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The PRRT2 knockout mouse recapitulates the neurological diseases associated with *PRRT2* mutations

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ABSTRACT

Heterozygous and rare homozygous mutations in PRoline-Rich Transmembrane protein 2 (PRRT2) underlie a group of paroxysmal disorders including epilepsy, kinesigenic dyskinesia episodic ataxia and migraine. Most of the mutations lead to impaired PRRT2 expression and/or function. Recently, an important role for PRTT2 in the neurotransmitter release machinery, brain development and synapse formation has been uncovered. In this work, we have characterized the phenotype of a mouse in which the PRRT2 gene has been constitutively inactivated (PRRT2 KO). β-galactosidase staining allowed to map the regional expression of PRRT2 that was more intense in the cerebellum, hindbrain and spinal cord, while it was localized to restricted areas in the forebrain. PRRT2 KO mice are normal at birth, but display paroxysmal movements at the onset of locomotion that persist in the adulthood. In addition, adult PRRT2 KO mice present abnormal motor behaviors characterized by wild running and jumping in response to audiogenic stimuli that are ineffective in wild type mice and an increased sensitivity to the convulsive effects of pentylentetrazol. Patch-clamp electrophysiology in hippocampal and cerebellar slices revealed specific effects in the cerebellum, where PRRT2 is highly expressed, consisting in a higher excitatory strength at parallel fiber-Purkinje cell synapses during high frequency stimulation. The results show that the PRRT2 KO mouse reproduces the motor paroxysms present in the human PRRT2-linked pathology and can be proposed as an experimental model for the study of the pathogenesis of the disease as well as for testing personalized therapeutic approaches.

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1. Introduction

Proline-rich transmembrane protein 2 (PRRT2)[1] is the causative gene for a variety of paroxysmal neurological disorders that can be grouped in three major phenotypes: Benign Familial Infantile Epilepsy (BFIE), Paroxysmal Kinesigenic Dyskinesia (PKD), and Infantile Convulsions with Choreoathetosis (ICCA)/Paroxysmal Kinesigenic Dyskinesia with Infantile Convulsions (PKD/IC) (Chen et al., 2011; Lee et al., 2012).

To date, some 1500 patients with 70 different PRRT2 mutations have been reported, 78% of which carrying the same frameshift mutation (c.649dupC). In addition to this frequent mutation, about 75% of all reported PRRT2 mutations involve the insertion of a precocious stop codon, leading to an unstable mRNA and/or a truncated protein that is degraded or mistargeted (Chen et al., 2011; Lee et al., 2012; Li et al., 2015; Liu et al., 2016; Valtorta et al., 2016). Of the 22 reported missense mutations, 16 were not found or were found only once in >60,000 exomes, indicating the high likelihood of their pathogenicity.

The core of PRRT2-associated disorders consists of three diseases BFIE, PKD and PKD/IC forming a continuous spectrum. PRRT2 mutations account for the majority of familial BFIE, PKD and PKD/IC patients and, conversely, 95% of PRRT2 patients have a diagnosis within the BFIE–PKD/IC–PKD spectrum. In addition, a low percentage (5%) of patients bearing PRRT2 mutations display other disorders such as episodic

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[1] AGS, audiogenic seizures; ACSF, artificial cerebrospinal fluid; BFIE = Benign Familial Infantile Epilepsy; β-gal, β-galactosidase; CNS, central nervous system; CS, conditioned stimulus; DG, dentate gyrus; EEG, electroencephalogram; eEPSC, evoked excitatory postsynaptic current; eIPSC, evoked inhibitory postsynaptic current; GC, granule cell; HET, heterozygous knockout; ICCA, Infantile Convulsions with Choreoathetosis; ISI, inter-stimulus interval; KO, homozygous knockout; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; NGS, normal goat serum; P, postnatal day; PB, phosphate buffer; PBS, phosphate buffered saline; PC, Purkinje cell; PF, parallel fiber; PKD, Paroxysmal Kinesigenic Dyskinesia; PKD/IC, Paroxysmal Kinesigenic Dyskinesia with Infantile Convulsions; PPR, paired-pulse ratio; PTZ, pentylentetrazol; PRRT2, proline-rich transmembrane protein 2; TTX, tetrodotoxin; US, unconditioned stimulus; WT, wild type

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ataxia, hemiplegic migraine, developmental delay and intellectual disability. The noticeable pleiotropy of the phenotypes associated with PRRT2 mutations is not reflected by specific genotype-phenotype correlations (Ebrahimi-Fakhari et al., 2015; Gardiner et al., 2015; Heron and Dibbens, 2013; Valtorta et al., 2016).

In BFIE, patients exhibit afebrile seizures starting from 6 months to about 2 years of age and including clusters of focal or generalized attacks with normal interictal EEG and MRI (Scheffer et al., 2012; Vigevano, 2005). PKD-affected patients are characterized by short and frequent episodes of dystonic or choreiform movements triggered by sudden voluntary movements or startle with onset in childhood or adolescence (Ebrahimi-Fakhari et al., 2015). In the ICCA syndrome, patients exhibit infantile seizures, movement disorders or both. All the above-described syndromes are characterized by paroxysmal attacks that occur periodically, suggesting the existence of common underlying pathophysiological mechanisms (Valtorta et al., 2016). The few patients bearing homozygous or compound heterozygous mutations in *PRRT2* show a severe encephalopathic phenotype, with paroxysmal dyskinesias, unusually prolonged ataxia attacks, seizures and intellectual disability (Delcourt et al., 2015; Huang et al., 2015; Labate et al., 2012). However, until very recently, knowledge concerning the molecular mechanisms by which PRRT2 mutations cause the disease and manifest with different phenotypes remained scarce. The association of PRRT2-linked pathologies with haploinsufficiency, as well as the more severe phenotype of homozygous mutations, indicate that the disorders are attributable to loss-of-function of the protein and gene-dosage effects and suggest that the silencing or the constitutive deletion of the PRRT2 gene in the mouse can be a valid model to study the pathogenesis of the PRRT2-linked diseases.

A series of very recent papers have shown that PRRT2 is involved in brain development, synapse formation and neurotransmitter release (Liu et al., 2016; Valente et al., 2016). PRRT2 is a type II transmembrane protein with a C-terminal anchor, resembling the SNARE proteins VAMP/synaptobrevin and syntaxin (Rossi et al., 2016). In humans and rodents, PRRT2 is neuron-specific and is expressed with the highest levels in cerebellum, basal ganglia and neocortex. In the cortex and hippocampus, PRRT2 is already expressed at early postnatal stages, a postnatal stages, and its expression increases to reach a plateau at 1 month of life over a period of synapse formation and rearrangement (Chen et al., 2011; Ebrahimi-Fakhari et al., 2015; Valente et al., 2016). In neurons, PRRT2 has a distal distribution with the highest levels at synapses, where it mostly codistributes with proteins associated with the presynaptic area and, to a much lower extent, in fractions enriched in post-synaptic densities (Lee et al., 2012; Liu et al., 2016; Valente et al., 2016).

Acute silencing of PRRT2 by RNA interference during *in vitro* development was shown to cause a decrease in the density of synaptic connections (Valente et al., 2016) that was paralleled *in vivo* by a delayed radial migration of neurons and a decreased density of synaptic spines (Liu et al., 2016). Interestingly, PRRT2 silencing in primary neurons was followed by a severe impairment in synchronous release, while the asynchronous release was relatively preserved (Valente et al., 2016). This cellular phenotype was supported by the observation that, at the presynaptic level, PRRT2 interacts with the fast Ca^{2+} sensors synaptotagmins 1 and 2 and with SNAP25 and VAMP/synaptobrevin, endowing the SNARE complex with Ca^{2+} sensitivity for fast synchronous neurotransmitter release (Boyken et al., 2013; Lee et al., 2012; Valente et al., 2016).

Here, we have for the first time characterized a genetically altered mouse constitutively lacking PRRT2 that also allowed us to analyze PRRT2 expression by means of a knocked-in lacZ sequence. We find that PRRT2 is abundantly expressed in the hindbrain neurons and in restricted populations of forebrain neurons. PRRT2 knockout (KO) mice display a pleiotropic paroxysmal phenotype during infancy and in the adulthood with paroxysmal dyskinesias in response to audiogenic stimuli and an increased sensitivity to the convulsive effects of pentylentetrazol (PTZ). Thus, the PRRT2 KO mouse reproduces multiple aspects of the human pathology associated with PRRT2 mutations and can therefore be used as a reliable experimental model of PRRT2-linked neurological disorders.

2. Materials and methods

2.1. Experimental animals and housing

Heterozygous PRRT2 KO mice were kindly provided by the IMPC European Consortium at the Sanger Institute (UK) in the frame of the European EMMA/Infrafrontier, and bred at the IIT SPF animal facility. The EUCOMM/KOMP targeting strategy was based on the "knockout-first" allele that contains an IRES:lacZ trapping cassette and a floxed neo cassette that were inserted into the intronic region among the exons 1 and 2 of the PRRT2 wild type locus (Fig. S1A). The presence of an Engrailed (En2) splice acceptor disrupts gene function, resulting in a lacZ fusion for studying gene expression localization (Skarnes et al., 2011). Genotyping was performed by PCR with primers Prrt2_F: AGGTAGACGGGCATTTGAGC, Prrt2_R: CGTGGGGAAGAGGAGACAAC; CAS_R1_Term: TCGTGGTATCGTTATGCGCC, that were used to detect the wild-type (WT) (Prrt2_F plus Prrt2_R product, 480 bp) and mutant (Prrt2_F plus CAS_R1_Term product, 200 bp) PRRT2 alleles and to genotype WT, heterozygous (HET) and KO mice. The primer Prrt2 F is common to wild type and mutant PCR and was designed in the intronic sequence between Prrt2 Exon 1 and Exon 2. The primer Prrt2 R was designed in the exon 2 of PRRT2 gene and the Cas_R1_Term was designed in the targeting cassette (Fig. S1A). The colony was maintained on a C57BL/6N background and propagated in heterozygosity. Two females were housed with one male in standard Plexiglas cages (33 × 13 cm), with sawdust bedding and a metal top. After two weeks of mating, male mice were removed and dams were housed individually in Plexiglas cages and daily checked for delivery. Mice were maintained on a 12∶12 h light/dark cycle (lights on at 7 a.m.). The temperature was maintained at 21 ± 1 °C, relative humidity (60 ± 10%). Animals were provided drinking water and a complete pellet diet (Mucedola, Settimo Milanese, Italy) *ad libitum*. Mouse genotypes were determined at weaning (at P25) by RT-PCR on tail samples. Mice were weaned into cages of same sex pairs. All experiments were carried out in accordance with the guidelines established by the European Communities Council (Directive 2010/63/EU of March 4th, 2014) and were approved by the Italian Ministry of Health (authorization n. 73/2014-PR and n. 1276/2015-PR).

2.2. X-gal (β-galactosidase) staining

Brains isolated from HET and PRRT2 KO mice at various postnatal day (P) stages (P4, P8 and P16 up to 2 months of age) were fixed with 2% paraformaldehyde/0.2% glutaraldehyde solution, permeabilized in wash buffer (1% sodium deoxycolate, 2% NP-40, $MgCl_2$ 0.1 M), included in 2.5% agarose and cut with a microtome (Bioptica). Coronal sections (120 μm) were incubated with X-Gal solution (X-Gal 100 mg/ml, ferrocyanide potassium 2 mg/ml, ferricyanide potassium 1.64 mg/ml in Wash Buffer) for 6 h (P4, P8 mice) and overnight (P16, P60 mice) at 37 °C as previously described (Gazzerro et al., 2012). The entire slices were acquired at 20× while higher magnifications were acquired at 40×. Sections were analyzed with Neurolucida software (MicroBrightField) connected to a Nikon E-800 microscope *via* a color CCD camera. The identification of the specific β-gal-positive areas was performed using the Paxinos mouse brain atlas (Paxinos and Franklin, 2012) and the interactive Allen Brain Explorer atlas (The Allen Institute, http://mouse.brain-map.org/static/brainexplorer).

2.3. Western blotting

Protein concentration of the samples was determined using the BCA assay and equivalent amounts of protein were subjected to SDS-PAGE

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