UNIVERSITY OF CALIFORNIA, SAN DIEGO

CMT2B Rab7V162M Mutations Induce Neuropathy in a Dosage-Dependent Manner

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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2017

The Thesis of Huayu Liu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2017

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ACKNOWLEDGEMENTS

I would like to thank Professor Chengbiao Wu for his support as the chair of my committee. His guidance has proved to be invaluable.

I would also like to thank Professor Susan Ackerman and Professor Gentry Patrick for their support throughout my research.

ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California, San Diego, 2017

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Charcot-Marie-Tooth type 2B (CMT2B) is a debilitating hereditary peripheral sensory neuropathy. Patients with this autosomal dominant disease lose pain sensation and experience muscle weakness and atrophy in distal limbs. Clinical manifestations of CMT2B include axonal dysfunction and degeneration of peripheral sensory neurons. However, the cellular and molecular pathogenic mechanisms remain undefined. CMT2B is caused by missense point mutations (L129F, K157N, N161T/I, V162M) in Rab7, a regulator of late endocytic transport. Strong evidence suggests that CMT2B mutations

enhance the cellular levels of activated Rab7 GTPase, thereby increasing lysosomal activity and autophagy. As a consequence, trafficking and signaling of neurotrophic factors such as nerve growth factor and epidermal growth factor in the long axons of peripheral sensory neurons are particularly vulnerable to premature degradation. A “gain of toxicity” model has, thus, been proposed based on these observations. In order to test the hypothesis that CMT2B mutations cause neural degeneration in a gene dosage-dependent, gain of toxicity manner, this study compares cutaneous autonomic innervation in footpad biopsies of wild-type, heterozygous and homozygous CMT2B mouse models. The results show significant neuronal degeneration associated with both disease genotypes compared to the wild-type control. In addition, disease phenotypes present in a gene dosage-dependent manner, with sensory deficits severely worsened in the homozygous model compared to the heterozygote. These results are consistent with data from previous behavioral tests showing a similar dosage-dependent effect of CMT2B mutant genes. Together, these findings affirm the gain of toxicity model of the disease and suggest that therapeutic development should aim to inhibit Rab7 hyperactivity in affected patients.

INTRODUCTION

Charcot Marie Tooth (CMT) neuropathies are clinically and genetically heterogeneous hereditary diseases with many subtypes (CMT1-4, CMTX) affecting motor and sensory nerves resulting in progressive distal muscle weakness, atrophy, sensory loss, and decreased or absent tendon reflexes [1-8]. Approximately 40 genes/loci have been identified to be associated with CMT [9], and no effective treatments are presently available [10-12]. CMT2 also has many subtypes (A, B, D, E, H, I) [2, 9, 13, 14]. These subtypes are clinically similar and classified based on molecular genetic findings. Specifically, we discuss CMT2B, a hereditary peripheral sensory neuropathy characterized by distal sensory loss, muscular weakness, and recurrent foot ulcers. Onset typically occurs between the first to third decade of life [14]. Affected limbs are prone to muscle atrophy and soft tissue infections, often leading to necessary amputation.

The primary pathological feature of CMT2B is chronic axonal degeneration caused by mutations in Rab7, a ubiquitously expressed GTPase that serves as the master regulator of vesicular trafficking, maturation, and fusion in the late endocytic pathway [15]. Primarily localized in acidic pre-degradative and degradative organelles, such as late endosomes, lysosomes, and autophagosomes, Rab7 presents on the cytosolic surface of the vesicle membrane and interacts with various downstream effectors to carry out its regulatory functions [16, 17]. Specifically, Rab7 orchestrates the transition of early endosomes into late endosomes, and the subsequent degradation of their associated cargos. This includes the lysosome-mediated degradation of epidermal growth factor (EGF) and its receptor EGFR, nerve growth factor (NGF), and

tropomyosin receptor kinase A (TrkA). It has, therefore, been proposed that neurodegeneration in CMT2B is attributable to disrupted neurotrophin trafficking by mutant Rab7 [18].

Conformational changes to the nucleotide binding pocket enable Rab7 to switch between its active (GTP bound) and inactive (GDP bound) forms. However, in CMT2B, five missense point mutations (L129F, K157N, N161T/I, and V162M) [7, 19-21] occurring near the nucleotide binding pocket decrease nucleotide affinity, causing unregulated nucleotide exchange [22-24]. The resulting mutants preferentially bind GTP and behave similarly to constitutively active mutant $Rab7^{Q67L}$ [25]. Numerous studies have proposed that disease pathogenesis is attributable to increased Rab7 activity [18, 23-26], suggesting a gain-of-toxicity model of disease.

Most studies on CMT2B have been performed using cell lines or fly models, making it difficult to interpret the physiological relevance of these findings to humans [22, 24, 27-29]. However, the Wu Lab at UC San Diego recently generated the first mammalian model of CMT2B as a $Rab7^{V162M}$ knock-in C57BL/6 mouse. A targeted knock-in via homologous recombination allows CMT2B mutant genes to achieve natural expression patterns and levels regulated by the endogenous promoter, an important characteristic when studying the disease in a developmental or degenerative context. Using this model, peripheral sensory function in CMT2B can now be characterized with behavioral and histopathological analyses.

In this study, the $Rab7^{V162M}$ mouse model is used to test the hypothesis that CMT2B mutations cause neural degeneration in a gene dosage-dependent, gain of toxicity manner. Specifically, the cutaneous innervation of sensory neurons will be

compared in footpad biopsies from wild-type, heterozygous and homozygous Rab7^{V162M} mouse models [26, 30, 31]. The results will be used to validate data from previously characterized sensory deficits in behavioral tests. The hypothesis will be supported if neural degeneration is more severe in the homozygous genotype compared to the heterozygous genotype.

MATERIALS AND METHODS

Tissue processing and immunostaining. Histopathological evaluation of skin biopsies was used to examine the progression of sensory axonal neuropathy [30]. 9-month-old Rab7^{V162M} knock-in C57BL/6 mice (+/+, KI/+, KI/KI) were anesthetized and skin samples from the hind paw were collected. The biopsy samples were fixed in a 1:1 mixture of methanol/acetone for 30 minutes at - 20°C. Samples were washed with 1x PBS and dehydrated overnight in 30% sucrose at 4°C. Following cryoprotection, tissues were embedded in Tissue-Tek Optimum Cutting Temperature compound (Thermo Fisher). 35 µm sections were collected using a cryostat (Leica, Wetzlar, Germany) and mounted onto glycine-coated Superfrost+ slides (VWR, Radnor, PA). Samples were then washed with 1x PBS and incubated in 50 mM glycine for 45 min at room temperature (RT). After incubation, samples were washed with a solution of 0.2% Triton in 1x PBS (0.2% PBST). Samples were blocked with a solution of 0.5% PBST, 10% goat serum and 1% BSA for 1 hour at RT. Samples were then incubated in primary antibody (UCHL1/PGP9.5 Proteintech #14730-1-AP 1:300 in 0.2% PBST, 10% goat serum, 1% BSA) overnight at 4°C. After primary incubation, samples were washed with 1x PBS and incubated in secondary antibody (Alexa Fluor 568 Life Technologies 1:500 in 1x PBS) for 1 hour at RT. Samples were then washed followed by DAPI (Sigma) nuclear staining. Fluorescence images were acquired with a Leica SP5 confocal scanning laser microscope.

Statistical Analysis. Quantitation and analysis of intraepidermal nerve fibers (IENF) were performed blindly with NIH ImageJ. Only IENF crossing the dermal-epidermal junction were quantified [26, 31]. The signal intensity of IENF profiles was

normalized to epidermal area to account for variations in epidermis thickness. The fluorescence of PGP 9.5-positive fibers in KI/+ and KI/KI mice were compared to +/+. Sample size of n=3 was used for each group. One-way ANOVA with post-hoc Dunnett tests was used to determine significance of differences among +/+, +/KI, and KI/KI genotypes.

RESULTS

To assess the neuropathy phenotype in Rab7^{V162M} mice, cutaneous innervation of sensory intraepidermal nerve fibers (IENFs) were compared in footpad skin biopsies from 9-month wild-type, heterozygous, and homozygous genotypes (Fig 1). IENFs were immunostained for PGP 9.5, a ubiquitin hydrolase and pan-neuronal marker. Samples from three different mice of each genotype were analyzed (n=3). Only IENF crossing the dermal-epidermal junction were quantified [26, 31]. The signal intensity of IENF profiles was normalized to epidermal area to account for variations in epidermis thickness. A decreased density of PGP 9.5 fibers in the epidermis was expected in disease phenotypes compared to the wild-type control. Indeed, immunostaining for both disease genotypes appeared to demonstrate severe small fiber neuropathy compared to the control (Fig 1A-F). Strong IENF signal intensities and extensive secondary branching were apparent in wild-type controls, verifying that the staining protocol had worked (Fig 1A, B). Rab7^{V162M} heterozygous mice showed reduced secondary branching and fewer PGP 9.5 signals in the epidermis, although some nerve structures were apparent (Fig 1C, D). In Rab7^{V162M} homozygotes, no secondary branching was observed and PGP-9.5 signals were scarce and localized to only a few regions on each skin sample (Fig 1E, F). A significant variation in PGP 9.5 signal intensity of stained IENF fibers was confirmed in all three groups, with a two-fold difference observed in the heterozygous genotype and a striking ten-fold difference in the homozygous genotype, compared to the wild-type control (Fig 1G).

The histopathological findings were compared with data from previous behavioral tests performed at 7 months. The Hargreaves nociception assay showed significant

differences between homozygous Rab7^{V162M} and control genotypes (Fig 2), consistent with the gene dosage-dependent effect observed in immunostaining. Statistical non-significance of the heterozygous genotype could be attributable to a small sample size (n=4) with high standard error of mean. As a control, body weight did not vary significantly between the three genotypes. Overall, the preliminary behavioral data is consistent with the histopathological findings showing increased sensory deficits with additional copies of mutant Rab7^{V162M}. Together, these findings suggest Rab7^{V162M} induces neuropathy in a gene dosage-dependent manner.

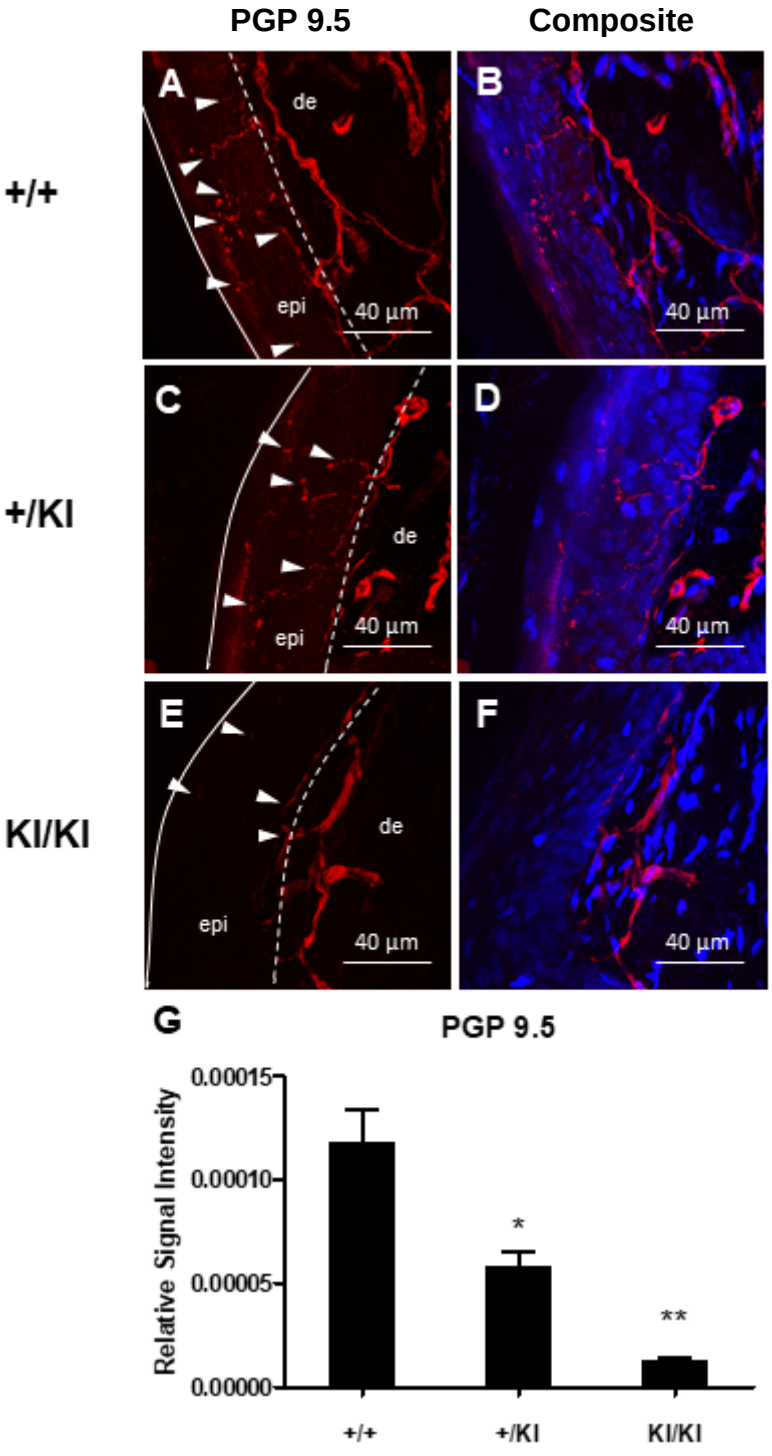

Figure 1: Footpad Histopathology (A-F) Immunolabeling of intraepidermal nerve fibers (white arrows) in footpad biopsies of 9-month-old Rab7^{V162M} mice. Nerve fibers stained for PGP 9.5 shown in red. Nuclei are stained with DAPI shown in blue. De, dermis; epi, epidermis. Dotted white lines denote dermo–epidermal boundaries. Solid white lines denote skin surface. (G) Relative fluorescence signal intensity was quantified using NIH ImageJ. Data are mean ±SEM, n = 3 for each group. * = $p<0.05$, ** = $p<0.01$ vs control (+/+) by ANOVA with Dunnett's test. (Unpublished)

Preliminary Behavioral Data

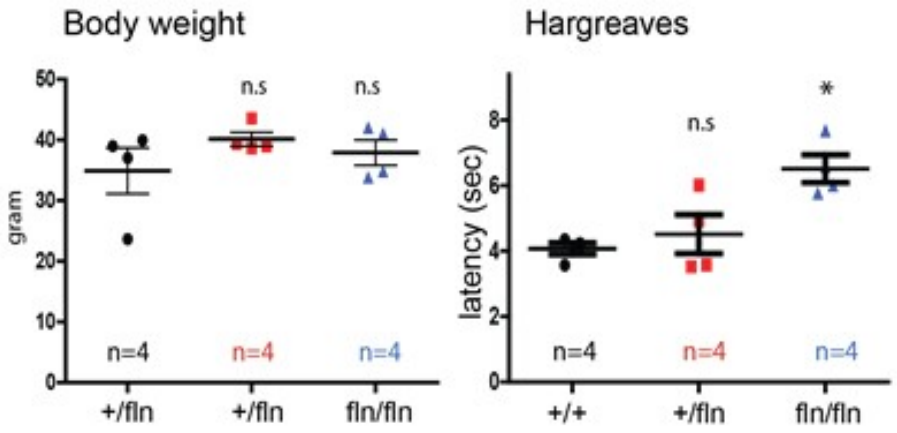

Figure 2: Nociception Assay Neuropathy phenotype at 7 months of age in control (black), heterozygote (red) and homozygote (blue) CMT2B mice. Data are mean ± SEM. * = $p<0.05$ vs control by ANOVA with Dunnett's test. (Unpublished)

DISCUSSION

An accurate functional characterization of CMT2B mutants is important for directing the focus of future therapeutic development. Because a total knockout of Rab7 (-/-) in mice is embryonically lethal [32], this mouse model enables the study of a homozygous CMT2B genotype for possible gene dosage effects. The results showed significant neuronal degeneration associated with both disease genotypes compared to the wild-type control. In addition, disease phenotypes presented in a gene dosage-dependent manner, with sensory deficits severely worsened in the homozygous model compared to the heterozygote. These results are consistent with data from previous nociception assays showing a similar dosage-dependent effect of CMT2B genes. The fact that homozygous mice survived to adulthood indicates that the Rab7^{V162M} mutation is unlikely to result in a total loss of function, a finding contradictory to a recent study in a *Drosophila* model of CMT2B [33]. This suggests possible fundamental differences in Rab7 function between the fly and mammalian CMT2B models. Together, these findings affirm a gain of toxicity model of the disease and suggest that therapeutic development should aim to inhibit Rab7 hyperactivity in affected patients.

While the histopathological findings in nine-month mutant mice were consistent with results from the Hargreaves test performed at seven months, showing a gene dosage-dependent effect on sensory neuropathy, the disease phenotypes need to be further examined at more timepoints throughout the model's lifespan in order to establish a developmental or neurodegenerative characterization of disease progression. Similar nociception assays and histological examination could be performed from one to three months of age in order to assess whether nerve fibers are

normal before the onset of disease. To further establish validity of the Rab7^{V162M} knock-in model, the experiment described in this paper can be incorporated into a longitudinal, multimodal study in which skin biopsies and behavioral testing are performed to assess disease onset and progression [34]. Because CMT2B is a sensory neuropathy, mechanical fibers can also be stained and compared to sensory fibers as a control. Furthermore, G-ratios of sciatic nerves can also be assessed [35, 36].

Because CMT2B Rab7 mutations cause pathology in heterozygosity, CMT2B is classified as an autosomal dominant disease [4]. Most dominant mutations lead to a gain of function, typically manifesting as increased normal activity, new functionality, or abnormal expression of the gene product. However, some dominant mutations are associated with a loss of function; these mutations are typically dominant negative or haploinsufficient. Prior experiments have demonstrated that CMT2B Rab7 mutations are not dominant negative. For example, CMT2B mutants can bind GTP similarly to wild-type and constitutively-active Rab7Q67L [25]. Compared to wild-type Rab7, CMT2B mutants L129F and V162M showed increased interactions with its specific effectors, including dynein-dynactin recruiting RILP, vacuolar protein sorting-associated protein 13 (Vps13C), and cholesterol sensor oxysterol-binding protein-related protein 1L (ORP1L) [23, 25]. CMT2B mutants also showed stronger affinity for clathrin heavy chain, intermediate filament protein peripherin, and increased phosphorylation of vimentin in HeLa and Neuro2A cells compared to wild-type Rab7 [23, 37, 38]. There is also evidence that CMT2B mutants could interact more frequently with effector Rabring7, a ubiquitin ligase that regulates EGFR degradation [39, 40].

Studies have also affirmed that CMT2B pathology is not attributable to Rab7

haploinsufficiency. CMT2B mutants demonstrated active functionality by rescuing Rab7 function after silencing of endogenous Rab7 expression in transfected HeLa cells [25]. In addition, CMT2B mutants were shown to rescue Rab7 haploinsufficiency in Rab7 null mutant *Drosophila* photoreceptor neurons [33]. Together these findings suggest CMT2B mutations likely cause a gain of normal Rab7 function. Ultimately, the dominant nature and functional ability of CMT2B mutants to rescue Rab7 haploinsufficiency further support a gain of toxicity characterization that is consistent with the gene dosage-dependent affect observed in this study.

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