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Development-related aberrations in Kv1.1 α-subunit exert disruptive effects on bioelectrical activities of neurons in a mouse model of fragile X syndrome

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ABSTRACT

Kv1.1, a Shaker homologue potassium channel, plays a critical role in homeostatic regulation of neuronal excitability. Aberrations in the functional properties of Kv1.1 have been implicated in several neurological disorders featured by neuronal hyperexcitability. Fragile X syndrome (FXS), the most common form of inherited mental retardation, is characterized by hyperexcitability in neural network and intrinsic membrane properties. The Kv1.1 channel provides an intriguing mechanistic candidate for FXS. We investigated the development-related expression pattern of the Kv1.1 α-subunit by using a *Fmr1* knockout (KO) mouse model of FXS. Markedly decreased protein expression of Kv1.1 was found in neonatal and adult stages when compared to age-matched wild-type (WT) mice. Immunohistochemical investigations supported the delayed development-related increases in Kv1.1 expression, especially in CA3 pyramidal neurons. By applying a Kv1.1-specific blocker, dendrotoxin-κ (DTX-κ), we isolated the Kv1.1-mediated currents in the CA3 pyramidal neurons. The isolated DTX-κ-sensitive current of neurons from KO mice exhibited decreased amplitude, lower threshold of activation, and faster recovery from inactivation. The equivalent reduction in potassium current in the WT neurons following application of the appropriate amount of DTX-κ reproduced the enhanced firing abilities of KO neurons, suggesting the Kv1.1 channel as a critical contributor to the hyperexcitability of KO neurons. The role of Kv1.1 in controlling neuronal discharges was further supported by the parallel developmental trajectories of Kv1.1 expression, current amplitude, and discharge impacts, with a significant correlation between the amplitude of Kv1.1-mediated currents and Kv1.1-blocking-induced firing enhancement. These data suggest that the expression of the Kv1.1 α-subunit has a profound pathological relevance to hyperexcitability in FXS, as well as implications for normal development, maintenance, and control of neuronal activities.

1. Introduction

Voltage-gated potassium (K+) channels (Kv) are major modulators of diverse excitatory events in the mammalian nervous system, such as neuronal firing, shaping of action potentials (APs), and neurotransmitter release (Jan and Jan 2012). Kv genes can be divided into four main subfamilies according to their homologous genes in *Drosophila*: Kv1 (*Shaker*), Kv2 (*Shab*), Kv3 (*Shaw*), and Kv4 (*Shal*) (Jan and Jan 1997). The *Shaker*-related Kv1 family, including at least six members (Kv1.1–1.6), mediate rapidly activating, slowly inactivating (delayed rectifier) outward K^+ currents. They have been localized to soma, axons, synaptic terminals, and proximal dendrites (Grosse et al. 2000; Monaghan et al. 2001; Trimmer and Rhodes 2004). The co-assembly of different Kv1 isoforms (mainly Kv1.1, Kv1.2 and Kv1.4) contributes to the functional diversity of Kv1 channels in various brain regions (Sokolov et al. 2007). These heteromeric Kv1 channels exquisitely mediate the characteristic electrophysiological properties of each subclass of neurons. Kv1 channels are characterized by low-threshold activation and rapid activation kinetics so that they effectively regulate the shape and rate of APs (Bean 2007). Alterations in the expression, distribution and function of Kv1 may impair the homeostatic regulation of neuronal excitability and eventually lead to neurological disorders.

Clinical and genetic findings highlight the key roles played by Kv1.1 in the modulation of neuronal excitability. In particular, distinct mutations in *KCNA1*, the gene encoding for Kv1.1, were found mostly in the patients with episodic ataxia-1 (EA1) (D'Adamo et al. 1999; Tan

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et al. 2013). EA1 is a dominant human neurological disorder characterized by phenotypes of brief episodes of ataxia, myokymia, neuromyotonia, paroxysmal dyskinesias, and associated epilepsy. Functional studies showed that the EA1-associated mutants exhibited different extents of impairments in Kv1.1 channel function including channel assembly, trafficking and biophysics (D'Adamo et al. 1999; Rea et al. 2002). The indispensable role of Kv1.1 channels in neuronal excitability has been evidenced in Kv1.1-null mice manifesting with severe epilepsy and even lethal seizures (Glasscock et al. 2010; Smart et al. 1998). Targeted knockdown of Kv1.1 protein in neurons leads to increased firing abilities (Ghelardini et al. 2003), while lentiviral overexpression of Kv1.1 reduces the probability of AP generation and neurotransmitter release (Wykes et al. 2012). Mutations in *KCNA1* or auto-antibodies against Kv1.1 can cause Morvan's syndrome characterized by central, autonomic, and peripheral hyperactivity (Irani and Vincent 2016; Kleopa et al. 2006). Clinical data from patients with KCNA1 mutations indicate that distinct neurological phenotypes, such as muscle spasms, cerebellar atrophy, cognitive delay, myokymia, epilepsy and migraine, are associated with functional deficits in the Kv1.1 channel (Brownstein et al. 2016; Eunson et al. 2000).

A theme emerging from these investigations is that Kv1.1 acts as a key and dynamic determinant of neuronal intrinsic excitability. Subtle changes in Kv1.1 function might be involved not only in the pathogenesis of EA1 and epilepsy, but also in the pathophysiological process of other neurological disorders featured by neuronal hyperexcitability. Fragile X syndrome (FXS), the most common form of inherited mental retardation, is characterized by elevated excitability in the brain at multiple levels: I) prominent symptoms such as hyperactivity, hypersensitivity to sensory stimuli, anxiety, seizures (Kazdoba et al. 2014); II) altered UP states and synaptic transmissions reflecting hyperexcitable circuitry (Contractor et al. 2015; Gibson et al. 2008; Goncalves et al. 2013); and III) altered intrinsic properties of neurons leading to the facilitation of burst firing (Zhang et al. 2016). Alterations in the expression or activity of Kv1.1 may be implicated in the mechanisms of hyperexcitability in FXS. FXS is caused by the functional absence of the fragile X mental retardation protein (FMRP), an RNA-binding protein that regulates the translation of a variety of mRNAs. FMRP directly targets approximately 5% of all mRNAs (Darnell and Klann 2013; Darnell et al. 2011). However, Kv1.1 mRNA has not been listed in these FMRP-binding targets. Kv1.1, a putative key determinant of neuronal excitability, has not yet attracted much attention concerning its roles in the pathophysiological processes of FXS. Nevertheless, it is possible that the loss of FMRP may result in secondary alterations of the Kv1.1 channel and subsequently contribute to changes in the membrane and discharge properties.

Although Kv1.1 may play a potential role in the pathophysiology of FXS, little is known about the expression profile of Kv1.1, and its specific current properties and impact on neuronal excitabilities. The present exploratory study thus attempts to provide an overview of Kv1.1-related abnormalities in FXS by using a *Fmr1* knockout mouse model.

2. Materials and methods

2.1. Animals

The *Fmr1* knockout (KO) mice of FVB strain and wild-type (WT) controls were from Prof. Oostra BA of Erasmus University of Netherlands. These *Fmr1* knockout mice have a neomycin resistance cassette replacing exon 5 of the *Fmr1* gene (detailed in (Gantois et al. 2001)). Offspring were bred in specific-pathogen-free facilities and housed in a 12-h light/dark cycle under standard conditions with 19–21 °C and with free access to food and water. Genotypes were confirmed by PCR analysis of the DNA extracted from tail samples before experiments. All animal protocols were reviewed and approved by the Ethics Committee for the Use of Experimental Animals of Guangzhou Medical University. All efforts were made to minimize the suffering and the number of animals used.

2.2. Western blot

The *Fmr1* KO mice at postnatal 10 (P10), 30, and 60 day and age-matched WT controls were used. After animals were anesthetized, brains were rapidly excised. Hippocampal formation and entorhinal cortex was further dissected from the brains and homogenized in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100) with protease inhibitors (Beyotime, China). The cellular debris was pelleted by centrifugation at 3000*g* for 10 min at 4 °C. The supernatant was retained and ultracentrifugated at 100,000 g for 1 h at 4 °C. The supernatant was discarded and the resulting pellet was resuspended in fresh homogenization buffer. The protein concentration was determined using BCA method. Aliquots from each sample were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidine fluoride membranes (Millipore). Membranes were blocked with Tris-buffered (50 mM) saline solution (pH 7.6) with 0.05% Tween containing 5% milk powder at room temperature for 1 h. The membranes were incubated with the primary antibodies and subsequently with the secondary HRP-conjugated antibodies. The primary monoclonal antibodies included rabbit anti-Kv1.1 antibody (Millipore, 1:1500). Immunoreactive bands were detected using enhanced luminol-based chemiluminescence (ECL). The membranes were stripped and then immunoblot for the enzyme glyceraldehydes 3-phosphate dehydrogenase (GAPDH, 1:5000, Sigma) was performed as a loading control. Blots were exposed to film to obtain clear bands. Bands were scanned into digital images and analyzed with the software of Image-Pro Plus (Media Cybernetics, USA).

2.3. Immunohistochemistry

Animals were deeply anesthetized with ether vapors and transcardially perfused with 0.9% saline followed by 4.0% paraformaldehyde (PFA). Following perfusion, brains were excised, postfixed for overnight at 4 °C, and sectioned on a cryotome (Leica, Germany) at a thickness of 30 μm along the coronal plane. For characterization of the expression of Kv1.1, selected sections were washed in phosphate-buffered saline (PBS; pH 7.4) and permeabilized in PBS and 0.2% Triton X-100 for 10 min. The free-floating sections were blocked with 5% normal goat serum for 1 h and incubated overnight at 4 °C with Kv1.1 antibody (Millipore, 1:1000 dilution in 1% normal goat serum in PBS containing 0.25% Triton X-100). Sections were then rinsed with PBS and incubated with a biotinylated secondary antibody. After washing, the sections were processed with the avidin biotinylated complex (ABC) and the reaction was developed using 3,3′-diaminobenzidine (DAB). After washes in PBS, sections were mounted onto slides. Images were obtained with a Leica DM5000B upright microscope. All experimental conditions were identical between KO and WT groups. In negative controls, the Kv1.1 antibody was replaced by the same volume of normal goat serum. For quantitative analysis of regional Kv1.1 immunohistochemistry, routine densitometry measurements were performed within the regions of CA1 and CA3 at high magnification (×400), by using two sets of anatomically matched brain slices taken from the KO and WT adult mice. Average grayscale values were normalized to the average obtained from CA1 region of adult WT mice.

2.4. RNA and cDNA preparation

To perform real-time PCR assays, different parts of brain tissues were isolated. For the isolation of CA3 subregion, brains were sectioned coronally at 400 μm using Leica vibratome, and then the CA3 tissues of dorsal hippocampus were dissected from the sections under stereo microscope. Total RNA of the brain tissues was extracted using TRIzol reagent (Invitrogen) and treated with 800 units of DNase I (Sigma).