Neurobiology of Disease 65 (2014) 211–219

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Human *Rab7* mutation mimics features of Charcot–Marie–Tooth neuropathy type 2B in *Drosophila*

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ARTICLE INFO

Article history:
Received 6 September 2013
Revised 23 December 2013
Accepted 30 January 2014
Available online 9 February 2014

Keywords:
Rab7
Drosophila melanogaster
Charcot–Marie–Tooth
Behavioural assay

ABSTRACT

Charcot–Marie–Tooth disease type 2B (CMT2B) is an inherited axonal peripheral neuropathy. It is characterised by prominent sensory loss, often complicated by severe ulcero-mutilations of toes or feet, and variable motor involvement. Missense mutations in *RAB7A*, the gene encoding the small GTPase *Rab7*, cause CMT2B and increase *Rab7* activity. *Rab7* is ubiquitously expressed and is involved in degradation through the lysosomal pathway. In the neurons, *Rab7* plays a role in the long-range retrograde transport of signalling endosomes in the axons. Here we developed the first animal model of CMT2B, modelling one of the mutations (L129F) in *Drosophila melanogaster*. Behavioural assays show that this model recapitulates several hallmarks of the human disease. Upon expression of mutant *Rab7* in the sensory neurons, larvae present with a reduction of temperature and pain perception. Furthermore, the larvae exhibit a crawling defect when the mutant protein is expressed in the motor neurons. Analysis of axonal transport of *Rab7* positive vesicles in sensory neurons of *Drosophila* larvae and in neurites of mammalian neuroblastoma cells demonstrates that mutant vesicles pause less than their wild-type counterparts. This latter finding indicates that alterations in vesicle transport might contribute to the pathomechanism of CMT2B.

Introduction

Inherited peripheral neuropathies (IPNs) are a clinically and genetically heterogeneous group of diseases that cause a length-dependent degeneration of the peripheral nerves. The IPNs are classified into three major disease categories, based on the degree of involvement of the motor, sensory and autonomic neurons. In the Hereditary Motor and Sensory Neuropathies (also collectively known as Charcot–Marie–Tooth Disease (CMT)), both afferent and efferent peripheral neurons can be affected. The resulting phenotype is muscular wasting and weakness in combination with impairment of some or all of the somatosensory modalities (pain, temperature, touch, vibration and proprioception) (Barisic et al., 2008). A distinction is further made between axonal IPNs, in which axonal degeneration is the prime hallmark, and demyelinating IPNs, which are caused by defects of the myelin sheet surrounding the axons.

CMT2B, an axonal form of IPN, is characterised by prominent sensory loss of all somatosensory modalities, ulcero-mutilations and variable motor abnormalities (Auer-Grumbach et al., 2000). So far, four missense mutations (L129F, K157N, N161T and V162M) in the *RAB7A* gene were reported as the cause for CMT2B (Houlden et al., 2004; Meggouh et al., 2006; Verhoeven et al., 2003). Genotype/phenotype correlations in CMT2B patients have been described in detail elsewhere (Rotthier et al., 2009). *Rab7* is a member of the family of small GTPases and is important in the intracellular transport and maturation of late endosomes, lysosomes, phagosomes and autophagosomes, vesicles whose contents are destined for lysosomal degradation (Bucci et al., 2000; Gutierrez et al., 2004; Harrison et al., 2004). Like all Rab GTPases, *Rab7* switches between a GDP-bound inactive cytosolic state and a GTP-bound active membrane-associated state; in the latter, *Rab7* will recruit downstream effector molecules through which it exerts its function. The four CMT2B-associated mutations affect amino acid clustering near the highly conserved G-loops that participate in the formation of the nucleotide-binding site. Structural analysis of the L129F mutant showed that this mutation alters the nucleotide-binding pocket, thereby decreasing the nucleotide affinity and deregulating nucleotide exchange. Consequently, the GDP–GTP exchange occurs independently of the guanine exchange factor and is accelerated, but this is partially counterbalanced

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Available online on ScienceDirect (www.sciencedirect.com).

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http://dx.doi.org/10.1016/j.nbd.2014.01.021

by unregulated, GTPase-independent inactivation. The net outcome is an increased Rab7-GTP binding, causing a subtle increase in the duration of mutant *Rab7* association with its target membranes (McCray et al., 2010). Importantly, mutant *Rab7* is still functionally active as shown by its ability to rescue *Rab7* function after silencing endogenous *Rab7* activity (Spinosa et al., 2008), indicating that the mutations do not act through haploinsufficiency.

As well as playing a role in lysosomal degradation, a neuron specific function has been described for *Rab7*: it is involved in the retrograde transport of neurotrophins (NGF, BDNF) and their receptors (TrkA, p75NTR and TrkB) from the axon tip to the cell body (Deinhardt et al., 2006). Recently, peripherin was identified as a new *Rab7* effector (Cogli et al., 2013). Peripherin is a type III intermediate filament of the peripheral neurons and is important for neurite outgrowth and axonal regeneration. Mutant *Rab7* was found to interact more strongly with peripherin, thereby altering neurofilament dynamics; this is thought to explain the restriction of the phenotype in CMT2B patients to the peripheral nervous system (Cogli et al., 2013).

The fruit fly *Drosophila melanogaster* has been used extensively to model neurodegenerative disorders of the central nervous system (Lessing and Bonini, 2009). Recently, we successfully demonstrated that *Drosophila* is a valuable organism to model disorders of the peripheral nervous system as well (Storkebaum et al., 2009). *Rab7* is a highly conserved protein; the human form shares 76% identity and 95% similarity with its single *Drosophila* ortholog (Suppl. Fig. 1), rendering CMT2B a prime candidate disease for modelling in the fly. The fly model presented here is the first animal model for CMT2B and the first fly model for CMT in general that recapitulates sensory deficits. Moreover, we demonstrate altered *Rab7* vesicle motility both in *Drosophila* sensory neurons and in neurites of human neuroblastoma cells expressing the *Rab7* L129F mutation, in line with the observed defects in axonal transport recently shown in cell models for CMT2B (Zhang et al., 2013). Our *Drosophila* model will serve as a resource for further in-depth characterisation of the pathomechanism underlying CMT2B.

Material and methods

Development of Rab7 transgenic flies

Full-length cDNA of *hRAB7A* and *dRab7* was amplified and ligated N-terminally with GFP. The L129F mutation was inserted with in vitro mutagenesis. Using the Gateway™ system (Life Technologies; Gent, Belgium), the constructs were subcloned into the pUASg.attB expression vector. In this vector, the gene of interest is placed under the UAS promotor, which, in combination with different Gal4 driver lines, provides a means to vary the expression of the transgenes in place and time (Brand and Perrimon, 1993). Furthermore, this vector allows the use of the Φ C31 system for site-directed transgenesis (Groth et al., 2004). For each construct, transgenic lines with insertion at chromosome 2 (landing site attP40) and chromosome 3 (landing site attP2) were created (BestGene; Chino Hills, CA, USA). Double transgenic flies, expressing the transgene from both chromosomes 2 and 3, were created starting from these single transgenic lines. Transgenic flies will be donated as a resource for further research to the Bloomington *Drosophila* Stock Center (Bloomington, Indiana, USA; http://flystocks.bio.indiana.edu/).

Drosophila stocks

All fly stocks were maintained on Nutri-Fly™ Bloomington Formulation (Flystuff; San Diego, CA, USA) in a humidified 25 °C incubator. The *221-* and *P0163-Gal4* driver lines were kindly provided by D. Schmucker (VIB and University of Leuven, Belgium) and C. Klämbt (University of Münster, Germany) respectively. The *D42-Gal4*, w_{1118}, *TrpA*^{1}[1] and w*; P{EP}pain^{EP2451} lines were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana, USA). *UAS-0N3R tau* was kindly provided by A. Mudher (University of Southampton, UK).

Determination of protein expression levels

Male flies from dWT, hWT and hL129F lines were crossed to *D42-Gal4* virgin flies. After 5 days, ten to fifteen third instar larvae were collected, washed in water and snap frozen in liquid N_2. The larvae were manually homogenized on liquid N_2; subsequently, 150 μl E1a lysis buffer (1% NP-40, 20 mM HEPES (pH 7.9), 250 mM NaCl, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 1 mM EDTA and a protease inhibitor cocktail) was added and the powder was further homogenized by using a pellet mixer (VWR International; Leuven, Belgium). Protein concentration was determined by using the Bradford protein assay (Bio-Rad; Nazareth Eke, Belgium). Sixty-five μg of lysate was separated on a 4–12% 10-well NuPAGE gel (Life Technologies; Gent, Belgium), transferred to a nitrocellulose membrane (Hybond ECL; GE Healthcare Life Sciences; Diegem, Belgium) and analysed by immunoblotting. Primary antibodies used were α-GFP (1/5000; Clontech; cat.no. 632380; Saint-Germain-en-Laye, Belgium) and α-actin (1/50; Developmental Studies Hybridoma Bank; cat.no. JLA20; Iowa City, Iowa, USA). For visualization, respectively mouse IgG2a and mouse HRP-tagged secondary antibodies (Jackson Immunoresearch; Newmarket, Suffolk, UK) were used in combination with the Enhanced Chemiluminescence system (ECL plus; Thermo Scientific Pierce; Aalst, Belgium).

Drosophila behavioural assays

To assay motor performance at the larval stage, third instar larvae expressing two copies of the transgene in all motor neurons (*D42-Gal4*) or in all sensory neurons (*P0163-Gal4*) were placed in the middle of a 15 cm petri dish containing 2% slightly moisturized agarose. The agarose was supplemented with 0.8% activated carbon in order to obtain a clear distinction between the dark background and the white larvae. Crawling was monitored at one frame per 2 s for 2 min. A one-way ANOVA was used to test the effect of genotype on the distance travelled. To test which genotypes differ from the hL129F genotype, a post hoc analysis using Dunnett's correction for multiple testing was performed.

To examine sensory deficits in third instar larvae, two assays were applied. In the thermotaxis assay, loss of temperature perception in larvae expressing two copies of the transgene in all sensory neurons (*P0163-Gal4*) was measured. Third instar larvae were placed in the middle of a petri dish containing 2% agarose (supplemented with 0.8% activated carbon) on which a 'warm' (30 °C) and a 'cold' (22 °C) side had been established. Larvae were assayed in complete darkness, by using an infrared camera. After 15 min, larval distribution across the plate was scored. In the case of normal thermotactic behaviour, most larvae will be on the cold side; upon complete loss of thermotactic behaviour, distribution of the larvae across the plate will be random. The effect of genotype on the distribution of the larvae was tested by fitting a logistic regression model, with the localization of the larvae (warm or cold side) as binary outcome and the genotype as a fixed effect. The effect of genotype on the distribution was tested by using a likelihood ratio test. Upon significance of the genotype, a Dunnett's post hoc analysis was performed to test which genotypes had a significantly different larvae distribution compared to the hL129F genotype.

For the nociception assay, we slightly adapted the protocol of Tracey et al. (2003). Individual third instar larvae expressing two copies of the transgene in all sensory neurons (*P0163-Gal4*) were placed on a 2% agarose plate and were laterally (abdominal segment 4 to 6) touched with a soldering iron (WMRP; Weller; Braine-l'Alleud, Belgium) to which a chisel-shaped tip (0.8 mm wide, 0.4 mm thick; RT9, Weller; Braine-l'Alleud, Belgium) had been attached. The temperature was kept constant at 43 °C by using a feedback system coupled to a power source. The touch, which is perceived as painful, evokes a rolling