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Defects in neural guidepost structures and failure to remove leptomeningeal cells from the septal midline behind the interhemispheric fusion defects in *Netrin1* deficient mice

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ABSTRACT

Corpus callosum (CC) is the largest commissural tract in mammalian brain and it acts to coordinate information between the two cerebral hemispheres. During brain development CC forms at the boundary area between the cortex and the septum and special transient neural and glial guidepost structures in this area are thought to be critical for CC formation. In addition, it is thought that the fusion of the two hemispheres in the septum area is a prerequisite for CC formation. However, very little is known of the molecular mechanisms behind the fusion of the two hemispheres. Netrin1 (NTN1) acts as an axon guidance molecule in the developing central nervous system and *Ntn1* deficiency leads to the agenesis of CC in mouse. Here we have analyzed *Ntn1* deficient mice to better understand the reasons behind the observed lack of CC. We show that *Ntn1* deficiency leads to defects in neural, but not in glial guidepost structures that may contribute to the agenesis of CC. In addition, *Nnt1* was expressed by the leptomeningeal cells bordering the two septal walls prior to fusion. Normally these cells are removed when the septal fusion occurs. At the same time, the Laminin containing basal lamina produced by the leptomeningeal cells is disrupted in the midline area to allow the cells to mix and the callosal axons to cross. In *Ntn1* deficient embryos however, the leptomeninges and the basal lamina were not removed properly from the midline area and the septal fusion did not occur. Thus, NTN1 contributes to the formation of the CC by promoting the preceding removal of the midline leptomeningeal cells and interhemispheric fusion.

1. Introduction

Corpus callosum (CC) axons connect the two cerebral hemispheres at the cortico-septal boundary (CSB) area of mammalian brain providing a pathway to coordinate and process information arising from different cortical regions. The first pioneering axons originate in the cingulate cortex and reach the midline between embryonic day (E) 14 and E15 in mice and between postconceptual weeks (PCW) 13 and 14 in humans (Koester and OíLeary, 1994; Rash and Richards, 2001; Edwards et al., 2014). Later, these pioneering axons assist the midline crossing of a subtype of mouse neocortical callosal axons projecting from cortical layers I, II/III, V and VI expanding the size of the CC by E17 (Polleux et al., 1998; Shu et al., 2003d; Edwards et al., 2014). In humans, CC expansion occurs during PCW 14 and 19, and the callosal axons originate from pyramidal neurons in layers II, III, V and VI (Edwards et al., 2014). Malformation of the CC has been associated with various congenital syndromes in humans causing neurological, psychiatric, cognitive and behavioral problems. In addition, more than 50 genes have been linked to a complete or partial agenesis of CC in humans and mice reflecting the complexity of the molecular network involved in the formation of CC (Richards et al., 2004; Paul et al., 2007; Edwards et al., 2014).

The formation of CC is thought to be driven by an integrative network of attractive and repulsive axon guidance molecules produced mainly by specific transient midline cellular structures at the CSB area (Lindwall et al., 2007). These structures are composed of so called guidepost cells that act as intermediate targets for axonal path-finding (Palka et al., 1992). In addition, the correct formation and location of the guidepost cells has been shown to be critical for CC formation (Lindwall et al., 2007; Donahoo and Richards, 2009).

The midline guidepost structures include the glial wedge (GW) and the midline zipper glia (MZG) composed of glial cells, the

Abbreviations: CC, corpus callosum; CR, calretinin; CSB, cortico-septal boundary; E, embryonic day; GW, glial wedge; IG, indusium griseum; MZG, midline zipper glia; PCW, postconceptual weeks; Pn, postnatal; SCS, subcallosal sling.

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indusium griseum (IG) area which includes both glia and neurons and the subcallosal sling (SCS) composed mainly of neurons in mice (Richards et al., 2004; Lindwall et al., 2007). These transient midline structures have also been detected in human brain (Jovanov-Milošević et al., 2009). The GW is formed ventrally to the forming CC by radial glial cells originating in the medial part of the lateral ventricular walls (Shu et al., 2003c). The IG glia is thought to originate from the dorso-medial part of the lateral ventricle wall radial glia and to translocate into the IG area at the midline dorsal to the CC (Shu et al., 2003c; Smith et al., 2006). The MZG can be detected as a continuous cell population with IG glia before the formation of the CC, which then separates these two cell populations. Therefore, these cells could have a similar origin (Shu et al., 2003c). The SCS neurons are thought to migrate from the medial aspect of the lateral ventricles and to form a sling-like structure below the forming CC (Silver and Ogawa, 1983; Hankin and Silver, 1988; Shu et al., 2003b). In addition, the CSB area is known to contain other guidepost neurons that are thought to guide callosal axons. These GABAergic and glutamatergic neurons have been proposed to migrate to the area from the ventral telencephalon along tangential routes and transiently populate the CC (Niquille et al., 2009; Niquille et al., 2013). Reduction and misplacement of these neurons in *Ascl1*-/- mice (formerly *Mash1*) lead to severe callosal axon guidance defects (Niquille et al., 2009).

The fusion of the interhemispheric midline in the septal area precedes the crossing of pioneering callosal axons (Richards et al., 2004; Edwards et al., 2014). Very little is known of the molecular and cellular players involved in the septal fusion although mutations in a few genes have been suggested to cause defective interhemispheric fusion accompanied by the agenesis of the CC (Stumpo et al., 1995; Demyanenko et al., 1999; Brouns et al., 2000; Páez et al., 2007). However, the mechanisms by which these genes influence fusion have remained unclear. From the midline cell populations, MZG has been proposed to contribute to the fusion (Silver et al., 1982, 1993). However, the role of these glial cells remains unclear since although the MZG was abnormal in *Nfia*-/- mice, no clear defects in the septal fusion was detected (Shu et al., 2003a). Regulatory Factor X3 (RFX3) and c-Jun N-terminal kinase (JNK) interacting protein JNK/stress-activated protein kinase associated protein1 (JSAP1) deficient mice also present disorganization of guidepost neurons in CSB region associated with defects in interhemispheric midline fusion (Ha et al., 2005; Benadiba et al., 2012). Therefore, it is possible that guidepost neurons at the interhemispheric midline may also be involved in fusion process.

Netrin1 (NTN1) is a well-known axon guidance molecule, which also participates in cell migration, survival and morphogenesis in the central nervous system (CNS) (Livesey and Hunt, 1997; Barallobre et al., 2005; Hakanen et al., 2011; Lai et al., 2011). In CNS, the cellular functions of NTN1 are mainly mediated through transmembrane receptors belonging to the Deleted in colorectal carcinoma (DCC), UNC-5 homolog (UNC5), Down syndrome cell adhesion molecule (DSCAM) and Integrin families (Hedgecock and Norris, 1997; Yurchenco and Wadsworth, 2004; Barallobre et al., 2005; Fuerst et al., 2008; Lai et al., 2011; Maynard and Stein, 2012). However, defects in the development of the CC have been reported only in *Ntn1* and *Dcc* deficient mice. Since the phenotype in these mice is highly similar, the general idea has been that NTN1 attracts DCC positive callosal axons towards the interhemispheric midline (Serafini et al., 1996; Fazeli et al., 1997). However, a recent study showed that NTN1-DCC signaling attracts mainly early pioneering axons towards the midline, whereas the later arriving neocortical axons are not guided in a NTN1-DCC dependent manner (Fothergill et al., 2014).

Very little is known of the molecular mechanisms behind the fusion of the two hemispheres which precedes the crossing of callosal axons in the CSB area. Moreover, the involvement of NTN1 in this process has remained unclear. Therefore, we analyzed the expression of *Ntn1* and the development of the CSB region in *Ntn1*-/- mice including the transient glial and neuronal structures thought to be involved in this process.

2. Material and methods

2.1. Mouse strains

The generation and genotyping of mice containing a gene trap insertion in *Ntn1* locus has been described before (Salminen et al., 2000). These mice carry an IRESβgeo cassette, from which the expression of *lacZ* follows the expression of the trapped *Ntn1* gene. *Ntn1*+/– mice were kept in a pure C57Bl6 background and crossed to generate *Ntn1*-/- embryos. Both wild type and Ntn1+/– embryos were used as littermate controls when compared to *Ntn1*-/- embryos, since no defects have been observed in *Ntn1*+/– animals (Salminen et al., 2000; Hakanen et al., 2011). Three mutant and control embryos were used for each stage for experiments except for the analysis in Fig. 6 where two embryos were used. In addition, the analyses on postnatal samples were performed on two animals. To generate animals that survived postnatally, *Ntn*+/– mice were bred to a mixed C57Bl6/ICR background and crossed to generate *Ntn1*-/- mice (Hakanen et al., 2011). All mouse work has been approved by the University of Helsinki ethical review board.

2.2. X-gal staining

For X-gal staining, brains were fixed in 0.02% NP40 containing 4% PFA for 10 min at 4 °C, cut into four pieces and fixation was continued for 50 min at 4 °C. The samples were washed 3 × 15 min in PBS and incubated overnight in X-gal solution (5 mM potassium ferrisyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 1 mg/ml X-gal) at 37 °C. Samples were

Table 1
Primary antibodies used.

Antibody	Host	Immunogen	Manufacturers information	Dilution
Calretinin	Mouse, IgG1	Recombinant rat Calretinin.	Millipore, MAB1568, monoclonal, clone 6B8.2, RRID:AB_94259	1:200
GFAP	Mouse, IgG1	Purified glial filament	Millipore, MAB3402, monoclonal, clone GA5, RRID:AB_94844	1:500
Laminin	Rabbit, IgG	Protein purified from the basement membrane of Englebreth Holm-Swarm (EHS) sarcoma (Mouse).	Abcam, Ab11575, polyclonal, RRID:AB_298179	1:1000
L1	Rat	Rats were immunized with a glycoprotein fraction from cerebellum of 8-10 day old C57BL/6J mice.	Millipore, MAB5272, monoclonal, clone 324, RRID:AB_2133200	1:50