Published in final edited form as:
Matrix Biol. 2012 January ; 31(1): 17–28. doi:10.1016/j.matbio.2011.09.002.

Laminin α1 is essential for mouse cerebellar development

Naoki Ichikawa-Tomikawa[1,5], **Junko Ogawa**[1,4], **Vanessa Douet**[1], **Zhuo Xu**[1], **Yuji Kamikubo**[2], **Takashi Sakurai**[2], **Shinichi Kohsaka**[4], **Hideki Chiba**[5], **Nobutaka Hattori**[3], **Yoshihiko Yamada**[6], and **Eri Arikawa-Hirasawa**[1,3]

[1]Research Institute for Diseases of Old Age, Juntendo University Graduate School of Medicine, Tokyo, Japan

[2]Department of Pharmacology, Juntendo University Graduate School of Medicine, Tokyo, Japan

[3]Department of Neurology, Juntendo University Graduate School of Medicine, Tokyo, Japan

[4]National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan

[5]Department of Basic Pathology, Fukushima Medical University School of Medicine, Fukushima, Japan

[6]National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland, U.S.A.

Abstract

Laminin α1 (Lama1), which is a subunit of laminin-1 (laminin-111), a heterotrimeric ECM protein, is essential for embryonic development and promotes neurite outgrowth in culture. Because the deletion of Lama1 causes lethality at early embryonic stages in mice, the in vivo role of Lama1 in neural development and functions has not yet been possible to determine. In this study, we generated conditional *Lama1* knockout (*Lama1*^{CKO}) mice in the epiblast lineage using *Sox2-Cre* mice. These *Lama1*^{CKO} mice survived, but displayed behavioral disorders and impaired formation of the cerebellum. Deficiency of Lama1 in the pial basement membrane of the meninges resulted in defects in the conformation of the meninges. During cerebellar development, Lama1 deficiency also caused a decrease in the proliferation and migration of granule cell precursors, disorganization of Bergmann glial fibers and endfeet, and a transient reduction in the activity of Akt. A marked reduction in numbers of dendritic processes in Purkinje cells was observed in *Lama1*^{CKO} mice. Together, these results indicate that Lama1 is required for cerebellar development and functions.

Keywords

Laminin α1 chain; meninges; and cerebellum

To whom correspondence should be addressed: Eri Arikawa-Hirasawa, Juntendo University Graduate School of Medicine, Research Institute for Diseases of Old Age, Building 10, Room 606, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, Tel & Fax: 81-3-3814-3016, ehirasaw@juntendo.ac.jp.

1. Introduction

Two principal neuronal cell types, Purkinje and granule cells, are essential for the normal development and functioning of the cerebellum. Purkinje cells, which develop first, migrate along the radial glial system from the germinative zone. In contrast, granule neurons migrate from the proliferative zone in the external granule layer (EGL) to the internal granule layer (IGL), guided by Bergmann glial processes (Hatten, 1999; Herrup and Kuemerle, 1997; Rakic and Sidman, 1973).

Extracellular matrix (ECM) proteins and their receptors closely participate in cerebellar development. For example, vitronectin, is expressed in differentiated granule neurons and acts to decrease sonic hedgehog-induced proliferation of granule cell precursors (GCPs) and to promote neural differentiation (Pons et al., 2001). In mice, CNS-specific knockout of integrin β1, one of the major receptors of ECM proteins, and of its downstream molecules, integrin-linked kinase (ILK) and focal adhesion kinase (FAK), causes defects in the formation of folia by decreasing the proliferation of GCPs and causes abnormalities in the formation of Bergmann glia and Purkinje cells (Blaess et al., 2004; Graus-Porta et al., 2001; Mills et al., 2006; Watanabe et al., 2008). Other ECM protein receptors, α- and β-dystroglycan, regulate the migration of cerebellar granule neurons (Qu and Smith, 2004; Satz et al., 2010).

Laminins comprise a family of heteromeric ECM proteins consisting of α, β, and γ chains (Aumailley et al., 2005; Miner and Yurchenco, 2004). Laminins are required for basement membrane assembly (Li et al., 2003; Li et al., 2005), and they regulate cellular behavior through interactions with cell surface receptors, including integrins, syndecans, and α-dystroglycan (Gullberg and Ekblom, 1995; Miner and Yurchenco, 2004). Laminin α1 (Lama1) is the first laminin to be expressed during mouse embryogenesis (Miner et al., 2004; Smyth et al., 1999) and in vitro studies have demonstrated it to have a number of biological activities, including promotion of cell adhesion, migration, neurite outgrowth, angiogenesis, and tumor metastasis (Ekblom et al., 2003; Ichikawa et al., 2009; Kleinman et al., 1990). Mouse embryos that are deficient in Lama1 lack Reichert's membrane and die by embryonic day 7 (E7) (Alpy et al., 2005; Miner et al., 2004). Mutant mice expressing a truncated Lama1 lack the C-terminal LG4 and LG5 subdomains and die before E6.5, despite the presence of both the embryonic basement membrane and Reichert's membrane (Scheele et al., 2005). A missense mutation and conditional knockout of the *Lama1* gene in mice disrupt retinal vascular development and inner limiting membrane formation (Edwards et al., 2010).

Although Lama1 is present in the meninges and in larger vessels in the late developmental stages of the CNS (Andrae et al., 2004), the in vivo role of Lama1 in the CNS is unknown. In this report, we created conditional null mice using epiblast-specific Sox2-Cre and found that Lama1 was essential for the proliferation and migration of GCPs in the cerebellum. Lama1 was also required for formation of Bergmann glial processes and for the localization and dendritic formation of Purkinje cells.

2. Results

2.1. Generation of conventional and conditional Lama1-knockout mice

To generate conventional ($Lama1^{KO}$) and conditional ($Lama1^{CKO}$) *Lama1 null* mice, two ES clones were isolated by transfecting the targeting vector, which contained the PGK-neo^r-PGK-tk cassette flanked by two loxP sequences in intron 15 and a third loxP sequence in intron 17 of the *Lama1* allele (supplementary material Fig. S1A). These cells were transiently transfected with a CMV-Cre expression plasmid, and ES clones containing either

the *Lama1*-deleted allele or floxed allele were obtained. The ES clones from each were injected into blastocysts to generate either $Lama1^{KO}$ or floxed-*Lama1* mice. Homozygous $Lama1^{KO}$ ($Lama1^{del/del}$) mice died by embryonic day 7 (E7) because of the absence of Reichert's membrane (data not shown), similar to *Lama1* null mice described in a previous report (Alpy et al., 2005; Miner et al., 2004). *Lama1* mRNA and protein were absent in homozygous $Lama1^{KO}$ mice (data not shown). Southern blotting and genomic PCR confirmed the genotype of $Lama1^{KO}$ mice (supplementary material Fig. S1B). The floxed-*Lama1* ($Lama1^{flox/flox}$) mice were fertile and phenotypically normal. To identify the role of Lama1 in tissue development and functions at later stages, we created $Lama1^{CKO}$ ($Lama1^{flox/del}$;$Sox2^{cre/+}$) mice by crossing floxed-*Lama1* mice with *Sox2-Cre* transgenic mice. The Sox2 gene is expressed in the inner cell mass, epiblast, and extraembryonic ectoderm prior to gastrulation, and in the prospective neural plate and chorion at the onset of gastrulation. All epiblast cells appear to have undergone a recombination event of *Sox2-Cre* by E6.5 (Hayashi et al., 2002). $Lama1^{CKO}$ mice underwent complete gestation and survived postnatally. Both genders of $Lama1^{CKO}$ mice showed normal sexual development and fertility. A genomic PCR analysis demonstrated that the floxed segment of the floxed-*Lama1* allele was almost completely deleted in the tail of $Lama1^{CKO}$ mice (supplementary material Fig. S1C), and that this deleted allele was detected in almost all adult tissues (supplementary material Fig. S1D). Subsequent RT-PCR analysis revealed the absence of *Lama1* mRNA in the cerebellum of $Lama1^{CKO}$ mice (supplementary material Fig. S1E).

2.2. $Lama1^{CKO}$ mice display abnormal behaviors

Three behavioral tests were conducted to determine whether $Lama1^{CKO}$ mice have abnormalities in behavior: the tail suspension test, the rotarod test, and footprint analysis. In the tail suspension test, $Lama1^{CKO}$ mice showed hugging behavior whereas control $Lama1^{flox/del}$ mice showed normal escape-oriented movements: running forward and backwards, body torsions with attempts to catch the suspended body (Fig. 1A). In the rotarod test, the movement time of $Lama1^{CKO}$ mice was slower than that of control mice, indicating that $Lama1^{CKO}$ mice have motor deficits (Fig. 1B). In the footprint test, $Lama1^{CKO}$ mice showed a significant reduction in stride length and an increase in interlimb coordination when compared with control mice, indicating the occurrence of locomotion disorder in $Lama1^{CKO}$ mice (Fig. 1C). These behavioral abnormalities suggest the occurrence of defects in neuronal functions, which result in the disequilibrium and lack of coordination seen in $Lama1^{CKO}$ mice.

2.3. Reduced size and structural defects in the cerebellum of $Lama1^{CKO}$ mice

For further investigation of the neurological dysfunction, we analyzed histological sections of the brains of adult $Lama1^{CKO}$ mice. The cerebellum was reduced in size and the superior colliculus was appreciably exposed to the surface of the brain compared to the situation in control $Lama1^{flox/del}$ mice (supplementary material Fig. S2). To analyze the morphological changes in the cerebellum in more detail, we examined sagittal sections stained with Luxol Fast Blue (Fig. 2A and B). In the cerebellum of $Lama1^{CKO}$ mice, the depth of folia decreased and aggregation of granule cells appeared at the cerebellum surface, along the line of fusion of adjacent cerebellar folia under the meninges (arrows in Fig. 2Ba and b). The meninges, which form the fissures of folia, were not in the correct location in $Lama1^{CKO}$ mice (asterisk in Fig. 2Bc). These results suggest that the behavioral dysfunctions seen in $Lama1^{CKO}$ mice are due at least in part to cerebellar defects.

2.4. Defects in the arrangement and dendritic processes in Purkinje cells

Purkinje cells, which are the main neuronal cells in the cerebellum, originate in the ventricular zone and migrate to the cortex along the radial glial fiber. After this migration, Purkinje cells form a cell layer and extend axons and dendrites (Goldowitz and Hamre,

1998). During postnatal development, Purkinje cell dendrites form synaptic contacts with parallel and climbing fibers in the molecular cell layer (Sotelo, 2004). Because of the remarkable abnormality in the cerebellar formation observed in *Lama1*^{CKO} mice, we examined the layer formation and neurite extension of Purkinje cells by staining 50 μm thick sections of the cerebellum with an antibody to calbindin, a marker of Purkinje cells (Fig. 3). In *Lama1*^{CKO} mice at P0, P10, and adult stages, the layer of Purkinje cells was distorted, and the localization of some Purkinje cells was away from the cell layer (Fig. 3A, asterisks). The formation of the dendritic tree of Purkinje cells in the folia of *Lama1*^{CKO} mice at P5, P10, and adult stages was reduced compared to that of control Purkinje cell dendrites (Fig. 3B, asterisks).

2.5. The expression of Lama1 and other ECM molecules in the meningeal basement membrane

Lama1 is expressed in the meningeal basement membrane at the surface of the brain, from the late embryonic stage to adulthood (supplementary material Fig. S3). Previous reports also indicated that other laminin α chains, including α2 (Lama2), α4 (Lama4), and α5 (Lama5), are expressed in the meningeal basement membrane (Andrae et al., 2004; Blaess et al., 2004). Because other ECM molecules may compensate for the deletion of Lama1 in *Lama1*^{CKO} mice, we examined the expression and localization of several ECM molecules in in normal looking cerebellar areas of P5 *Lama1*^{CKO} mice by immunostaining. In *Lama1*^{CKO} mice, no expression of Lama1 was detected, consistent with the RT-PCR and immunostaining results (supplementary material Fig. S1E and Fig. 4A), but Lama2 and Lama5 were present in the meningeal basement membrane of *Lama1*^{CKO} mice (supplementary material Fig. S4A and C). The expression level of Lama2 was slightly increased, whereas that of Lama5 was decreased in the mutant mice. Lama4 was present in the blood vessel basement membrane and its expression level did not change between *Lama1*^{CKO} and control *Lama1*$^{flox/del}$ mice (supplementary material Fig. S4B). Nidogen-1, another ECM molecule, was expressed in both the meningeal and blood vessel basement membrane of *Lama1*^{CKO} mice, and its expression level was the same as that in control mice (supplementary material Fig. S4B). Integrin β1, a main receptor of laminin α chains, was present in the endothelial cells of *Lama1*^{CKO} mice, and no significant differences were noted between mutant and control mice (supplementary material Fig. S4C). These results indicate that the deletion of Lama1 did not substantially affect the expression of other laminin α chains, nidogen-1, or integrin β1.

2.6. Lama1 is critical for proliferation of granule cell precursors

The reduced cerebellar size observed in *Lama1*^{CKO} mice might possibly result from a reduction in proliferation of granule cell precursors (GCPs) and decreased migration of proliferated GCPs. We therefore measured the number of proliferating cells in cerebellar sections from P0, P5, and P10 mice during the growth phase of the cerebellar cortex (Fig. 4A, upper panel, immunostaining of P5 cerebellum; bottom panel, quantitative data from P0, P5, and P10 cerebellums). Proliferating cells within the external granular layer (EGL) were identified by staining with phospho-histone 3 (Ser10), a marker for the M-phase of the cell cycle. We confirmed that phospho-histone 3-positive cells were GCPs by double staining with antibody to Pax6, a marker of GCPs (Fig. 4B). The number of proliferating GCPs in *Lama1*^{CKO} mice was significantly reduced during cerebellar development compared to that in control *Lama1*$^{flox/del}$ mice (Fig. 4). In addition, TUNEL assays in the cerebellum at P0, P5, P10, P21, and adulthood revealed no differences in cell death between *Lama1*^{CKO} and control mice (data not shown).

We next examined signaling molecules involved in cerebellar development. Akt is a downstream molecule of PI3-kinase and functions in both the survival and proliferation of

progenitor cells in the brain (Barnabe-Heider and Miller, 2003; Groszer et al., 2001). The activation of Akt in P0 and P5 *Lama1*^{CKO} mice was significantly reduced compared to control. This reduction persisted during P0–P5, but the decrease was repaired at P10 (Fig. 5A). The expression levels of integrin β1 did not change (Fig. 5B). Integrin-linked kinase (ILK1) was increased at P0, but this increase disappeared at P5 (Fig. 5B). We also found no significant differences in the expression and phosphorylation levels of focal adhesion kinase (FAK) and Src family kinase (SFK) in the cerebellum of *Lama1*^{CKO} and control mice (Fig. 5B). These results suggest that Lama1 is essential for the proliferation of GCPs via activation of the Akt signaling pathway.

2.7. Lama1 regulates migration of proliferating GCPs

The abnormal cerebellar formation and granule ectopies in *Lama1*^{CKO} mice may be caused in part by defects in GCP migration. We therefore examined migration of proliferating GCPs in culture using cerebellar slices from *Lama1*^{CKO} mice, either in the absence or presence of exogenous laminin-1 (Lam-111) (Fig. 6). Sagittal slices of the cerebellum from P10 control and *Lama1*^{CKO} mice were cultured and labeled with BrdU for 30 min and stained with antibodies for Lama1 and BrdU (Fig. 6A). Lama1 was expressed in the meninges in control cerebellum slices, but was absent from *Lama1*^{CKO} cerebellum slices. The addition of exogenous laminin-1 in the culture restored Lama1 staining in the meninges of *Lama1*^{CKO} mice (Fig. 6A). BrdU-positive cells were observed in both control and *Lama1*^{CKO} mice. After three days of culture, BrdU-positive cells within the external granular layer had moved to the inner layer in control slices, whereas no cell migration was observed in *Lama1*^{CKO} slices; the BrdU-positive cells remained in the periphery (Fig. 6B). When laminin-1 was added to the culture medium of *Lama1*^{CKO} cerebellum slices, BrdU-positive cells were again able to migrate to the inner layer (Fig. 6B). Next, we used a TUNEL assay to examine whether cell death affects the migration of GCPs in a culture system. No difference was noted in the number of apoptotic cells in *Lama1*^{CKO} slices compared to control slices (Fig. 6C). We therefore concluded that the difference in the localization of BrdU-positive cells was due to defects in cell migration. These results suggest that Lama1 is essential for the migration of proliferating GCPs.

2.8. Abnormal radial and Bergmann glial fibers in Lama1^{CKO} mice

Proliferating GCPs in the external granule layer migrate to the internal granule layer using glial guidance systems (Edmondson and Hatten, 1987; Rakic, 1971; Solecki et al., 2004). In the cerebellum, Bergmann glial cells are first seen in the cortex at the late embryonic period, and they extend their processes to the pial surface. The migration of postmitotic GCPs is guided by surface-mediated interactions with Bergmann glial fibers (Komuro and Rakic, 1998). We therefore investigated the formation of radial glial fibers at E16 and P0, and Bergmann glial fibers at P10 (this is the peak period of postmitotic GCP migration), as well as at P5 and the adult stage, by staining with antibody to radial glial cell marker-2 and glial fibrillary acidic protein (GFAP), a marker for astrocytes (Fig. 7). We found that, in *Lama1*^{CKO} mice, radial glial fibers and Bergmann glial fibers directed to the meninges were short in length and disoriented (Fig. 7A–E). Bergmann glial endfeet, which run along the meninges, were fragmented and discontinuous compared to those in control mice (Fig. 7D and E). These results suggest that distorted the layer of Purkinje cells and the reduced migration of GCPs in *Lama1*^{CKO} mice are due to impaired glial guidance and that Lama1 is required for proper glial fiber formation.

2.9. Disruption of pial basement membranes and abnormal meningeal structure

Connexin 26 (Cx26) and 43 (Cx43), gap junction family proteins, are expressed in the cerebellar arachnoid and pia of the meninges (Mercier and Hatton, 2000, 2001; Nagy et al., 2001). To delineate the role of Lama1 in the conformation of the meninges, we examined

the localization of Cx26 and Cx43 by immunostaining the meninges in control and *Lama1*^{CKO} mice. We also examined the pial basement membrane by immunostaining laminin γ1 (Lamc1), which is a subunit of laminin-1 (Lama-111) and some of the other laminins, and a marker of the basement membrane. In control meninges, both Cx26 and Cx43 were expressed in the arachnoid and pia (Fig. 8Aa, b, and d). However Cx26 expression was less prominent in the arachnoid barrier (Fig. 8Ae and h, upper part) than in the arachnoid trabeculae (bottom part). Cx43 expression was nearly absent in the arachnoid of *Lama1*^{CKO} mice (Fig. 8Af and h). A thin and continuous basement layer at the bottom of the pia was stained by anti-Lamc1 antibody (arrow, Fig. 8Ac and d). The meninges of *Lama1*^{CKO} mice were thinner and the expression of both Cx26 and Cx43 was reduced (Fig. 8Ae, f, and h). The Lamc1 staining revealed that the pial basement membrane was irregular (Arrows, Fig. 8Ag and h). We also analyzed the area where granule cell ectopies were observed at the cerebellum surface, as shown in Fig. 2Ba (arrows). In the cerebellum of *Lama1*^{CKO} mice, aggregated granule cells were observed under the meninges by DAPI staining (Fig. 8Be). Some of the ectopically accumulated granule cells near the cerebellum surface were positive for Cx26 staining (Fig. 8Bd, e, and f, asterisks).

We next used electron microscopy to examine the disruption of the pial basement membrane in *Lama1*^{CKO} mice at P0 (Fig. 9A) and the adult stage (Fig. 9B). In *Lama1*^{CKO} mice, the density of glial cells was remarkably low compared with the control and no glial cells were found in the places where they were normally located in control mice (Fig. 9A, asterisk). Moreover, the pial basal lamina in *Lama1*^{CKO} was curved and discontinuous (Fig. 9A, arrows). Similar results were observed in adult mice (Fig. 9B). These results suggest that Lama1 is required for the formation of the pial basement membrane and the meningeal structure.

3. Discussion

Lama1 has various biological functions, such as promotion of cell adhesion, migration, neurite outgrowth, angiogenesis, and tumor metastasis (Ekblom et al., 2003; Ichikawa et al., 2009; Kleinman et al., 1990). However, the significance of Lama1 in terms of in vivo cerebellar development and function has been difficult to elucidate because Lama1 knockout mice die in early embryonic stages (Miner et al., 2004). In this report, using conditional knockout mice for Lama1, we demonstrate that Lama1 is important for cerebellar development. Our data show that Lama1 is required for proper conformation of the meninges and for proper formation of the glial fibers.

During cerebellar development, two kinds of glial cells, radial glia and Bergmann glia, function in the migration of Purkinje cells and granule cells, which are different neuron types (Hatten, 1999; Rakic, 1971). During development, the radial glial cells disappear and are replaced by Bergmann glia until birth (Yuasa, 1996; Yuasa et al., 1996). It is conceivable that the observed defects in Bergmann glial fibers cause the aggregated granule cells under the pia and reduced GCP migration in adult *Lama1*^{CKO} mice. The interaction between GCPs and Bergmann glial fibers is necessary for GCP migration, and several adhesion molecules, such as NCAM, L1, and thrombospondin, contribute to these interactions (Chuong et al., 1987; O'Shea et al., 1990). Because laminin-1 containing Lama1 can interact with the HNK-1 carbohydrates of NCAM and L1 (Hall et al., 1997), Lama1 may be required to organize the contact between GCPs and Bergmann glia under the pia, in concert with these adhesion molecules.

Lama1 also regulates the distribution and dendritic elongation of Purkinje cells. In the mouse cerebellum, Purkinje cell precursors are generated in the primary rhombic lip between the E11 and E13 stages and these precursors migrate to the cortex along the radial

glial fibers. Then, during the postnatal 3 weeks, dendrites of the Purkinje cells extend, branch, and form synapses with other neurons. Reelin, a large extracellular protein, promotes migration of Purkinje cells through radial glial guidance and lamination of Purkinje cells (Tissir and Goffinet, 2003). Since Lama1 has epidermal growth factor-like repeats similar to reelin, the abnormal lamination of Purkinje cells in *Lama1*^{CKO} mice might be due to an impaired radial glial system.

Various endogenous and microenvironmental factors, such as granule cells, growth factors, steroids, thyroid hormone, coricotroprotein-releasing factor, calcium-related molecules, PKCγ, RORα and pleiotrophin-PTP ζ, are involved in dendrite formation and extension in Purkinje cells (Tanaka, 2009). Granule cells contribute to the formation of Purkinje cell dendrites via granule-Purkinje cell interactions, while electrical activity affects dendritic differentiation through calcium signaling. The poor dendritic formation of Purkinje cells in *Lama1*^{CKO} mice may be due to a reduction in granule cell migration and to abnormal Bergmann glial processes.

Integrin β1, α-dystroglycan, FAK, and ILK, which are receptors and signaling molecules of Lama1, are critical for the proliferation and migration of granule cells and GCPs during cerebellar development (Blaess et al., 2004; Graus-Porta et al., 2001; Mills et al., 2006; Watanabe et al., 2008). Laminin-1 is also reported to modulate sonic hedgehog (SHH) function and to increase proliferation of GCPs (Pons et al., 2001). We showed that Lama1 is essential for the proliferation and migration of GCPs. This Lama1 activity is similar to the activity of the Lama1 receptors, but the defects observed in cerebellar size and motor functions in *Lama1*^{CKO} mice were considerably more severe compared to those observed in other mutant mice. This may be because Lama1 interacts with both integrin β1 and α-dystroglycan. We found that the expression and activation of integrin β1 signaling were unchanged in *Lama1*^{CKO} mice. In contrast, the activation of Akt was transiently decreased in the *Lama1*^{CKO} cerebellum. The PI-3 kinase-Akt pathway is a signaling cascade that involves both integrin β1 and α-dystroglycan, and activation of PI-3 kinase-Akt signaling promotes neural cell proliferation and survival (Barnabe-Heider and Miller, 2003; Groszer et al., 2001). Therefore, the observed decrease in proliferation of GCPs may be due to the reduction in Akt activation at the early postnatal stages. At later stages, Akt phosphorylation levels reached normal levels, similar to those found in WT mice, probably because different external signals induced Akt activation for other cell functions, such as survival. In *Lama1*^{CKO} mice, the expression levels of Lama4 did not change, but Lama2 expression was slightly increased and Lama5 expression was reduced in the *Lama1*^{CKO} cerebellum. Double knockout mice for *Lama2* and *Lama4* showed a reduction in cortical sizes, an increase in apoptotic cells at the ventricular neuroepithelium, and detachment of radial glial cell processes in the cerebral cortex (Radakovits et al., 2009). However, these abnormalities are not observed in single gene knockouts for *Lama2* and *Lama4* (Radakovits et al., 2009). The meninges have been implicated in the regulation of radial glial survival using a slice culture that lacks the meninges (Radakovits et al., 2009) and in proper morphology of radial glia cells and migration of Cajal-Retzius cells in the cortex(Halfter et al., 2002). Lama1 deficiency resulted in discontinuous pia basement membranes (Fig. 9), although Lama2, Lama5 and nidogen were present in non-disrupted areas of the basement membrane (Supplementary material Fig. S4). These results suggest that Lama1 is required for the formation of the pial basement membrane and the meningeal structure. Thus, Lama1 may serve an important function among laminin α chains expressed in the meninges, as *Lama1*^{CKO} mice show remarkable phenotypic changes in the cerebellum following a single knockout.

The meninges consist of three membranous layers composed of fibroblasts, extracellular matrix, and a well-organized fluid-containing space. These membranes are the dura mater,

arachnoid, and pia mater. These structures serve to protect the central nervous system and to entrap a fluid-filled space that is important for brain function and survival. In the adult brain, gap junctions are abundant in the meninges and can mediate cell-cell communication and cellular morphogenesis, proliferation, and differentiation (Mercier and Hatton, 2001; Nagy et al., 1997). Connexins (Cxs) are implicated in these biological processes through their gap junction and hemichannel activities. Cx43 is expressed in the arachnoid and pia mater of the meninges (Nagy et al., 1999). Glia-specific Cx43 knockout mice display a reduction in size of the cerebellum, granule cell ectopies, and dislamination of Purkinje cells, granule cells, and Bergmann glia (Clark and Barbour, 1997; Muller et al., 1996; Wiencken-Barger et al., 2007). Cx43 is required for cerebellum-dependent motor coordination and motor learning (Tanaka et al., 2008a; Tanaka et al., 2008b). Interestingly, these morphological defects observed in Cx43 knockout mice are similar to those seen in *Lama1*^{CKO} mice. We found that a deficiency in Lama1 results in the reduction and altered localizations of Cx43 in the cerebellum. Knockout mice for laminin 5 (Lam-332) also show altered expression and localization of Cx43 via laminin-integrin interactions in epithelial cells (Lampe et al., 1998; Isakson et al., 2006). In neuronal cells, laminin has been reported to affect the populations of postnatal neural stem and progenitor cells by altering the expression of connexins (Imbeault et al., 2009). Thus, Lama1 may be required for functional localization of connexins in the cerebellum through a similar mechanism and may have a similar function in cerebellar development.

4. Experimental procedures

4.1. Generation of conventional and conditional Lama1 knockout mice

A mouse genomic clone encoding the 5′ part of *Lama1* was isolated from a mouse 129SvJ genomic library by screening with mouse *Lama1* cDNA (Sasaki et al., 1988). A DNA fragment containing exons 14 to 18 of *Lama1* from the genomic clone was used for gene targeting. The PGKneo^{r}-PGKtk cassette from pPNT was flanked with two loxP sequences at each end and inserted into intron 15, and a third loxP sequence was cloned into intron 17 of *Lama1*. The tk-DTa cassette, which encodes the diphtheria toxin A subunit, was attached to the short arm of the targeting vector for negative selection. The *Lama1*-loxP construct (supplementary material Fig. S1A) was linearized by *Cla*I and electroporated into R1 ES cells. The homologous recombinant cells were isolated by drug selection and transiently transfected with the CMV-cre expression plasmid (pBS185, Gibco Life Technology). Two different types of targeted ES clones were isolated, one containing the *Lama1*-deficient allele and the other containing the floxed-*Lama1* allele (supplementary material Fig. S1A).

Two independent clones from each were injected into blastocysts to generate chimeric mice. These chimera were mated with C57BL/6 mice to generate heterozygous null (*Lama1*$^{del/+}$) or floxed-*Lama1* (*Lama1*$^{flox/+}$) mice. Genotypes of the mutant alleles were confirmed by genomic PCR and Southern blotting (supplementary material Fig. S1B and C). These mice were further intercrossed to generate homozygous null (*Lama1*$^{del/del}$), heterozygous null/floxed (*Lama1*$^{del/flox}$), or floxed-*Lama1* (*Lama1*$^{flox/flox}$) offspring. Heterozygous *Lama1* null mice containing the *Sox2-Cre* gene (*Lama1*$^{del/+}$;*Sox2-Cre*$^{cre/+}$) were created by crossing with *Lama1*$^{del/+}$mice with *Sox2-Cre*$^{cre/+}$ mice from The Jackson Laboratory, in which the Cre recombinase gene was inserted in the *Sox2* locus (Hayashi et al., 2002). Mice with a conditional *Lama1*-deficiency (*Lama1*^{CKO}) specifically in epiblasts were created by crossing *Lama1*$^{del/+}$;*Sox2-Cre*$^{cre/+}$) with floxed-*Lama1* (*Lama1*$^{flox/flox}$) mice. *Lama1*$^{flox/del}$ mice developed normally and were used as controls.

Cre-mediated recombination in both null and conditional *Lama1* alleles was identified by Southern blotting and genomic PCR. DNA was prepared from mice, digested with *Xba*I, and hybridized with the probe indicated in Figure 1A, the WT *Lama1* allele. For PCR, DNA was

denatured at 95°C for 15 min and then amplified for 30 cycles (30 sec at 94°C, 60 sec at 60°C, and 90 sec at 72°C) with ExTaq (Takara). The following primer sets were used: wild-type and floxed alleles: E85 (5′-TACTTCAACGTGGTTAGACTTGTGCCTG-3′) and E100 (5′-ATTAGGGCTTGCTATGTCCAGAGAGACAG-3′); null allele, E85 (5′-TACTTCAACGTGGTTAGACTTGTGCCTG-3′) and E114 (5′-TTGTGCCTTTACTAAGCCCTGCTCC-3′); cre allele, E350 (5′-AAAATTTGCCTGCATTACCG-3′) and E360 (5′-ATTCTCCCACCGTCAGTACG-3′). Deficiency of *Lama1* mRNA expression was confirmed by RT-PCR using E85 primer and 5′-CACACAGAGCAAATTCCATGA-3′. Both male and female mice were used for analyses.

4.2. Locomotion analyses

Mice were tested for neuropathic defects with the tail suspension test at 8–15 weeks of age. Mice were suspended for 5 min and observed. For the rotarod analysis, mice were trained for 5 min on a rod rotating at 10 rpm. This training was performed 2 times at 45-min intervals. After the training, a trial was performed on a rod that was accelerated linearly from 10 to 35 rpm over 5 min, and the time taken to fall from the rod was analyzed. The footprint analysis was modified as described previously (Karimi-Abdolrezaee et al., 2006). The mice were trained to walk on a narrow paper-covered runway (50 cm long and 5.5 cm wide). Their forelimbs and hindlimbs were dipped in black and red ink, respectively, and the mice walked on the paper 3 times. For the measurements, the first and last 10 cm of the prints were excluded. If the mouse stopped in the middle of the track, the trial was repeated. The data were analyzed by calculating the average of all steps per print in all trials.

4.3. Histological analysis

Mice at 8 weeks age were fixed by perfusion with 4% paraformaldehyde (PFA) under anesthesia. Samples of brain tissue were fixed in 4% PFA and embedded in paraffin. Sections (4 microns thick) were deparaffinized with xylene, hydrated in a graded series of ethanol, and washed with PBS. Tissue sections were stained with Luxol Fast Blue.

4.4. Immunohistochemistry

The brains were fixed with 4% PFA in 0.1 M PBS, pH 7.4 overnight at 4°C and then cryoprotected in 30% sucrose in PBS for 72 h at 4°C. Sections (20 μm) were cut with a cryostat and mounted onto glass slides. For calbindin staining, the brain was again fixed by perfusion with 4% PFA in 0.1 M PBS, pH 7.4 but sections (50 μm) were prepared using a vibratome. For immunostaining, the frozen sections were air-dried and washed with PBS. The paraffin-embedded sections were deparaffinized and incubated with either proteinase K (DAKO) for GFAP or 10 mM citrate buffer, pH 6.0 under microwave irradiation. The sections were incubated with 0.1% Triton X-100 in PBS for 15 min. After washing, the sections were blocked with blocking buffer (5% normal donkey serum and 2% BSA in PBS) for 30 min, and incubated with dilutions of the primary antibody in the blocking buffer overnight at 4°C. The following primary antibodies were used: rabbit anti-laminin α1 (Lama1) (Sasaki et al., 2002); rabbit anti-laminin α2 (Lama2), rabbit anti-laminin α3 (Lama3), rabbit anti-laminin α4 (Lama4), rabbit anti-laminin α5 (Lama5) (a gift from Dr. Sasaki); rat anti-nidogen-1 (Millipore); mouse anti-phospho-histone 3 (Ser10)(Cell Signaling); rat anti-BrdU (Serotec); rabbit anti-GFAP (DAKO); rabbit anti-Pax6 (Covance); mouse anti-radial glial cell marker-2 (Millipore) and anti-calbindin D28K (Millipore). The slides were washed with PBS and incubated with the secondary antibodies, Alexa 488 donkey anti-rabbit (Molecular Probes), Cy3 donkey anti-mouse, and Cy3 donkey anti-rat (Jackson Immuno) for 1 h. The slides were washed with PBS, coverslipped with mounting medium containing DAPI, and observed using a Zeiss 510 confocal laser microscope. For connexin staining, the frozen sections were fixed in acetone and incubated with primary

antibodies in 0.2% gelatin PBS for 3 h. The primary antibodies included rabbit anti-connexin 26 (Invitrogen), monoclonal mouse anti-connexin 43 (Invitrogen), and rat monoclonal anti-laminin γ1 (Lamc1) chain (Millipore). The secondary antibodies were AlexaFluor 488 goat anti-rabbit, AlexaFluor 488 and 546 goat anti-mouse (Invitrogen), and Cy™5-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories) and were incubated for 1 h. In each experiment, several sections were incubated without the primary antibody to serve as controls. When the primary antibody was omitted from the staining, no immunoreactivity was observed.

4.5. Slice culture

Slice culture of cerebellum tissue was established as described previously (Streit et al., 1993). Cerebella from postnatal day 10 (P10) mice were sliced into 300 μm thick pieces using a tissue chopper (Muromachi Kikai Co., Ltd). The slices were put onto Millicell tissue culture inserts (Millipore) and incubated with 5 mM bromodeoxyuridine (BrdU) in culture medium comprising 50% Minimum essential medium, 25% Hanks' balanced salt solution, and 25% horse serum (Invitrogen) for 30 min at 37° C in 5% CO_2. The slices were washed with the culture medium and cultured with or without 5 μg/ml laminin-1 (Sigma). After incubation, the slices were frozen and cut in a sagittal orientation. The sections were fixed with 4% PFA in 0.1 M PBS, pH 7.4, and incubated with 0.5% Triton X-100 in PBS for 15 min. The sections were subsequently incubated with 2 N HCl for 30 min at 37° C, neutralized with 0.1 M borate buffer for 10 min and then stained as described above.

4.6. Western blotting

Lysate from the cerebellum was prepared in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1.5 mM $MgCl_2$, 10% glycerol, complete mini protease inhibitor mixture (Roche), 1 mM PMSF, 1 mM Na_3VO_4, and 100 mM NaF, pH 7.4. The lysate was subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen). For immunoblotting, the gels were transferred onto PVDF membranes, blocked with 5% non-fat milk (Cell Signaling), and incubated with primary antibodies in 5% BSA overnight at 4° C. The primary antibodies used were mouse anti-phospho Akt (Ser473), rabbit anti-Akt, phospho-Src family kinase (Tyr416), rabbit anti-ILK1 (Cell Signaling), rabbit anti-FAK (Tyr397), mouse anti-FAK, mouse anti-GAPDH, mouse anti-Src family kinase (Millipore), and rabbit anti-integrin β1 (Santa Cruz).

4.7. Electron microscopy

The cerebellum of P0 mice was dissected and fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4). The cerebellum of adult mice was dissected and perfusion-fixed with 2% PFA/2% glutaraldehyde in phosphate buffer (pH 7.4). The cerebellum was postfixed with 1% OsO_4 in PBS for 1 hour at 4°C, dehydrated through a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were stained with 4% uranyl acetate and lead citrate and then examined with a JEM1230 (JOEL) electron microscope.

4.8. Statistical analyses

Data are presented as the mean and SD. The minimum level of statistical significance was set at P=0.05. Differences were analyzed using the two-sided Student's t test with unequal variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Glenn Longenecker and Ashok B. Kulkarni for their help in creating the mutant mice, Takako Sasaki for the laminin α1 antibody, and Hynda K. Kleinman for critical reading. This work was supported by the Intramural Program of the NIDCR, National Institutes of Health (Y.Y.) and grants from the Ministry of Education, Culture, Sports Science and Technology of Japan (17082008 and 2230023 to E. A-H.).

Abbreviations

CKO	conditional *Lama1* knockout
CNS	central nervous system
Cont	control
ECM	extracellular matrix
EGL	external granule layer
GCPs	distorted the layer; granule cell precursors
IGL	internal granule layer

References

Alpy F, Jivkov I, Sorokin L, Klein A, Arnold C, Huss Y, Kedinger M, Simon-Assmann P, Lefebvre O. Generation of a conditionally null allele of the laminin alpha1 gene. Genesis. 2005; 43:59–70. [PubMed: 16100707]

Andrae J, Afink G, Zhang XQ, Wurst W, Nister M. Forced expression of platelet-derived growth factor B in the mouse cerebellar primordium changes cell migration during midline fusion and causes cerebellar ectopia. Mol Cell Neurosci. 2004; 26:308–321. [PubMed: 15207855]

Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, Engel J, Engvall E, Hohenester E, Jones JC, Kleinman HK, Marinkovich MP, Martin GR, Mayer U, Meneguzzi G, Miner JH, Miyazaki K, Patarroyo M, Paulsson M, Quaranta V, Sanes JR, Sasaki T, Sekiguchi K, Sorokin LM, Talts JF, Tryggvason K, Uitto J, Virtanen I, von der Mark K, Wewer UM, Yamada Y, Yurchenco PD. A simplified laminin nomenclature. Matrix Biol. 2005; 24:326–332. [PubMed: 15979864]

Barnabe-Heider F, Miller FD. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci. 2003; 23:5149–5160. [PubMed: 12832539]

Blaess S, Graus-Porta D, Belvindrah R, Radakovits R, Pons S, Littlewood-Evans A, Senften M, Guo H, Li Y, Miner JH, Reichardt LF, Muller U. Beta1-integrins are critical for cerebellar granule cell precursor proliferation. J Neurosci. 2004; 24:3402–3412. [PubMed: 15056720]

Chuong CM, Crossin KL, Edelman GM. Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers. J Cell Biol. 1987; 104:331–342. [PubMed: 3805122]

Clark BA, Barbour B. Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. J Physiol. 1997; 502 (Pt 2):335–350. [PubMed: 9263914]

Edmondson JC, Hatten ME. Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. J Neurosci. 1987; 7:1928–1934. [PubMed: 3598656]

Edwards MM, Mammadova-Bach E, Alpy F, Klein A, Hicks WL, Roux M, Simon-Assmann P, Smith RS, Orend G, Wu J, Peachey NS, Naggert JK, Lefebvre O, Nishina PM. Mutations in Lama1 disrupt retinal vascular development and inner limiting membrane formation. J Biol Chem. 2010; 285:7697–7711. [PubMed: 20048158]

Ekblom P, Lonai P, Talts JF. Expression and biological role of laminin-1. Matrix Biol. 2003; 22:35–47. [PubMed: 12714040]

Goldowitz D, Hamre K. The cells and molecules that make a cerebellum. Trends Neurosci. 1998; 21:375–382. [PubMed: 9735945]

Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, Orban P, Klein R, Schittny JC, Muller U. Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron. 2001; 31:367–379. [PubMed: 11516395]

Groszer M, Erickson R, Scripture-Adams DD, Lesche R, Trumpp A, Zack JA, Kornblum HI, Liu X, Wu H. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. Science. 2001; 294:2186–2189. [PubMed: 11691952]

Gullberg D, Ekblom P. Extracellular matrix and its receptors during development. Int J Dev Biol. 1995; 39:845–854. [PubMed: 8645569]

Halfter W, Dong S, Yip YP, Willem M, Mayer U. A critical function of the pial basement membrane in cortical histogenesis. J Neurosci. 2002; 22:6029–6040. [PubMed: 12122064]

Hall H, Carbonetto S, Schachner M. L1/HNK-1 carbohydrate- and beta 1 integrin-dependent neural cell adhesion to laminin-1. J Neurochem. 1997; 68:544–553. [PubMed: 9003039]

Hatten ME. Central nervous system neuronal migration. Annu Rev Neurosci. 1999; 22:511–539. [PubMed: 10202547]

Hayashi S, Lewis P, Pevny L, McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mech Dev. 2002; 119(Suppl 1):S97–S101. [PubMed: 14516668]

Herrup K, Kuemerle B. The compartmentalization of the cerebellum. Annu Rev Neurosci. 1997; 20:61–90. [PubMed: 9056708]

Ichikawa N, Iwabuchi K, Kurihara H, Ishii K, Kobayashi T, Sasaki T, Hattori N, Mizuno Y, Hozumi K, Yamada Y, Arikawa-Hirasawa E. Binding of laminin-1 to monosialoganglioside GM1 in lipid rafts is crucial for neurite outgrowth. J Cell Sci. 2009; 122:289–299. [PubMed: 19118221]

Imbeault S, Gauvin LG, Toeg HD, Pettit A, Sorbara CD, Migahed L, DesRoches R, Menzies AS, Nishii K, Paul DL, Simon AM, Bennett SA. The extracellular matrix controls gap junction protein expression and function in postnatal hippocampal neural progenitor cells. BMC Neurosci. 2009; 10:13. [PubMed: 19236721]

Isakson BE, Olsen CE, Boitano S. Laminin-332 alters connexin profile, dye coupling and intercellular Ca2+ waves in ciliated tracheal epithelial cells. Respir Res. 2006; 7:105. [PubMed: 16884540]

Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Morshead CM, Fehlings MG. Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. J Neurosci. 2006; 26:3377–3389. [PubMed: 16571744]

Kleinman HK, Sephel GC, Tashiro K, Weeks BS, Burrous BA, Adler SH, Yamada Y, Martin GR. Laminin in neuronal development. Ann N Y Acad Sci. 1990; 580:302–310. [PubMed: 2186693]

Komuro H, Rakic P. Distinct modes of neuronal migration in different domains of developing cerebellar cortex. J Neurosci. 1998; 18:1478–1490. [PubMed: 9454856]

Lampe PD, Nguyen BP, Gil S, Usui M, Olerud J, Takada Y, Carter WG. Cellular interaction of integrin alpha3beta1 with laminin 5 promotes gap junctional communication. J Cell Biol. 1998; 143:1735–1747. [PubMed: 9852164]

Li S, Edgar D, Fassler R, Wadsworth W, Yurchenco PD. The role of laminin in embryonic cell polarization and tissue organization. Dev Cell. 2003; 4:613–624. [PubMed: 12737798]

Li S, Liquari P, McKee KK, Harrison D, Patel R, Lee S, Yurchenco PD. Laminin-sulfatide binding initiates basement membrane assembly and enables receptor signaling in Schwann cells and fibroblasts. J Cell Biol. 2005; 169:179–189. [PubMed: 15824137]

Mercier F, Hatton GI. Immunocytochemical basis for a meningeo-glial network. J Comp Neurol. 2000; 420:445–465. [PubMed: 10805920]

Mercier F, Hatton GI. Connexin 26 and basic fibroblast growth factor are expressed primarily in the subpial and subependymal layers in adult brain parenchyma: roles in stem cell proliferation and morphological plasticity? J Comp Neurol. 2001; 431:88–104. [PubMed: 11169992]

Mills J, Niewmierzycka A, Oloumi A, Rico B, St-Arnaud R, Mackenzie IR, Mawji NM, Wilson J, Reichardt LF, Dedhar S. Critical role of integrin-linked kinase in granule cell precursor proliferation and cerebellar development. J Neurosci. 2006; 26:830–840. [PubMed: 16421303]

Miner JH, Li C, Mudd JL, Go G, Sutherland AE. Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. Development. 2004; 131:2247–2256. [PubMed: 15102706]

Miner JH, Yurchenco PD. Laminin functions in tissue morphogenesis. Annu Rev Cell Dev Biol. 2004; 20:255–284. [PubMed: 15473841]

Muller T, Moller T, Neuhaus J, Kettenmann H. Electrical coupling among Bergmann glial cells and its modulation by glutamate receptor activation. Glia. 1996; 17:274–284. [PubMed: 8856324]

Nagy JI, Li X, Rempel J, Stelmack G, Patel D, Staines WA, Yasumura T, Rash JE. Connexin26 in adult rodent central nervous system: demonstration at astrocytic gap junctions and colocalization with connexin30 and connexin43. J Comp Neurol. 2001; 441:302–323. [PubMed: 11745652]

Nagy JI, Ochalski PA, Li J, Hertzberg EL. Evidence for the co-localization of another connexin with connexin-43 at astrocytic gap junctions in rat brain. Neuroscience. 1997; 78:533–548. [PubMed: 9145808]

Nagy JI, Patel D, Ochalski PA, Stelmack GL. Connexin30 in rodent, cat and human brain: selective expression in gray matter astrocytes, co-localization with connexin43 at gap junctions and late developmental appearance. Neuroscience. 1999; 88:447–468. [PubMed: 10197766]

O'Shea KS, Rheinheimer JS, Dixit VM. Deposition and role of thrombospondin in the histogenesis of the cerebellar cortex. J Cell Biol. 1990; 110:1275–1283. [PubMed: 2182649]

Pons S, Trejo JL, Martinez-Morales JR, Marti E. Vitronectin regulates Sonic hedgehog activity during cerebellum development through CREB phosphorylation. Development. 2001; 128:1481–1492. [PubMed: 11290288]

Qu Q, Smith FI. Alpha-dystroglycan interactions affect cerebellar granule neuron migration. J Neurosci Res. 2004; 76:771–782. [PubMed: 15160389]

Radakovits R, Barros CS, Belvindrah R, Patton B, Muller U. Regulation of radial glial survival by signals from the meninges. J Neurosci. 2009; 29:7694–7705. [PubMed: 19535581]

Rakic P. Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus. J Comp Neurol. 1971; 141:283–312. [PubMed: 4101340]

Rakic P, Sidman RL. Weaver mutant mouse cerebellum: defective neuronal migration secondary to abnormality of Bergmann glia. Proc Natl Acad Sci U S A. 1973; 70:240–244. [PubMed: 4509657]

Sasaki M, Kleinman HK, Huber H, Deutzmann R, Yamada Y. Laminin, a multidomain protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. J Biol Chem. 1988; 263:16536–16544. [PubMed: 3182802]

Sasaki T, Giltay R, Talts U, Timpl R, Talts JF. Expression and distribution of laminin alpha1 and alpha2 chains in embryonic and adult mouse tissues: an immunochemical approach. Exp Cell Res. 2002; 275:185–199. [PubMed: 11969289]

Satz JS, Ostendorf AP, Hou S, Turner A, Kusano H, Lee JC, Turk R, Nguyen H, Ross-Barta SE, Westra S, Hoshi T, Moore SA, Campbell KP. Distinct functions of glial and neuronal dystroglycan in the developing and adult mouse brain. J Neurosci. 2010; 30:14560–14572. [PubMed: 20980614]

Scheele S, Falk M, Franzen A, Ellin F, Ferletta M, Lonai P, Andersson B, Timpl R, Forsberg E, Ekblom P. Laminin alpha1 globular domains 4–5 induce fetal development but are not vital for embryonic basement membrane assembly. Proc Natl Acad Sci U S A. 2005; 102:1502–1506. [PubMed: 15668394]

Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, Edgar D. Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J Cell Biol. 1999; 144:151–160. [PubMed: 9885251]

Solecki DJ, Model L, Gaetz J, Kapoor TM, Hatten ME. Par6alpha signaling controls glial-guided neuronal migration. Nat Neurosci. 2004; 7:1195–1203. [PubMed: 15475953]

Sotelo C. Cellular and genetic regulation of the development of the cerebellar system. Prog Neurobiol. 2004; 72:295–339. [PubMed: 15157725]

Streit A, Nolte C, Rasony T, Schachner M. Interaction of astrochondrin with extracellular matrix components and its involvement in astrocyte process formation and cerebellar granule cell migration. J Cell Biol. 1993; 120:799–814. [PubMed: 7678837]

Tanaka M. Dendrite formation of cerebellar purkinje cells. Neurochem Res. 2009; 34:2078–2088. [PubMed: 19821027]

Tanaka M, Yamaguchi K, Tatsukawa T, Nishioka C, Nishiyama H, Theis M, Willecke K, Itohara S. Lack of Connexin43-mediated bergmann glial gap junctional coupling does not affect cerebellar long-term depression, motor coordination, or eyeblink conditioning. Front Behav Neurosci. 2008a; 2:1. [PubMed: 18958191]

Tanaka M, Yamaguchi K, Tatsukawa T, Theis M, Willecke K, Itohara S. Connexin43 and bergmann glial gap junctions in cerebellar function. Front Neurosci. 2008b; 2:225–233. [PubMed: 19225596]

Tissir F, Goffinet AM. Reelin and brain development. Nat Rev Neurosci. 2003; 4:496–505. [PubMed: 12778121]

Watanabe F, Miyazaki T, Takeuchi T, Fukaya M, Nomura T, Noguchi S, Mori H, Sakimura K, Watanabe M, Mishina M. Effects of FAK ablation on cerebellar foliation, Bergmann glia positioning and climbing fiber territory on Purkinje cells. Eur J Neurosci. 2008; 27:836–854. [PubMed: 18279360]

Wiencken-Barger AE, Djukic B, Casper KB, McCarthy KD. A role for Connexin43 during neurodevelopment. Glia. 2007; 55:675–686. [PubMed: 17311295]

Yuasa S. Bergmann glial development in the mouse cerebellum as revealed by tenascin expression. Anat Embryol (Berl). 1996; 194:223–234. [PubMed: 8849669]

Yuasa S, Kawamura K, Kuwano R, Ono K. Neuron-glia interrelations during migration of Purkinje cells in the mouse embryonic cerebellum. Int J Dev Neurosci. 1996; 14:429–438. [PubMed: 8884376]

Highlights

Laminin a1 (Lama1) knockout mice in the epiblast lineage developed behavioral disorders and displayed impaired formation of the cerebellum. Deficiency of Lama1 in the pial basement membrane of the meninges resulted in a decrease in the proliferation and migration of granule cell precursors, disorganization of Bergmann glial fibers and endfeet. Lama1 is required for cerebellar development and functions.

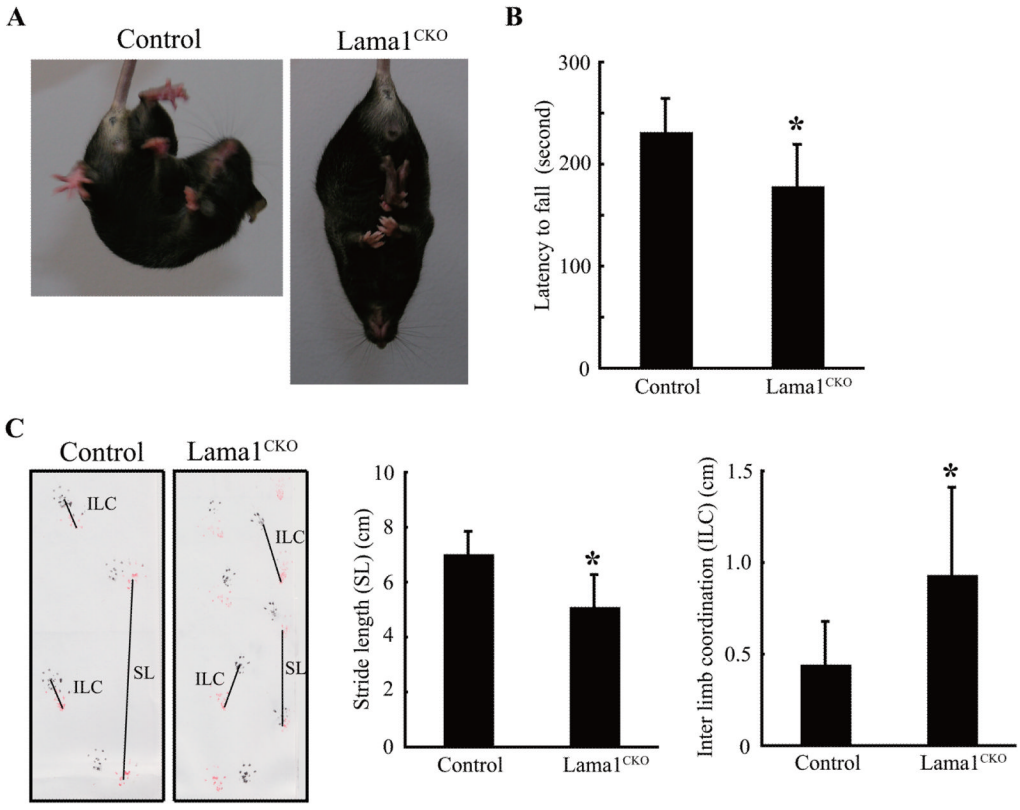

Fig 1.

Impaired behavior of Lama1^{CKO} mice. (A) Tail suspension test of adult mice. *Lama1*^{CKO} mice exhibit hugging behavior. (B) RotaRod performance at 8–15 weeks of age. *Lama1*^{CKO} mice show a marked decrease in latency to fall as compared with control *Lama1*$^{flox/del}$ mice. The bar graph shows the mean and SD of time to fall (n=10) (*, $P < 0.001$; two-sided t-test) (C) Representative footprints of control and *Lama1*^{CKO} mice. *Lama1*^{CKO} mice showed a significant reduction in stride length (SL) and increase in interlimb coordination (ILC) compared with control *Lama1*$^{flox/del}$ mice. The graphs show the mean and SD of length. (n=5) (*, $P < 0.001$; two-sided t-test).

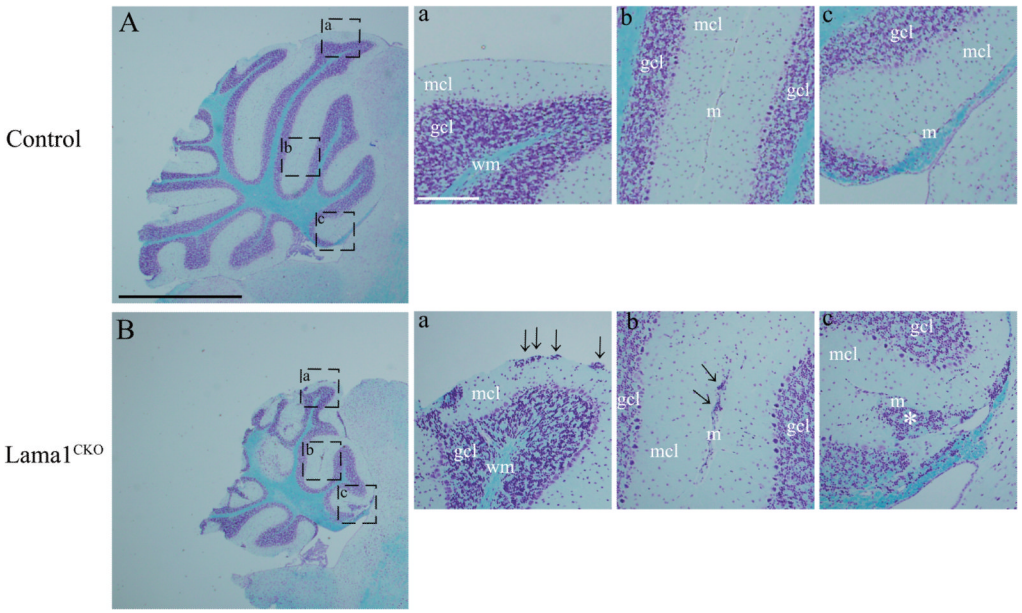

Fig. 2.

Cerebellar defects in Lama1^{CKO} mice. Sagittal sections of adult control (A) and *Lama1*^{CKO} mice at 8 weeks age (B) were stained with Luxol Fast Blue. The cerebellum of *Lama1*^{CKO} mice is smaller than that of control mice, and the formation of some folia is disrupted. (a–c) High magnification view of areas indicated. In *Lama1*^{CKO} mice, the aggregation of granule cells in the molecular layer was observed under meninges of the cerebellar surface and fusion lines of folia (arrows). The meninges, which form the fissure of the folia, were completely lacking (asterisk). mcl, molecular cell layer; gcl, granule cell layer; wm, white matter; m, meninges. Black scale bars: 1.0 mm, White scale bars: 100 μm.

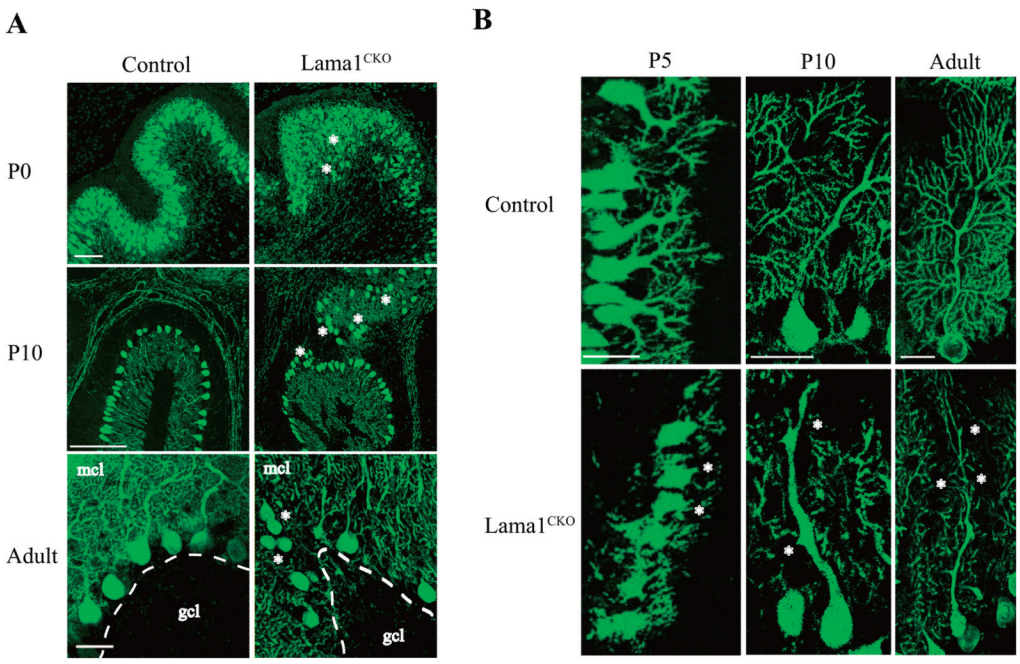

Fig. 3.

Abnormal arrangement and loss of processes in Purkinje cells in Lama1^{CKO} mice. Brains from P0, P5, P10, and adult (8 weeks old) mice were cut into sections of 50 μm using a vibratome. The sections were stained with anti-calbindin D28K. Images were obtained every 2 μm at a thickness of 30 μm using a confocal laser microscope and staked using LSM510 software. (A) In the lobules of *Lama1*^{CKO} mice, the arrangement of the Purkinje cell layer between the molecular layer (mcl) and granule cell layer (gcl) was disordered at P0, P10 and adult (asterisks). Scale bars: 50 μm. (B) The branching of dendritic processes of Purkinje cells decreased remarkably in *Lama1*^{CKO} compared to control *Lama1*$^{flox/del}$ mice at P5, P10 and adult (asterisks). Scale bars: 50 μm.

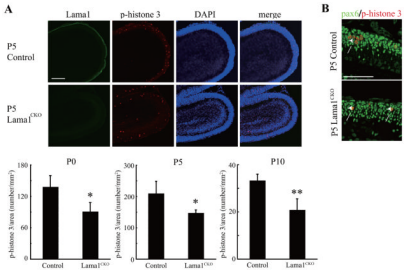

Fig. 4.

Reduction in granule cell precursor (GCP) proliferation in Lama1^{CKO} mice. (A) Sagittal cerebellar sections at postnatal day 5 (P5) were analyzed by staining with phospho-histone 3 (p-H3). Proliferation of GCPs in *Lama1*^{CKO} mice was markedly decreased. Fifteen 20 μm thick sections were successively prepared from the midline of the cerebellum. The number of p-H3-positive GCPs was counted and the area of the cerebellum was measured by Image J software. The bar graph shows the mean and SD of the number of p-H3- positive cells per area (n=5) (*, $P < 0.02$, **, $P < 0.005$; two-sided t-test). Scale bars: 100 μm. (B) Sagittal cerebellar sections at P5 were analyzed by staining with Pax6 and phospho-histone 3. Phospho-histone 3-positive cells were also positive for Pax6 (arrows). Scale bars: 50 μm

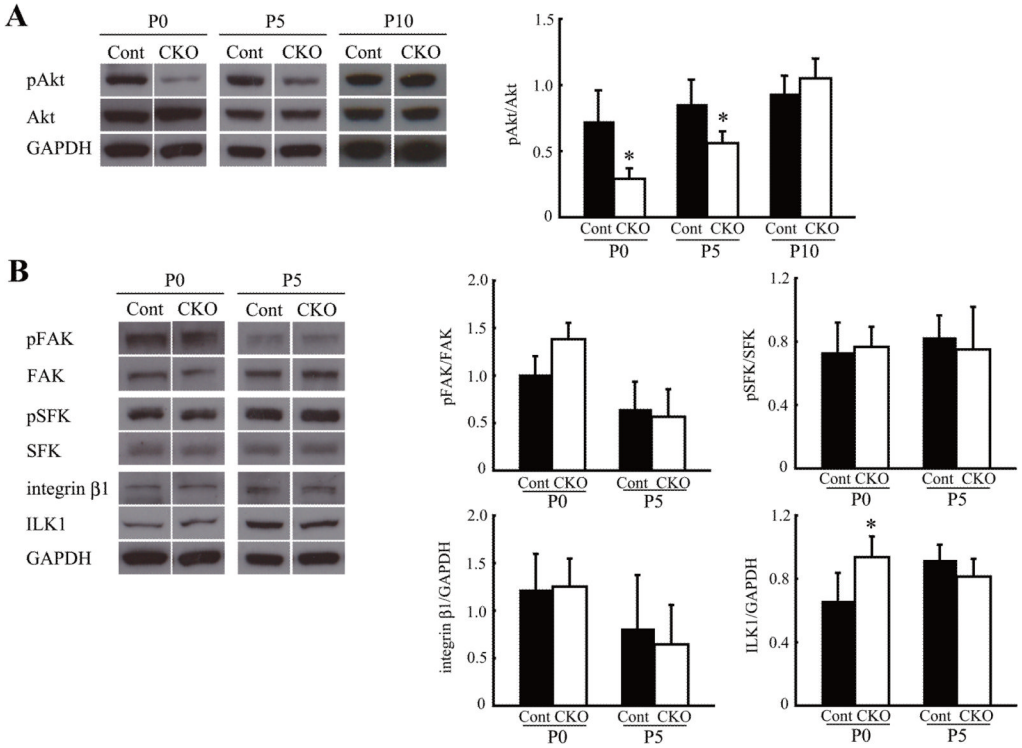

Fig. 5.

Reduced proliferation signaling in Lama1^{CKO} mice. Cerebellum of control (Cont) and *Lama1*^{CKO} mice (CKO) was analyzed by immunoblotting. (A) The phosphorylated Akt level was significantly decreased at P0 and P5 in *Lama1*^{CKO} mice, compared with control mice, but this reduction was not observed at P10. (B) The expression of ILK1 was increased at P0 in *Lama1*^{CKO} mice. The activation or expression of other integrin β related molecules was unchanged. The bar graphs show the mean and SD, which was calculated for each signaling density (n=5). (*, $P < 0.05$; two-sided t-test).

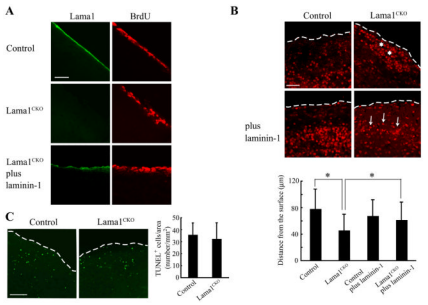

Fig. 6.

Reduced migration of proliferating granule cells in Lama1^{CKO} mice. Sagittal slices of the cerebellum of P10 control and *Lama1*^{CKO} mice were labeled with bromodeoxyuridine (BrdU) and then cultured. After culture, the slices were stained with anti-BrdU antibody and anti-Lama1. (A) At 1h after culture, lama1 was not expressed in the meninges in the slices of *Lama1*^{CKO} mice, but the expression was recovered by adding 5 μg/ml laminin-1. Scale bars: 20 μm. (B) The migration of BrdU-positive granule cells to the inner layer in *Lama1*^{CKO} mice decreased after a 72 h culture period (asterisks). However, the decrease could be rescued by adding 5 μg/ml laminin-1 (arrows). The dashed lines show the surface of the cerebellum. Scale bars: 20 μm. The bar graphs show the calculated mean and SD, (n=500 cells). (*, $P < 0.001$; two-sided t-test). (C) After culturing for 72h, the slices were stained using the TUNEL method. The dashed lines show the surface of the cerebellum. No differences were observed in the number of apoptotic cells in the control or *Lama1*^{CKO} mice. The bar graphs show the calculated mean and SD. Scale bars: 100 μm.

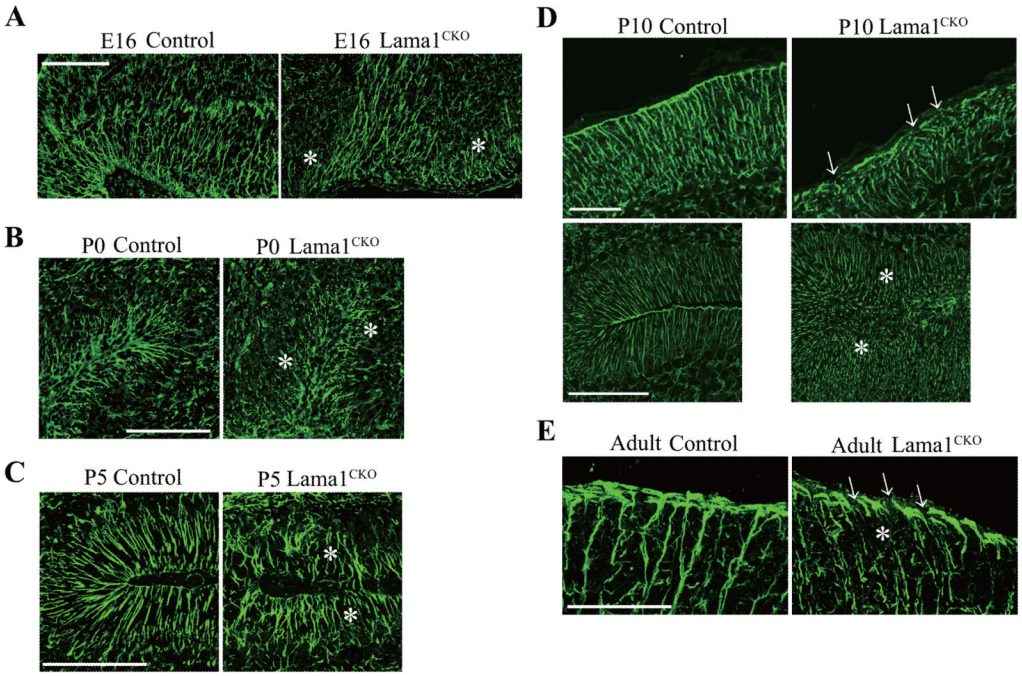

Fig. 7.

Aberration of radial and Bergmann glial fibers and endfeet in Lama1^{CKO} mice. Cerebellar sections from E16 (A), P0 (B), P5 (C), P10 (D) and adult (E) mice were stained with anti-radial glial marker-2 or anti-GFAP antibody. In control mice, radial glia and Bergmann glial fibers extended to the meninges in the fissure of folia, whereas in *Lama1*^{CKO} mice, the glial fibers were discontinuous and fragmented (asterisks). (D) At the surface of the cerebellum, Bergmann glial endfeet are continuous along the meninges in control mice. Some endfeet are disrupted in *Lama1*^{CKO} mice (D and E, arrows). Scale bars: 100 μm. Scale bars: 200 μm (A and B), 50 μm (C and E), 100 μm (D).

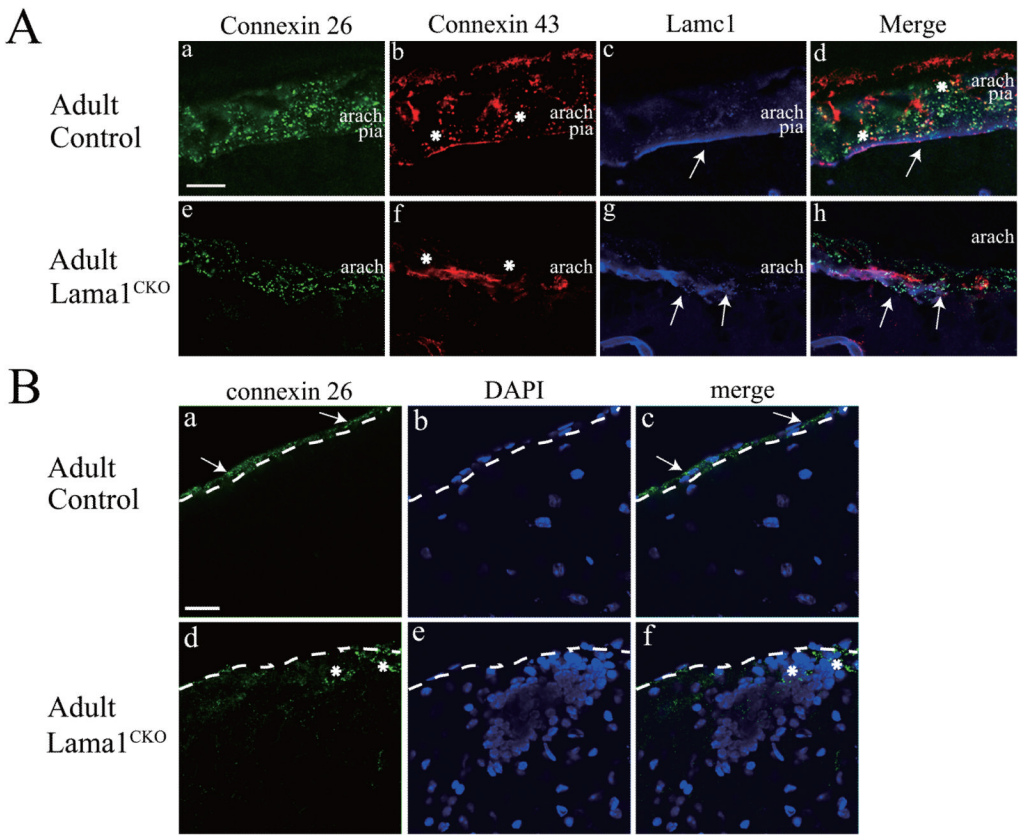

Fig. 8.

Abnormal expression of connexin proteins in the meninges of *Lama1*^{CKO} mice. (A) Sagittal sections of the cerebellar surface of control (a–d) and *Lama1*^{CKO} (e–h) animals were immunostained with antibodies to connexin 26 (a and c, green), connexin 43 (b and f, red), and Lamc1 (laminin γ1, c and g, blue). Merged images in d and h. Both Cx26 and Cx43 were expressed in the arachnoid and pia of control meninges (a, b, and d). In *Lama1*^{CKO} mice, Cx26 expression was reduced in the arachnoid barrier (e and h) and Cx43 was almost entirely absent from the arachnoid (f and h, asterisks). Lamc1 was stained in a thin and continuous layer (arrows) of the pial basement membrane in control mice (c and d) but its staining was discontinuous (g and h, arrows) in *Lama1*^{CKO} mice. Scale bar: 20 μm. (B) Connexin 26 expression in granule ectopies. Sagittal sections of the cerebellar surface of control (a–c) and *Lama1*^{CKO} (d–f) mice were immunostained with antibodies to connexin 26 (green) with DAPI (blue). Cx26 puncta were present in the pial fibroblastic layer of control mice (a and c, arrows). In *Lama1*^{CKO} mice, Cx26 were stained in ectopic granule cells near the surface (d and f, asterisks). Aggregated granule cells were observed under the meninges in *Lama1*^{CKO} mice (e and f). The dashed lines show the borderline between the pial membrane and the surface of the cerebellum. Scale bar: 10 mm.

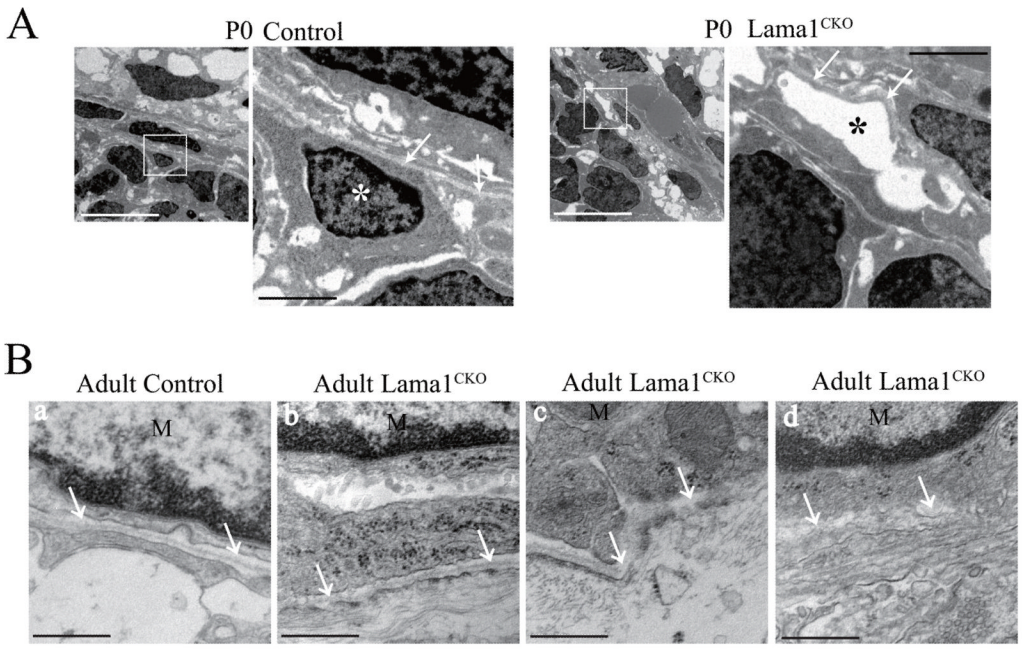

Fig. 9.

Disruption of the pial basement membranes in Lama1^{CKO} mice.

(A) Electron micrographs of the cerebellar surface of mice at P0. White boxed areas are enlarged in the right panels. In *Lama1*^{CKO} mice, the density of glial cells was remarkably low compared with that of the control mice (white and black asterisk). The pial basal lamina of *Lama1*^{CKO} mice was distorted, curved and discontinuous (arrows). White scale bar: 10 μm, Black scale bar: 2 μm. (B) Electron micrograph of the cerebellar surface of adult mice. Control (a) and *Lama1*^{CKO} mice (b–d). The pial basal lamina in *Lama1*^{CKO} mice was often irregular (b, arrows), disorganized (c, arrows) and discontinuous (d, arrows). M: Meningeal cells. Scale bar: 500 nm.