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Age-dependent kainate sensitivity in heterozygous *rolling Nagoya* Cav2.1 channel mutant mice

Tae Yeon Kim, Takuro Yoshimoto, Yo Aoyama, Kimie Niimi, Eiki Takahashi *, Chitoshi Itakura

Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

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ABSTRACT

Cav2.1α_1 is involved in glutamate release. The kainate-induced intensive firing of neurons via glutamate receptors causes seizure and neuronal damage, especially in the hippocampus. Cav2.1α_1 mutation in homozygous *rolling Nagoya* (*rol*/*rol*) mice caused reduced Ca^{2+} permeability compared to wild-type mice. The *rol*/*rol* mice exhibited ataxia approximately after 2 weeks of age. Although we have reported that heterozygous *rolling Nagoya* (*rol*/+) mice show age-dependent behavioral changes, sensitivity to kainate has not been examined. To examine the relationship between Cav2.1 function and neurological disease, we investigated how Cav2.1 is related to kainate-induced seizure and neuronal damage using 2- and 18-month-old *rol*/+ mice. The seizure scores of 18-month-old *rol*/+ mice that received 20 mg/kg kainate intraperitoneally were significantly lower than those of wild-type mice. As a consequence of seizure, kainate induced delayed neuronal damage along with astrocytic growth in the hippocampus in wild-type mice, with a moderate effect observed in *rol*/+ mice. In the hippocampus of 18-month-old rol/+ mice, the levels of mutant-type Cav2.1α_1 were increased compared to +/+ mice. The phosphorylation of p38, a mitogen-activated protein kinase (MAPK) activated by kainate, was not increased after kainate injection compared to +/+ mice. No difference was observed between 2-month-old *rol*/+ and wild-type mice intraperitoneally injected with 20 mg/kg kainate in these analyses. These findings suggest that *rol*/+ mice experience age-related changes in sensitivity to kainate due to changes in the p38 MAPK signaling pathway via a mutant Cav2.1 channel. Hence, *rol*/+ mice may represent a novel model to delineate the association between Cav2.1 function, synaptic transmission, and the postsynaptic signaling cascade.

1. Introduction

Neuronal voltage-dependent Ca^{2+} (Cav) channels mediate the presynaptic machinery for neurotransmitter release (Evans and Zamponi, 2006; Jarvis and Zamponi, 2007). Given the pivotal role of Cav2.1 (P/Q-type) channels in controlling neurotransmitter release, defects in the expression, localization, structure, or modulation of presynaptic Cav2.1 channels may result in aberrant synaptic signaling leading to various patterns of neural network dysfunction (Catterall and Few, 2008). The glutamatergic system is one of the neurotransmitter systems regulated by Cav2.1 (Lee et al., 2009).

Glutamate is a major signaling molecule that binds to specific receptors including the ionotropic glutamate, kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA) receptors (Rojas and Dingledine, 2013). Kainate directly stimulates five subtypes of kainate receptor, namely, GluK1 (Grik1), GluK2 (Grik2), GluK3 (Grik3), GluK4 (Grik4), and GluK5 (Grik5), and four subtypes of AMPA receptor, namely, GluA1 (Gria1), GluA2 (Gria2), GluA3 (Gria3), and GluA4 (Gria4) (Bassani et al., 2013; Contractor et al., 2011). Each receptor subunit is the product of a distinct gene. The overactivation of these receptors by excessive kainate evokes excitatory postsynaptic currents (EPSCs) at hippocampal synapses closely related to epilepsy. Indeed, the administration of excessive kainate to animal subjects induces persistent spikes of high amplitude and frequency discharges (Williams et al., 2006) and the resulting excitotoxicity can cause seizure and neuronal cell death (Watkins and Jane, 2006). Therefore, kainate is used as an experimental model to reveal the precise mechanism of excitotoxicity, and the kainate/AMPA receptors are an appropriate drug target for an antiepileptic drug (Bassani et al., 2013; Contractor et al., 2011; Faught, 2014).

The α_1 subunit is an essential subunit for Cav functions and properties (Catterall and Few, 2008). The Cav2.1α_1 subunit (Cav2.1α_1) has four homologous transmembrane domains (I–IV), each containing six membrane-spanning helices (S1–S6), plus a reentrant p-loop motif that lines the channel pore, enabling the passage of Ca^{2+}. The four domains are connected through cytoplasmic linkers, and both the C- and N-termini are cytoplasmic and interact with regulatory proteins (Catterall and Few, 2008). The Cav2.1α_1 mutant strains

* Corresponding author at: Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Tel.: +81 48 467 5871; fax: +81 48 467 9692.
E-mail address: etakahashi@brain.riken.jp (E. Takahashi).

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have been detected in *tottering*, *rolling Nagoya*, and *leaner* mice (Takahashi, 2012). The *rolling* Nagoya mouse has a mutation in the voltage-sensing S4 segment of the third repeat in the Cav2.1α_1 (Mori et al., 2000). The Ca^{2+} current amplitude exhibits a 40% reduction in homozygous *rolling Nagoya* (*rol*/*rol*) compared to wild-type controls (Mori et al., 2000). Although *rol*/*rol* mice have been reported to exhibit ataxia but not seizures, after about 2 weeks of age (Oda, 1973), heterozygous *rolling Nagoya* (*rol*/+) mice have no apparent behavioral deficits at 2 months of age. However, systemic or intra-hippocampal injection of the NMDA receptor blocker MK-801 decreased spatial cognition in 2-month-old *rol*/+ mice (Takahashi et al., 2010). On the other hand, it has not been elucidated whether Cav2.1α_1 dysfunctions in *rol*/+ mice affect kainate-induced signaling leading to seizure and neuronal damage. In previous studies, male mice with C57BL/6 background were resistant to excitotoxin sensitivity when injected with kainate (McCord et al., 2008; McKhann et al., 2003). Female mice are more susceptible to kainate-induced excitotoxicity than males, similar to humans (Zang et al., 2008).

In the present study, as a first step to investigate the role of Cav2.1 in kainate-regulated excitotoxicity, we examined kainate/AMPA receptor signaling-induced seizure and neuronal damage in 2- and 18-month-old *rol*/+ and wild-type (+/+) female mice with a C57BL/6 background. We also compared differences in expression patterns of Cav2.1α_1; p38, one of the mitogen-activated protein kinases (MAPK) activated by kainate (Namiki et al., 2007); and phosphorylated p38 (p-p38) in the hippocampus using in situ hybridization (ISH), real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), histochemistry, and Western blot analyses.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Animal Experiments Committee of RIKEN and all animals were cared for and treated humanely in accordance with institutional guidelines for animal experimentation. All of the mice used were female *rol*/*rol*, *rol*/+, and +/+ mice with a C57BL/6 genetic background (Tian et al., 2013). Two-month-old (5- to 8-week-old) and 18-month-old (69- to 72-week-old) mice were used. We used separate groups of female mice for each study. The mice were given ad libitum water and food pellets (CRF-1; Oriental Yeast, Tokyo, Japan) and kept at room temperature (23 ± 1 °C) and 55 ± 5% humidity under a 12 h:12 h light–dark cycle (light from 8:00 am to 8:00 pm).

2.2. In situ hybridization

The mice were anesthetized with an overdose of pentobarbital sodium. Paraffin-embedded blocks and sections of mouse brain tissue for ISH were obtained from Genostaff Co. Ltd. (Tokyo, Japan). Each mouse brain was dissected after perfusion, fixed with tissue fixative (Gonostaff) and embedded in paraffin according to proprietary procedures in 6 μm sections. The hybridization protocol was conducted as previously reported (Sakuraoka et al., 2012). The probe for a 504 bp cDNA fragment was designed from positions 3229 to 3732 of the Cav2.1α_1 subunit cDNA and labeled with a digoxygenin RNA labeling kit (Roche Diagnostics, Mannhein, Germany). Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, USA) overnight and washed with PBS. The sections were counterstained with Kernechtrot stain solution (Mutoh Pure Chemicals, Tokyo, Japan) and mounted with CC/Mount (Diagnostic Biosystems Inc., Pleasanton, CA, USA).

2.3. Real-time qRT-PCR analysis

The mice were anesthetized with an overdose of pentobarbital sodium. Total RNA was isolated from the hippocampus and liver using TRIzol reagent, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). To quantify the mRNA level of the gene of interest, we performed qRT-PCR in duplicate assays using an ABI7700 sequence detection system (Applied Biosystems, Carlsbad, CA, USA), as previously described (Takahashi and Niimi, 2009). First-strand cDNA was synthesized from 1 μg total RNA using a TaqMan reverse transcription reagent system (Applied Biosystems). The primers and probes used to determine wild-type Cav2.1α_1 gene expression, mutant Cav2.1α_1 gene expression and the total amount of Cav2.1α_1 gene expression including both wild-type and mutant mice are described in our previous study (Takahashi and Niimi, 2009). The mRNA levels of kainate receptors (Grik1, Grik2, Grik3, Grik4, and Grik5) and AMPA receptors (Gria1, Gria2, Gria3, and Gria4) were measured using Applied Biosystems TaqMan Gene Expression Assays (Grik1, Assay ID Mm00446882_m1; Grik2, Assay ID Mm00599860_m1; Grik3, Assay ID Mm01179716_m1; Grik4, Assay ID Mm00615472_m1; Grik5, Assay ID Mm00433774_m1; Gria1, Assay ID Mm00433753_m1; Gria2, Assay ID Mm00442822_m1; Gria3, Assay ID Mm00497506_m1; Gria4, Assay ID Mm00444754_m1). The 18S ribosomal RNA (Assay ID Hs99999901_s1, Applied Biosystems TaqMan Gene Expression Assay) was used for normalization. The PCR conditions were 94 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All samples were analyzed in duplicate and the threshold cycle (Ct) value, which reflects the amount of PCR product, was calculated. The relative levels of expression were determined based on the Ct values (Takahashi and Niimi, 2009).

2.4. Seizure assessment based on behavioral analysis

The mice with the C57BL/6 background were monitored continuously every 30 min for 3 h to evaluate seizure activity after intraperitoneal injection of kainate (Sigma-Aldrich). The drug dose (20 mg/kg) was determined according to a previous report (Namiki et al., 2007). The test was conducted between 9:00 am and 4:00 pm by a well-trained experimenter who was blinded to the mouse strains. Seizure classification was as follows: score 1, mice became motionless after exploring, sniffing, and grooming ceased; score 2, forelimb and/or tail extension, giving the appearance of a rigid postural tone; score 3, myoclonic jerks of the head and neck, with brief twitching movements; score 4, forelimb clonus and partial rearing; score 5, forelimb clonus, rearing, and falling; and score 6, generalized tonic–clonic activity with loss of postural tone, often resulting in death.

2.5. Histology

The mice were intracardially perfused with ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia 1 week after kainate or vehicle injection. Then the brains were removed, placed in fixative for 1 day, and cut coronally into samples 20 μm thick using a cryostat (Leica, Heidelberg, Germany). The sections were stained with hematoxylin and eosin (H&E) and double-stained with Fluoro Jade B (FJB, Chemicon, Temecula, CA, USA) and anti-glial fibrillary acidic protein (1:500; Cy3-labeled anti-GFAP, Millipore Corporation, Billerica, MA, USA). To assess the severity and extent of neurodegeneration in the CA1 and CA3 regions of the hippocampus according to H&E staining, sections were scored using a semiquantitative grading system: score 1, normal; score 2, slight shrinkage of neurons (1–4% pyknotic neurons in CA1 or CA3); score 3, moderate shrinkage of neurons (5–15% pyknotic neurons in CA1 or CA3); score 4, severe shrinkage of neurons (more than 15% pyknotic neurons in CA1 or CA3); score 5, slight loss of neurons (5–10% neuronal loss in CA1 or CA3); score 6, moderate loss of neurons (11–40% neuronal loss in

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