Essential role of α6 integrins in cortical and retinal lamination

Elisabeth Georges-Labouesse, Manuel Mark, Nadia Messaddeq and Anne Gansmüller

Extracellular matrix (ECM) is believed to play important roles in many aspects of nervous system development [1]. The laminins are ECM glycoproteins expressed in neural tissues and are potent stimulators of neurite outgrowth *in vitro* [1–3]. Genetic approaches using *Drosophila* and *Caenorhabditis elegans* have demonstrated a role for laminin and a laminin receptor *in vivo* in axon pathfinding and fasciculation, respectively [4,5]. In higher organisms, however, the role of laminins in the development of the nervous system is poorly understood. Integrins α6β1 and α6β4 are major laminin receptors. A role for the α6 integrin in neurulation has been reported in amphibians [6]. We previously described mice lacking integrin α6; these mice died at birth with severe skin blistering [7]. Detailed analyses of integrin α6$^{-/-}$ mice reported here revealed abnormalities in the laminar organization of the developing cerebral cortex and retina. Ectopic neuroblastic outgrowths were found on the brain surface and in the vitreous body in the eye. Alterations of laminin deposition were found in mutant brains. Thus, this study provides evidence for an essential role of integrin–laminin interactions in the proper development of the nervous system. These observations are particularly significant given the recent report that human patients suffering from epidermolysis bullosa can carry mutations in *ITGA6*, the gene encoding the α6 integrin chain [8,9].

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Results and discussion

Cerebral cortical and retinal lamination defects in α6$^{-/-}$ mice

At autopsy, the brain surface of embryonic day (E) 18.5 α6$^{-/-}$ fetuses displayed abnormal bulges. These bulges were readily visible by scanning electron microscopy on the cerebral hemispheres (Figure 1a–c), but also on the brain stem, indicating that this type of defect is not restricted to a particular region of the brain. From E13.5 to E18.5, defects were detected in all mutant brains (n = 16) by histological analysis (Figure 1d,e). During normal brain development, postmitotic neuroblasts migrate in an 'inside-out' manner from the ventricular zone to generate the cortical plate [10] by E14.5 in the mouse. The final architecture of the cortex with its six distinct zones is laid down postnatally from the cortical plate. At E13.5 and E14.5, the defects consisted of small clumps of ectopic neuroblasts continuous with the cortical plate and protruding at the brain surface. At later stages (E15.5–E18.5), mutant brains showed disorganization of the cortical plate manifested by a wavy aspect of this structure and/or by cortical plate outgrowths crossing the basement membrane (BM) surrounding the brain as well as the pia mater, and forming multiple large clumps of ectopic neuroblasts within the subarachnoidal space (Figure 1d,e). As many as 30–40 ectopic outgrowths were observed in mutant brains.

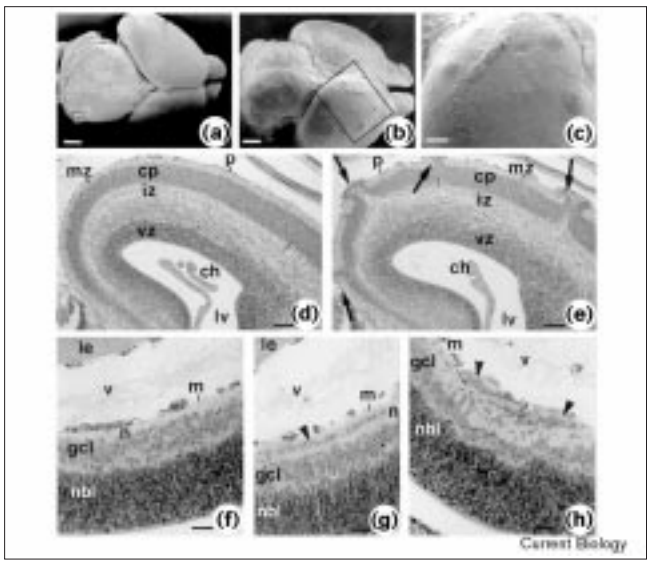
Ectopias of neuroblasts were also observed in the retina. At E15.5–E16.5, when the outer neuroblastic layer and the ganglion cell layer start to separate, ectopic cells were found either in the nerve fiber layer or, in the form of small clusters, in the vitreous body (Figure 1f–h). Here also, some ectopic neuroblasts had crossed the inner limiting membrane of the retina to localize within the vitreous body. A laminin-containing basal lamina is present at the level of the inner limiting membrane [11].

Integrin α6β1 is expressed in developing brain

The integrin α6 chain can associate with the β1 or β4 subunits. To determine which integrin heterodimer is implicated in the α6$^{-/-}$ brain and eye phenotype, we analyzed by *in situ* hybridization the expression of both β subunits in fetal brains from E13.5 to E16.5, stages at which the α6$^{-/-}$ phenotype is already apparent (Figure 2). At these stages, only β1 could be detected in different regions of the brain (Figure 2c). The expression pattern of β1 partially colocalized with that of α6, but also showed some differences, consistent with the fact that β1 can associate with other α subunits (Figure 2a–c). For integrin β4, no signal was detected in the cerebral cortex (Figure 2d). Integrin β1 was also highly expressed in the retina (data not shown). Thus, brain and most likely retinal abnormalities are caused by the loss of function of α6β1.

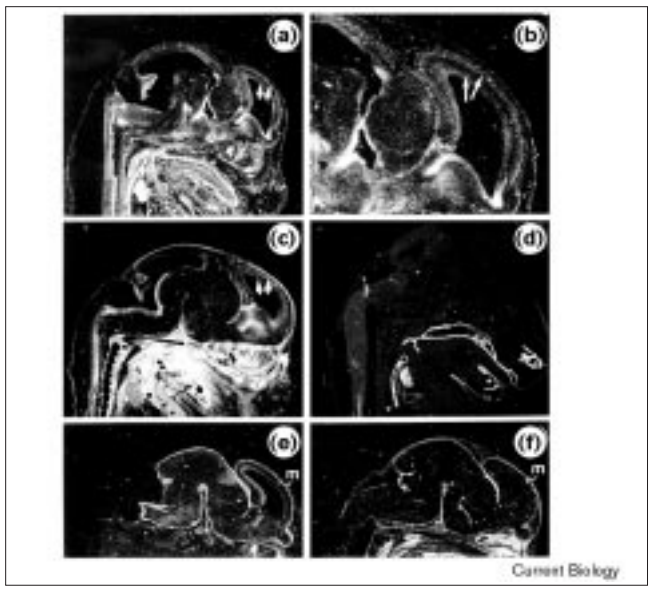
Several forms of laminin are recognized by integrin α6β1. To determine which laminin was present in the cortex when the defects occur, we analyzed the expression of two laminin α chains, α1 and α2 — constituents of laminin 1

Figure 1



(a–c) Scanning electron micrographs of (a) wild-type and (b,c) $\alpha6^{-/-}$ E18.5 brains. The area boxed in (b) is shown at higher magnification in (c). Note the presence of several outgrowths on the surface of a cerebral hemisphere. (d–h) Histological analysis of cortex and retina. (d,e) Coronal sections through the cerebral cortex of (d) wild-type and (e) $\alpha6^{-/-}$ fetuses at E16.5. Arrows points to ectopic clusters of cells that have crossed the pia mater. (f–h) Coronal sections through the retina of (f) wild-type and (g,h) $\alpha6^{-/-}$ E16.5 fetuses. Arrowheads point to an ectopic layer of nuclei in the nerve fiber layer (g) or to ectopic clusters of cells in the vitreous body (h). The ganglion cell layer appears disorganized. Abbreviations: ch, choroid plexus; cp, cortical plate; gcl, ganglion cell layer; iz, intermediate zone; le, lens vesicle; lv, lateral ventricle; m, inner limiting membrane; mz, marginal zone; n, nerve fiber layer; nbl, outer neuroblastic layer; p, pia mater; v, vitreous body; vz, ventricular zone. Scale bar = 500 μm (a,b), 200 μm (c), 120 μm (d,e) or 30 μm (f–h). For (a–c), brains were dissected from E18.5 fetuses, fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.3) and processed for scanning electron microscopy. For (d–h), fetuses were fixed in Bouin's fluid and embedded in paraffin. Serial sections (7 μm thick) were prepared and stained with Mallory's tetrachrome.

Figure 2



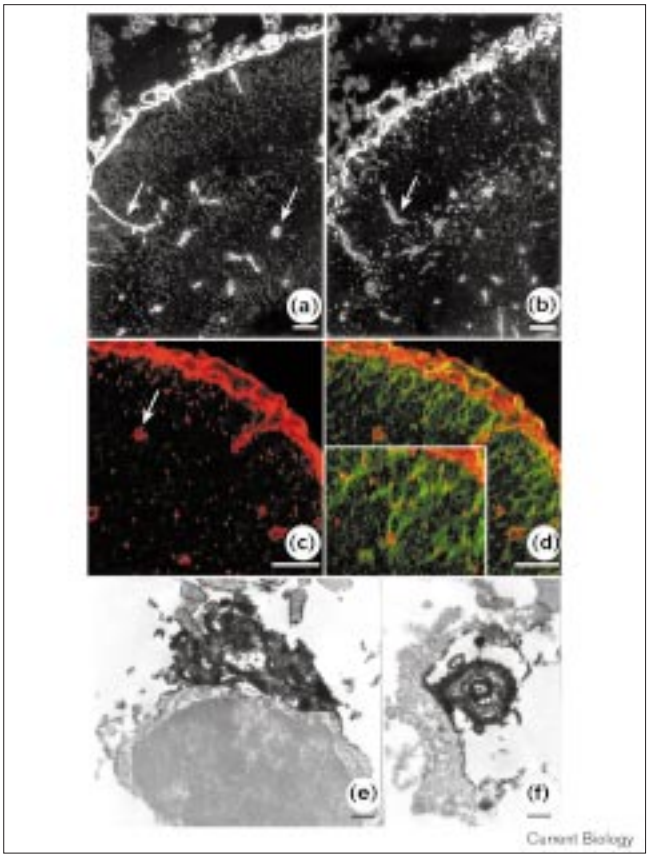
Expression of integrin and laminin chains in E14.5 wild-type embryos. (a) Expression of the integrin α6 chain and (b) a higher magnification of (a). Note α6 expression in the ventricular zone and cortical plate (arrows) in the cerebral cortex. (c) The integrin β1 chain is expressed similarly in the ventricular zone and cortical plate (arrows) in the cerebral cortex. (d) Integrin β4 is not detected in brain; strong expression is observed in epithelial layers of oral and nasal cavities. (e) The laminin α1 chain is expressed in the meninges (m) and in the ventricular zone in the forebrain. (f) Laminin α2 is detected in the meninges. Cryostat sections (10–15 μm thick) were processed for *in situ* hybridization with ^{35}S-labeled RNA probes [20]. Probes used were a fragment of the 5′ region of the α6 cDNA, and cDNA fragments of integrins β1 (1 kb; gift from R. Fässler), and β4 (gift from S. Kennel), laminin α1 chain (gift from K. Kuhn) and laminin α2 chain (gift from L. Sorokin).

and 3, and of laminin 2 and 4, respectively. Both laminin 1 and 2 have been reported to promote neurite outgrowth and neuronal migration [1,2]. Moreover, laminin 2 (or merosin) has recently been implicated in congenital muscular dystrophies, associated in some cases with brain abnormalities [12]. Both laminin chains were present in the meninges at the stages tested (E13.5–E16.5; Figure 2e,f). A signal for the α1 chain was also detected in the ventricular zone (Figure 2e). In the retina, laminin α1 was found (data not shown) as already reported [11]. No differences in the pattern or level of expression were found between wild-type and mutant brains (data not shown).

Analysis of the glial limiting membranes

The meninges are a set of membranes surrounding the central nervous system, the most internal being the pia mater. Underneath the pia mater, and in direct contact with the brain surface, lies the BM, to which glial endfeet are closely apposed [13]. A possible explanation for the appearance of neuroblastic outgrowths in α6 mutants would be that alterations of the BM are responsible for the absence of arrest of migrating neuroblasts. Such BM defects could be due to a failure of proper basal lamina assembly in the absence of a major laminin receptor, or to an impairment of glial endfeet attachment to the basal lamina. To look for possible alterations, immunofluorescence experiments were carried out using antibodies against laminin. A polyclonal antibody against Engelbreth–Holm–Swarm (EHS) laminin gave a strong and broad signal in the meninges of fetal brains from E12.5 to E18.5 (Figure 3a); a weak signal was observed in the ventricular zone (data not shown). When the same antibody was applied on mutant brains, no obvious differences were found at the pial level outside the lesions. The laminin signal was also broad, and no clear evidence for BM abnormality was found (Figure 3b). In contrast, intense punctate staining of laminin was observed within the ectopias suggestive of BM degradation/remodeling in these lesions (data not shown). This may, however, be viewed as a consequence and not a cause of the outgrowths.

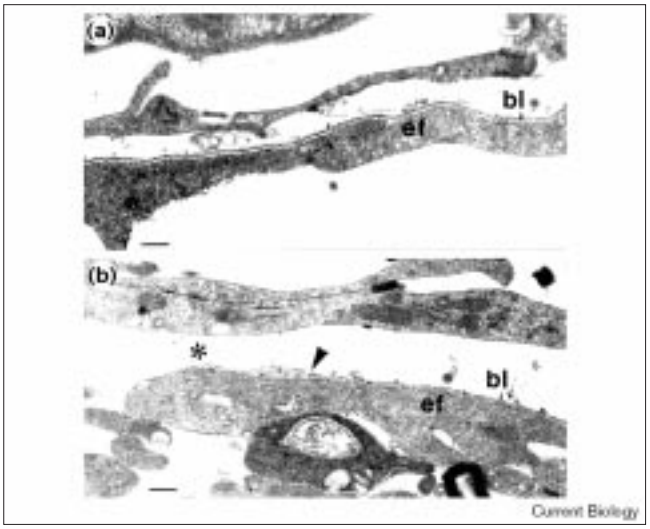
Figure 3



Localization of laminin in wild-type and mutant brains. (a,b) Sections from (a) wild-type and (b) $\alpha6^{-/-}$ E15.5 brains stained with an antibody against EHS laminin. Note the punctate laminin staining in $\alpha6^{-/-}$ brain. (c,d) Section from a $\alpha6^{-/-}$ E12.5 brain stained with (c) the antibody against EHS laminin and with (d) the antibody against EHS laminin and an antibody (RC2) specific for radial glial fibers; confocal analysis showing (c) punctate deposits of laminin (red) and (d) laminin deposits (red) aligned with RC2-positive radial glial fibers (green). Arrows indicate blood capillaries. (e,f) Electron microscopic immunoperoxidase detection of laminin in $\alpha6^{-/-}$ E18.5 brains. The dark laminin signal is in the extracellular space, associated with a cell plasma membrane (e), or a glial cell process (f). Scale bar = 50 μm (a–d) or 0.3 μm (e,f). For (a–d), after fixation in 4% paraformaldehyde for 5 min, frozen sections were incubated with antibodies as previously described [7]. Antibodies were a polyclonal antibody against laminin EHS (Sigma) or mouse monoclonal RC2 antibody (gift from P. Gressens). For (e,f), sections were incubated with the anti-laminin antibody overnight at 4°C, then with a peroxidase-conjugated anti-rabbit antibody (Sigma) for 3 h. Detection was performed with the DAB substrate kit (Vector Laboratories). Sections were then processed for transmission electron microscopy.

To further analyze the integrity of the glial limiting membrane, fetal brains from mutant and wild-type animals were observed by transmission electron microscopy. In eight ectopias from three mutant brains observed, gaps of the BM were found at the edges of the ectopias (Figure 4). In the parts of the cortex which were morphologically normal, however, the BM was continuous, although thinner than normal in some areas. As in wild-type brains, endfeet

Figure 4



Electron micrograph of the brain surface. (a) Wild-type and (b) $\alpha6^{-/-}$ brain surface at E14.5 at the edge of an ectopic outgrowth (asterisk). The arrowhead shows disorganization of the BM; bl, basal lamina; ef, glial endfoot. Scale bar = 0.4 μm. Brains were dissected, immediately fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, in cacodylate buffer containing 1% tannic acid for 24 h at 4°C and processed for transmission electron microscopy.

presumably corresponding to glial cell processes were found closely apposed to the BM (Figure 4). Therefore, the immunohistochemical and electron microscopic techniques employed here failed to reveal a generalized defect of the BM or attachment of the glial endfeet which could readily account for the neuroblastic ectopias. This situation contrasts with that described for Fukuyama congenital muscular dystrophy patients, in whom ectopias of neurons arise due to defects in the pial–glial barrier [14]. It is interesting to note, though, that precisely at the edges of the ectopias, the BM appeared highly disorganized (Figure 4b), suggestive of an active BM degradation process.

Laminin is found as punctate deposits in mutant brain

Whereas, in the meninges, laminin staining was indistinguishable between wild-type and mutant mice, a clear difference was observed in the cerebral cortex itself. Underneath the pia mater and at a depth of about one-third of the telencephalic wall, intense punctate staining was observed with the antibody against EHS laminin (Figure 3b,c). This punctate staining was seen as early as E12.5 and was present in all mutant brains at whatever developmental stage. This punctate aspect of laminin signal was absent from the brains of wild-type animals.

Laminin deposits are associated with glial cell processes

Punctate laminin deposits have been described previously by Liesi [15] in developing rat and mouse brains. These were shown to be associated with radial glial fibers along

which neuroblasts migrate [15] and it was therefore proposed that this laminin could act as a neuron guidance cue. These deposits normally disappear at later developmental stages. The punctate signal that we observe in $\alpha6^{-/-}$ mutant animals could correspond to this glia-associated laminin, which in the mutant brain would persist. In order to assess the spatial relationship between laminin deposits and radial glia, we performed a double immunostaining with anti-laminin antibody and the radial glia-specific antibody RC2 [16]. The punctate deposits revealed by the antibody against laminin were found to be associated with RC2-positive fibers (Figure 3c,d), strongly suggesting that these deposits indeed correspond to the glial laminin described by Liesi [15]. The localization of laminin at E18.5 was further examined by immunoperoxidase labeling at the electron microscopical level. In the $\alpha6^{-/-}$ ventricular zone, more cells showed cytoplasmic staining than in the wild type, suggestive of a continued laminin synthesis in the mutant brain. In the peripheral layers, as already mentioned, punctate staining was observed only in the mutant brain. This signal was clearly extracellular, often associated with processes probably corresponding to glial cell processes (Figure 3e,f). All these results are in keeping with the possibility of an altered biosynthesis or deposition of laminin in the mutant brain. We thus propose that the persistence of glial laminin is causally related to the failure of neuroblasts to arrest their migration and localize in the appropriate cortical layer, leading to the formation of ectopias. In a previous study, a role had been proposed for integrin β1 in the initiation of radial migration of neuroblast in chick optic tectum [17]. The formation of a cortical plate in $\alpha6^{-/-}$ mutant brains, however, indicates that radial migration takes place in the absence of $\alpha6\beta1$. It is thus possible that other integrin α chains are involved in the initial steps of migration from the ventricular zone while the $\alpha6\beta1$ heterodimer would be required at later stages of migration, when neuroblasts reach the cortical plate.

Conclusions

We have shown that a laminin receptor is essential for the formation of layers in both the cerebral cortex and the retina during development. Ongoing experiments are aimed at understanding the cellular mechanism, the defect in the organization of basal lamina, or the impairment of neuroblast migration involved in these particular phenotypes. Importantly, these results suggest that patients carrying mutations in the *ITGA6* gene may present abnormalities in the nervous system. Recently, a few mutations have been shown to alter cortical laminar organization in mouse and in human patients [18]. Mice mutant for MARCKS, a protein kinase C substrate, present leptomeningeal ectopias very similar to those described here, and basal lamina alterations [19]. The similarity of the brain phenotypes raises the interesting possibility of a link between protein kinase C and integrins.

Supplementary material

Additional observations on the $\alpha6^{-/-}$ phenotype are provided as Supplementary material published with this article on the internet.

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Supplementary material

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Additional observations on the $\alpha6^{-/-}$ phenotype

Ectopic neuroblasts are not restricted to the cortex, but are also found in other areas, notably within the subarachnoid space surrounding the mesencephalon.

Complete penetrance of the phenotype was observed both on a 129Sv/C57B16 mixed background and after one cross onto a CD1 genetic background.

Antibodies against laminin α1 and α2 (gift from L. Sorokin) were also used to label mutant brains. Both laminin α1 and α2 immunoreactivities were present in the meninges. Only in the case of laminin α1 were foci of punctate staining consistently observed. These foci were less numerous than those detected with the polyclonal antibody against EHS laminin, suggesting that an abnormal distribution of an α1-containing laminin may be involved in the generation of the defect, together with other forms of laminin.