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ABSENCE OF THE BASILAR PONTS IN MICE LACKING A FUNCTIONAL *LARGE* GLYCOSYLTRANSFERASE GENE SUGGESTS A DEFECT IN PONTINE NEURON MIGRATION

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Abstract

Several forms of congenital muscular dystrophy result from mutations in glycosyltransferases that modify α -dystroglycan. As pontine hypoplasia has been reported in some clinical cases of congenital muscular dystrophy, we have begun to examine whether these glycosyltransferases are required for the normal development of the basilar pons, one of several precerebellar nuclei of the hindbrain. In *veils* (*Large*^{vl}) mice, which carry a loss-of-function mutation in the *Large* glycosyltransferase gene, the basilar pons is absent. Instead, ectopic clusters of pontine neurons are found lateral to their normal site, suggesting that these neurons are unable to migrate to their appropriate site. Two other precerebellar nuclei, the lateral reticular nucleus and the inferior olive, are present in *Large*^{vl} mice. In addition, the basilar pons forms normally in dystrophin-deficient mice. These results demonstrate that the *Large* glycosyltransferase but not dystrophin is required for normal basilar pontine development.

Section: Nervous system development, regeneration, and aging

Keywords

Muscular dystrophy; glycosyltransferase; basilar pons; migration; dystroglycan; dystrophin; precerebellar nucleus

INTRODUCTION, RESULTS, DISCUSSION, AND EXPERIMENTAL PROCEDURES

Many forms of congenital muscular dystrophy, such as Walker-Warburg syndrome and Fukuyama congenital muscular dystrophy, result from mutations in glycosyltransferases that normally modify α -dystroglycan. Collectively known as dystroglycanopathies, one hallmark of these disorders is the appearance of brain malformations such as type II lissencephaly (“cobblestone cortex”) and cerebellar hypoplasia (Grewal and Hewitt, 2003; Muntoni, 2004). At the histological level, breaches in the glia limitans, a disordered basal lamina, and glioneuronal heterotopia have been observed (Saito et al., 1999). This disrupted organization

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is also found in brains from *myodystrophy* (*Large^{myd}*) mice, which carry a mutation in the *Large* glycosyltransferase gene (Holzfeind et al., 2002; Michele et al., 2002). Mice deficient in another glycosyltransferase, POMGnT1, exhibit similar but not identical defects (Liu et al., 2006). The cortical and cerebellar malformations observed in dystroglycanopathies are consistent with defects in neuronal migration along radial glia, suggesting functions for specific glycosyltransferases and glycosylated α -dystroglycan in regulating radial migration.

Like other precerebellar nuclei of the hindbrain, the basilar pons (BP) is derived from the rhombic lip (Rodriguez and Dymecki, 2000). Once generated, BP neurons undergo a circumferential ventrally-directed migration just beneath the pial surface of the brain, through a pathway known as the anterior extramural stream. Observations of pontine hypoplasia and basilar pontine heterotopias in clinical cases of congenital muscular dystrophy suggest that the underlying affected genes may regulate pontine neuron migration during development (Saito et al., 2003; van der Knaap et al., 1997). However, as pontine neurons undergo tangential migration, not in association with a radial glial scaffold, the mechanism of such regulation is unknown. To begin to address the potential functions of glycosyltransferases related to congenital muscular dystrophy and of glycosylated α -dystroglycan in regulating pontine development, we examined the BP in *veils* (*Large^{vl}*) mice (Lee et al., 2005), which, like *Large^{myd}* mice, possess a loss-of-function mutation in the *Large* gene.

Pontine morphology was examined in serial thionin-stained sections of *vl* mice and wildtype littermates. Sagittal sections of wildtype brains reveal the normal position of the BP (Fig. 1A). In similar sections of *Large^{vl}* brains, however, no significant nucleus is observed, although a small amount of thionin-stained material and some white matter is still present (Fig. 1B). Furthermore, the reticulotegmental nucleus (RTN), another precerebellar nucleus whose neurons migrate through the anterior extramural stream, is absent (Figs. 1A,B). In more lateral sections, where normally no BP would be observed (Fig. 1C), ectopic cells are found in *Large^{vl}* hindbrain overlying the usually external white matter (Fig. 1D). Similar observations were made in coronal sections of *Large^{vl}* hindbrain (Figs. 1E,F), which exhibit large ectopic clusters of cells (asterisks in Fig. 1F) extending from the midline to the nucleus of the lateral lemniscus, lateral to the normal site of the BP. The more lateral clusters appeared both denser and larger. During the course of the experiments presented in this report (Figs. 1 and 2, and data not shown), the BP defect was consistently observed in *Large^{vl}* mice (n=5), but not in wildtype (n=3) or heterozygous (n=3) controls.

The loss of the BP in the *Large^{vl}* hindbrain, accompanied by the appearance of ectopic cellular clusters, suggests a mispositioning of BP neurons. To assess the identity of ectopic cells, serial hindbrain sections from P7 *Large^{vl}* hindbrain (n=1) were immunostained for the neuronal marker neuN. The expression of neuN in the ectopic clusters (Figs. 2A,B) indicates that they contain neurons, although we cannot rule out that they contain glia as well. Ectopic cells from P0.5 *Large^{vl}* hindbrain (n=1) also express NFI-B, a transcription factor expressed in the BP (Chaudhry et al., 1997) (Fig. 2C,D). These results support the hypothesis that ectopic cells are in fact mispositioned BP neurons.

Like pontine neurons, other precerebellar neurons are generated at the rhombic lip and then undergo tangential migration. We therefore tested whether these other nuclei fail to develop as well. While inferior olivary (IO) neurons do not express high levels of neuN, we were nevertheless able to identify the IO in serial neuN-stained sections (Fig. 2E). No obvious difference existed between the IO in control (n=1) and *Large^{vl}* (n=1) hindbrain (Figs. 2E,F). In addition, Nissl staining revealed normal lamellae in both wildtype brains (n=2) and mutants (n=2; Fig. 1 and data not shown). Similarly, the lateral reticular nucleus (LRN), identified by NFI-B staining, was observed in the *Large^{vl}* hindbrain (n=1; Figs. 2G,H). The LRN could also be discerned in Nissl-stained material from mutant hindbrain (Fig. 1, and data not shown; n=2).

While we cannot rule out partial phenotypes in either nuclei, it is apparent neither mirror the almost complete loss of the BP. We were also able to observe several other mid- and hindbrain nuclei at their correct locations in *Large^{vls}* mice, including the facial nucleus, the motor trigeminal nucleus, the nucleus of the lateral lemniscus, the nucleus of the trapezoid body, the hypoglossal nucleus, the superior olive, the interpeduncular nucleus, and the red nucleus (Fig. 1, and data not shown).

Dystroglycan is a component of the dystrophin-glycoprotein complex that, at least in muscle, links the extracellular matrix to the cytoskeleton. Dystrophin participates in this complex by associating with the cytoplasmic domain of dystroglycan, and dystrophin mutations lead to Duchennes muscular dystrophy. We therefore investigated pontine morphology in serial thionin-stained hindbrain sections from the *mdx* mouse, which carries a mutation in dystrophin. We found that the *mdx* BP (n=4) is morphologically indistinguishable from that of wildtype (n=3) (Fig. 3), although we cannot rule out more subtle alterations. We therefore conclude that dystrophin is not required for BP development. The RTN appears similarly unaffected. This result is consistent with the absence of severe neurological phenotypes in *mdx* mice or in Duchennes muscular dystrophy (Mehler, 2000). One possibility, to be investigated in future studies, is that utrophin may compensate for the lack of dystrophin in brain (Culligan et al., 2001).

The disappearance of the BP accompanied by the appearance of neuN- and NFI-B-positive heterotopia along the remnants of the anterior extramural stream supports the idea that the Large glycosyltransferase is required for BP neuron migration during development. During the review of this manuscript, an independent study was published confirming this BP phenotype using *Large^{myd}* (Qu et al., 2006), which possess an independent mutation in the *Large* gene distinct from that found in *Large^{vls}* mice. The idea that pontine neuron migration is regulated by Large and potentially by other glycosyltransferases that target α -dystroglycan is consistent with the appearance of pontine heterotopias in clinical cases of Fukuyama congenital muscular dystrophy (Saito et al., 2003) and with the findings that neuronal migration is affected in the cerebral cortex and cerebellum of *Large^{myd}* mice (Holzfeind et al., 2002; Michele et al., 2002; Qu and Smith, 2005). In these two latter regions, the loss of Large results in the hypoglycosylation of α -dystroglycan, which is normally localized to the endfeet of radial or Bergmann glia. This results in defective interactions with the overlying subpial basal lamina, and is associated with the retraction of glial endfeet and breaches in the glia limitans. Disorganized radial glia may also fail to properly support cortical and cerebellar neuron migration.

While both the cerebral cortex and cerebellar phenotypes in Large-deficient mice are related to defects in radial neuronal migration, to our knowledge the BP is the first example of tangentially-migrating neurons mistargeting in any dystroglycanopathy. BP neurons do not migrate along a radial glial scaffold, and no role for glia in regulating the migration of BP neurons has yet been reported. It is therefore possible that Large acts in a novel manner in BP neurons. Alternatively, as the pontine migratory stream contacts the glia limitans virtually throughout its entire length (Ono and Kawamura, 1990), any breaches in the glia limitans could lead to the formation of pontine heterotopias. The latter possibility is supported by Qu et al., (2006), who report such breaches in the ventral region of the pontine migratory stream in *Large^{myd}* hindbrain.

Interestingly, although precerebellar neurons all derive from the rhombic lip and undergo tangential migration to reach their final destinations (Rodriguez and Dymecki, 2000), the BP and RTN, the only precerebellar populations to migrate through the anterior extramural stream, were the only precerebellar nuclei affected in the *Large^{vls}* mouse. It is not surprising that the IO appears to develop normally, for while its neurons undergo tangential migration, they do

so deep to the surface of the brain, and are therefore not in contact with the glia limitans. The appearance of LRN neurons in their normal location is more surprising, as they, like BP neurons, migrate directly beneath the surface of the brain (Ono and Kawamura, 1989).

The distinct BP phenotypes observed in mice lacking genes such as *Pax6* (Engelkamp et al., 1999) and *Cxcr4* (Vilz et al., 2005) suggest that BP migration is regulated by different molecular mechanisms at different points along the anterior extramural stream. Migration along the ventral portion of the stream, as BP neurons approach the midline, is influenced by the floor plate-derived chemoattractant netrin-1 and its receptor Dcc. In the absence of either, BP neurons migrate normally along the dorsal portion of the anterior extramural stream emanating from the rhombic lip, but subsequently fail to reach the ventral midline, and instead form ectopic clusters (Alcantara et al., 2000; Yee et al., 1999). Similar phenotypes have also been reported for mice deficient in *Rig1* (Marillat et al., 2004) and *presenilin-1* (Louvi et al., 2004), both of which may regulate netrin signaling. The appearance of large ectopic cellular clusters along the ventral surface of the *Large^{vtls}* brain suggests that BP neurons arrest migration at a similar point. Indeed, preliminary data in *Large^{vtls}* mice suggest that the dorsal portion of the anterior extramural stream is normal (E.D.L., unpublished observations). This is supported by similar results in *Large^{myd}* mice (Qu et al., 2006). While it is not known whether netrin-1 signaling is regulated directly or indirectly by *Large*, the ability of α -dystroglycan to act as a laminin-1 receptor (Miner and Yurchenco, 2004) along with the phenotype observed in this report makes it tempting to hypothesize that *Large* and α -dystroglycan regulate netrin-1 signaling in migrating BP neurons.

Animals used in these experiments were bred in the Research Animal Facility at The Jackson Laboratory (Lee et al., 2005) or at the University of Maryland, Baltimore animal facility under the care and approval of the accredited Institutional Animal Care and Use Committee. Brains from postnatal mice were fixed in 4% paraformaldehyde, and 40-50 μ m serial sections were made on a vibratome. For Nissl staining, sections were mounted on gelatin-subbed slides and stained with 0.1% thionin. For immunostaining, floating sections were blocked for 2 hours in 2% goat serum (Sigma, S. Louis, MO) in PBST (phosphate-buffered saline + 0.2% Triton X-100) and then incubated overnight at room temperature in antibodies to mouse anti-neuN (1:2000; Chemicon, Temecula, CA) or rabbit anti-NFI-B (1:50,000; Active Motif, Carlsbad, CA). The next day, sections were incubated for one hour in either biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) or biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) secondary antibody, and then for 1 hour at room temperature in ABC reagent (Vector) diluted 1:500 in PBST. Sections were developed for 20 minutes in 2.5% (w/v) nickel sulfate, 0.02% (w/v) 3,3'-diaminobenzidine (Sigma), 0.01% (v/v) H_2O_2 in 0.175 M sodium acetate, and then mounted on gelatin-subbed slides. All slides were dehydrated through increasing concentrations of ethanol followed by xylenes, and coverslipped in DPX mounting media (Electron Microscopy Sciences, Hatfield, PA). Sections were photographed on an upright microscope (Leica, Deerfield, IL) using a digital camera, and brightness and contrast adjusted using Adobe Photoshop CS. To compare the BP between control and mutant animals, sections were matched based on the similar position and morphology of other hindbrain nuclei (see labeled nuclei in Figs. 1 and 3), particularly those that sit near the BP (e.g., the nucleus of the lateral lemniscus). Matched sections were considered to be from the same position along the rostrocaudal or mediolateral axis.

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Fig. 1. Absence of the BP in *Large^{vls}* mice. Sagittal thionin-stained sections of P4 (A) wildtype and (B) *Large^{vls}* hindbrain demonstrate an absence of the *Large^{vls}* BP and RTN. Arrowheads indicate the normal position of the BP. The IO and Tz are present at this sagittal level in both animals. Sagittal sections of (C) wildtype and (D) *Large^{vls}* brain, lateral to those in panels A and B, show that while the BP is not normally observed at this level (arrowhead in C), an ectopic cell mass is found in *Large^{vls}* mice (arrowhead in D). Several other hindbrain nuclei (7, LRN, Mo5, nLL, and SO) are present at this sagittal level in both animals. Coronal sections of P4 (E) wildtype and (F) *Large^{vls}* hindbrain demonstrate ectopic clusters of cells (asterisks in F) extending lateral to the normal site of the BP. At this coronal level, the IP, nLL, and RN

are present in both animals. Arrowheads in E and F indicate the midline. In A-D, rostral is to the left; in E and F, lateral is to the left. Abbreviations: 7, facial nucleus; BP, basilar pons; Cb, cerebellum; IO, inferior olive; IP, interpeduncular nucleus; LRN, lateral reticular nucleus; Mo5, motor trigeminal nucleus; nLL, nucleus of the lateral lemniscus; RN, red nucleus; RTN, reticulotegmental nucleus; SO, superior olive; Tz, nucleus of the trapezoid body. Scale bars: D = 0.5 mm (for A-D); E = 0.25 mm (for E,F).

Fig. 2.

Identification of neuN- and NFI-B-positive populations in the *Large^{vls}* hindbrain. All panels are horizontal sections, with lateral to the left. (A) The heterozygote P7 BP normally expresses neuN, a neuronal marker. (B) Ectopic cellular clusters (arrows) in P7 *Large^{vls}* hindbrain express neuN. (C) The P0.5 heterozygote BP expresses NFI-B. (D) Ectopic cellular clusters (arrows) in the P0.5 *Large^{vls}* hindbrain express NFI-B. (E)-(H) are horizontal sections, with rostral at the bottom. (E) While not highly expressing neuN, the IO can be discerned in neuN-stained P7 heterozygote sections. (F) The IO appears normal in the P7 *Large^{vls}* hindbrain. (G) The P0.5 heterozygote LRN expresses NFI-B. (H) NFI-B staining reveals that the LRN is present in the P0.5 *vls* hindbrain. Scale bar in A = 200 μ m (for A-H).

Fig. 3.

The basilar pons forms normally in *mdx* mice. Thionin stained sagittal sections of P7 (A) wildtype and (B) *mdx* hindbrain demonstrate the presence of the BP and the RTN in the absence of dystrophin. The Tz is also present in both sections. Thionin stained sagittal sections of P7 (C) wildtype and (D) *mdx* hindbrain are more lateral to those in A and B, and also demonstrate the presence of a normal BP. The PO is also observed in both sections. Rostral is to the left in all panels. Abbreviations: BP, basilar pons; PO, periolivary nucleus; RTN, reticulotegmental nucleus; Tz, nucleus of the trapezoid body. Scale bar in D = 0.5 mm (for A-D).