ERAD components Derlin-1 and Derlin-2 are essential for postnatal brain development and motor function
in vitro. Derlin-1 or Derlin-2 deficiency reduced neurite outgrowth in brain development, particularly of the cerebellum and striatum, and induced motoneuronal loss and motor function.

Cholesterol biosynthesis, which is essential for appropriate postnatal brain development and motor control deficits. Derlin-1 deficiency reduced neurite outgrowth due to Derlin-1 deficiency was rescued by SREBP-2-mediated brain cholesterol biosynthesis. In addition, reduced neurite outgrowth due to Derlin-1 deficiency was rescued by SREBP-2 pathway activation. Overall, our findings demonstrate that Derlins sustain brain cholesterol biosynthesis, which is essential for appropriate postnatal brain development and function.

**INTRODUCTION**

To prevent misfolding of proteins and ensuing pathological endoplasmic reticulum (ER) stress, cells activate the unfolded protein response (UPR), which restores ER protein homeostasis by refolding or degrading unfolded proteins. The Derlin family members, Derlin-1, Derlin-2, and Derlin-3, are ER membrane proteins that associate with various other ER proteins, such as Sel1L, Hrd1, Herp, and p97, to form the ER-associated degradation (ERAD) complex, which eliminates unfolded proteins (Christianson et al., 2008; Lilley and Ploegh, 2004; Ye et al., 2004). In addition, Derlins contribute to ER protein quality control by facilitating the degradation of newly synthesized ER-targeted proteins, termed ER stress-induced pre-emptive quality control (ERpQC) (Kadowaki et al., 2015, 2018). In rodents, Derlin-1 and Derlin-2 mRNAs are ubiquitously expressed, including throughout the central nervous system (CNS), whereas Derlin-3 mRNA expression is restricted to specific tissues except the brain (Oda et al., 2006). Whole-body deletion of Derlin-1 causes lethality at embryonic day 7 (E7) to E8 (Eura et al., 2012), whereas most Derlin-2-deficient mice demonstrate perinatal lethality due to feeding failure, resulting in only 4% survival at weaning (Dougan et al., 2011). Unlike Derlin-1- or Derlin-2-deficient mice, Derlin-3-deficient mice are normally born and grow as well as wild-type mice (Eura et al., 2012). Therefore, Derlin-1 and Derlin-2 appear to be essential during ontogeny. We have reported that the interactions of Derlin-1 with amyotrophic lateral sclerosis-related superoxide dismutase 1 (SOD1) mutants trigger a pathological UPR, leading to motor neuron dysfunction (Nishitoh et al., 2008). However, the contributions of Derlin-1 to normal brain development have not been established.

Cellular cholesterol level is tightly controlled by transcriptional as well as posttranscriptional regulation of biosynthetic enzymes (Luo et al., 2020). Genes encoding many cholesterol biosynthetic enzymes are induced by activation of the ER membrane-anchored transcription factor sterol regulatory element binding protein 2 (SREBP-2). Under cellular cholesterol depletion, the ER transmembrane protein Scap escorts SREBP-2 from the ER to the Golgi apparatus, where it is sequentially cleaved by the Golgi-resident site-1 and site-2 proteases. Following cleavage, the amino-terminal form of SREBP-2 translocates to the nucleus, where it induces the transcription of cholesterol biosynthesis-related genes. In contrast, when...
excess cholesterol accumulates in the ER membrane, Scap interacts with the insulin-induced gene (Insig)-1 and Insig-2, resulting in the inhibition of Scap/SREBP-2 transport to the Golgi apparatus. In addition, the ERAD-related-E3 ubiquitin ligases RNF145, gp78, Hrd1, and MARCH6 ubiquitinate and degrade the cholesterol biosynthetic enzymes (van den Boomen et al., 2020). Another ER-resident E3 ligase TRC8 interacts with the Scap/SREBP-2 complex, which in turn inhibits cholesterol biosynthesis (Irisawa et al., 2009). Insigs are ubiquitinated by TRC8 and gp78 and degraded by the ERAD pathway only when unbound from Scap (Liu et al., 2012; Luo et al., 2020). Hence, it is still uncertain whether the ERAD pathway positively or negatively regulates cholesterol biosynthesis, including in the brain, which contains ~20%–25% of all cholesterol in the body (Dietschy and Turley, 2004).

In the present study, we generated CNS-specific Derlin-1- or Derlin-2-deficient mice and investigated changes in brain development and function. Both mice models exhibited widespread postnatal brain atrophy, which was particularly severe in the cerebellum and striatum, as well as reduced neurite outgrowth and motor function deficits. Both Derlin-1 and Derlin-2 deficiency, surprisingly, also suppressed SREBP-2-mediated cholesterol biosynthesis in the cerebellum, and activation of the SREBP-2 pathway rescued neurite outgrowth from Derlin-1-deficient neurons. Altogether, these findings illustrate that Derlins are indispensable for postnatal brain development and function by sustaining the SREBP-2-mediated cholesterol biosynthetic pathway.

RESULTS

Developmental defects in mouse brain due to deletion of Derlin-1 or Derlin-2

Derlin-1 and Derlin-2 are widely expressed in the mature CNS (Figure 1A), suggesting essential roles in physiological brain functions. We established CNS-specific deletion mutants by crossing mice harboring Derl1 and Derl2 genes flanked by loxP sites (Derl1flo/flo and Derl2flo/flo) with mice expressing Cre recombinase driven by the nestin promoter [Tg(Nes-Cre)1Kag mice] (Dougan et al., 2011; Isaka et al., 1999) (Figures S1A–S1D). The resulting Derl1flo/flo;Tg(Nes-Cre)1Kag (Derl1cKO mice) and Derl2flo/flo;Tg(Nes-Cre)1Kag (Derl2cKO mice) exhibited markedly reduced expression levels of Derlin-1 and Derlin-2 in the striatum, hippocampus, cerebral cortex, thalamus, midbrain, and cerebellum (Figure 1A). In addition, Derlin-1 deletion reduced the expression of Derlin-2 (Dougan et al., 2011; Kadowaki et al., 2015). The gross structure of the brain on postnatal day 0 (P0) appeared normal in both Derl1NesCre mice (Figures S1E and S1F) and systemic Derl2 deletion mice (Derl2−/−) (Dougan et al., 2011); however, Derl1NesCre and Derl2NesCre mice demonstrated substantial microcephaly with significant reductions in brain size, weight, and whole volume at 37 weeks of age (Figures 1B–1D). Serial sections through whole brains of these mice aged 37 weeks revealed particularly dramatic volume loss in cerebellum and striatum (Figure 1D). Atrophy of the cerebellum and striatum was observed in both Derl1NesCre and Derl2NesCre mice as early as 4 weeks of age and increased progressively up to 12 weeks of age compared with age-matched Derl1flo/flo and Derl2flo/flo control mice (Figures 1E and 1F). Derlin-1- or Derlin-2-deficient mice exhibited both lower brain and body weight compared with the control (Figures S1G and S1H). The brain and body weight correlated with each other in the Derl1NesCre but not in the Derl2NesCre mice (Figures S1I and S1J). Although we cannot exclude the possibility that brain atrophy depends on body growth inhibition, there might be another mechanism by which CNS-specific Derlin-1 or Derlin-2 deficiency induces brain atrophy. Taken together, Derlin-1 and Derlin-2 are both critical for postnatal brain development, particularly for normal growth of the cerebellum and striatum, whereas gross embryonic development appears to progress normally in the absence of these proteins.

Requirement of Derlin-1 and Derlin-2 for neurite outgrowth

Immunohistological staining using an anti-NeuN antibody was conducted to demonstrate the extent of cerebellar atrophy in Derl1NesCre and Derl2NesCre mice (Figure S2A). Quantitative analysis of the entire cerebellum revealed developmental defects in the molecular layer containing dendrites of Purkinje cells (Figure S2B). In several neurodegenerative disorders (e.g., spinocerebellar ataxia), the number of Purkinje cells is reduced owing to enhanced death rate, leading to cerebellar atrophy and functional deficits (Koeppen, 1998). In contrast, the number of calbindin-positive Purkinje cells per unit length of the cell body layer was not reduced in Derl1NesCre and Derl2NesCre mice compared with corresponding Derl1flo/flo and Derl2flo/flo control mice (Figures S2C and S2D). Similarly, the number of NeuN-positive neurons per unit area of striatum was not affected by Derlin-1 or Derlin-2 deficiency (Figures S2E and S2F). Hence, we hypothesized that Derlin-1 and Derlin-2 deficiencies may induce regional atrophy by suppressing the morphological maturation of individual neurons rather than by reducing cell number. We then investigated possible failure of...
Figure 1. Developmental defects in the brains of Derlin-1- and Derlin-2-deficient mice

(A) Expression of Derlin-1 and Derlin-2 in the brains of Derl1NesCre and Derl2NesCre mice at 4 weeks of age. Tissue extracts were analyzed by immunoblotting (IB) with the indicated antibodies. NesCre (+), Derl1NesCre or Derl2NesCre, NesCre (-), Derl1f/f or Derl2f/f.

(B) Representative gross brain images of mice aged 37 weeks.

(C) Brain weights of mice aged 37 weeks.

(D) Estimated volume (fold) of different brain regions:
- Whole
- Hippocampus
- Cerebral cortex
- Striatum
- Cerebellum

(E, F) Estimated volume of cerebellum and striatum over time (4w, 12w):
- Derl1f/f (n=3)
- Derl1NesCre (n=3)
- Derl2f/f (n=3)
- Derl2NesCre (n=3)
Derlin-1 deficiency-induced atrophy was also observed in the cerebral cortex (Figure 1D), which provides a convenient source of embryonic neurons to examine the effects of Derlins on neurite length more precisely under controlled in vitro conditions. Indeed, cultured primary cortical neurons derived from Derlin-1-deficient mice also exhibited shorter neurites compared with neurons derived from Derl1f/f mice (Figures 2H and 2I). This in vitro primary neuron culture contained only neurons without other cell types, such as astrocytes and microglia, suggesting that Derlin-1 might be required for a cell-autonomous dendritic outgrowth. Although Derlin-1-deficient neurons could potentially exhibit a delay in the dendritic outgrowth, our results further demonstrate that Derlin-1 promotes the elongation of dendrites, a process necessary for postnatal development of functional neural circuits.

Many degenerative diseases are associated with neuroinflammation as evidenced by the appearance of reactive astrocytes and activated microglia in atrophied brain regions. The cerebellum and striatum of Derl1NesCre mice exhibited more S100β- and glial fibrillary acidic protein (GFAP)-double-positive (immunoreactive) astrocytes and Iba1-positive (active) microglia than those of the control (Figures S3A–S3F). However, Derl2NesCre mice did not exhibit enhanced numbers of reactive astrocytes and microglia in the cerebellum or striatum (Figures S3G–S3L); they demonstrated both regional brain atrophy (Figure 1D) and functional motor deficits (see Figure 3). Hence, neuroinflammation does not appear necessary for the observed abnormalities in regional brain structure.

**Motor dysfunction due to Derlin-1 or Derlin-2 deficiency in the brain**

Neural circuits encompassing striatal and cerebellar neurons are critical mediators of motor control; therefore, regional atrophy may impair performance on motor function tests. In the rotarod test, Derl1NesCre and Derl2NesCre mice demonstrated significantly shorter latencies to lose balance and fall off compared with Derl1f/f and Derl2f/f control mice (Figures 3A and 3B), suggesting impaired motor coordination. A previous study reported that rotarod performance is negatively correlated with body weight (McFadyen et al., 2003), which may account for performance differences among some transgenic lines. However, Derl1NesCre and Derl2NesCre mice were lighter than control mice in body weight (Figures 5G and S1H); hence, the reduction in rotarod performance was likely weight independent.

To further assess motor coordination deficits, we conducted beam-walking tests in which mice were required to traverse an elongated cylindrical rod. Both Derl1NesCre and Derl2NesCre mice fell off more frequently than corresponding control mice (Figures 3C and 3D), and Derl1NesCre and Derl2NesCre mice moved more slowly across the rod than control mice during all three trials (Figures 3E and 3F). Together, these results indicate that neuron-specific Derlin-1 or Derlin-2 deficiency impairs motor function and coordination. However, Cre recombinase driven by the nestin promoter also deletes the target genes flanked by loxp sites in astrocytes and oligodendrocytes. To examine the unique contributions of striatal and cerebellar neurons to these functional deficits, we established neuron-specific deletion mutants by crossing Derl1f/f mice with mice expressing Cre recombinase driven by the CaMKII promoter (Karpati et al., 2019). The Derl1f/f; C57BL/6-TgN(a-CaMKII-nlCre)/10 (Derl1CaMKII-nlCre) mice exhibited reduced Derlin-1
Figure 2. Requirement of Derlin-1 for neurite outgrowth

(A–C) Morphological analysis of cerebellar Purkinje cells at 30 weeks of age. (A) Representative images of Golgi-stained Purkinje cells, (B) quantification of the dendritic area, and (C) the number of branches. The dendritic area was measured using ImageJ software. Ten Purkinje cells were measured in each mouse, and the average from three unrelated mice per genotype are presented.

(D–G) Morphological analysis of striatal MSNs at 30 weeks of age. (D) Representative images of Golgi-stained MSNs. (E) Sholl analysis showing the number of dendritic intersections at the indicated distances from the soma. (F) Quantification of total dendritic length and (G) number of branches. Ten MSNs were measured from each mouse, and the average from three unrelated mice per genotype is presented.

(H and I) Morphological analysis of primary cultured cortical neurons derived from mouse embryos at 6 days in vitro. (H) Representative immunofluorescence images of βIII-tubulin- and MAP2-positive neurons. (I) The dendritic length was measured using ImageJ software. More than 25 primary cultured neurons from each embryo were measured, and the average from three unrelated embryos per genotype are presented.

Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 by Student’s t test (B, C, F, G, and I) or repeated measures ANOVA (E). See also Figures S2 and S3.
Figure 3. Motor dysfunction of CNS-specific Derlin-1- and Derlin-2-deficient mice

(A and B) Rotarod test performance at 11–12 weeks of age. Each mouse performed three trials per day for a total of six trials over 2 days.

(C–F) Beam-walking performance at 32 weeks of age (C and E) and 26–27 weeks of age (D and F). Shown are the number of mice that fell off one or more times of three trials (Fell) and the number that safely reached the platform three of three trials (Clear). The time required to walk across the beam was also recorded to calculate movement speed.

(G) Volumetric analysis of brain regions at 12 weeks of age. Volumes were estimated as described in Figure 1D.
expression levels markedly in the striatum, hippocampus, cerebral cortex, thalamus, and midbrain, and partially in the cerebellum (Figure S4A). Consistent with observations in Derl1NesCre mice, Derl1CaMKIICre mice also exhibited brain atrophy (Figures S4B and S4C) and significant volume loss in the cerebellum and striatum (Figure 3G). Moreover, Derl1CaMKIICre mice fell off the beam more frequently during the beam-walking test than control mice (Figure 3H) and were significantly slower than control mice (Figure 3I). Of interest, neuroinflammation was observed in the striatum, but not in the cerebellum, of Derl1NesCre mice (Figures S3M and S3N). Together, our findings suggest that neuronal Derlin-1 contributes to brain development and motor function.

ER stress response in the cerebella of Derlin-1- and Derlin-2-deficient mice

To elucidate the molecular mechanisms by which Derlin-1 or Derlin-2 deletion reduces neurite outgrowth and disrupts brain development, we compared cerebellar gene expression profiles among Derl1NesCre, Derl2NesCre, and control (Derl1f/f and Derl2f/f) mice at P28 using DNA microarrays. In cerebella of Derl1NesCre and Derl2NesCre mice, expression levels of 4,017 and 1,984 genes were upregulated (>1.5-fold) and 1,854 and 2,015 downregulated (<0.67-fold) compared with control mice, respectively (Tables S1 and S2). In both Derl1NesCre and Derl2NesCre mice, 741 genes showed higher than 1.5-fold expression (Figure S5A). Among them, 13 genes were categorized as ER stress-responsive genes by gene ontology (GO) annotation (Figures 4A, 4B, and S5A). To examine whether Derlin-1 or Derlin-2 deficiency activates the UPR pathway, we analyzed Derl1NesCre, Derl2NesCre, and control mice cerebellar total RNA samples using quantitative real-time PCR (qPCR). Derlin-1 or Derlin-2 deficiency increased the spliced Xbp1 (Xbp1s) and Chop mRNA levels (Figures S5B and S5C). Moreover, the immunoblotting analysis revealed the increased expression of the ERAD complex, SEL1L, HRD1, and OS9, just as well as the activation of the IRE1α-XBP1 and PERK-eIF2α pathways, suggesting that Derlin deficiency may alter the ERAD function (Figures 4C and S5D–S5K). To investigate whether ER stress could contribute to the dendrite shortening of the Derlin-1-deficient neurons, we treated primary cortical neurons with the chemical chaperon 4-phenylbutyric acid (4-PBA). The 4-PBA treatment significantly mitigated the UPR in the Derl1NesCre mouse neurons (Figures 4D and 4E). However, surprisingly, the treatment with 4-PBA had no effect on the reduced neurite outgrowth of Derlin-1-deficient neurons (Figures 4F and 4G), suggesting that impaired ER quality control may not contribute to the reduced neurite outgrowth and disrupted brain development observed in Derl1NesCre and Derl2NesCre mice.

Requirement of Derlin-1 and Derlin-2 for cholesterol biosynthesis in the cerebellum

In addition to the ER stress-related genes, we found significant enrichment of cholesterol biosynthesis-related genes among downstream targets of Derlin-1 (Figures S5A and S5B). Efficient cholesterol biosynthesis is essential for neurite elongation because neuronal membranes contain high levels of cholesterol (Heacock et al., 1984). To examine whether Derlin-1 and Derlin-2 regulate the transcription of cholesterol biosynthesis-related genes, total RNAs harvested from the cerebella of Derl1NesCre, Derl2NesCre, and control mice were analyzed using qPCR. Derlin-1 or Derlin-2 deficiency reduced the mRNA levels of many genes encoding components of the cholesterol biosynthetic pathway, *Hmgcs1*, *Hmgcr*, *Mvk*, *Fdft1*, *Cyp51*, and *Dhcr24* (Figure 5C). Moreover, total cholesterol was significantly reduced in the cerebella of Derl1NesCre and Derl2NesCre mice (Figures 5D and 5E).

Many cholesterol biosynthetic enzyme genes are induced by the transcription factor SREBP-2, which resides in the ER until translocation to the nucleus, suggesting that Derlins in the ER may normally serve to enhance cholesterol production by promoting nuclear SREBP-2 activity. To address this question directly, we compared expression of ER (precursor) and nuclear (active) forms of SREBP-2 in cerebellar membrane and nuclear extracts from knockout and control mice. To test the feasibility of this approach, we first examined expression in extracts from human hepatoma HepG2 cells, a line demonstrating robust SREBP-2 activity, under excess cholesterol and cholesterol depletion. The 125 kDa band in the membrane fraction and the 68 kDa band in the nuclear fraction from HepG2 cell extracts were also detected in cerebellar extract from wild-type C57BL/6 mouse (Figure S6A). The amount of precursor (p)SREBP-2 was...
A

Response to ER stress

Den1^[1st] vs Den1^[1st]Eos
Upregulated: Den1^[1st]Eos
Normalized enrichment score = 2.4604
P value < 0.0001

B

Response to ER stress

Den2^[2nd] vs Den2^[2nd]Eos
Upregulated: Den2^[2nd]Eos
Normalized enrichment score = 1.5217
P value = 0.0019

C

| Protein  | Den1^[1st] | Den1^[1st]Eos | Den1^[1st]EosKOE |
|----------|------------|---------------|------------------|
| SEL1L    |            |               |                  |
| HRD1     |            |               |                  |
| P-JIP1a  |            |               |                  |
| P-JIP2a  |            |               |                  |
| BIP      |            |               |                  |
| Actin    |            |               |                  |
| IRE1a    |            |               |                  |
| PERK     |            |               |                  |
| OS9      |            |               |                  |
| gp78     |            |               |                  |
| Actin    |            |               |                  |

D

Relative gene expression (fold)

Xbp1s

E

Relative gene expression (fold)

Chop

F

MAP2

Den1^[1st] + vehicle

MAP2

Den7^[1st]Eos + 4PBA

G

Relative dendrite length (fold)

P = 0.963
in the cerebellum and striatum also exhibited reduced neurite outgrowth, and consistent with neural circuit dysfunction in these regions, both mouse lines demonstrated impaired motor performance. We speculate that these motor deficits are due to insufficient processing capacity by motor control circuits comprising these maldeveloped striatal and cerebellar neurons. An alternative explanation is that Derlin perturbs

Figure 4. ER stress response in the cerebella of Derlin-1- and Derlin-2-deficient mice

(A and B) Heatmap (left) and gene set enrichment analysis (GSEA, right) showing differential expression of 235 genes related to the GO term “Response to ER stress.” Genes upregulated by Derlin deletion are indicated in red and downregulated genes in green. GSEA shows gene expression changes in the cerebellum of Derl1NesCre or Derl2NesCre mice relative to control mice. The enrichment plot shows the distribution of genes in the set of “Response to ER stress” that are positively (red) or negatively (blue) correlated with Derlin-1 or Derlin-2 deficiency. (C) Expression levels of ER stress-related molecules in the cerebellum at 4–5 weeks of age. Whole tissue lysates from cerebella of Derl1f/f, Derl1NesCre, Derl2f/f, Derl1CaMKIICre, Derl2f/f, and Derl2NesCre mice were analyzed by IB with the indicated antibodies. (D–G) A chemical chaperon 4-PBA does not mitigate the reduced neurite outgrowth observed in Derlin-1-deficient neurons. Cortical neurons derived from Derl1f/f and Derl1NesCre embryos were treated with vehicle (water) or 1 mM 4-PBA and cultured for 3 days. Gene expression levels of Xbp1s and Chop in neurons were estimated by qPCR and normalized to that of S18 (D and E). Neurons were stained with anti-MAP2 antibody (F). Dendritic length of MAP2-positive neuron was quantified using ImageJ software (G). One hundred neurons were measured in each culture dish and averaged to obtain n = 1. Bar graphs are presented as mean ± SEM. *P < 0.05 by Student’s t test. n indicates independent dishes (D, E, and G).

Requirement of SREBP-2-mediated cholesterol biosynthesis for neurite outgrowth

Derl1NesCre embryo-derived cultured primary cortical neurons also exhibited reduced mRNA levels of several cholesterol biosynthesis-related genes (Figure S7A) and significantly lower cholesterol per milligram protein compared with Derl1f/f mouse-derived neurons (Figure 6A). Taken together, these results suggest that Derlin contributes to the maintenance of brain cholesterol biosynthesis. To investigate whether cholesterol biosynthesis would be required for neurite outgrowth, we treated the primary cultured cortical neurons with lovastatin, a strong cholesterol biosynthesis inhibitor that blocks HMG-CoA reductase. The treatment with lovastatin significantly increased cholesterol biosynthetic genes, suggesting that cholesterol was depleted in primary cortical neurons (Figure S7B). Lovastatin-treated neurons exhibited shortened neurite outgrowth (Figures S7C and S7D). To investigate whether SREBP-2 activation is sufficient to restore normal neurite outgrowth, primary cultured Derl1NesCre neurons were transfected with lentivirus-encoded control Venus (GFP variant) or Venus-tagged human SREBP-2 (1–481), which activates the cholesterol biosynthetic pathway (Figure S7E), and expression levels of cholesterol biosynthetic genes and neurite length were compared. Consistent with impaired cholesterol synthesis under Derlin deficiency contributing to reduced neurite outgrowth, exogenously expressed SREBP-2 (1–481) significantly restored the reduced expression levels of biosynthetic genes (Hmgcs1, Hmgcr, Mvk, Fdft1, and Cyp51) in Derl1NesCre neurons (Figure 6B) and increased neurite length (Figures 6C and 6D). Although there is a possibility that the overexpression of Derlins induces the neurite outgrowth independently of cholesterol biosynthesis, exogenously expressed Derlin-1 (Figure S7F) or Derlin-2 (Figure S7G) had no effect on the neurite outgrowth of wild-type cortical neurons in which cholesterol biosynthesis is not inhibited (Figures S7H and S7I). Collectively, our findings indicate that inhibition of SREBP-2-mediated cholesterol biosynthesis by deletion of Derl1 or Derl2 reduces neurite outgrowth and disrupts normal brain development, whereas we cannot exclude the possibility that the altered ERAD function by deletion of Derlins may contribute to shorter neurites to some extent. This abnormal development was particularly pronounced in the cerebellum and striatum, resulting in motor dysfunction.

DISCUSSION

In this work, we provide evidence that Derlin-1 and Derlin-2 have an unexpected function required for normal postnatal brain development and function. Both Derlin-1- and Derlin-2-deficient mice exhibited widespread postnatal brain atrophy, which was particularly severe in the cerebellum and striatum. Neurons in the cerebellum and striatum also exhibited reduced neurite outgrowth, and consistent with neural circuit dysfunction in these regions, both mouse lines demonstrated impaired motor performance. We speculate that these motor deficits are due to insufficient processing capacity by motor control circuits comprising these maldeveloped striatal and cerebellar neurons. An alternative explanation is that Derlin perturbs
An important question is how Derlin-1 and Derlin-2 regulate the SREBP-2 pathway. When excess cholesterol accumulates in the ER membrane, Insigs prevent recruitment of the Scap/SREBP-2 complex to COPII-coated vesicles and, thereby, halt SREBP-2 transport to the Golgi apparatus. Insig-1 is ubiquitinated by the UPR, which has also been reported to induce severe brain maldevelopment (Alimov et al., 2013; Passe-mard et al., 2019). However, we suggest that an ER stress response triggered by impaired ER quality control does not contribute to the observed postnatal brain atrophy in Derlin-deficient mice because the treatment of primary Derlin-1-deficient neurons with 4-PBA did not mitigate the shortened neurite outgrowth (Figure 4F).

In conclusion, our findings demonstrate an unexpected function of Derlins in brain development via regulation of cholesterol biosynthesis. Although further investigation is necessary to clarify the precise mechanisms by which Derlin-1 and Derlin-2 regulate SREBP-2 activation, we propose that Derlins may be a therapeutic target to ameliorate or delay the progression of neurodegenerative diseases.

Limitations of the study
An important question is how Derlin-1 and Derlin-2 regulate the SREBP-2 pathway. When excess cholesterol accumulates in the ER membrane, Insigs prevent recruitment of the Scap/SREBP-2 complex to COPII-coated vesicles and, thereby, halt SREBP-2 transport to the Golgi apparatus. Insig-1 is ubiquitinated by the UPR, which has also been reported to induce severe brain maldevelopment (Alimov et al., 2013; Passe-mard et al., 2019). However, we suggest that an ER stress response triggered by impaired ER quality control does not contribute to the observed postnatal brain atrophy in Derlin-deficient mice because the treatment of primary Derlin-1-deficient neurons with 4-PBA did not mitigate the shortened neurite outgrowth (Figure 4F).

In conclusion, our findings demonstrate an unexpected function of Derlins in brain development via regulation of cholesterol biosynthesis. Although further investigation is necessary to clarify the precise mechanisms by which Derlin-1 and Derlin-2 regulate SREBP-2 activation, we propose that Derlins may be a therapeutic target to ameliorate or delay the progression of neurodegenerative diseases.
by gp78 and degraded by the ERAD pathway during cholesterol depletion, whereas the sterol-induced binding of Insig-1 to Scap prevents degradation (Gong et al., 2006; Tsai et al., 2012). Because Derlin-1 interacts and coordinates with gp78 in the ERAD complex (Bernardi et al., 2010; Kadowaki et al., 2018), Derlin deficiency may stabilize Insig-1 and anchor Scap/SREBP-2 to the ER membrane. Indeed, SREBP-2 immunoreactivity was elevated in the membrane fraction but reduced in the nuclear fraction of cerebellar lysates from Derlin-deficient mice (Figures 5F and 5I). Since the significant down-regulation of the SREBP-1 pathway was not observed in the cerebella of Derl1NesCre mice (Figures S6B–S6E), there might be a specific Derlin-mediated regulatory mechanism for SREBP-2 activation. Further studies are needed to determine the precise molecular mechanisms by which Derlins regulate SREBP-2 nuclear translocation and brain cholesterol biosynthesis.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

Figure 6. Rescue of shortened neurite outgrowth of Derlin-1-deficient cortical neuron by exogenously expressed active form of SREBP-2

(A) Quantification of the amount of cholesterol per milligram protein in 6 DIV primary cultured cortical neurons derived from Derl1f/f and Derl1NesCre embryos.

(B–D) Rescue of shortened neurite outgrowth of Derlin-1-deficient neuron by active form of SREBP-2. Gene expression levels of cholesterol biosynthesis-related genes in neurons were estimated by qPCR and normalized to that of S18 (B). Neurons were stained with anti-MAP2 and GFP antibodies (C). Dendritic length of MAP2-positive neuron and MAP2- and GFP-double-positive neuron was quantified using ImageJ software (D). More than 80 neurons were measured in each culture dish and averaged to obtain n = 1.

Bar graphs are presented as mean ± SEM. *P < 0.05 and **P < 0.01 by Student’s t test. n indicates the number of unrelated culture dishes. See also Figure S7, Table S3.
RESOURCE AVAILABILITY
- Lead contact
- Materials availability
- Data and code availability

EXPERIMENTAL MODEL AND SUBJECT DETAILS
- Animals
- Primary cultures of cortical neurons

METHOD DETAILS
- Cell culture
- Tissue preparation for biochemical analysis
- Tissue preparation for immunofluorescence
- Immunohistochemistry
- Golgi staining
- Volumetric analysis and cell counting
- Immunoblotting
- Evaluating SREBP-2 processing in cultured cells
- Lentivirus production and infection
- Immunocytochemistry
- Morphological analysis of cultured cortical neurons
- Quantitative real-time PCR analysis
- DNA microarray analysis
- Cholesterol assay
- Rotarod test
- Beam-walking test

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102758.

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AUTHOR CONTRIBUTIONS
T.S. conceptualization, investigation, and writing-original draft. N.M. conceptualization, funding acquisition, investigation, and writing-original draft. H.Kadowaki conceptualization, funding acquisition, investigation, supervision, and writing-review and editing. K.Takao, T.M., and T.K. resources and methodology. Y.M. investigation and methodology. A.F., Y.S., H.Kawasaki, K.Takeda, K.M., and H.L.P. resources. J.S. methodology. K.S. and M.N. supervision. H.I. resources, funding acquisition, and supervision. H.N. conceptualization, data curation, supervision, funding acquisition, project administration and writing-original draft, review, and editing.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We worked to ensure diversity in experimental samples through the selection of the cell lines. We worked to ensure diversity in experimental samples through the selection of the genomic datasets. The author list
of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit-anti-Derlin-1| (Nishitoh et al., 2008) | N/A         |
| Rabbit-anti-Derlin-2| MBL International | PM019, RRID: AB_593007 |
| Mouse-anti-Actin    | Sigma-Aldrich | A4700, RRID: AB_476730 |
| Rabbit-anti-SREBP2  | Abcam   | ab30682, RRID: AB_779079 |
| Mouse-anti-Lamin B1 | Thermo Fisher Scientific | 33-2000, RRID: AB_2533106 |
| Rabbit-anti-Calnexin| Abcam   | ab22595, RRID: AB_2069006 |
| Mouse-anti-NeuN     | Millipore | MAB377, RRID: AB_2298772 |
| Mouse-anti-Calbindin-D-28K | Sigma-Aldrich | C9848, RRID: AB_476894 |
| Chicken-anti-Glial Fibrillar Acidic Protein | Millipore | AB5541, RRID: AB_177521 |
| Mouse-anti-S-100 (α-Subunit) | Sigma-Aldrich | s2532, RRID: AB_477499 |
| Rabbit-anti-Iba1    | Wako    | 019-19741, RRID: AB_839504 |
| Rabbit-anti-Tubulin β-3 | Covance | PRB-435P, RRID: AB_2564645 |
| Guinea pig-anti-MAP2| Synaptic System | 188 004, RRID: AB_2138181 |
| Mouse-anti-MAP2     | Sigma-Aldrich | M4403, RRID: AB_477193 |
| Mouse-anti-GFP      | MBL International | M048-3, RRID: AB_591823 |
| Rabbit-anti-SEL1L   | Abcam   | ab78298, RRID: AB_2285813 |
| Rabbit-anti-HRD1    | Sigma-Aldrich | H7915, RRID: AB_1840939 |
| Rabbit-anti-P-eIF2α | Invitrogen | 44-728G, RRID: AB_1500038 |
| Mouse-anti-BiP      | MBL International | M181-3, RRID: AB_10693914 |
| Rabbit-anti-IRE1α   | Cell signaling | 3294, RRID: AB_823545 |
| Rabbit-anti-PERK    | Cell signaling | 3192, RRID: AB_2095847 |
| Rabbit-anti-OS9     | Abcam   | ab109510, RRID: AB_2864354 |
| Rabbit-anti-gp78    | Proteintech | 16675-1-AP, RRID: AB_2226463 |
| **Anti-rabbit IgG, HRP-linked antibody** | Cell signaling Technology | 7074, RRID: AB_2099233 |
| **Anti-Mouse IgG, HRP-linked antibody** | GE Healthcare | NA931, RRID: AB_772210 |
| CF®555, Donkey Anti-Mouse IgG (H+L), Highly Cross-Adsorbed | Biotium | 20037, RRID: AB_10559035 |
| CF®555, Donkey Anti-Rabbit IgG (H+L), Highly Cross-Adsorbed | Biotium | 20038, RRID: AB_10558011 |
| CF®647, Donkey Anti-Rabbit IgG (H+L), Highly Cross-Adsorbed | Biotium | 20047, RRID: AB_10559808 |
| CF®488A, Donkey Anti-Chicken IgY (H+L), Highly Cross-Adsorbed | Biotium | 20166, RRID: AB_10854387 |
| CF®488A, Donkey Anti-Mouse IgG (H+L), Highly Cross-Adsorbed | Biotium | 20014, RRID: AB_10561327 |
| Cy®5 AffiniPure Donkey Anti-Guinea Pig IgG (H+L) | Jackson ImmunoResearch Labs | 706-175-148, RRID: AB_2340462 |
| **Bacterial and Virus Strains** |        |            |
| LV-pRRL-Ven-HA      | Nishitoh et al. (2008) | N/A         |
| LV-pRRL-Ven-hSREBP2(1481)-HA | This paper | N/A         |
| LV-pRRL-mDerlin-1-HA | This paper | N/A         |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| LV-pRRL-mDerlin-2-HA | This paper | N/A |
| Biological Samples | | |
| Mouse brain tissue | Strains listed in this table | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Tissue Tek | Sakura Finetek | Cat# 4583 |
| Immu-Mount | Thermo Scientific | Cat# 9990402 |
| Dulbecco’s modified Eagle’s medium | Nacalai Tesque | Cat# 08459-64 |
| Minimal essential medium (MEM) | Nacalai Tesque | Cat# 21443-15 |
| Neurobasal | Gibco | Cat# 21103-049 |
| MEMx | Gibco | Cat# 12571-063 |
| Penicillin-streptomycin solution | Nacalai Tesque | Cat# 09367-34 |
| Bisbenzimide H33258 Fluorochrome Trihydrochloride Solution | Nacalai Tesque | Cat# 19173-41 |
| Poly-L-lysine hydrobromide | Sigma-Aldrich | Cat# P2636 |
| Papain from papaya latex | Sigma-Aldrich | Cat# F3125 |
| DNase I | Sigma-Aldrich | Cat# D4527 |
| B-27 supplement | Gibco | Cat# 17504-044 |
| Glutamax supplement | Gibco | Cat# 35050-061 |
| Cytosine β-D-arabinofuranoside crystalline | Sigma-Aldrich | Cat# C1768 |
| 4-phenylbutyric acid | MERCK | Cat# 8.20986.0025 |
| Bovine lipoprotein deficient serum | Alpha Diagnostic Int | Cat# LDL46-S |
| Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts | Nacalai Tesque | Cat# 25955-11 |
| ALLN | Calbiochem | Cat# 208719 |
| Leupeptin | Nacalai Tesque | Cat# 43449-62 |
| Lovastatin | AdipoGen | Cat# AG-CN2-0051 |
| 25-hydroxycholesterol | Sigma-Aldrich | Cat# H1015 |
| Synthetic cholesterol | Sigma-Aldrich | Cat# 55442 |
| Polyethylenimine (PEI)-Max | Polysciences | Cat# 24765-1 |
| Lipofectamine RNAiMAX reagent | Invitrogen | Cat# 13778150 |
| Critical Commercial Assays | | |
| FD Rapid GolgiStain Kit | FD NeuroTechnologies | Cat# PK401 |
| RNeasy Plus Mini Kit | QiAGEN | Cat# 74104 |
| NucleoSpin RNA kit | Takara Bio | Cat# 740955 |
| RevaTra Ace qPCR RT Master Mix with gDNA Remover | TOYOBO | Cat# FSQ-301 |
| SYBR Green PCR Master Mix | Kapa Biosystems | Cat# KK4602 |
| SurePrint G3 Mouse GE Ver2 platform | Agilent Technologies | Cat# G4852A, G4852B |
| Colorimetric total cholesterol assay kit according to manufacturer instructions | Cell Biolabs | Cat# STA-384 |

**Deposited Data**

| Raw and analyzed data | This paper | GEO: GSE155425 ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155425](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155425)) |

GEO: GSE171796 ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171796](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171796))

(Continued on next page)
## REAGENT or RESOURCE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental Models: Cell Lines** | | |
| Human: HEK293T cells | ATCC | Cat# CRL-3216 |
| Human: HepG2 cells | ATCC | N/A |
| **Experimental Models: Organisms/Strains** | | |
| Mouse: C57BL/6 Derl1f/f | This paper | N/A |
| Mouse: C57BL/6 Derl2f/f | (Dougan et al., 2011) | N/A |
| Mouse: C57BL/6 Tg(Nes-Cre)1Kag | (Isaka et al., 1999) | N/A |
| Mouse: C57BL/6 TgN(a-CaMKII-nlCre)/10 | (Karpati et al., 2019) | RBRC00153 |
| **Oligonucleotides** | | |
| Stealth RNA™ siRNA SREBF2-35H110189, target sequence: 5‘−GAAGCGAGGCUUUGAAGACGAAGCUA−3’ | Invitrogen | Cat# 1299001 |
| Stealth RNA™ siRNA Negative Control Med GC Duplex #2 | Invitrogen | Cat# 12935112 |
| Primers for quantitative real-time PCR | See Table S3 | N/A |
| **Recombinant DNA** | | |
| pRRL-Venus-HA | (Nishitoh et al., 2008) | N/A |
| pRRL-Venus-hSREBP2(1-481)-HA | This paper | N/A |
| pRRL-mDerlin1-HA | This paper | N/A |
| pRRL-mDerlin2-HA | This paper | N/A |
| pMD2.G | Addgene | Cat# 12259 |
| psPAX2 | Addgene | Cat# 12260 |
| pcDNA3.0 | Invitrogen | N/A |
| **Software and algorithms** | | |
| ImageJ | (Schneider et al., 2012) | https://imagej.nih.gov/ij/ |
| Fiji | (Schindelin et al., 2012) | https://imagej.net/Fiji |
| Adobe Photoshop Elements 14 | Adobe | N/A |
| ImageQuant TL Ver.8.1 | GE Healthcare | N/A |
| GeneSpring software version 13.0, 14.9, 14.9.1 | Agilent Technologies | N/A |
| DAVID 6.8 | (Huang da et al., 2009) | http://david.ncifcrf.gov/ |
| GSEA v4.0.3 | (Mootha et al., 2003; Subramanian et al., 2005) | https://www.gsea-msigdb.org/gsea/index.jsp |
| MeV | Center for Cancer Computational Biology at Dana-Farber Cancer Institute | http://mev.tm4.org/ |
| Mouse Genome Informatics | The Jackson Laboratory | http://www.informatics.jax.org/ |
| EZR software version 1.30 | (Kanda, 2013) | http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html |
| **Other** | | |
| Micro Smash | TOMY | Cat# MS-100 |
| Freezing microtome | Leica Microsystems | Cat# CM3050S |
| Confocal laser microscope | Leica Microsystems | Cat# TSC-SP8 |
| Fluorescence microscope | Keyence | Cat# BZ-9000 |
| ROTA-ROD FOR MICE | UGO Basile | Cat# 47600 |
| ROTA-ROD TREADMILL FOR MICE | Muromachi | Cat# MK-610A |
| Balanced beam test | O’Hara | N/A |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hideki Nishitoh (nishitoh@med.miyazaki-u.ac.jp)

Materials availability
All unique reagents (plasmids, antibodies, and Derl1<sup>f/f</sup> mice) generated in this study are available from the Lead Contact. Derl2<sup>f/f</sup> mice are available from Hidde L. Ploegh. C57BL/6-TgN(a-CaMKII-nlCre)/10 mice are available from Katsuhiko Mikoshiba. Tg(Nes-Cre)1Kag mice are available from Ryoichiro Kageyama (Kyoto University).

Data and code availability
DNA microarray data generated in this study are deposited with the NCBI Gene Expression Omnibus archive as series GSE155425 and GSE171796 and are publicly available as of the date of publication. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Wild-type C57BL/6 mice were raised under specific pathogen-free conditions and housed under a 12-h/12-h light/dark cycle with free access to food and water. Derl1<sup>f/f</sup> mice were generated by conventional gene targeting (Figure S1A) as previously described, while mice expressing Cre recombinase driven by the nestin promoter [Tg(Nes-Cre)1Kag mice] were obtained from Dr. Ryoichiro Kageyama (Kyoto University) (Isaka et al., 1999). Mice expressing Cre recombinase driven by the CaMKII<sub>a</sub> promoter [C57BL/6-TgN(a-CaMKII-nlCre)/10] have been described (Karpati et al., 2019). Mice expressing Cre recombinase were intercrossed with Derl1<sup>f/f</sup> mice and Derl2<sup>f/f</sup> mice to generate Derl1<sup>NesCre</sup>, Derl2<sup>NesCre</sup>, and Derl1<sup>CaMKII<sub>a</sub>Cre</sup> mice. Both male and female mice were used, as mice up to 5 weeks old are sexually immature and do not affect the results. After 6 weeks of age, male mice were mainly used to avoid the influences of the female sex cycle, including behavioral analysis. All mice experiments were approved by the Animal Research Committee of the University of Miyazaki, National Institute for Physiological Sciences, and University of Toyama and performed in accordance with the institutional guidelines. The experiments were performed in accordance with the institutional guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Primary cultures of cortical neurons
Cortical neurons were isolated from E17 wild-type, Derl1<sup>f/f</sup> and Derl1<sup>NesCre</sup> mice (female and male). In brief, cerebral cortices were dissociated with papain (Sigma-Aldrich; P3125) at 37°C for 20 min, and triturated in the presence of DNase I (Sigma-Aldrich; D4527) and 10% fetal bovine serum (FBS). Dissociated cells were plated on culture dishes pre-coated with poly-L-lysine (Sigma-Aldrich; P2636) in minimal essential medium (MEM) (Gibco; 12571-063) supplemented with 5% FBS and 0.6% glucose. After allowing neurons to adhere for 3 to 4 h, the plating medium was replaced with Neurobasal medium (Gibco; 21103-049) supplemented with 20 µL/mL B27 (Gibco; 17504-044) and 0.5 mM GlutaMAX (Gibco; 35050-061). To eliminate non-neuronal cells, 5 µM cytosine β-D-arabinofuranoside crystalline (Sigma-Aldrich; C1768) was added. 4-phenylbutyric acid (4-PBA) (1 mM) (MERCK; 8.20986.0025) and lovastatin (1 µM) (AdipoGen; AG-CN2-0051) were treated at the first medium replace timing. A half-volume of medium was replaced every three days. The cells were maintained in a 5% CO2 atmosphere at 37°C.

METHOD DETAILS

Cell culture
Human hepatoma HepG2 cells were cultured in MEM (Nacalai Tesque; 21443-15) supplemented with 10% FBS and penicillin–streptomycin solution (Nacalai Tesque; 09367-34). Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Nacalai Tesque; 08459-64) supplemented with 10% FBS and penicillin–streptomycin solution. All cells were maintained under a 5% CO2 atmosphere at 37°C.
**Tissue preparation for biochemical analysis**

Mice were sacrificed by cervical dislocation and brains rapidly dissected for immunoblotting (IB), qPCR, and DNA microarray analysis. Each brain region was frozen immediately on dry ice and stored at −80°C.

**Tissue preparation for immunofluorescence**

Mice were deeply anesthetized by intraperitoneal injection of a 4 mg/kg midazolam/0.3 mg/kg medetomidine/5 mg/kg butorphanol mixture, and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were dissected and post-fixed overnight in the same fixative at 4°C. Fixed brains were incubated in 15% sucrose solution at 4°C overnight followed by incubation in 30% sucrose solution at 4°C overnight. The size of fixed brains was measured before embedding. Brains were then cut into two pieces along the midline, and each half was embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek; 4583) and stored at −80°C. Embedded frozen brains were serially sectioned in the coronal plane at 40-μm thickness using a freezing microtome (Leica Microsystems; CM3050S), and every sixth section was sequentially transferred to 6-well plates in PBS for subsequent immunohistochemical staining (below).

**Immunohistochemistry**

The brain sections were washed with PBS and incubated in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100) for 1 h at room temperature (RT) followed by overnight incubation at 4°C with the indicated primary antibody diluted in blocking solution. Sections were washed thrice with PBS and incubated for 2 h at RT with secondary antibody diluted in blocking solution. After a final wash with PBS, the sections were mounted on glass slides with Immuno-Mount (Thermo Scientific; 9990402). Immunofluorescence images were obtained using a fluorescence microscope (Keyence; BZ-9000) or confocal laser microscope (Leica Microsystems; TSC-SP8) and processed using Adobe Photoshop Elements (Adobe). Nuclei were counterstained using bisbenzimide H33258 fluorochrome trihydrochloride solution (Hoechst; 1:500; Nacalai Tesque, 19173-41). Antibodies are listed in the key resources table.

**Golgi staining**

Coronal half brain sections from 30-week-old Derl1NESCre and Derl2NESCre mice and their respective controls were stained using the FD Rapid GolgiStain Kit (FD NeuroTechnologies; PK401) following the manufacturer’s recommendations and then cut into 150-μm thick coronal sections on a cryostat (Leica Microsystems). All sections were visualized by confocal laser microscopy (Leica Microsystems). Ten cerebellar Purkinje cells and 10 striatal MSNs were analyzed from 3 unrelated animals per genotype. For MSNs, dendrite length, branch number, and number of branches at given distances from the soma were measured using the Sholl analysis plug-in of Fiji software (National Institutes of Health) after neuronal reconstruction with the plug-in Simple Neurite Tracer. The dendritic tree size of Purkinje cells was measured using ImageJ software (National Institutes of Health), and the number of branches was counted manually.

**Volumetric analysis and cell counting**

Volumetric analyses were conducted using every sixth 40-μm coronal half brain section stained with NeuN. The areas of each brain region were measured using ImageJ and volume (V) calculated as V = ΣA x i x d according to the Cavalieri’s principle, where A is the sum of target areas in each section, i is the interval between the sections, and d is the section thickness. Fold changes between Derl1NESCre or Derl2NESCre mice and respective controls were calculated as measures of regional brain atrophy. Marker-positive cell numbers in the cerebellum and striatum were calculated using every sequential hemisphere section. The number of Purkinje cell somata per 200 μm of the Purkinje cell layer was manually counted at 12 sites, and the average was compared among genotypes. The numbers of marker-positive cells were also manually counted within twelve 150 × 150 μm areas of the cerebellar molecular layer and twelve 200 × 200 μm areas of the striatum. These cell numbers are reported per mm².

**Immunoblotting**

Whole cell lysates were prepared by homogenizing brain and other tissues for 60 s in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EGTA, and 1% Triton X-100) supplemented with 5 μg/mL leupeptine (Nacalai Tesque; 43449-62) on ice using a Micro Smash (TOMY; MS-100) (4,500 rpm 4°C). Cellular nuclear and membrane fractions were isolated as described previously with minor modifications (Sakai et al., 1996). Briefly, brain tissues were homogenized in 0.5 mL of buffer A (10 mM HEPES at pH 7.6, 10 mM KCl, 1.5 mM
MgCl₂, 1.0 mM EDTA, 1.0 mM EGTA) containing protease inhibitor cocktail (Nacalai Tesque; 25955-11) and ALLN (Calbiochem; 208719). Cells were allowed to swell in the homogenate at 4°C for 30 min and then passed through a 23-gauge needle 30 times. The obtained lysate was centrifuged at 1,000 x g for 7 min at 4°C. The supernatant from this 1,000 x g centrifugation was used to prepare the membrane fraction. First, the supernatant was centrifuged at 100,000 x g for 30 min at 4°C, followed by resuspension of the pellet in 0.25 mL of lysis buffer containing 5 µg/mL leupeptine. The new suspension was centrifuged at 20,400 x g at 4°C for 15 min, and the supernatant was used as the membrane fraction. Alternatively, the pellet obtained from the 1,000 x g centrifugation above was used to isolate the nuclear fraction. The pellets were resuspended in 0.25 mL of buffer B (20 mM HEPES at pH 7.6, 2.5% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 1.0 mM EDTA, and 1.0 mM EGTA) containing protease inhibitor cocktail and ALLN. The suspension was centrifuged at 20,400 x g at 4°C for 15 min. The supernatant from this spin was used as the nuclear fraction.

Whole cell lysates, nuclear fractions, and the membrane fractions were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween-20), the membranes were probed with the indicated antibodies and immunolabeling detected with an enhanced chemiluminescence (ECL) system. Antibodies are listed in the key resources table. Band intensity was measured by ImageQuant TL (GE Healthcare).

Evaluating SREBP-2 processing in cultured cells

HepG2 cells were plated in MEM supplemented with 10% FBS and penicillin–streptomycin solution (Nacalai Tesque), and transfected with a small interfering RNA (siRNA) targeting SREBF2 (SREBF2-HSS110189 Stealth siRNA; Invitrogen; 1299001) or control siRNA (Negative Control Medium GC Duplex; Invitrogen; 12935112) using Lipofectamine RNAiMAX reagent (Invitrogen; 13778150). After two days (defined as DIV), each culture was washed twice with PBS and switched to medium for inducing SREBP-2 activation (minus sterols) or suppressing SREBP-2 activation (plus sterols) as described previously with minor modifications (Hua et al., 1995; Sakai et al., 1996). Medium for inducing SREBP-2 activation included 5% bovine lipoprotein-deficient serum (LPDS) (Alpha Diagnostic Int; LDLD46-S) and 10 µM lovastatin (Adipogen; AG-CN2-0051) in MEM (Nacalai Tesque), while the medium for suppressing SREBP-2 activation included 5% LPDS, 1 µg/mL 25-hydroxycholesterol (Sigma-Aldrich; H1015), and 10 µg/mL cholesterol (Sigma-Aldrich; S5442) in MEM. After incubation for 16–20 h in induction or suppression medium (3 DIV), 25 µg/ml ALLN (Calbiochem) was added to each dish and the cells were harvested 2–4 h later.

Lentivirus production and infection

Lentiviruses were produced by co-transfecting HEK293T cells with the lentivirus constructs pRRL-Venus-HA, pRRL-Venus-hSREBP-2(1–481)-HA, pRRL-mDerlin-1-HA or pRRL-mDerlin-2-HA and lentivirus packaging vector constructs pMD2.G (Addgene; 12259) and psPAX2 (Addgene; 12260) using Polyethylenimine (PEI)-Max (Polysciences; 24765-1). The culture medium was changed at 16–24 h after transfection. The supernatants were collected at 24 and 48 h after medium change, and virus was introduced into cortical neurons by adding these supernatants to the culture at the first medium change.

Immunocytochemistry

Primary cultured cortical neurons were fixed with 4% PFA in PBS for 20 min at the indicated times (DIV), washed thrice in PBS, permeabilized and blocked with blocking solution (PBS containing 3% FBS and 0.1% Triton X-100) for 30 min at RT, and incubated for 1.5 h at RT with the indicated primary antibody diluted in blocking solution. Cells were then washed thrice with PBS and incubated for 1.5 h at RT with the secondary antibody diluted in blocking solution. After a final wash with PBS, cells were mounted on glass slides with Immu-Mount (Thermo Scientific). Nuclei were counterstained using Hoechst (1:500; Nacalai Tesque). Immunofluorescence images were obtained using a confocal laser microscope (Leica Microsystems) and processed using Adobe Photoshop Elements. The antibodies are listed in the key resources table.

Morphological analysis of cultured cortical neurons

For analysis of dendrite development in vitro, cortical neurons were immunostained with antibodies against MAP2 at 3 DIV (Figures 4E, 6C, and S7E) and 6 DIV (Figure 2H). Dendrites were defined as MAP2-positive neurites. Dendritic length was quantified using ImageJ.
Quantitative real-time PCR analysis
Total RNA was isolated from the cerebellum at P28 or cultured cortical neurons at 3 DIV using RNAiso Plus (Takara Bio; 9109) or the RNeasy Plus Mini Kit (QIAGEN; 74104) and reverse transcribed using RevaTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO; FSQ-301). Quantitative (q)PCR was performed using SYBR Green PCR Master Mix (Kapa Biosystems; KK4602) and a StepOnePlus Real-Time PCR System (Applied Biosystems). Expression levels were normalized to expression of S18 mRNA. The primer sequences are shown in Table S3.

DNA microarray analysis
Total RNA was extracted from the cerebellum at P28 using a NucleoSpin RNA kit (Takara Bio; 740955) according to the manufacturer’s instructions. A total of 150 ng total RNA from each sample was amplified and Cy3-labeled. Next, 600 ng Cy3-labeled cRNA was fragmented, hybridized onto the SurePrint G3 Mouse GE Ver2 platform (Agilent Technologies; G4852B and G4858A) and then incubated with rotation at 65°C for 17 h. Data were analyzed using GeneSpring software version 13.0 and 14.9 (Agilent Technologies) as previously described (Komatsu et al., 2013). In brief, the microarray data were normalized by quantile normalization, and baseline transformed the signal values to the median in all samples. Then, quality control and filtering steps were performed based on flags and expression levels. Mean signal intensities were measured in duplicate and averaged for identification of genes differentially expressed among mouse lines. A fold-change <0.67 was considered downregulation and a fold-change >1.5 as upregulation. Data from this microarray analysis have been submitted to the NCBI Gene Expression Omnibus archive as series GSE155425 and GSE171796. Functional enrichment analysis of differentially expressed genes was performed using DAVID online tools (version DAVID 6.8; http://david.ncifcrf.gov/). Heat map analysis was performed using MeV (multiple experimental viewer; http://mev.tm4.org/). On the heat map, red indicates higher expression and green lower expression. Gene set enrichment analysis (GSEA) was performed using GSEA v4.0.3 (https://www.gsea-msigdb.org/gsea/index.jsp). The Enrichment plot shows the distribution of genes in each set that are positively (red) and negatively (blue) correlated with Derlin-1 or Derlin-2 deficiency. The gene ontology (GO) terms for heat map analysis and GSEA were obtained from the Mouse Genome Informatics (MGI) GO project (http://www.informatics.jax.org/), which provides functional annotations for mouse gene products using Gene Ontology (http://www.informatics.jax.org/vocab/gene_ontology).

Cholesterol assay
Total cholesterol was measured from the cerebellum and primary cultured cortical neurons using a colorimetric assay kit according to the manufacturer’s instructions (Cell Biolabs; STA-384).

Rotarod test
Derl1f/f and Derl1NesCre mice were examined using an accelerating rotarod [ROTA-ROD FOR MICE (UGO Basile; 47600)], while Derl2f/f and Derl2NesCre mice were examined using the ROTA-ROD TREADMILL FOR MICE (Muromachi; MK-610A). In each trial, a mouse was placed on a rotating drum (3 cm diameter) and the time required to lose balance (as indicated by falling off or splaying out on the drum) was recorded. The speed of the rotarod was increased from 4 to 40 rpm over a 5 min period during the test. Each mouse performed 3 trials per day for a total of 6 trials over two days.

Beam-walking test
The balance beam apparatus (O’Hara) used for testing Derl1f/f, Derl1NesCre, and Derl1CaMKIIaCre mice consisted of a 1-m cylindrical rod suspended horizontally 50 cm above the floor and connected to a safe platform, while the apparatus used for Derl2f/f and Derl2NesCre mice consisted of a 1-m long steel pipe suspended 30 cm above the floor and connected to a safe platform. In each test, a mouse was placed on the rod or pipe at the starting end, and the numbers of animals per genotype able to traverse to the safe platform as well as the time required was recorded. Derl1NesCre and Derl2NesCre mice and their respective control mice were tested on 3 trials while Derl1CaMKIIaCre mice and controls underwent 1 trial.
QUANTIFICATION AND STATISTICAL ANALYSIS

All data are presented as means ± standard error. Student’s t-test and Fisher’s exact test were performed to compare two group means. One-way ANOVA and repeated measures ANOVA followed by post hoc tests were used to compare three or more group means. All statistical analyses were performed by using EZR software version1.30 (Kanda, 2013). A P < 0.05 (two-tailed) was considered significant for all tests.