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Motor discoordination of transgenic mice overexpressing a microtubule destabilizer, stathmin, specifically in Purkinje cells

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Abstract

The proper regulation of microtubule (MT) structure is important for dendritic and neural circuit development. However, the relationship between the regulation of the MTs in dendrites and the formation of neural function is still unclear. Stathmin is a MT destabilizer, and we have previously reported that the expression and the activity of stathmin is downregulated during cerebellar Purkinje cell (PC) development. In this study, we generated transgenic mice that specifically overexpress the constitutively active form of stathmin in the PCs. These mutant mice did not show any obvious morphological or excitatory transmission abnormalities in the cerebellum. In contrast, we observed a decline in the expression of MAP2 and KIF5 signal in the PC dendrites and a discoordination of motor function in the mutant mice, although they displayed normal general behavior. These data indicate that the overexpression of stathmin disrupts dendritic MT organization, motor protein distribution, and neural function in PCs.

Keywords: Microtubule; Stathmin; MAP2; Kinesin; Purkinje cell; Dendrite; Motor coordination

1. Introduction

Microtubules (MTs) are an essential structural component of dendrites. MTs are highly dynamic structures that are regulated by various associating proteins and post-translational modifications. MTs serve as rails for the transport of macromolecules by various motor proteins. Hence, it is thought that the proper MT organization in neurons is required for the regulation of either the morphology or the function of the neurons.

The dendritic morphology of the cerebellar Purkinje cell (PC) is the most complicated among the various neuronal cell types. The function of the PC is important for the cerebellar neural function, including motor coordination and motor learning. The PCs receive two major inputs from the parallel fibers (PFs) and the climbing fibers (CFs). The PFs are axons of granule cells in the cerebellar granule cell layer. The CFs originate from the inferior olive of the medulla. Early in postnatal development, the PCs are innervated by multiple CFs. The supernumerary CFs are then eliminated so that only a single CF innervates each PC by the end of the 3rd postnatal week (Crepel, 1982; Kano et al., 1995, 1997, 1998). These processes of synapse formation between either the PFs or CFs and the PCs are required for the acquisition of proper cerebellar functions.

Stathmin is an MT destabilizer. The four-serine residues (Ser16/25/38/63) of stathmin are phosphorylated by various kinases, which reduces the MT-destabilizing activity (Cassimeris, 2002; Wittmann et al., 2004). The 4A mutant stathmin, in which all four serine residues are replaced

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with alanine residues, has a constitutive MT-destabilizing activity regardless of the extracellular stimuli (Gavet et al., 1998). Indeed, the overexpression of the 4A stathmin targets the dendritic development of cultured PCs *in vitro*, and causes a severe phenotype compared with the overexpression of the wild type protein (Ohkawa et al., 2007). Hence, stathmin is a useful genetic tool to modulate the dendritic MT states.

MT associated proteins contribute to the morphological and functional regulation of the neurons. MAP2 is one of the major MT associated proteins, and its association stabilizes the structure of the MTs (Goedert et al., 1991; Matus, 1994). In addition, members of the kinesin super family are also MT associated proteins that function as a MT-dependent motor. Several kinesins (KIFs) transport macromolecules on the dendritic MTs (Hirokawa and Takemura, 2005). The stability of the MTs mediates the neuronal function through a mechanism that is dependent on the KIF-based dendritic transport (Yuen et al., 2005). It is predicted that the expression pattern of these proteins in dendrites correlates with the stabilized state of the MTs and neuronal function.

In this study, we generated transgenic mice in which a constitutively active form of stathmin, 4A stathmin, was specifically expressed in the PCs. In the PC dendrites of these mice, the MAP2 and KIF5 signal were reduced. In addition, we observed the motor discoordination in the mutant mice, although the mice showed a normal appearance and a normal excitatory synaptic transmission in the cerebellum. Therefore, we concluded that a proper microtubule organization in the PCs is required for the formation of cerebellar neural function.

2. Materials and methods

2.1. Transgene construction

A mouse 4A *stathmin-myc* cDNA was obtained by PCR as previously described (Ohkawa et al., 2007). For the construction of the transgene, 4A *stathmin-myc* and *DsRed2* fragments were introduced into the EcoRI site and the SalI/ NotI site of the pIRES vector (Clontech), respectively. The *stathmin-myc*-IRES-*DsRed2* fragment was then cut out from the pIRES plasmid by XhoI and NotI, blunted with a T4 DNA polymerase reaction, and subcloned into exon 4 of the *L7* gene cassette (Oberdick et al., 1990; Ichise et al., 2000). The *L7* gene cassette is kindly donated from Dr. A. Aiba (Kobe University, Japan). The transgene was prepared by elimination of the plasmid region and microinjected into fertilized eggs.

2.2. Animals and brain sections

All procedures involving the use of the animals complied with the guidelines of the National Institute of Health and were approved by the Animal Care and Use Committee of Mitsubishi Kagaku Institute of Life Sciences (MITILS). C57BL/6 mice were purchased from CLEA Japan Inc. (Tokyo, Japan). To generate the transgenic mice, the transgene was injected into fertilized eggs of C57BL/6 mice and the eggs were transplanted into pseudopregnant females (Hogan et al., 1994). For the preparation of frozen brain sections, mice were sacrificed, and their brains were dissected and immediately frozen on dry ice. Cryosections (10 μm thick) were air-dried and stored at −80 °C until required for *in situ* hybridization and immunohistochemistry.

2.3. Antibodies and immunohistochemistry

Immunohistochemistry was performed as previously described (Ohkawa et al., 2007). The primary antibodies that were used include a mouse anti-MAP2 antibody (1:250, Chemicon), a rabbit anti-calbindin antibody (1:1000, Chemicon), a rabbit anti-neuron-specific class III β-tubulin (Mβ6) antibody (1:500, kindly donated by Dr. Y. Arimatsu at MITILS), a mouse anti-KIF5 antibody (1:200, Chemicon), and/or a mouse anti-Myc antibody (1:100, Santa Cruz), which were added to a blocking buffer and incubated at 4 °C overnight. After the PBS washes, the sections were incubated with FITC-, rhodamine-, and/or Cy5-conjugated secondary antibodies (Chemicon) at 4 °C overnight. The sections were washed and then mounted with ProLong Gold antifade reagents (Molecular Probes) and the fluorescent signals were examined with a laser-scanning confocal microscope (LSM 5 PASCAL, Carl Zeiss, Jena, Germany).

2.4. In situ hybridization

The *DsRed2* cDNA was amplified by PCR and subcloned into the vector pCRII-TOPO (Invitrogen). The vector was digested with SpeI or EcoRV to generate a template for the *in vitro* transcription of an antisense or sense cRNA probe, respectively. Digoxigenin-labeled cRNA probes were produced by transcription using T7 or Sp6 RNA polymerase. *In situ* hybridization was performed as previously described (Matsuo et al., 2000).

2.5. Electrophysiology

Parasagittal cerebellar slices (250-μm thickness) were prepared from mice aged P22–P59 days as described (Hashimoto et al., 2001; Hashimoto and Kano, 2003). Whole-cell recordings were made from visually identified PCs using an upright microscope (Olympus BX51WI) at 31 °C. The resistance of the patch pipettes were 3–6 MΩ when filled with an intracellular solution composed of: 60 mM CsCl, 10 mM Cs D-gluconate, 20 mM TEA–Cl, 20 mM BAPTA, 4 mM $MgCl_2$, 4 mM ATP, 0.4 mM GTP, and 30 mM HEPES (pH 7.3, adjusted with CsOH). The pipette access resistance was compensated by 70–80%. The composition of the standard bathing solution was: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 20 mM glucose, bubbled with 95% O_2 and 5% CO_2. Bicuculline (10 μM) was always added to the buffer to block the inhibitory synaptic transmission. Ionic currents were recorded with an Axopatch 1D (Axon Instruments). The signals were filtered at 2 kHz and digitized at 20 kHz. The on-line data acquisition and the off-line data analysis were performed using PULSE software (HEKA). The stimulation pipettes (5–10 μm tip diameter) were filled with standard saline and used to apply square pulses for focal stimulation (duration, 0.1 ms; amplitude, 0–90 V). The CFs were stimulated in the granule cell layer 50–100 μm away from the Purkinje cell soma. The PFs were stimulated in the molecular layer.

2.6. Behavior tests

Open field test. Each mouse was placed in the center of a 50 cm × 50 cm enclosure. The walking route of the mouse was traced with a behavioral tracing analyser (Muromachi Kikai, Tokyo, Japan). The total walking distance was recorded for 30 min.

Traction test. The grip strength of each mouse was measured using a traction apparatus (O'Hara and Co. Ltd., Tokyo, Japan). Each mouse was made to grasp the attached bar with the forefeet and was slowly pulled back by the tail. The maximum tension before release was recorded.

Rod-walking test. Each mouse was placed at the center of an elevated wooden rod (diameter, 12 mm or 7 mm; length, 60 cm). There were scaffolds on each side of the thin rod. The time spent moving from the start point to the end of the rod (length 30 cm) was measured.

Rota-rod test. Motor coordination was assessed with a rotating rod apparatus (O'Hara and Co. Ltd.), which consists of a plastic rod (3 cm diameter, 8 cm long) with a textured surface flanked by two large discs (40 cm diameter). Each mouse was placed on the rod, which began to rotate at a speed of 3 rpm. The retention time, which is the time that the mouse remains on the rod during rotation, was measured using an acceleration protocol in which the rotation speed was elevated from 3 rpm to 30 rpm in a linear manner over 10 min,