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ALS2/alsin deficiency in neurons leads to mild defects in macropinocytosis and axonal growth

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ABSTRACT

Loss of function mutations in the *ALS2* gene account for a number of juvenile/infantile recessive motor neuron diseases, indicating that its gene product, ALS2/alsin, plays a crucial role in maintenance and survival for a subset of neurons. ALS2 acts as a guanine nucleotide exchange factor (GEF) for the small GTPase Rab5 and is implicated in endosome dynamics in cells. However, the role of ALS2 in neurons remains unclear. To elucidate the neuronal ALS2 functions, we investigate cellular phenotypes of ALS2-deficient primary cultured neurons derived from *Als2*-knockout (KO) mice. Here, we show that ALS2 deficiency results not only in the delay of axon outgrowth in hippocampal neurons, but also in a decreased level of the fluid phase horseradish peroxidase (HRP) uptake, which represents the activity for macropinocytic endocytosis, in cortical neurons. Thus, ALS2 may act as a modulator in neuronal differentiation and/or development through regulation of membrane dynamics.

ALS2 was identified as a causative gene for a number of recessive motor neuron diseases (MNDs), including autosomal recessive juvenile form of amyotrophic lateral sclerosis (ALS) called ALS2, juvenile primary lateral sclerosis (PLSJ) and infantile-onset ascending hereditary spastic paralysis (IAHSP) [1–3]. It is currently appreciated that loss of the ALS2 functions accounts for motor neuron dysfunction and/or degeneration in the *ALS2*-linked MNDs [3]. ALS2/alsin is a 184 kDa protein of 1657 amino acid residues and contains three putative guanine nucleotide exchange factor (GEF) domains; the regulator of chromosome condensation 1-like domain (RLD), the Dbl homology and pleckstrin homology (DH/PH), and the vacuolar protein sorting 9 (VPS9) [1–3]. In particular, the VPS9 domain is a hallmark of GEFs for the Rab5 GTPase family [4]. Indeed, it has been shown that ALS2 is a GEF for Rab5 *in vitro* and involved in endosome fusion through its Rab5GEF activity in the cells [5–7]. Further, ALS2 interacts with Rac1, a member of the Rho-type GTPase family, via its DH/PH domain [6,9] and acts as an effector for Rac1, rather than a Rac1GEF [8].

Recent studies have reported that *Als2*-knockout (KO) mice display age-dependent mild and subclinical levels of axonal degeneration in the spinal cord and the spinal ventral root [10–14]. ALS2-deficient cells from the *Als2*-KO mice also exhibit the delayed fusion of epidermal growth factor (EGF)-positive endosomes in fibroblasts [11] and the alteration of trafficking for neurotrophic factor receptors in neurons [12]. On the other hand, a reduction in ALS2 by a small interfering RNA (siRNA)-mediated ALS2 knockdown accounts for the cell death and severe inhibition of axonal growth in rat cultured spinal motor neurons [15]. Therefore, there is some discrepancy in phenotypes between ALS2-knocked-down rat neurons in the cell biological experiments and *Als2*-KO mice. Thus, it remains a matter of conjecture whether ALS2 has active implications in neuronal differentiation and development.

We have recently found that ALS2 is recruited to membranous compartments through Rac1-activated macropinocytosis, thereby mediating endosome fusion between macropinosomes and classical early endosomes in non-neuronal cells [8]. Rab5 is a key regulator for early endocytic pathway, such as various forms of endocytosis (internalization step), endosome fusion and trafficking [4]. Moreover, macropinocytosis is known to be a mode of Rab5-dependent endocytosis [16], which is induced by growth factors

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or mitogenic agents [17]. Since ALS2 is an activator for Rab5, it may regulate the internalization step of macropinocytic pathway in addition to endosome fusion in macropinocytosis-mediated membrane/vesicular trafficking. However, the contribution of ALS2 to the internalization step of macropinocytic pathway in cells including neurons remains unknown.

In the present study, by utilizing primary cultured neurons from *Als2*-KO mice, we examine whether ALS2 takes part in macropinocytosis and axonal outgrowth. We here show that ALS2 contributes to macropinocytosis and is implicated in axonal growth in neurons. Since the regulated membrane dynamics is required for axonal growth [18], ALS2 may play a role as a modulator in neuronal differentiation and/or development via regulation of membrane dynamics, such as macropinocytosis, vesicle trafficking, and endosome fusion.

Materials and methods

Antibodies and reagents. Anti-ALS2 antibody (Ab) was described in the previous study [5]. Anti-synaptophysin and anti-MAP2 Abs were obtained from Carbiochem (Darmstadt, Germany) and Sigma–Aldrich (Missouri, USA), respectively. Other reagents used were of analytical grade.

Animals. All animal experimental procedures were approved by the Tokai University Medical School Committee on Animal Care and Use. *Als2*-KO mice were described previously [11]. Mice expressing human ALS2 under the control of the human *ALS2* promoter (*ALS2*-tg mice; Hadano et al., unpublished) were also used in this study.

Cell cultures. Mouse embryonic fibroblasts (MEFs) and primary hippocampal neurons were cultured as previously described [11].

Measurement of axon length. A total of 1.5×10^4 cells were plated onto a poly-D-lysine-coated 12 mm round glass cover slip, and were incubated at 37 °C, 5% CO_2 for 36 h or 72 h. Then, the cells were fixed, permeabilized, and stained with anti-MAP2 Ab and Alexa 594-Phalloidin. The longest neurite of the cell with a subsided-MAP2 staining was defined as axon. Four individual cultures from the different pups with the same genotypes were subjected to the analysis. Axon length was determined by measuring the distance tracking along the longest neurite between the edge of nucleus and the tip of phalloidin-positive growth cone. At 36 h- and 72 h-points, 30 and 10 cells from each individual culture were analyzed, respectively. Each cell image was captured by Leica TCS-NT system and processed by ImageJ 1.37v (NIH, USA).

HRP uptake assay. Horseradish peroxidase (HRP) uptake was measured as previously described [16]. In brief, serum starved MEFs were cultured in Dulbecco-modified eagle's medium (DMEM) containing 1 mg/ml HRP and 10% (v/v) FBS for the designated period of times. After the incubation, the cells were washed with ice-cold PBS(−) containing 0.2% (w/v) bovine serum albumin (BSA), and then once again with ice-cold PBS(−). Collected cells were lysed with lysis buffer (150 mM NaCl, 0.1% (w/v) Triton X-100 (TX), 5% (w/v) glycerol). The resulting lysates were used for HRP activity assays. In the case of cultured neurons, $1–1.5 \times 10^6$ cells were plated onto a poly-D-lysine-coated 35 mm round dish and incubated for 36 h. The serum starvation was conducted for 30 min before inducing HRP uptake. The cells were then cultured in DMEM:F-12 1:1 medium containing 1 mg/ml HRP and 5% FBS with 1× B27 supplement for indicated periods. After the HRP uptake, the cells were washed with cooled-DMEM:F-12 1:1 medium containing 0.2% BSA twice and once again with ice-cold PBS(−) containing 0.2% BSA. The cells were transferred to a 1.5 ml tube followed by the centrifugation. After the rinse with ice-cold PBS(−) containing 0.2% BSA twice and once with ice-cold PBS(−), the cells were collected by the centrifugation and lysed with lysis buffer, followed by the re-centrifugation. The resulting supernatant from neuronal cultures was used for quantitative analysis of HRP enzymatic activity. The enzyme assay was conducted in a 96-well plate using *O*-phenylenediamine (SIGMA, Missouri, USA) as the chromogenic substrate. Results were expressed as OD490/μg protein.

Immunohistochemistry, immunocytochemistry, and statistical analysis. See Supplemental methods.

Results

Subcellular localization of ALS2 in primary cultured hippocampal neurons at an immature stage

To clarify the intracellular sites where ALS2 functions, we analyzed the subcellular localization of ALS2 in developing cultured hippocampal neurons. After several hours of plating, neurites begin to sprout from the cell body (Stage 2), and then a single axon and shorter processes are elongated within 48 h (Stage 3) [19]. At Stage 2–3, ALS2 was colocalized with F-actin, particularly in membrane ruffles at the edge of the growth cones (Fig. 1A–C). ALS2 was also localized onto F-actin-coated vesicles in the growth cones (Fig. 1D–F). Due to the lower sensitivity of the anti-ALS2 Ab used, we were unable to define the detailed subcellular localization of endogenous ALS2 molecules. To overcome this difficulty, we used hippocampal neurons from *ALS2*-tg mice. The *ALS2*-tg hippocampal neurons normally developed *in vitro* and were morphologically indistinguishable from wild-type (WT) neurons. Exogenously expressed ALS2 was predominantly present in the growth cones as well as in the cell bodies (Fig. 1G–I), consistent with the distribution of endogenous ALS2 in WT neurons. Notably, ALS2 was localized to membrane ruffles and the F-actin-coated large vesicles in the growth cones (Fig. 1J–L). These vesicles appear to share the features with those of macropinosomes [20], which are F-actin-coated, large (diameter; >0.2 μm) and phase-bright organelles [21] formed via a mode of endocytosis called macropinocytosis at a base of membrane ruffles [22,23].

It has been demonstrated that the recruitment of early endosome antigen 1 (EEA1) to the nascent macropinosome membrane occurs immediately after the dissociation of F-actins [8,24]. Further, we have previously shown that ALS2 is localized not only to F-actin-positive vesicles, but also to F-actin-negative/EEA1-positive matured macropinosomes in non-neuronal cells [8]. Consistently, ALS2 was present on EEA1-positive large endosomes in the growth cones (Fig. 1M–S), indicating that ALS2 is localized onto matured macropinosomes in developing neurons. We also analyzed the ALS2 localization at Stage 4–5, and found that ALS2 was also present on a subpopulation of tubulovesicular endosomes as previously reported (see Supplemental Figs. 1 and 2) [5,6].

ALS2 regulates axon outgrowth

siRNA-mediated ALS2 knockdown induces cell death and severe impairment of axonal growth in primary cultured rat spinal neurons [15]. Inconsistent with these findings, *Als2*-KO mice do not exhibit severe neurodegenerative phenotypes [10–14]. Thus, this discrepancy raises the question as to whether the complete loss of murine ALS2 affects on neuronal differentiation and development. To address this issue, we compared the length of axons (the MAP2-negative longest neurite, a typical image of neuron is shown in Fig. 2A) between *Als2*-KO and WT primary cultured hippocampal neurons at Stage 3. Compared with other neuronal cultures, such as cortical neurons, hippocampal cultures consist of a relatively homogenous population of large neurons with a constant number and length of axon, and thus are the most appropriate means to precisely measure the effect of the ALS2-deficiency on axonal outgrowth. After 36 h of culture, the ALS2-deficient neurons extended slightly but significant shorter axon ($p < 0.01$, Student's *t*-test) (Fig. 2B and C), although survival rates after plating and neurite number of the cell were not affected by the ALS2 deficit (data not shown). Notably, in our conditions, the axons of ALS2-deficient neurons ultimately grew to a comparable length with those of WT after 72 h of culture (Fig. 2C). Therefore, the lack of ALS2 does not halt but significantly slows the axonal growth. These results indicate that although the impact of complete depletion of ALS2 by *Als2*-KO on axonal growth was much smaller than the siRNA-mediated knockdown approaches on rat cultured spinal neurons [15], ALS2 plays a limited but discernible regulatory role in axonal growth in mouse neurons.

ALS2 plays an active role in macropinocytosis in neurons

The ALS2-localizing vesicles (>0.2 μm diameter, F-actin-coated or EEA1-positive) are close resemblance to nascent or maturated macropinosomes. Moreover, macropinocytosis is known to be

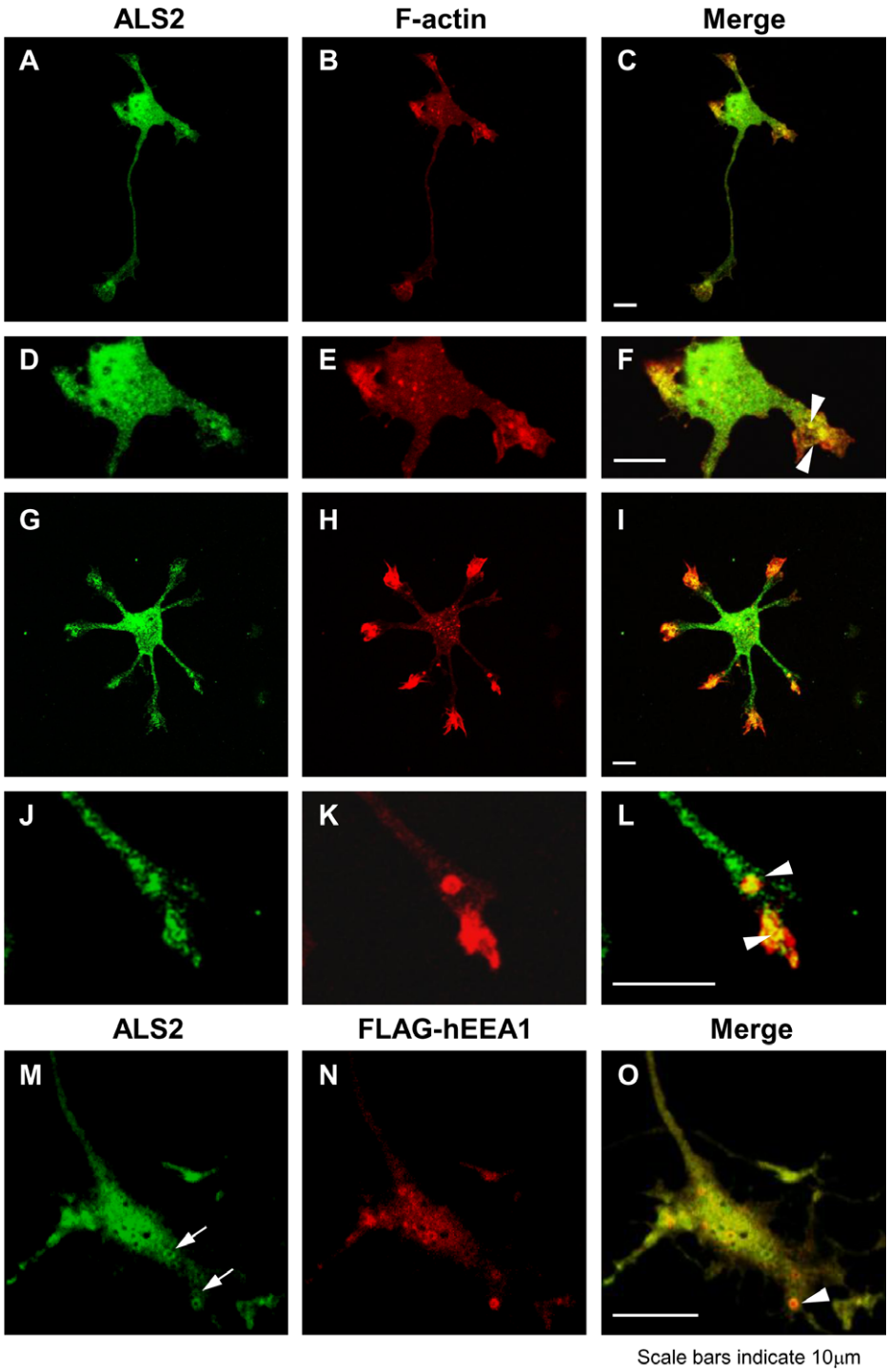

Fig. 1. Localization of ALS2 to lamellipodia and actin-positive vesicles and EEA1-labeled large endosomes in growth cones of immature cultured neurons. Primary cultured hippocampal neurons at either Stage 2 or Stage 3, which were derived from WT and *ALS2*-tg mice, were fixed and costained with anti-ALS2 polyclonal Ab (HPF1-1680) and phalloidin. ALS2 was enriched in growth cones of immature hippocampal neurons derived from both WT (A–F) and *ALS2*-tg mice (G–L) and showed a colocalization with F-actin (C, F, I, and L). In addition, ALS2 was present in vesicular structures throughout the cell bodies (D–F). Notably, the enlarged image revealed that ALS2 was localized onto F-actin-positive vesicles with variable sizes (F and L; arrowheads). ALS2-localizating large vesicles were either EEA1-positive (M–O; arrowhead) or EEA1-negative (M; arrows). Each confocal image was digitally-reconstructed by merging with 8 serial optical sections of 0.2 μm thickness. Each scale bar indicates 10 μm.

Rab5-dependent [16], and is induced by growth factors or mitogenic agents [21]. Since ALS2 is not only an activator for Rab5 [5,6], but also the molecules to be recruited to membranous compartments (ruffles) upon the stimulation by growth factor [8] and then onto macropinosomes (Fig. 1), it is conceivable that ALS2 directly enhances macropinocytosis.

To clarify the ALS2 contribution to macropinocytosis, we quantified HRP uptake in cortical neurons and fibroblasts from *Als2*-KO and WT mice. Fluid phase uptake of HRP was measured with or without the serum stimulation, since HRP uptake is primarily dependent on macropinocytosis under growth factor-stimulating conditions. As a result, the ALS2-deficient cortical neurons at the

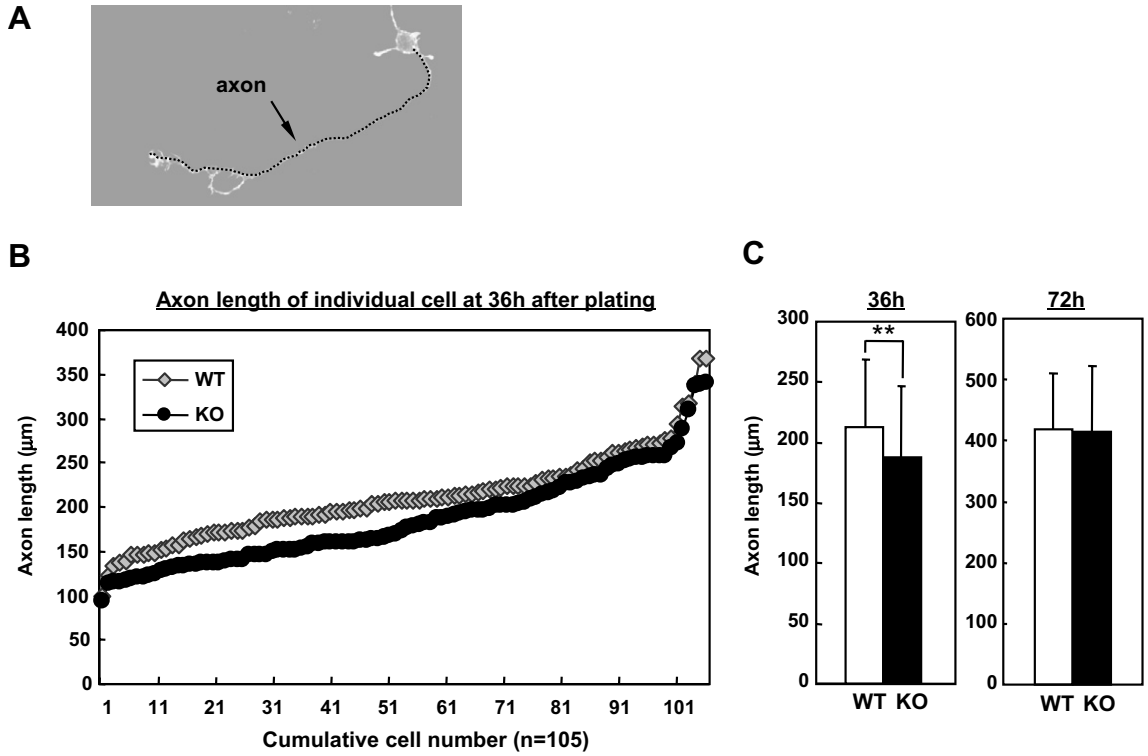

Fig. 2. Loss of ALS2 results in a mild defect in axon outgrowth. (A) Representative image of hippocampal neurons cultured for 36 h (axon was indicated as a broken line; arrow). (B) Measured axon lengths of *Als2*-KO (KO; black circle) and WT (WT; gray diamond) hippocampal neurons are displayed in an ascending order in the graph ($n = 105$). (C) A quantitative analysis of the axon length of hippocampal neurons at 36 and 72 h after plating are indicated. *Als2*-KO hippocampal neurons ($n = 105$) exhibited a mild but statistically significant defect in axonal growth compared with WT hippocampal neurons ($n = 105$) at 36 h ($p < 0.01$; between WT and KO). At 72 h, both wild-type ($n = 35$) and *Als2*-KO hippocampal neurons ($n = 36$) extended a comparative length of axons. Values of the axon length (μm) are presented as means ± SD, and analyzed for statistical significance using a Student's *t*-test (two-tailed, unpaired).

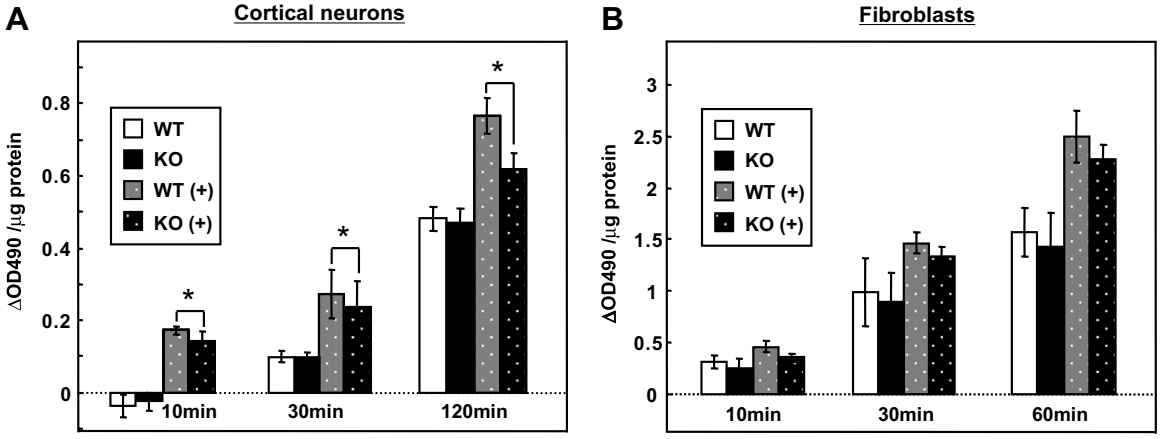

Fig. 3. ALS2 plays a role in fluid phase HRP uptake. (A) The HRP uptake in primary cultured cortical neurons (Stage 3) derived from *Als2*-KO and WT mice. The endocytic activities were measured after the incubation with HRP for 10, 30, and 120 min in the presence or absence of serum. Data are presented as means ± SEM (OD490/μg protein) ($n = 7$). At 120 min, the endocytic activity with serum stimulation of *Als2*-KO primary cultured cortical neurons was significantly lower than that of WT neurons ($^{*}p < 0.05$; ANOVA followed by Fisher's PLSD test). (B) The HRP uptake in *Als2*-KO and WT fibroblasts. Cells were incubated with HRP in the presence (KO(+); black column or WT(+); white column) or the absence of serum (KO; polka-dotted black column or WT; gray column) in the media, and harvested at indicated time points. The endocytic activity at each time point is presented as means ± SEM (OD490/μg protein) of five independent assays ($n = 5$). In fibroblasts, ALS2-deficiency did not affect the HRP uptake under experimental conditions used (ANOVA followed by Fisher's PLSD test).

early Stage 3 showed a lower level of HRP uptake than that of WT cells ($p < 0.05$, ANOVA followed by a Fisher's PLSD test, 19.27% down at 120 min) (Fig. 3A). On the other hand, in fibroblasts, there were no significant differences in the levels of the fluid phase HRP uptake at any time points assayed between *Als2*-KO and WT groups (Fig. 3B). These findings indicate that ALS2 contributes to neuronal macropinocytosis.

Discussion

Continuous supply of the plasma membrane to growing axon is required for axon outgrowth [18]. Further, endocytosis and recycling of membrane proteins (e.g., receptors and cell adhesion molecules) in the growth cone contributes to the growth cone motility, thereby regulating axonal growth [23]. Interestingly, it has been implied that

the Rab5 activation is required for endocytosis of the membrane proteins linked to neurite outgrowth [25]. Therefore, Rab5 and its activators, Rab5GEFs, may have implications in neurite outgrowth. Consistently, we (Fig. 2) and others [9,15] have shown that ALS2, a Rab5GEF, is involved in axonal growth. Further, overexpression of the Rab5GEF activity-defective ALS2 mutant significantly inhibits neurite outgrowth in PC12 cells (Otomo et al., unpublished). Thus, ALS2 may modulate membrane dynamics through its Rab5GEF activity which is functionally associated with axonal growth.

Another novel finding presented here was that ALS2 was involved in macropinocytosis in neurons (Fig. 3). Recently, the implication of several factors in macropinocytosis has been reported. Simultaneous activation of phosphatidylinositol-3-OH kinase, Rac1, and Rab5 induces dorsal ruffles through membrane ruffles, which is prerequisite for macropinocytosis [22]. Thus, Rab5, Rac1, and their activating factors; i.e., GEFs, localizing onto membrane ruffles and macropinosomes, may take part in macropinocytosis and the following macropinosome trafficking. Consistent with this notion, ALS2 is localized onto membrane ruffles and macropinosomes, and is involved in macropinocytosis. Although macropinocytosis is crucial for antigen presentation in dendritic cells [21], its role in other cell types including neurons remains to be elucidated. Our findings here might shed light on the macropinocytosis-mediated cellular process in neuronal cells.

It has been reported that the Rac1 activity is essential not only for macropinocytosis [21,22], but also for axonal growth [26]. As a notable ALS2 nature, ALS2 interacts with Rac1 [6,8,9], and acts as an effector for Rac1 during macropinocytic pathway in non-neuronal cells [8]. Although molecular mechanisms by which ALS2 is implicated in the axonal growth are not fully understood, the Rac1-mediated direct activation of ALS2 and the following ALS2-mediated Rab5 activation [8] may explain the ALS2 involvement in axonal growth. Further studies are needed to clarify the molecular interplay between ALS2 and Rac1 in axonal growth.

We here showed that ALS2-deficient cultured hippocampal neurons displayed minor but distinct abnormalities, which was consistent with the mild neuronal phenotypes in *Als2*-KO mice. On the other hand, a previous study has reported that siRNA-treated rat spinal neurons exhibit a severe impairment of axonal growth [15]. It is possible that this discrepancy is simply due to differences in the levels of ALS2 expression in each cell type. However, *Als2*-deficiency does not result in the impairment of axonal growth in cerebellar granule cells, where ALS2 is most highly expressed in central nervous system [11]. Thus, the level of the ALS2 expression is not a major factor for the differential effect of the ALS2 deficiency on axonal growth in particular neuronal cell types. Alternatively, by taking into consideration the fact that unlike severe neuronal dysfunctions in *ALS2*-linked MNDs in humans, ALS2-deficient mice merely showed mild neuronal phenotypes, a difference in species may account for the ALS2 contribution on axonal growth.

Although little is known about the molecular pathology and mechanisms for the ALS2-linked MNDs, infantile and/or juvenile dysfunction of motor neurons observed in patients imply that the affected neurons exhibit an abnormal development or premature degeneration. Our observations indicating the involvement of ALS2 in axonal growth are consistent with the view of such earlier neuronal dysfunctions in patients. Thus, the functional loss of ALS2 might result in the alteration of membrane dynamics, such as macropinocytosis and endosome trafficking, which may in turn lead to earlier neuronal dysfunction in the ALS2-linked diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.177.

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