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Breaches of the pial basement membrane are associated with defective dentate gyrus development in mouse models of congenital muscular dystrophies

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ABSTRACT

A subset of congenital muscular dystrophies (CMDs) has central nervous system manifestations. There are good mouse models for these CMDs that include *POMGnT1* knockout, *POMT2* knockout and Large^{myd} mice with all exhibiting defects in dentate gyrus. It is not known how the abnormal dentate gyrus is formed during the development. In this study, we conducted a detailed morphological examination of the dentate gyrus in adult and newborn *POMGnT1* knockout, *POMT2* knockout, and Large^{myd} mice by immunofluorescence staining and electron microscopic analyses. We observed that the pial basement membrane overlying the dentate gyrus was disrupted and there was ectopia of granule cell precursors through the breached pial basement membrane. Besides these, the knockout dentate gyrus exhibited reactive gliosis in these mouse models. Thus, breaches in the pial basement membrane are associated with defective dentate gyrus development in mouse models of congenital muscular dystrophies.

Congenital muscular dystrophies (CMDs) with central nervous system manifestations are a group of heterogeneous genetic diseases, including Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain (MEB) disease, Walker–Warburg syndrome (WWS), and congenital muscular dystrophy 1D (CMD1D) [1–8]. Their mutated genes are *FKTN* (encoding fukutin), *POMGnT1* (encoding protein O-mannose N-acetylglucosaminyltransferase 1, POMGnT1), *POMT1* (encoding protein O-mannosyltransferase 1, POMT1), POMT2 (encoding POMT2), and *LARGE* (encoding like-glycosyltransferase, LARGE), [9–17], respectively. All of these proteins contain glycosyltransferase domains. The main implicated structures of the central nervous system include the cerebrum, the cerebellum, and the pons; manifesting micropolygyria, hypoplasia of the pons, and hydrocephalus. Mental retardation is one of the prominent symptoms.

Dentate gyrus, which is one of the components of the hippocampus formation, plays critical role in processing memory and learning. Mouse models of CMD, *POMGnT1* knockout mice [18], brain-specific knockout mice of *POMT2* [19] and Large^{myd} mice [20] exhibit abnormal dentate gyrus morphologies. However, no further investigation concerning this has been performed and it is still unknown how the abnormal dentate gyrus is formed during the development. In this study, detailed morphological investigation of the dentate gyrus of adult and newborn mice was performed in *POMGnT1* knockout mice, *POMT2* knockout mice, and Large^{myd} mice.

POMGnT1 knockout mice were generated in collaboration with Lexicon Genetics [18]. *POMT2*-floxed mice were generated at the Transgenic and Knockout Mouse Facility at the University of Connecticut Health Sciences Center [19]. Large^{myd} and Emx1-Cre knock-in mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Adult (>2 months old) and newborn (postnatal day 3) homozygous mice were used in the study. Littermate wild-types (+/+) were used as controls. Animal care and usage were approved by the Institutional Animal Care and Use Committee of SUNY Upstate Medical University and adhered to the guidelines of the National Institutes of Health.

Antibodies were obtained as follows: Rabbit poly clonal anti-laminin and anti-GFAP (used at 1:1000 dilutions) from Sigma–Aldrich (St. Louis, MO). Polyclonal anti-Prox1 (used at 1:500 dilutions) from Abcam (Cambridge, MA). Anti-rabbit IgG antibody produced in goat conjugated with Rhodamine and anti-rabbit IgG antibody produced in goat conjugated with FITC (used at 1:500 dilutions) from Vector Laboratories (Burlingame, USA).

Mice were anesthetized and decapitated. Whole brain was removed from the skull and the forebrain was dissected and embedded in optimal cutting temperature (OCT) compound in cryomolds and frozen in dry ice/2-methylbutane bath. The frozen tissues were cut into 10 μm sections in a coronal plane and mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). After the sections were air-dried, they were fixed with 4% paraformaldehyde for

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15 min. To stain with antibodies, the sections were incubated with 3.0% bovine serum albumin in Tris-buffered saline (TBS, 50 mM Tris, pH 7.4, 150 mM NaCl) to block non-specific binding. The sections were then incubated with the indicated primary antibody overnight at 4 °C. After washing with TBS containing 0.1% Triton X-100, the sections were incubated with FITC-conjugated goat anti-mouse IgG or anti-rabbit IgG for 2 h. After washing, all sections were counterstained for 10 min with 0.10% DAPI (Sigma–Aldrich, St. Louis, MO) before being mounted with coverslips. Fluorescence was visualized with a Zeiss Axioskop upright fluorescence microscope equipped with a digital camera (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Electron microscopic analysis of the dentate gyrus was carried out essentially as previously described [21]. The samples were observed, and photographs were taken with a Tecnai T12 transmission electron microscope (FEI Company, Salem, MA).

To examine the integrity of the pial basement membrane in the dentate gyrus of *POMGnT1* knockout mice and other available mouse models of CMDs, we carried out laminin immunofluorescence staining on sections of adult brains (Fig. 1). In the wildtype, there were two lines of immunofluorescence separating the dentate gyrus and the midbrain, one representing the pial basement membrane of the hippocampus (arrows in Fig. 1A) and the other representing the pial basement membrane of the midbrain (arrowheads in Fig. 1A). In *POMGnT1* knockout (Fig. 1B), disruptions of the pial basement membrane were apparent in addition to the characteristic architectural abnormality of the inferior blade (Fig. 1B). In some areas, only a single pial basement membrane (belonging to the midbrain, arrowhead in Fig. 1B) was seen. In other areas, pial basement membrane was absent (asterisks in Fig. 1B). Similar results were obtained for *POMT2*$^{f/f;Emx1\text{-}Cre(+)}$ mice (Fig. 1C), where only the pial basement membrane of the midbrain remained in an area with apparent dysmorphological appearance of the inferior blade and the pial basement membrane of the dentate gyrus was absent. Also, similar results were obtained for Large^{myd} mice (Fig. 1D). These results suggest that the pial basement membrane overlying the dentate gyrus was disrupted in these mouse models of CMDs.

To confirm that the pial basement membrane of the dentate gyrus in the mouse models of CMDs was defective, we performed electron microscopic analysis. In the wildtype (Fig. 2A), two basement membranes were identified at the border of the dentate gyrus and the midbrain. One of the basement membranes belonged to the dentate gyrus (arrows in Fig. 2A); the other to the midbrain (arrowheads in Fig. 2A). Between the two pial basement membranes, fibroblasts of the pial mater were identified. Blood vessels were also occasionally observed between the two pial basement membranes (not shown). In *POMGnT1* knockout mice, basement membrane was sometimes completely missing (Fig. 2B). In *POMT2*$^{f/f;Emx1\text{-}Cre(+)}$ mice, there was only a single basement membrane at the border of the dentate gyrus and the midbrain (arrowheads in Fig. 2C). Similar results were obtained from Large^{myd} mice (Fig. 2D). These results indicate that the pial basement membrane is disrupted between the dentate gyrus and the midbrain in the mouse models of CMDs.

The neocortex of *POMGnT1* knockout mice exhibited extensive reactive gliosis [22]. To examine whether reactive gliosis existed in the dentate gyrus of the mouse models of congenital muscular dystrophy, we carried out immunofluorescence staining with an

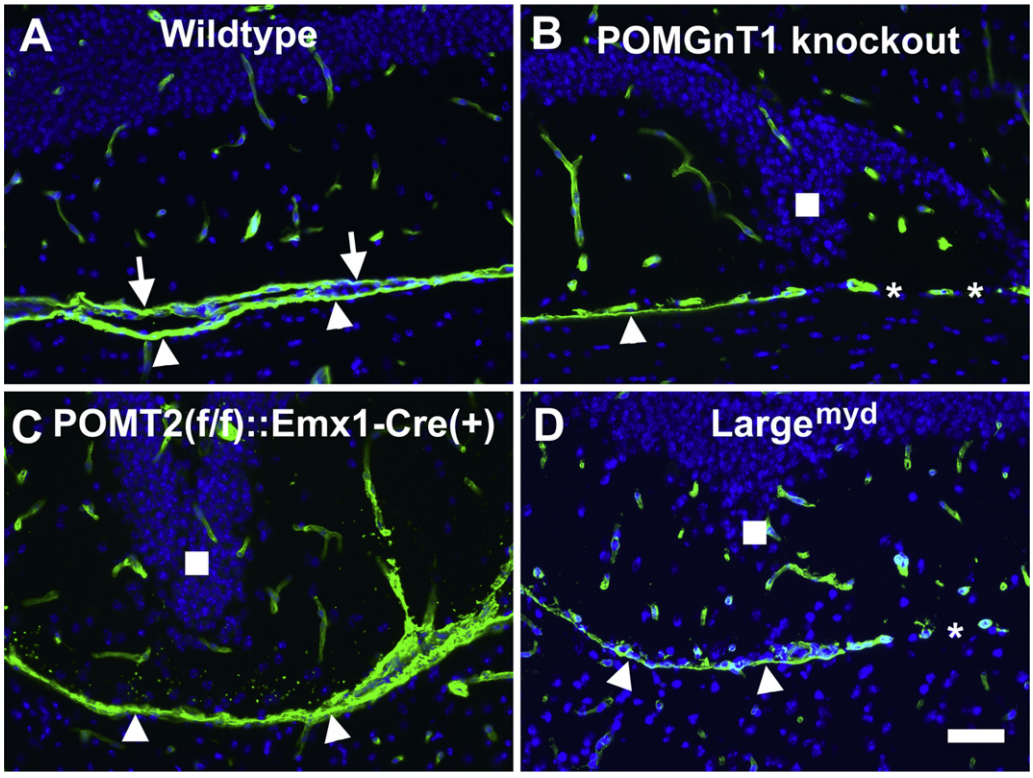

Fig. 1. Laminin immunofluorescence staining suggests disruption of the pial basement membrane in the dentate gyrus of CMD mouse models. Coronal sections of adult forebrain were immunostained with anti-laminin (green fluorescence) and counterstained with DAPI (blue fluorescence). (A) Wildtype: laminin immunofluorescence was observed in blood vessels and pial basement membrane. The pial basement membrane of the dentate gyrus (arrows) and the midbrain (arrowheads) were clearly discerned. (B) POMGnT1 knockout: in some regions only a single pial basement membrane, presumably of the midbrain, was observed (arrowhead). There were regions devoid of any pial basement membrane separating the dentate gyrus and the midbrain (asterisks). (C) POMT2$^{f/f;Emx1\text{-}Cre(+)}$: a single pial basement membrane is observed between the dentate gyrus and the midbrain (arrowheads). (D) Large^{myd} mice: regions of single pial basement membrane (arrowheads) and regions with no pial basement membrane were observed. Scale bar in D: 50 μm.