistical testing of 3rd tail suspension score proportions amongst male mice of different four-week age groups and genotypes

Male results of chi squared test			
age (weeks)	+/+ vs +/R	+/+ vs R/R	+/R vs R/R
4 to 7 P =	Not Significant 0.088081512	Significant 3.04E-42	Significant 1.63E-32
8 to 11 P =	Not Significant 0.107896678	Significant 1.23E-108	Significant 3.73E-116
12 to 15 P =	Significant 0.000549252	Significant 2.14E-139	Significant 3.94E-173
16 to 19 P =	Significant 0.007021339	Significant 1.50E-245	Significant 1.13E-282
20 to 23 P =	Not Significant 0.146986208	Significant 3.64E-216	Significant 2.67E-225
24 to 27 P =	Significant 0.003697864	Significant 4.91E-30	Significant 8.73E-05
28 to 31 P =	Significant 0.000173167	Significant 2.23E-41	Significant 5.26E-56
32 to 35 P =	Significant 0.000302072	Significant 8.63E-49	Significant 5.82E-03
36 to 39 P =	Significant 1.76013E-06		
40 to 43 P =	Significant 1.25892E-05		
44 to 47 P =	Significant 0.00070117		
48 to 51 P =	Significant 0.003110539		
52 to 55 P =	Significant 9.63194E-06		
56 to 59 P =	Significant 1.65768E-06		
60 to 63 P =	Significant 9.11609E-05		
64 to 67 P =	Significant 0.029298294		

Table 4: Statistical testing of score 3rd tail suspension score proportions amongst female mice of different four-week age groups and genotypes

Female results of chi squared test			
age (weeks)	+/+ vs +R	+/+ vs R/R	+/R vs R/R
4 to 7	Not Significant	Significant	Significant
P =	0.536676976	6.20E-62	4.10353E-53
8 to 11	Not Significant	Significant	Significant
P =	0.601110927	5.28E-47	3.75E-39
12 to 15	Not Significant	Significant	Significant
P =	0.193772626	1.36E-61	3.77E-49
16 to 19	Not Significant	Significant	Significant
P =	0.078463766	2.84E-47	2.94E-37
20 to 23	Not Significant	Significant	Significant
P =	0.076023799	7.62E-86	9.34E-69
24 to 27	Not Significant	Significant	Significant
P =	0.084383763	1.71E-22	5.65E-26
28 to 31	Not Significant	Significant	Significant
P =	0.402021383	4.51E-31	3.53E-21
32 to 35	Not Significant	Significant	Significant
P =	0.086133931	4.26287E-06	9.44E-23
36 to 39	Not Significant		
P =	0.167851419		
40 to 43	Significant		
P =	0.001701135		
44 to 47	Not Significant		
P =	0.464145699		
48 to 51	Not Significant		
P =	0.154354757		
52 to 55	Not Significant		
P =	0.084394188		
56 to 59	Not Significant		
P =	0.430081974		
60 to 63	Significant		
P =	0.007130928		
64 to 67	Not Significant		
P =	0.924719037		

Aim 2. Characterize the neuromuscular junctions of H304R animals

With a timeline established through behavioral testing as a way to monitor disease onset and progression, one obvious next question was to investigate the histological effects of the H304R mutation in way that rationally could explain the symptoms observed in human patients and the phenotypic changes observed in the tail suspension behavioral test. To answer this question, the neuromuscular junctions from sections of the gastrocnemius muscle of +/+ and +/R mice were observed and characterized by their structure and the extent of innervation. The gastrocnemius muscle is located on the back of the lower leg and is one of two major muscles that make up the calf, the other being the soleus muscle. The gastrocnemius is a skeletal muscle that attaches to the Achilles tendon in humans. The neuromuscular junction is the region where the nervous system connects to the muscular system through synapses. In the neuromuscular junctions of skeletal muscles, motor neuron axons extend between folds of the sarcolemma, the membrane of the muscle cell. Upon the release of acetylcholine from the motor neurons, skeletal muscle cells contract (Sanes and Lichtman, 1999). On the muscle cell around the axon are acetylcholine receptors at a density of about 10,000 receptors per micrometer^2, forming a region known as the end plate (Sine, 2012). Studying this interface between the nervous system and the muscular system may provide insight into the root cause of the reflex and muscle fatigue phenotypes observed in the behavioral test. This further elucidates the pathogenesis of CMT.

Normally, the axon from motor neurons completely penetrates the muscle in a complex manner with many different branches, completely filling out the space occupied by the neuromuscular junction. However, in preliminary investigation it was observed that in H304R

mice, some of the neuromuscular junctions were less innervated than others or contained no innervation whatsoever. To study the extent of innervation of neuromuscular junctions, each neuromuscular junction was sorted into one of three categories - Full, Partial, or None – based on how far the stained neurofilament penetrates the junction. The three different categories are shown in example in Figure 5. 2H3 is an antibody that recognizes both mouth and rat neurofilaments and α-Bungarotoxin is a neurotoxin that binds irreversibly to the nicotinic acetylcholine receptor found at the neuromuscular junction. For a neuromuscular junction to qualify as fully innervated, the stained neurofilament had to extend into the entire main body of the neuromuscular junction, although not every branch from the neuromuscular junction was innervated. The difference between partial and full innervations is that in partial innervation, some main body regions (or "stems") are not innervated, whereas with full innervation only short, terminal branches are not innervated. In the category without innervation, the neurofilament staining still did work and possibly only the base of the neuromuscular junction shows signal, but the vast majority of the space of the neuromuscular junction does not appear innervated. Only +/+ and +/R male animals were used for the histological portion of this study. Tissue was collected from 6 month old animals, 9 month old animals and 12 month old animals.

Because of the categorical nature of this analysis, a 2-tailed Pearson's chi-squared test was performed (Table 5). No difference was found in the extent of innervation when comparing 6 month-old male heterozygous animals to their wildtype littermates ($P = 0.3947$). At 9 months age however, heterozygous males have significantly less innervation than the 9 month old wildtypes ($P < 0.0001$) with heterozygous animals having less fully innervated neuromuscular

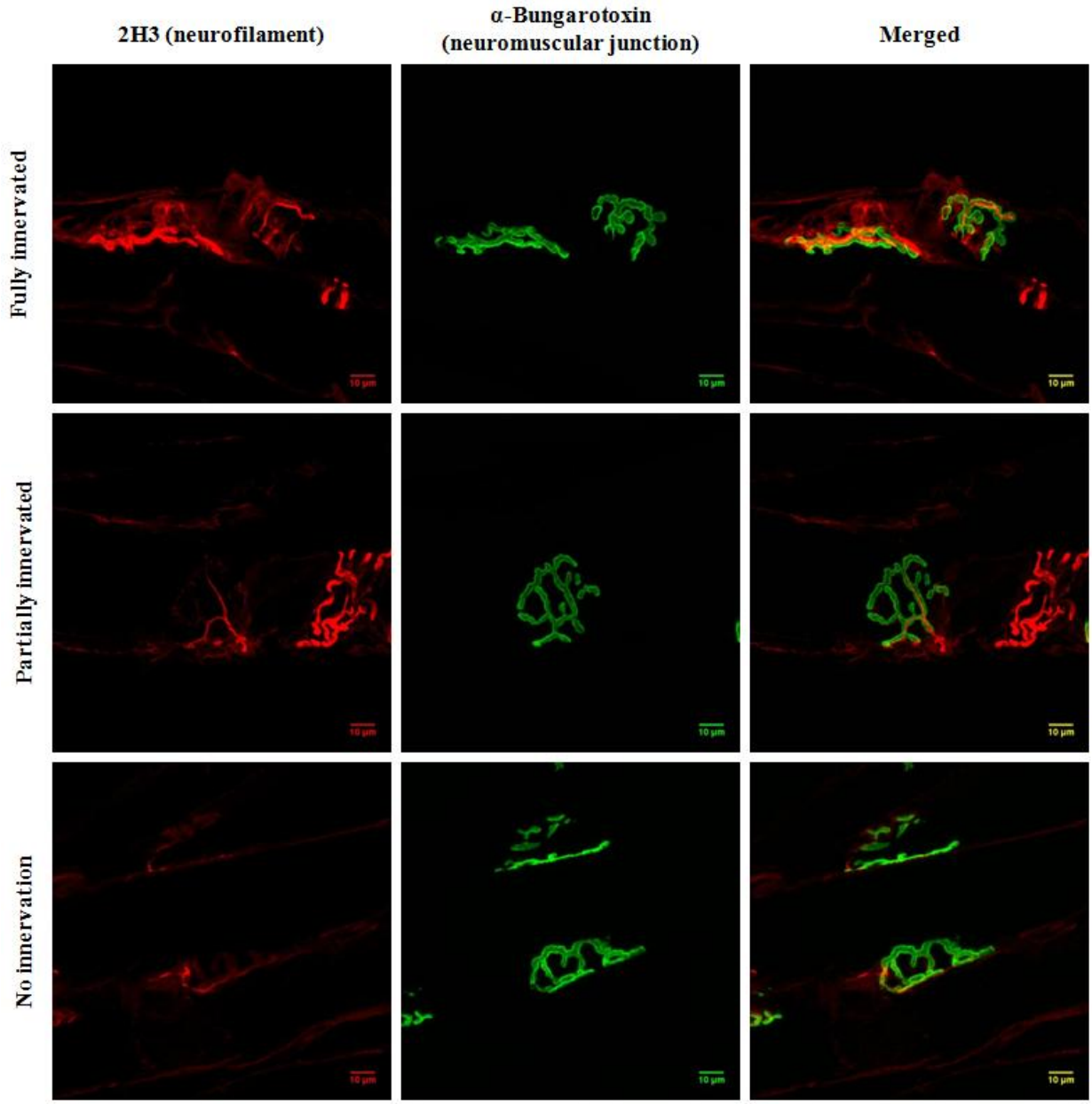

Figure 5: The variable extent of innervation in neuromuscular junctions: full, partial, and none

Table 5: Results of chi-squared statistical testing on innervation differences in neuromuscular junctions

	+/+				+/R			Chi-squared test of significance +/+ vs +/R
	type	#	percent		type	#	percent	
6 months *n* = 4	full	40	47.06%	6 months *n* = 4	full	33	44.59%	P = 0.3947
	partial	34	40%		partial	34	45.95%	
	none	11	12.94%		none	7	9.46%	
	total NMJs	85			total NMJs	74		
	type	#	percent		type	#	percent	
9 months *n* = 2	full	13	65%	9 months *n* = 2	full	4	15.38%	P < 0.0001
	partial	5	25%		partial	12	46.15%	
	none	2	10%		none	10	38.46%	
	total NMJs	20			total NMJs	26		
	type	#	percent		type	#	percent	
12 months *n* = 5	full	46	55.42%	12 months *n* = 4	full	22	28.95%	P < 0.0001
	partial	32	38.55%		partial	38	50%	
	none	5	6.02%		none	16	21.05%	
	total NMJs	83			total NMJs	76		

junctions and more neuromuscular junctions without any innervation. Similarly, 12 month old male heterozygous mutants differed significantly different from their wildtype littermates ($P < 0.0001$) with less fully innervated neuromuscular junctions and more neuromuscular junctions without any innervation. This means that starting at 9 months, the neuromuscular junctions in heterozygous male animals become less innervated when compared to non-mutant littermates.

These neuromuscular junctions were also studied by their structures. Table 6 shows the results of statistical testing performed on various structural criteria. While Figures 5 and 6 show two-dimensional images (maximum projections), these neuromuscular junctions were in fact captured through confocal microscopy in a series of z-stacks with 1 micrometer resolution, forming three-dimensional image stacks. Because of this, taking the volume and surface area of the neuromuscular junctions was possible. As a measure for complexity, the surface area to volume ratio was taken for each neuromuscular junction and compared at 6 months, 9 months and 12 months for both +/+ and +/R males. As the neuromuscular junctions become thicker or shorter (as is depicted in the low surface area / volume column in Figure 6), this ratio is lower, while the ratio is higher with more complex and convoluted neuromuscular junctions. The surface area to volume ratio was found to be significantly different comparing 6 month old heterozygous males to wildtype males ($P = 0.0336$). The difference is also significant at 9 months ($P < 0.0001$) and 12 months ($P < 0.0001$), with heterozygous males having lower surface area to volume ratios than their wildtype counterparts.

The specific structural elements of the neuromuscular junctions were also studied through skeleton analysis. In skeleton analysis, the shape of the neuromuscular junction is reduced to a

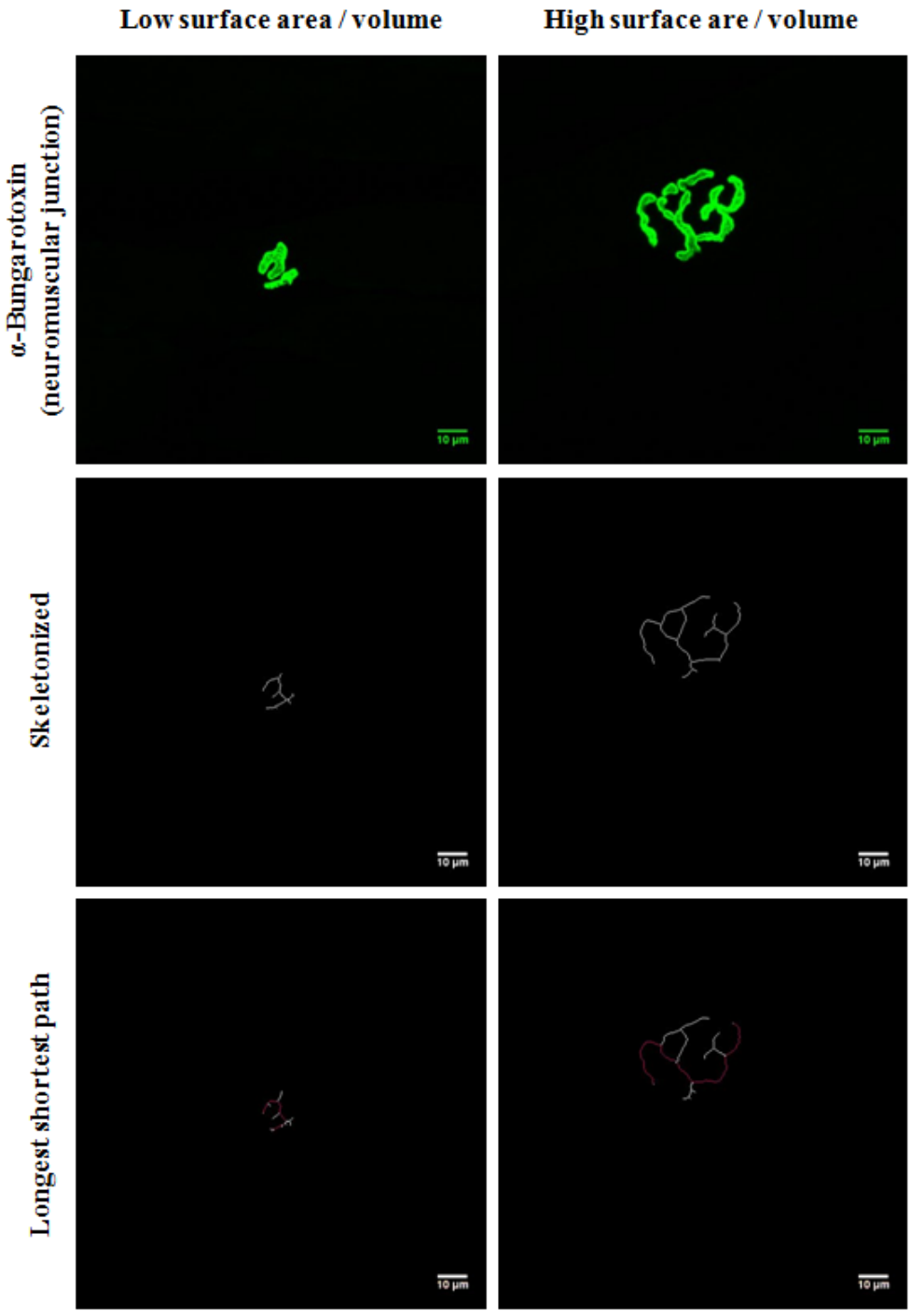

Figure 6: Complexity differences observed between neuromuscular junctions

Table 6: Results of student's t-test statistical testing of neuromuscular junction structures

Average branch length (micrometers)								
Group	6+/+	6+/R	Group	9+/+	9+/R	Group	12+/+	12+/R
Mean	8.96	8.58	Mean	8.30	8.04	Mean	7.84	8.12
SD	6.06	3.48	SD	2.02	2.48	SD	2.46	2.80
N	85.00	71.00	N	21.00	26.00	N	102	69
	P = 0.6303			P = 0.7041			P = 0.5042	
Maximum branch length (micrometers)								
Group	6+/+	6+/R	Group	9+/+	9+/R	Group	12+/+	12+/R
Mean	25.40	24.82	Mean	30.43	25.97	Mean	25.39	23.65
SD	8.97	9.73	SD	11.07	11.76	SD	9.53	9.38
N	85	71	N	21	26	N	102	69
	P = 0.7018			P = 0.1913			P = 0.2419	
Number of branches								
Group	6+/+	6+/R	Group	9+/+	9+/R	Group	12+/+	12+/R
Mean	18.72	17.39	Mean	25.57	20.15	Mean	22.74	15.23
SD	8.95	9.72	SD	9.7	11.44	SD	11.77	8.27
N	85	71	N	21	26	N	102	69
	P = 0.3780			P = 0.0912			P = 0.0001	
Surface area / volume								
Group	6+/+	6+/R	Group	9+/+	9+/R	Group	12+/+	12+/R
Mean	1.87	1.48	Mean	1.69	1.35	Mean	1.68	1.44
SD	1.52	0.27	SD	0.27	0.21	SD	0.34	0.29
N	87	74	N	21	26	N	101	85
	P = 0.0336			P < 0.0001			P < 0.0001	
Longest shortest path (micrometers)								
Group	6+/+	6+/R	Group	9+/+	9+/R	Group	12+/+	12+/R
Mean	67.87	67.34	Mean	85.71	66.55	Mean	76.41	58.38
SD	22.58	22.20	SD	27.54	19.71	SD	23.80	24.33
N	85	71	N	21	26	N	102	69
	P = 0.8837			P = 0.0013			P < 0.0001	

single skeleton with distinct branches, junctions, and endpoints. Figure 6 shows examples of skeletonized neuromuscular junctions. First, in number of branches, the only statistically significant difference manifests at 12 months of age ($P = 0.0001$) in which neuromuscular junctions from heterozygous males have on average 15.23 ± 8.27 branches, while wildtype neuromuscular junctions have 22.74 ± 11.77. This difference is not present at 6 months or 9 months of age. For both genotypes and across all age groups, average branch lengths were consistently around 8 micrometers. Furthermore, the maximum branch length did not change as animals aged, nor did it differ between the genotypes.

What did however have a significant difference was the longest shortest path. A shortest path is the distance between any two points where a branch ends. The longest shortest path is the longest of all the shortest paths in a single skeleton. Figure 6 shows an example of two longest shortest paths generated for the two example skeletons, where the longest shortest path is colored purple. At 6 months age, there was no difference between the longest shortest path between heterozygous males and wildtype littermates. However, there was a significant difference between the two genotypes at 9 months age ($P = 0.0013$) and 12 months age ($P < 0.0001$) with heterozygous males.

A conclusion of the analysis of the neuromuscular junctions can be seen in Table 7. At 6 months age, the neuromuscular junctions from heterozygous males have significantly lower surface are to volume ratios. At 9 months age, the neuromuscular junctions from heterozygous males are significantly less innervated and have shorter longest shortest paths. At 12 months age, the neuromuscular junctions in heterozygous male mice also have a significantly smaller number

of branches than the neuromuscular junctions in their wildtype littermates. These data suggest that the innervation and structural changes in the neuromuscular junctions of heterozygous mutant mice progress over time. Further analysis and interpretation can be found in the discussion section.

Table 7: Comparing neuromuscular junction structure between +/R males and +/+ males

	Innervation	Surface area / volume	Number of branches	Longest shortest path
6-month	Not Significant	Significant	Not Significant	Not Significant
9-month	Significant	Significant	Not Significant	Significant
12-month	Significant	Significant	Significant	Significant

DISCUSSION

The tail suspension test was developed because of two reasons. Mice with similar mutations in the DYNC1H1 gene were known to have very prominent leg posture phenotypes when suspended by their tails. Although the H304R mutation is thought to be less drastic when compared to *Cra1* and *Loa*, we initially observed trends with how heterozygous mutant animals from the H304R colony held their legs. We subsequently designed a behavioral test, initially scoring legs just by their angle relative to their bodies from only a ventral view and suspending animals from their tails for one minute. This system was changed by adding a second camera in order to get a view from the side of the animal. The test was also changed by adding three tail suspensions each lasting fifteen seconds, instead of one minute-long tail suspension. We believe that this introduces the factor of fatigue into the test and speculate that the change in phenotype from the first to third tail suspension is due to the fatigue that the test gives on the animal. We believe that a mouse holding their legs further away from the body while suspended by their tails (the normal reflex, score of 3) takes the most energy and is the most physically demanding posture.

The third tail suspension showed the greatest difference in phenotypes between heterozygous mutant males and wildtype littermates. Between ages 12 and 15 weeks, male heterozygous mutant animals had significantly different scores than wildtype animals (+/+ 2.94 average score, +/R 2.81 average score). Further, the average scores for heterozygous males continued to lower as the animals aged. This correlates with CMT, where the disease symptoms

progress throughout a patient's life, gradually getting worse. The detection between 12 and 15 weeks age similarly coincides with the onset of CMT, where patients will usually be diagnosed in adolescence or early adulthood. Homozygous mutant males show an even stronger rate of change when examining their average scores.

Curiously, the behavioral data is different with female mice. The third tail suspension scores did not significantly differ between the heterozygous mutant female mice and their wildtype littermates until ages 40 to 43 weeks, then 60 to 63 weeks. Behavioral testing will have to continue for these mice at later timepoints in order to determine if this infrequent difference is the onset of a scoring difference that only affects older mice. Presently, there are too few ($n < 10$) animals in the older female age groups to make any conclusions about their behavioral data. It is possible that at later timepoints, female heterozygous mutants would score consistently differently than their wildtype littermates in a similar manner to how the male heterozygotes and wildtype animals differ. It is also possible that the test itself does not show a phenotype but that one is still there, and the onset of disease symptoms can be measured through other criteria.

Differences between males and females in neurodegenerative diseases is not unprecedented, but is however poorly understood. In Parkinson's disease, there is a greater prevalence and earlier symptomatic onset observed in men. It is believed that the sex hormone estradiol provides resilience to loss of dopamine (Gillies et al., 2014). In Alzheimer's disease, several studies show a higher risk in women than men and observational studies show that postmenopausal women treated with estrogen had a lower risk (Musicco, 2009). Estrogen replacement therapy was similarly investigated as a therapy for amyotrophic lateral sclerosis in

women. ALS occurs more in men than in women, and women get the disease later in life than men. Postmenopausal women were given estrogen, but no evidence for a neuroprotective role was found (Rudnicki, 1999).

Neuromuscular junctions have been investigated in many studies on neurodegenerative disease. The morphology of the neuromuscular junction has been studied by many different methods, most frequently by studying the area of acetylcholine receptor expression as a measure of neuromuscular junction size. A study investigating the effects of resistance training on neuromuscular junction morphology in rats found that after a 7-week training program, neuromuscular junctions examined in two-dimensional images of the soleus muscle (another muscle in the calf, lower leg) increased in perimeter by 15% and in area by 16% and acetylcholine receptor dispersion was significantly increased (Deschenes et al., 2000). Research investigating the structure of neuromuscular junction structure in mice lacking the P2X2 receptor (an ion channel receptor subunit expressed during neuromuscular junction development) similarly observed acetylcholine receptor expression area in confocal images of the soleus muscle (Ryten et al., 2007). Ryten et al. performed their analysis on maximum projections – these are two-dimensional images made from a three-dimension stack of images, where the maximum pixel value in the z series at a fixed x-y position is kept, generating a map of all the maximum intensities.

The methods used in this research instead measured the expression of the acetylcholine receptor in three dimensions using z-stacked images taken through confocal microscopy without reducing them to maximum projections. We believe that this allows for a better representation of

the space taken up by the neuromuscular junction and allows for better comparisons to be made between heterozygous mutants and wildtype littermates. We found that the most informative quantitative measurement we not solely comparing surface areas or volumes, but instead comparing the ratio between the two. This was done as a way to standardize for variable neuromuscular junction sizes and we believe provides a better metric.

Classifying neuromuscular junctions by the extent of innervation is a fairly common technique, but the definitions of each category are not standardized. In a study examining synaptic and axonal degeneration in canine motor neuron disease, neuromuscular junctions of the gastrocnemius were classified into three categories with respect to innervation: intact, partially occupied or denervated (Carrasco et al., 2004). A study using mouse models for Spinal Muscular Atrophy examined the morphological characteristics of motor neurons and attempted to link different phenotypes to Spinal Muscular Atrophy susceptibility. Here, neuromuscular junctions were categorized by innervation as occupied (greater than 50% neuromuscular junction area occupied by neurofilament), partially occupied (less than 50%) or vacant (no overlapping) (Thomson et al., 2012). The criteria we used to are comparable to the previous ones. Our methods grouped neuromuscular junctions into three similar categories. Further, images were categorized after rotating the image in all three dimensions in order to best determine the extent of innervation rather than relying on a maximum projection or an inaccurate two-dimensional image.

The neuromuscular junctions in heterozygous mutant males had significantly lower surface are to volume ratios at 6 months age. At 12 months age, the neuromuscular junctions in

heterozygous male mice were also significantly less innervated, had significantly fewer branches and a significantly smaller longest shortest path. These data suggest that differences were gained as the mice aged, meaning that they started with more wildtype morphologies and gradually changed. However, because earlier timepoints were not taken, it cannot be definitively said that the surface are to volume ratio difference seen in the 6 month old heterozygous males is the result of a neurodevelopmental defect. At the age of 12 to 15 weeks (3 months), the first difference in behavioral phenotypes is observed, whereas with earlier ages there was no difference.

Neuromuscular junctions from mice at 3 months age and younger would have to be examined in order to investigate the onset of this surface area to volume ratio difference. Further, the histological study would have to continue to include the homozygous mutant animals in order to best translate the histological observations to the H304R mutation. In cases where only H304R mutant dynein is present, what histological effects are observed? How do the heterozygous animals compare to the homozygous mutant animals in terms of neuromuscular junction? Tissues from female mice at all time points should also be investigated to see how their neuromuscular junctions change as they age. Because the behavioral data for the females indicate that heterozygous mutants are not significantly different from wildtypes, what do their neuromuscular junctions look like?

Future experiments with more timepoints are required. While results certainly indicate in males that heterozygous mutant neuromuscular junctions do change significantly between 6 and 12 months age when compared to wildtype animals, earlier timepoints are needed to determine

the onset of morphological phenotypes and to determine if there are neurodevelopmental symptoms of the H304R mutation.

This research finds that the H304R mutant mouse displays behavioral phenotypes in male heterozygotes that correlate with the progression of CMT in humans. The neuromuscular junctions of these mice become less innervated as they age (characteristic of neuromuscular degeneration) and also have various morphological differences. Further, there is a behavioral difference observed with the female mutant mice that indicates a delayed onset of disease symptoms.

MATERIALS AND METHODS

The tail suspension test was performed with +/+, +/R and R/R male and female mice beginning at 4 weeks of age. Each individual mouse was only tested once in each 4-week period. Mice were suspended by their tails for 15 seconds and then returned to rest on the tester's hand for 5 seconds. This was repeated for a total of 3 suspensions. During the test, two USB computer cameras were recording the mouse's leg posture from two different angles, one ventral to the suspended mouse and the other lateral. During analysis, both videos were watched and each of the three suspensions was scored a 3, 2, or a 1 depending on leg posture (see Figure 1). All data sorting and statistical analysis was performed using custom scripts written in Python, with functions for P-value calculation for the chi squared statistical testing coming from the SciPy: Open Source Scientific Tools for Python library (Jones et al., 2001--).

Cardiac perfusion was performed for all animals used for histological studies. Animals were given a lethal dose of Somnasol intraperitoneally. In order to proceed with the cardiac perfusion, the efficacy of the anesthesia had to be deemed sufficient through a tail pinch test. Before proceeding, it was required that the animal has a lack of response to the tail pinch, in addition to slowed breathing movements and a cessation of whisker movements. Upon successful anesthesia, a thoracotomy was performed by opening the skin over the thoracic cavity. Two incisions were then made bilaterally to the rib cage, and a single incision through the diaphragm. While the sternum was held back with a hemostatic clamp, a small needle was inserted into the left ventricle while the heart was still beating. Following this, a cut was

performed to the right atria to allow blood to be flushed out. Using a perfusion pump, approximately 20 mL of 0.9% NaCl solution was perfused, followed by a 0.9% NaCl + 4% paraformaldehyde solution to preserve tissues. The gastrocnemius muscle was then removed from both legs of the animal and cryopreserved in 30% sucrose overnight. Tissues were then embedded in OCT cryoembedding media before being sectioned into 20 µm slices and adhered to a slide. These slides were then placed in acetone for 20 minutes at room temperature to fix the tissue. Slides are then stored at -20°C until staining.

Slides containing muscle sections were washed with phosphate-buffered saline for 10-15 minutes before being permeabilized for 10 minutes in 0.5% Triton X-100 solution. In a humidified chamber, slides were blocked for 1.5 to 2.5 hours in blocking solution (5% bovine serum albumin and 0.1% Triton X-100). A mouse 2H3 antibody (staining for neurofilaments) was added at a 1:25 dilution into blocking solution, 300 µL per slide. Parafilm was applied over the slide and this was allowed to incubate over night at 4°C. Slides were then washed twice with blocking solution before applying α-mouse 546 and α-Bungaro toxin-488 (directly stains acetylcholine receptors). This stain was applied for 60 minutes before being washed twice with blocking solution, washed twice with phosphate-buffered saline. Cover slips were then applied to the slides overtop Prolong-Gold with DAPI mounting media. These were allowed to sit overnight in a dark environment before nail polish was applied to seal the cover slip. Once dry, they were imaged using confocal microscopy.

Images of neuromuscular junctions were analyzed using the public domain, Java-based image processing program ImageJ (Abramoff, 2004). Images were split into individual channels

and innervation was examined through inspecting assembled 3D images of red and green channels (neurofilament and acetylcholine receptors). The area and volume of neuromuscular junctions were quantified using the 3D Object Counter plugin for ImageJ after applying a binary threshold (Bolte and Cordelieres, 2006). Skeletonization of neuromuscular junctions and skeleton analysis was performed using the Skeletonize and Analyze Skeleton plugins for ImageJ (Arganda-Carreras et al., 2010).

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