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Altered cerebellar function in mice lacking $Ca_V2.3$ Ca^{2+} channel

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Abstract

Voltage-dependent Ca^{2+} channels play important roles in cerebellar functions including motor coordination and learning. Since abundant expression of $Ca_V2.3$ Ca^{2+} channel gene in the cerebellum was detected, we searched for possible deficits in the cerebellar functions in the $Ca_V2.3$ mutant mice. Behavioral analysis detected in delayed motor learning in rotarod tests in mice heterozygous and homozygous for the $Ca_V2.3$ gene disruption ($Ca_V2.3+/-$ and $Ca_V2.3-/-$, respectively). Electrophysiological analysis of mutant mice revealed perplexing results: deficit in long-term depression (LTD) at the parallel fiber Purkinje cell synapse in $Ca_V2.3+/-$ mice but apparently normal LTD in $Ca_V2.3-/-$ mice. On the other hand, the number of spikes evoked by current injection in Purkinje cells under the current-clamp mode decreased in $Ca_V2.3$ mutant mice in a gene dosage-dependent manner, suggesting that $Ca_V2.3$ channel contributed to spike generation in Purkinje cells. Thus, $Ca_V2.3$ channel seems to play some roles in cerebellar functions.

Keywords: Voltage-dependent Ca^{2+} channel; $Ca_V2.3$ channel; R-type Ca^{2+} channel; Knock-out mouse; Rotarod; Long-term depression; Discharge property

Cerebellar circuit is essential for motor coordination and learning [1]. It is well known that synaptic plasticity such as long-term depression (LTD) at parallel fiber (PF)-Purkinje cell synapse (so-called cerebellar LTD), which is induced by conjunction of PF and climbing fiber inputs, is a critical event underlying some forms of motor learning [1]. In this process, climbing fiber input is assumed to induce an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which is essential for expression of the cerebellar LTD, via activation of voltage-dependent Ca^{2+} channels (VDCCs) [2]. Thus, VDCCs seem to play pivotal roles in the control of cerebellar functions including motor learning. VDCCs are classified into several groups termed L-, N-, P-, Q-, R-, and T-types based on their electrophysiological and pharmacological properties, and also classified into three families (Ca_V1, Ca_V2, and Ca_V3) according to the sequence similarities of the pore-forming α_1 subunits [3]. Ca_V1 family members are thought to underlie L-type channels and Ca_V3 family members T-type channels. Ca_V2 family is comprised of $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$, corresponding to P/Q-type, N-type, and R-type VDCCs, respectively. Of these VDCCs, $Ca_V2.1$ channel is most abundantly expressed in the cerebellum, and this channel is thought to be critical for normal cerebellar functions: mice mutant for this channel displayed ataxic phenotypes to various degrees depending on the mutation [4–6]. However, VDCCs other than $Ca_V2.1$ channel are also suggested to play important roles in cerebellar functions. For example, Ni^{2+}-sensitive low-threshold current is also detected in the Purkinje cells [7–9], and the VDCC underlying this current is thought to contribute to determination of the somato-dendritic firing properties [10,11]. However, the molecular counterpart of this low-threshold channel is not clear, since R-type and T-type VDCCs are similar in

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the voltage dependencies and pharmacological properties in some cases [12].

In the present study, we focused on $Ca_V2.3$ (also referred to as α_{1E}) channel, which underlies at least some of the R-type currents in neurons [13–15]. We previously established a mouse strain carrying a targeted mutation in *Cacna1e* gene encoding $Ca_V2.3$ channel and reported several abnormalities of the mutant mice related to pain, spatial cognition, ischemic neuronal injury, and anesthesia [16–19]. We report here the abnormalities of the $Ca_V2.3$ mutant mice in behavioral and electrophysiological properties.

Materials and methods

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) staining. Generation of $Ca_V2.3$ mutant mice was reported previously [16]. $Ca_V2.3+/-$ mice were anesthetized with pentobarbital sodium and transcardially perfused with a fixative solution (0.2% glutaraldehyde, 1.2% formaldehyde, and 0.02% Nonidet P-40 in phosphate-buffered saline [PBS]). Brains were removed and sagittal slices (400 μm thick) were prepared with a vibratome slicer. After a postfixation with the same fixative for 2–3 h, the slices were rinsed with PBS and then stained with X-gal solution (8.4 mM KCl, 1 mM $MgCl_2$, 3 mM $K_4Fe(CN)_6$, 3 mM $K_3Fe(CN)_6$, 0.05% Nonidet P-40, and 0.05% X-gal in 84 mM phosphate buffer, pH 7.5) overnight [20].

Rotarod test. Mice were individually housed 1 week before the start of the test and were handled every day. Mice were placed on a rotarod apparatus (MK-660A, Muromachi Kikai, Tokyo, Japan) with a constant speed of rotation (15 rpm). Five trials were given each day with the inter-trial interval of 1–2 min for 5 consecutive days. The latency to fall was recorded with the cut-off time 300 s. Data from second to fifth trials were used for the statistical analysis, because the data of the first trial tended to deviate from those of the others.

Preparation of brain slices. $Ca_V2.3+/+$, $+/-$, and $-/-$ mice (14–28 days old) were anesthetized with pentobarbital sodium (50 mg/kg) and were decapitated. The cerebellum was rapidly isolated and placed in ice-cold normal artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2–5% CO_2. Sagittal slices, 300 μm thick in electrophysiological studies were prepared with a vibratome tissue slicer (Leika, VT-1000). Slices were incubated at room temperature in a submerged chamber containing gassed ACSF for at least 90 min. One slice was then transferred to a recording chamber, in which it was continuously perfused with the oxygenated ACSF that was maintained at 30 ± 1 °C at a rate of 2 ml/min. The composition of normal ACSF is as follows (in mM): 137 NaCl, 2.5 KCl, 0.58 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 21 $NaHCO_3$, and 10 glucose. In all experiments, (+)-bicuculline (10 μM; Sigma) was added to the perfusate in order to eliminate inhibitory transmission.

Electrophysiological experiments. All experiments were performed using whole-cell patch-clamp recording under direct visualization using a 40× water immersion objective (NA 0.8) attached to a fixed-stage upright microscope (BX50WI; Olympus, Tokyo, Japan). Borosilicate glass pipets (4–8 MΩ) were used for patch-clamp recordings and were filled with an intracellular solution containing the following (in mM): 135 K-methanesulfonate, 5 KCl, 0.5 EGTA, 5 Mg-ATP, 0.4 Na-GTP, and 10 Hepes (pH 7.2, adjusted with KOH). Currents and membrane potential were recorded with an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany), low-pass-filtered at 3 kHz, and digitally sampled at 10 kHz. For stimulation of PFs, a glass pipet with 10–20 μm tip diameter filled with ACSF was placed on the molecular layer, within 100 μm from the Purkinje cell layer. Bipolar square pulses (duration, 100 μs; strength, 0–10 V) were applied for focal stimulation. Recordings of PF-excitatory postsynaptic currents (EPSCs) were carried out under voltage-clamp configuration. In all recordings of PF-EPSC, paired-pulse stimuli were applied. The paired-pulse interval was 50 ms and the inter-stimulus interval was 20 s unless otherwise stated. Membrane potential was held at −70 mV. To induce LTD, we used a conjunction protocol that is composed of 480 stimuli to PF in conjunction with a depolarizing pulse (100 ms, −70 to 0 mV) repeated at 1 Hz. Conjunctive stimulation was applied after stable recording of PF-EPSCs for 10 min. To monitor changes in Rm and Rs, a hyperpolarizing square pulse (−5 mV, 20 ms, 60 ms prior to the PF stimulation) was applied through the patch pipet. In all experiments, the data were discarded if the holding current exceeded −500 pA and Rs was greater than 40 MΩ. In addition, LTD experiments were accepted, if Rs changed by <20%, and Rm and the holding current remained constant. To investigate the firing properties of Purkinje cells, recordings were carried out under current-clamp configuration. Membrane potential was maintained at −70 mV by injecting steady-state current. Cells were depolarized by injecting steps of currents (0–450 pA, 100 ms).

Statistical analysis. All data are presented as means ± SEM and n means the number of cells. Group differences were analyzed using the ANOVA and Tukey post hoc test for multiple comparisons unless otherwise stated. Difference was considered significant at $p < 0.05$. All the experiments were performed in a blind manner.

Results

Expression pattern of $Ca_V2.3$ channel in the cerebellum

We first examined the expression of $Ca_V2.3$ channel in the cerebellum from $Ca_V2.3+/-$ mice by assessing the β-galactosidase (β-gal) activity by X-gal staining. The gene coding for β-gal with a nuclear localization signal has been inserted into *Cacna1e* gene and the β-gal activity is thought to represent the native expression of the gene encoding $Ca_V2.3$ channel [16]. X-gal staining revealed that $Ca_V2.3$ channel was expressed in the cerebellar granule cell layer strongly and in the Purkinje cells weakly (Fig. 1A).

Motor learning in $Ca_V2.3$ mutant mice

If $Ca_V2.3$ channel contributes to the cerebellar function, the mutant mice may show some kind of deficit in motor coordination or learning. To test this possibility, we conducted a rotarod test using $Ca_V2.3$ mutant mice. At the rotation speed of 15 rpm, poor performance in the $Ca_V2.3+/-$ mice as well as $Ca_V2.3-/-$ mice was apparent. On day 2 and 3, the retention time of the $Ca_V2.3+/-$ and $-/-$ mice was much shorter than that of the $Ca_V2.3+/+$ mice (Fig. 1B). However, the retention time of the $Ca_V2.3+/-$ and $-/-$ mice on day 5 is not significantly different from that of $Ca_V2.3+/+$ mice.

Short-term plasticity in $Ca_V2.3$ mutant mice

To explore the physiological significance of $Ca_V2.3$ channel in cerebellum, we conducted several electrophysiological analyses. First we tested whether $Ca_V2.3$ channel is involved in the short-term plasticity at the PF-Purkinje cell synapse, because a possible correlation between short-term plasticity at the PF-Purkinje cell synapse and motor performance has been demonstrated in several mutant mice studies [21–23]. Using patch-clamp recordings from Purkinje cells, we investigated short-term plasticity at PF-Purkinje cell synapses in normal and $Ca_V2.3$ channel-deficient mice.