Neurobiology of Disease 71 (2014) 270–279

Contents lists available at ScienceDirect

Neurobiology of Disease

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

KCNC3^{R420H}, a K^+ channel mutation causative in spinocerebellar ataxia 13 displays aberrant intracellular trafficking

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ARTICLE INFO

Article history:
Received 6 June 2014
Revised 29 July 2014
Accepted 13 August 2014
Available online 22 August 2014

Keywords:
Spinocerebellar ataxia
Dominant inheritance
SCA13
KCNC3
Voltage-gated potassium channel
Golgi
Protein trafficking

ABSTRACT

Spinocerebellar ataxia 13 (SCA13) is an autosomal dominant disease resulting from mutations in KCNC3 (Kv3.3), a voltage-gated potassium channel. The KCNC3^{R420H} mutation was first identified as causative for SCA13 in a four-generation Filipino kindred with over 20 affected individuals. Electrophysiological analyses in oocytes previously showed that this mutation did not lead to a functional channel and displayed a dominant negative phenotype. In an effort to identify the molecular basis of this allelic form of SCA13, we first determined that human KCNC3^{WT} and KCNC3^{R420H} display disparate post-translational modifications, and the mutant protein has reduced complex glycan adducts. Immunohistochemical analyses demonstrated that KCNC3^{R420H} was not properly trafficking to the plasma membrane and surface biotinylation demonstrated that KCNC3^{R420H} exhibited only 24% as much surface expression as KCNC3^{WT}. KCNC3^{R420H} trafficked through the ER but was retained in the Golgi. KCNC3^{R420H} expression results in altered Golgi and cellular morphology. Electron microscopy of KCNC3^{R420H} localization further supports retention in the Golgi. These results are specific to the KCNC3^{R420H} allele and provide new insight into the molecular basis of disease manifestation in SCA13.

Introduction

The spinocerebellar ataxias (SCAs), a heterogeneous group of neurologic diseases bound by the shared phenotypes of ataxia and motor incoordination, are characterized both by their mode of inheritance and, when known, the particular causative mutation. We previously demonstrated that two different point mutations in the *Shaw* subtype *KCNC3* (*Kv3.3*) voltage-gated potassium channel located at 19q13.33 segregate within a four generation Filipino kindred [KCNC3^{R420H} (c.1259G>A)] and a three generation French pedigree [KCNC3^{F448L} (c.1344C>A)] causing SCA13 (Subramony et al., 2013; Waters et al., 2006). The KCNC/Kv3 family contains four related genes (*KCNC1*/*Kv3.1*, *KCNC2*/*Kv3.2*, *KCNC3*/*Kv3.3*, and *KCNC4*/*Kv3.4*), that can assemble into both homotetrameric and heterotetrameric channels (Chang et al., 2007; Rudy and McBain, 2001). This family of channels has large voltage-dependent K^+ currents evident upon membrane depolarization at −20 mV, values more positive than any other known mammalian voltage-gated K^+ channels (Rudy and McBain, 2001).

KCNC3^{R420H} causes a late-onset neurodegenerative phenotype similar to other SCA subtypes, while patients with KCNC3^{F448L} present with early-onset ataxia accompanied by delayed motor milestones, cognitive delay, and epilepsy. Analyses of the electrophysiological profile for KCNC3^{R420H} in a *Xenopus* oocyte heterologous system was consistent with a non-functional channel, whereas the KCNC3^{F448L} mutation altered the gating of KCNC3 thus prolonging the open state (Waters et al., 2006) with these biophysical profiles for these mutants duplicated in the zebra fish *KCNC3* genes (Mock et al., 2010). Autosomal dominant inheritance in the Filipino family is characterized by cerebellar signs including gait and limb ataxia, brisk tendon reflexes and mild cognitive impairment (Subramony et al., 2013), as well as abnormal sound localization cueing (Middlebrooks et al., 2013). The most pronounced gross pathological feature in both the Filipino and French kindred was severe, isolated cerebellar atrophy revealed by magnetic resonance (MR) imaging (Subramony et al., 2013; Waters et al., 2006). This pathological

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

http://dx.doi.org/10.1016/j.nbd.2014.08.020
0969-9961/

hallmark in SCA13, as well as other SCAs is in sharp contrast to the complete lack of alterations in brain anatomy and motor function in any single mouse gene ablation models (Espinosa et al., 2001; McMahon et al., 2004; Zagha et al., 2010) or studies in the zebrafish (Issa et al., 2011; Mock et al., 2010).

We therefore sought to understand the underlying mechanisms that might explain the dominant inheritance of the *KCNC3*^{R420H} allele in SCA13 patients, given that this channel mutation is electrophysiologically non-functional (Mock et al., 2010; Waters et al., 2006; Waters and Pulst, 2008). Our studies reveal that KCNC3^{R420H} displays altered post-translational modifications and aberrant intracellular trafficking with associated changes in Golgi and cellular morphology and dominant phenotypes in the *Drosophila* wing and eye.

Materials and methods

Cell culture, transient and stable transfection

COS-1 cells (ATCC, CRL 1650) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Omega) with 10% FBS (Omega), 4 mM glutamine, and antibiotic/antimycotic (ABAM) (Gibco) at 37 °C in humidified air with 5% CO_2. Human neuroblastoma cells, SH-SY5Y (ATCC, CRL 2266) were cultured similarly in 1:1 Eagle's Minimum Essential Medium: F12 Medium (ATCC, 30-2003). The human *KCNC3*^{WT} cDNA was kindly provided by Dr. James L. Rae (Mayo Foundation) (Rae and Shepard, 2000) and subcloned into pcDNA1 (Invitrogen). *KCNC3*^{WT} and individual mutants (*KCNC3*^{R420H} or *KCNC3*^{F448L}) were generated by Quikchange Mutagenesis (Stratagene) and used for transient transfection in COS-1 cells. For co-transfection, equimolar amounts (1:1) of each plasmid were used. cDNAs for KCNC3^{WT}, KCNC3^{R420H} or KCNC3^{F448L} were subcloned into a pEF-IRES-puro vector for stable selection in SH-SY5Y cells using puromycin and individual colonies selected for clonal expression. Transfections were performed using Lipofectamine LTX (Invitrogen) as per manufacturer's instructions.

Immunoblotting

Total cellular protein (30 μg, bicinchoninic acid (BCA) assay (Pierce)) was separated by SDS-PAGE (4–12% gradient, Invitrogen) and electrotransferred to nitrocellulose membrane (Millipore). The membranes were then blocked for 1 h with 5% non-fat dry milk in TBST [10 mM Tris–HCl (pH 7.5), 200 mM NaCl, 0.1% (v/v) Tween 20] at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies to KCNC3 (Alomone, APC-102, 1:200) or β1 integrin (Chemicon 1952-20, 1:500) washed three times with TBST, incubated with a peroxidase-conjugated secondary antibody for 1 h, washed again three times, and subjected to ECL (Perkin Elmer). To address glycosylation from total cellular protein isolated from SH-SY5Y cells stably expressing KCNC3^{WT} and KCNC3^{R420H}, protein isolates were treated with either PNGase F or Endo H per manufacturer's instructions (New England Biolabs) followed by immunoblot analysis.

Immunohistochemistry

For immunofluorescence microscopy, transiently transfected COS-1 cells were permeabilized with Dulbecco's PBS containing 0.1% Triton X-100 for 20 min and blocked with 1% bovine serum albumin and 5% goat serum (Sigma) for 1 h. PBS washed cells were incubated with antibodies to KCNC3 (Alomone, APC-102, 1:500), cadherin (Abcam, ab6528, 1:500), calnexin (Abcam, ab2798, 1:200), GM130 (BD Bioscience, 1:200), 58K/FTCD (Abcam, ab27043, 1:50), β-tubulin (Sigma, T 4026, 1:200) followed by corresponding secondary antibodies conjugated with Alexa Fluor 488 or with Alexa Fluor 568 fluorescent dyes (Molecular Probes). Antigen retrieval was performed in 10 mM sodium citrate and control reactions were devoid of primary antibodies or stained with blocking antibodies. Samples were imaged with a Leica TCS/SP spectral confocal scanner (Leica Microsystems, Mannheim, Germany) in dual emission mode to distinguish autofluorescence from specific staining.

Membrane protein surface biotinylation

To study surface localization of membrane proteins, transiently transfected COS-1 cells were subjected to surface biotinylation as per manufacturer's protocols (Cell Surface Protein Isolation Kit, Pierce). Isolated protein was quantified by BCA assay and then subjected to immunoblot analysis for KCNC3 and β1 integrin as described above. Densitometry on the resulting immunoblots was performed using ImageJ software (v1.48) to determine the proportion of unmodified to glycosylated proteins for both mutant and WT KCNC3.

Acceptor photobleaching fluorescence resonance energy transfer (apFRET)

COS-1 cells were grown on 1 cm coverslips and fixed and mounted for processing. Cells transiently transfected with KCNC3^{WT} or KCNC3^{R420H} were labeled with fluorescently tagged antibodies to KCNC3 (rhodamine, acceptor fluorophore) and GM130 or 58K/FTCD (FITC, donor fluorophore). After 6 h, cells were rinsed in PBS and covered with a thin layer of 50% PEG solution (Sigma) with gentle rocking for 90 s. The PEG solution was removed by repeated rinses with media and the cells were allowed to recover for 4 h. Pre-bleach images were obtained by laser excitation at 488 nm (FITC) or 543 nm (rhodamine). Acceptor bleaching (acceptor) was performed within a region of interest by repeated pulses at 543 nm. A post-bleach FITC (donor) image excited with 543 nm is also detected. apFRET efficiency was calculated by pixel density as the difference between maximal and absorbed output of the donor (post-bleach minus pre-bleach) divided by the maximal (post-bleach) output of the donor. The data analysis resulted from n = 9 for R420H, n = 8 for WT and n = 6 F448L.

Electron microscopy

COS-1 cells transiently transfected with *KCNC3*^{WT} and *KCNC3*^{R420H} were trypsinized, pelleted, fixed in 1% osmium tetroxide in H_2O for 1 h, and then dehydrated with subsequent immersion in 30–100% acetone and embedded in Eponate (Ted Pella). After overnight polymerization at 56 °C, samples were stained with methylene blue, counterstained with 5% uranyl acetate in methanol and in Reynold's lead citrate for 10 min each and viewed with a JEOL 100CX transmission electron microscope at 80 kV. Digital images were collected with a XR40 Digital Camera (Advance Microscopy Techniques).

Generation of transgenic flies and Drosophila genetics

The cDNAs encoding KCNC3^{WT} and KCNC3^{R420H} were first subjected to Quikchange Mutagenesis (Stratagene) to optimize the Kozak sequence for appropriate translation in flies. The 3.2 kb cDNAs were sub-cloned into the fly expression vector pUAST (Brand and Perrimon, 1993) via EcoRI (5′) and NotI (3′) ligation. The resulting constructs were sequenced to verify the integrity of the DNAs and injected into *yw* embryos by standard procedures (Rainbow Transgenics, Inc.). The driver strains *da-Gal4* (ubiquitous) and *gmr-Gal4* (all eye cells), and the reporter *UAS-LacZ* were obtained from the Bloomington Drosophila Stock Center. Fly stocks were maintained on standard *Drosophila* medium at 25 °C. For experiments, homozygous females for the *Gal4* strains were crossed with *UAS* males to generate progeny expressing KCNC3 in the desired tissue. All crosses were initially placed at 25 °C for two days and the progeny was raised at 28 °C to maximize expression.