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The first knockin mouse model of episodic ataxia type 2

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ABSTRACT

Episodic ataxia type 2 (EA2) is an autosomal dominant disorder associated with attacks of ataxia that are typically precipitated by stress, ethanol, caffeine or exercise. EA2 is caused by loss-of-function mutations in the *CACNA1A* gene, which encodes the α_{1A} subunit of the $Ca_V2.1$ voltage-gated Ca^{2+} channel. To better understand the pathomechanisms of this disorder *in vivo*, we created the first genetic animal model of EA2 by engineering a mouse line carrying the EA2-causing c.4486T>G (p.F1406C) missense mutation in the orthologous mouse *Cacna1a* gene. Mice homozygous for the mutated allele exhibit a ~70% reduction in $Ca_V2.1$ current density in Purkinje cells, though surprisingly do not exhibit an overt motor phenotype. Mice hemizygous for the knockin allele (EA2/— mice) did exhibit motor dysfunction measurable by rotarod and pole test. Studies using Cre-flox conditional genetics explored the role of cerebellar Purkinje cells or cerebellar granule cells in the poor motor performance of EA2/— mice and demonstrate that manipulation of either cell type alone did not cause poor motor performance. Thus, it is possible that subtle dysfunction arising from multiple cell types is necessary for the expression of certain ataxia syndromes.

Introduction

Episodic ataxia type 2 (EA2) is an autosomal dominant disorder characterized by a relatively normal neurological baseline and attacks of ataxia that are typically precipitated by stress, ethanol (EtOH), caffeine or exercise (Baloh, 2012; Jen et al., 2007; Rajakulendran et al., 2012). EA2 is caused by specific mutations in the *CACNA1A* gene, which encodes the α_{1A} subunit of the $Ca_V2.1$ (P/Q-type) voltage-gated Ca^{2+} channel. Dozens of EA2 mutations have been identified in the *CACNA1A* gene including nonsense, deletion or splice site mutations that abrogate $Ca_V2.1$ channel function and missense mutations encoding hypoconductive $Ca_V2.1$ channels (Pietrobon, 2010; Tomlinson et al., 2009).

Although it has been well established that EA2 mutations most often lead to a loss of channel function and reduction in whole-cell $Ca_V2.1$ Ca^{2+} current, the pathomechanisms are still debated. It has been suggested that EA2 mutant channels suppress normal $Ca_V2.1$ channel function through a dominant-negative mechanism, comparable to a total knockout of channel function (Jeng et al., 2006, 2008; Mezghrani et al., 2008; Raike et al., 2007). However, other studies suggest that EA2 is caused by mere haploinsufficiency of $Ca_V2.1$ currents (Imbrici et al., 2004, 2005; Wappl et al., 2002). Indeed even where mutant channel properties are well characterized by multiple laboratories *in vitro*, such as the c.4486T>G (p.F1406C) missense mutation (Jen et al., 2001; Jeng et al., 2006, 2008), the disease-causing mechanism is unresolved.

Consistent with the association between ataxic disorders and cerebellar dysfunction, $Ca_V2.1$ channels are highly expressed in cerebellar Purkinje cells (PCs) and cerebellar granule cells (GCs) and both cell types are sensitive to changes in $Ca_V2.1$ conductance (Randall and Tsien, 1995; Regan, 1991). Furthermore, PCs are the sole output neurons of the cerebellar cortex and PC-specific lesions induce ataxia in humans and animal models (Diener and Dichgans, 1992; Feddersen et al., 1992; Holmes, 1917). Indeed, selective elimination of $Ca_V2.1$ channels from PCs causes a severe and chronic motor syndrome in mice that includes ataxia (Mark et al., 2011; Todorov et al., 2012). In other spontaneous mouse mutants with point mutations within *Cacna1a* that lead to moderately hypoconductive $Ca_V2.1$ channels, the motor dysfunction is somewhat less severe with both chronic and episodic components (Kodama et al., 2006; Shirley et al., 2008; Wakamori et al., 1998).

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However, in the heterozygous state, where one wild-type *Cacna1a* allele remains, mutant mice have normal motor function. Yet, heterozygsity in humans with EA2 is sufficient to cause attacks of ataxia. Therefore, to specifically address questions of EA2 pathomechanisms *in vivo*, we generated the first EA2 knockin mice bearing the human pathogenic p.F1406C missense mutation, which results in a severely hypoconductive channel with only 10–20% residual current (Jen et al., 2001).

Materials and methods

EA2 knockin mice

Recombineering methods and vectors from NCI-Frederick (http://ncifrederick.cancer.gov/research/brb/reagents/recombineeringReagent.aspx) were used to prepare the targeting construct. A C57BL/6 BAC clone was identified from the RPCI23 BAC library. RP23–275N4, which encompassed exons 2–46 of mouse *Cacna1a*, was confirmed by endonuclease restriction digest and diagnostic PCRs (not shown). The EA2-causing F1406C mutation was introduced into exon 26 by site-directed mutagenesis. The targeting vector also contained, a PGK-driven neomycin (neo^r) cassette flanked by two loxP sites downstream of exon 26. C57BL/6-derived embryonic stem cells were electroporated with the targeting construct and clones were screened for homologous recombination by Southern blot. The presence of the c.4486T>G mutation was confirmed by polymerase chain reaction (PCR) amplification of exon 26 with forward and reverse primers 5′-GGAAACCAGAAGCTGAACCA-3′ and 5′-CCCTGAATTCCTCCATTTC-3′, and endonuclease digestion of the PCR product with the enzyme *BstAPI*, or sequencing of the PCR product. Targeted ES cells were injected into blastocysts to produce chimeric mice. Mice with germline transmission were identified and long PCR from genomic DNA of F1 progeny was used as an additional screen to confirm homologous recombination of the targeting construct in the mice (Fig. 1A). The 5′ insertion site was verified by two reactions (P1–P2 and P1–P3) that used a common forward primer specific to the genomic DNA upstream of and outside of the 5′ end of the construct, P1, 5′-TCCTGCCCAGTACAGAGATTGA-3′, and a reverse primer upstream of the neo^r, P2, 5′-GAATTCAAGCTTCACTGG GAGACTAG-3′, and one specific to the neo^r, P3, 5′-AGGCCAGAGGCCAC TTGTGTAG-3′. The 3′ insertion site was confirmed by two reactions (P4–P6 and P5–P6) that used a common reverse primer specific to the genomic DNA downstream and outside of the 3′ end of the construct, P6, 5′-CCCTAAACTTTTTCAGCCCAAAGG-3′, and one forward primer specific to the neo^r, P4, 5′-GACGAGTTCTTCTGAGGGGATCAA-3′, and one downstream of the neo^r, P5, 5′-GGAGGATCACCCTGAGTTTTGAGA-3′. The PCR product of a short amplicon (325 bp) encompassing the c.4486T>G mutation was also sequenced, verifying that mice testing positive for the long PCR assay also carried the point mutation. Mice carrying the unresolved knockin allele were crossed with the Cre-deleter strain C57BL/6-Tg(Zp3-Cre)93Knw/J (The Jackson Laboratory, Bar Harbor, ME) to remove the neomycin cassette. Progeny carrying the resolved allele were crossed with C57BL/6J mice to segregate the Cre allele. These EA2 knockin mice, which were coisogenic with C57BL/6J, were bred and maintained on a C57BL/6J background. For subsequent genotyping of EA2 knockin mice, PCR amplicons were sequenced as above or quantitative PCR was used (not shown).

Other mouse lines

Mice carrying a knockout *Cacna1a* allele (+/−) on a mixed C3H-C57BL/6J background were kindly provided by Dr. David Yue (Johns Hopkins University). Mice carrying a floxed *Cacna1a* allele (flox/+) on a C57BL/6J background were previously described (Todorov et al., 2006). Mice carrying the Tg(Pcp2-Cre)2Mpin/J (L7-Cre/−) transgene on a C57BL/6J background were obtained from The Jackson Laboratory and were used to drive recombination specifically in PCs. Mice carrying the Math1-CreER^{T2} (Math1-Cre/−) transgene on a C57BL/6J background were kindly provided by Dr. Rob Machold (New York University). The Math1-Cre transgene expresses Cre recombinase in GCs between embryonic days 13.5 and 16.5 upon activation with tamoxifen (Machold and Fishell, 2005). For experiments involving this transgene, dams carrying E16.5 pups were injected i.p. with 100 mg/kg tamoxifen (Sigma-Aldrich, St. Louis, MO), dissolved in corn oil. Because the tamoxifen injections interfered with parturition, pups were delivered by cesarean section at E19.5 and cross-fostered to CD-1 dams. All mice were bred at Emory University vivaria, housed on a 12 h light/dark cycle and had access to food and water *ad libitum*. 2–3 week-old mice of both sexes were used for electrophysiological studies. 2–3 month-old mice of both sexes were used for behavioral

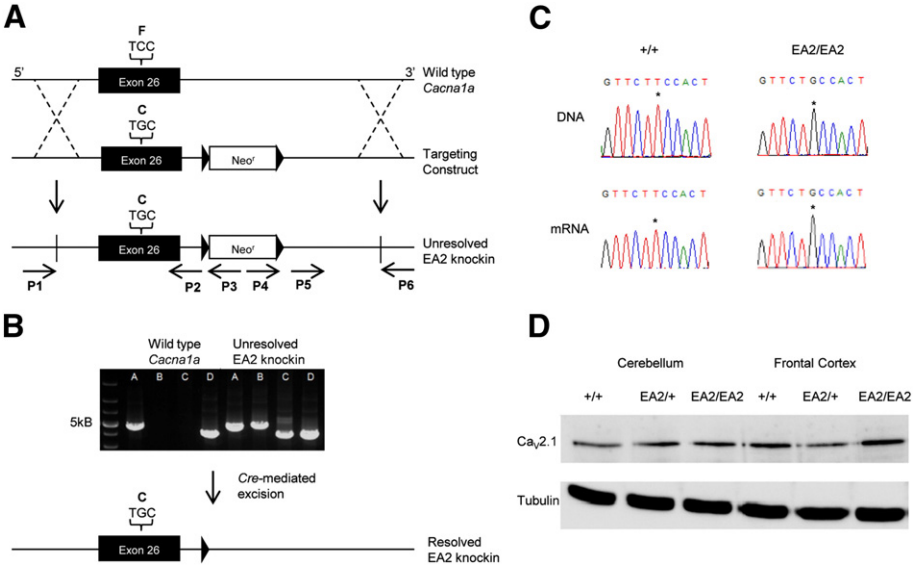

Fig. 1. Generation and molecular characterization of EA2 knockin mice. (A) The EA2 knockin construct contained a c.4486T>G substitution, coding a p.F1406C missense in exon 26 of *Cacna1a* and a neomycin resistance cassette (neo^r), flanked by two loxP sites, upstream of exon 27. The targeting construct was introduced into C57BL/6J-derived ES cells *via* homologous recombination and cells carrying the construct were injected into blastocysts. (B) A series of PCRs from the DNA of F1 mice was used to verify correct homologous recombination. Reactions from primers P1 and P3 illustrate proper insertion of the 5′ end of the construct and reactions from primers P4 and P6 verify 3′ recombination. (C) DNA and mRNA sequences illustrate the presence of the c.4486T>G substitution in the PCR product of genomic DNA and RT-PCR product of brain mRNA from +/+ and EA2/EA2 mice. (D) Western blot shows the normal expression of $Ca_V2.1$ in frontal cortex and cerebellum.