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Critical role of JSAP1 and JLP in axonal transport in the cerebellar Purkinje cells of mice

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ABSTRACT

JNK/stress-activated protein kinase-associated protein 1 (JSAP1) and JNK-associated leucine zipper protein (JLP) are structurally related scaffolding proteins that are highly expressed in the brain. Here, we found that JSAP1 and JLP play functionally redundant and essential roles in mouse cerebellar Purkinje cell (PC) survival. Mice containing PCs with deletions in both JSAP1 and JLP exhibited PC axonal dystrophy, followed by gradual, progressive neuronal loss. Kinesin-1 cargoes accumulated selectively in the swollen axons of *Jsap1*/*Jlp*-deficient PCs. In addition, autophagy inactivation in these mice markedly accelerated PC degeneration. These findings suggest that JSAP1 and JLP play critical roles in kinesin-1-dependent axonal transport, which prevents brain neuronal degeneration.

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades transmit signals through protein-phosphorylation relays. The specificity of MAPK signaling is mediated, at least in part, by scaffolding proteins, such as c-Jun NH_2-terminal kinase (JNK)-interacting protein 1 (JIP1) and JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JIP3 or Sunday Driver) [1–6]. Recent in vitro studies have suggested that JSAP1 and the structurally related JNK-associated leucine zipper protein (JLP, also known as JIP4 or SPAG9) [7–9] may be multifunctional proteins involved in diverse cellular processes, such as cell signaling, intracellular transport, and cytokinesis. JSAP1 and JLP were first identified as scaffolding proteins for mammalian JNK and p38 MAPK signaling modules [4,5,7,8]. They were subsequently shown to interact with the kinesin light chain (KLC) subunit of kinesin-1 (which contains two KLCs and two kinesin heavy chains [KHCs]), and were suggested to function as adaptor proteins that link cargoes and kinesin-1 [10,11]. JSAP1 also plays important roles in neuronal morphogenesis, including axonal elongation (through its interaction with KHC) [12,13], and branching [14,15]. Furthermore, in cycling cells, JSAP1 and JLP have been suggested to function as either effectors [16] or regulators of the subcellular localization [17] of the small GTPase, ADP-ribosylation factor 6, during cytokinesis. In some cases, such as axonal transport [18] and cytokinesis [17], JSAP1 and JLP have overlapping functions.

JSAP1 and JLP are highly expressed in the brain, and recent studies involving mouse knockouts have begun to uncover their physiological roles. *Jsap1* knockout (KO) mice die shortly after birth,

Abbreviations: APP, amyloid precursor protein; cDKO, conditional double KO; cKO, conditional KO; cTKO, conditional triple KO; Cyt *c*, cytochrome *c*; DAPI, 4,6-diamidino-2-phenylindole; DCN, deep cerebellar nuclei; floxed, *loxP*-flanked; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; IHC, immunohistochemistry; JIP, JNK-interacting protein; JLP, JNK-associated leucine zipper protein; JNK, c-Jun NH_2-terminal kinase; JSAP1, JNK/stress-activated protein kinase-associated protein 1; KHC, kinesin heavy chain; KLC, kinesin light chain; KO, knockout; MAPK, mitogen-activated protein kinase; PC, Purkinje cell; Phospho, phosphorylated; SYP, synaptophysin; SYT1, synaptotagmin 1

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most likely due to neural defects [19–21]. These mice exhibit various developmental brain defects, including an axon guidance defect of the corpus callosum, although abnormal vesicle accumulation is not observed in the axons [19,20]. In contrast, *Jlp* KO mice are viable and grow normally, exhibiting a lightened coat color and pale skin [22,23]. These deleterious and nearly normal phenotypes of *Jsap1* and *Jlp* KO mice, respectively, may hinder the functional analysis of JSAP1 and JLP in vivo. Recently, however, the conditional double KO (cDKO) of both *Jsap1* and *Jlp* revealed that they play crucial and functionally redundant roles in the developing mouse brain [18].

In this study, we show that JSAP1 and JLP play essential and functionally redundant roles in cerebellar Purkinje cell (PC) survival in mice, and that their deletion causes axonal degeneration followed by gradual, progressive neuronal loss. Our findings also indicated that JSAP1 and JLP regulate kinesin-1-dependent axonal transport in PCs, and that disruption of this process leads to neurodegeneration. Furthermore, we found that the deletion of JSAP1 and JLP in PCs resulted in the activation of macroautophagy (hereafter referred to as autophagy), which appeared to be a mechanism for controlling the extent of neurodegeneration in these mice.

2. Materials and methods

2.1. Animals

Jsap1$^{f/f}$ mice [21], *Jlp*$^{f/f}$ mice [18], and *Atg5*$^{f/f}$ mice [24] were generated previously. To generate PC-specific conditional knockout (cKO) mice, *Pcp2-Cre* transgenic mice [25] were obtained from the Jackson Laboratory and crossed with *Jsap1*$^{f/f}$, *Jlp*$^{f/f}$, and/or *Atg5*$^{f/f}$ mice. The animal experiments were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and approved by the Committee on Animal Experimentation of Kanazawa University.

2.2. Antibodies

The following primary antibodies were used for this study: anti-JSAP1 (1 μg/ml) [26], anti-JLP (2 μg/ml) [22], anti-calbindin D-28K (1:2000; C9848, Sigma–Aldrich, St. Louis, MO, USA or 1:1000; AB1778, Millipore, Bedford, MA, USA), anti-amyloid precursor protein (APP) (1:50; MAB348, Millipore), anti-cytochrome *c* (Cyt *c*) (1:500; #556432, BD Biosciences, San Jose, CA, USA), anti-synaptophysin (SYP) (1:200; S5768, Sigma–Aldrich), anti-synaptotagmin 1 (SYT1) (1:100; #105002, Synaptic Systems, Göttingen, Germany), anti-ubiquitin (1:100; Z0458, Dako, Glostrup, Denmark), anti-glial fibrillary acidic protein (GFAP) (1:500; G3893, Sigma–Aldrich), anti-LC3 (1:5000; #010-22841, Wako, Osaka, Japan), anti-phosphorylated (Phospho) JNK (1:100; #9251, Cell Signaling, Boston, MA, USA), and anti-α-tubulin (1:3000; T5168, Sigma–Aldrich) antibodies. The following secondary antibodies were used: Alexa 488- and 568-conjugated anti-mouse or anti-rabbit IgG (1:1000; Invitrogen, Rockville, MD, USA) and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (1:5000; GE Healthcare, Buckinghamshire, UK) antibodies.

2.3. Immunohistochemistry

Mice were deeply anesthetized and fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4. Then, brain samples were collected and post-fixed overnight at 4 °C. The samples were cryoprotected in 30% sucrose and embedded in Tissue-Tek OCT compound (Sakura Finetek, Kyoto, Japan). Immunohistochemistry (IHC) was performed as previously described [27]. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich). IHC images were acquired with a confocal laser scanning microscope (LSM510 META, Carl Zeiss, Oberkochen, Germany) with a 20× or 40× objective lens. To generate Z-stacking images, ten single-plane images (2-μm optical thickness) were acquired at 2-μm intervals. To capture overviews of the cerebellum, a fluorescent microscope with a 10× objective lens (BZ-9000, Keyence, Osaka, Japan) was used, and the images were combined using the image-joiner function in the BZ-Analyzer software (Keyence). All images were captured below saturation level.

2.4. Quantification of PC numbers

Two midsagittal 25-μm-thick frozen serial sections were prepared from control, single *Jsap1* or *Jlp* cKO, and *Jsap1*:*Jlp* cDKO mice as described above. The sections were then stained with the Nissl stain, cresyl violet. Optical images of Nissl-stained lobules (II to IX) were captured using an inverted microscope with a 20× objective lens (IX71, Olympus, Tokyo, Japan), attached to a CCD camera (DP50, Olympus). The Nissl-stained PCs in the lobules were then counted. Segments connecting the center of the soma of each PC along the entire length of the PC layer were created, and the sum of their lengths was determined. Using this length, the average number of PCs per μm was calculated from two serial sections per mouse.

2.5. Immunoblotting

Cerebellar total cell lysates were prepared and analyzed by immunoblotting as described previously [27]. Protein bands were detected with the Immobilon Western Chemiluminescence HRP Substrate (Millipore). Image J software was used to quantify the intensity of the protein bands.

2.6. Rotarod analysis

To investigate motor coordination and balance, an accelerating rotarod analysis was performed using 8- to 9-week-old mice. The mice were placed on a horizontal rubber-coated rod (30-mm diameter) and tested in three trials with a 300-s accelerating program (from 5 to 40 rpm) using a rotarod (RRAC-3002; O'Hara & Co. Ltd., Tokyo, Japan). The length of time each mouse remained on the rod before falling (retention time) was measured. The average retention time for each mouse was calculated from the results of three trials.

2.7. Statistical analysis

Significance was determined using the two-tailed unpaired Student's *t-test*. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Targeted deletion of Jsap1 and Jlp in PCs

To investigate the role of JSAP1 and JLP in cerebellar PCs, we generated mice with PC-targeted conditional deletions in *Jsap1*, *Jlp*, or both, using *loxP*-flanked (floxed) alleles of these genes, in combination with the *Pcp2-Cre* transgene. PC-specific deletion of *Jsap1* and *Jlp* was confirmed by the immunohistochemical analysis of 4-week-old control (*Jsap1*$^{f/f}$:*Jlp*$^{f/f}$) and cDKO (*Jsap1*$^{f/f}$:*Jlp*$^{f/f}$:*Pcp2-Cre*) mice (Fig. 1A). Notably, both the control and cDKO mice exhibited intense JSAP1-positive immunosignals in the cerebellar pinceau (see Fig. 1A)