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Gli3 is required in Emx1$^+$ progenitors for the development of the corpus callosum

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ABSTRACT

The corpus callosum (CC) is the largest commissure in the forebrain and mediates the transfer of sensory, motor and cognitive information between the cerebral hemispheres. During CC development, a number of strategically located glial and neuronal guidepost structures serve to guide callosal axons across the midline at the corticoseptal boundary (CSB). Correct positioning of these guideposts requires the *Gli3* gene, mutations of which result in callosal defects in humans and mice. However, as *Gli3* is widely expressed during critical stages of forebrain development, the precise temporal and spatial requirements for *Gli3* function in callosal development remain unclear. Here, we used a conditional mouse mutant approach to inactivate *Gli3* in specific regions of the developing telencephalon in order to delineate the domain(s) in which *Gli3* is required for normal development of the corpus callosum. Inactivation of *Gli3* in the septum or in the medial ganglionic eminence had no effect on CC formation, however *Gli3* inactivation in the developing cerebral cortex led to the formation of a severely hypoplastic CC at E18.5 due to a severe disorganization of midline guideposts. Glial wedge cells translocate prematurely and *Slit1/2* are ectopically expressed in the septum. These changes coincide with altered Fgf and Wnt/β-catenin signalling during CSB formation. Collectively, these data demonstrate a crucial role for *Gli3* in cortical progenitors to control CC formation and indicate how defects in CSB formation affect the positioning of callosal guidepost cells.

Introduction

The corpus callosum (CC) is the largest transverse axon fibre tract in the forebrain and mediates the interhemispheric transfer of sensory, motor and cognitive information between the cerebral hemispheres (Paul et al., 2007; Richards et al., 2004). Malformations of the corpus callosum are amongst the most common brain anomalies found at birth and are thought to occur in up to 7/1000 of the total newborn population (Bedeschi et al., 2006). Complete or partial agenesis of the CC is associated with over 50 human congenital syndromes and is a cause of mental retardation having a wide range of cognitive, behavioural and neurological consequences (Paul et al., 2007; Richards et al., 2004). However, while the clinical implications of CC agenesis are known, the developmental mechanisms determining the formation of the CC are yet to be fully elucidated.

During development of the corpus callosum, callosal axons from each cortical hemisphere must cross the midline to reach the contralateral hemisphere. This crossing occurs at the corticoseptal boundary (CSB) which separates cortex and septum and involves complex interactions between callosal axons and several midline guidance structures. Glial cells of the glial wedge (GW) and the indusium griseum are located adjacent to callosal axons (Shu and Richards, 2001) and prevent them from migrating into the septum by producing the repellent axon guidance molecule Slit2 (Bagri et al., 2002; Shu et al., 2003a). In addition, these midline glial populations act in combination with several neuronal populations. GABAergic neurons derived from the medial ganglionic eminence populate the corpus callosum and channel axons across the midline (Niquille et al., 2009). Moreover, Calretinin+ and Calbindin+ neurons located in the indusium griseum and in the cingulate cortex express the Sema3c guidance factor which is required for callosal development (Niquille et al., 2009; Piper et al., 2009). Calretinin+ neurons are also detected within the corpus callosum where they delineate its dorsal and ventral components (Niquille et al., 2009). Taken together, these findings indicate that CC development is based on complex cellular and molecular interactions between callosal axons, midline glia and neuronal populations and guidance molecules produced by these cells. Moreover, these interactions require

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a precise spatial arrangement of the cellular guidance cues at the CSB but very little is known about the mechanisms by which these cells acquire their position.

Acrocallosal syndrome patients carry mutations in the *GLI3* gene and among other symptoms show complete absence of the CC (Elson et al., 2002). *Gli3* encodes a zinc finger transcription factor with crucial roles in early patterning of the dorsal telencephalon through controlling the expression of several signaling molecules (Fotaki et al., 2011; Grove et al., 1998; Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000). $Gli3^{Xt/Xt}$ mice which carry a *Gli3* loss of function allele have very severe cortical defects, precluding their use to study corpus callosum development. However, the *Gli3* hypomorphic mutant *Polydactyly Nagoya* ($Gli3^{Pdn/Pdn}$) displays corpus callosum agenesis (Naruse et al., 1990) and presents an interesting model to study *Gli3* function in callosal development. In these mutants, altered Fgf and Wnt/β-catenin signaling leads to the ectopic formation of glial fascicles which interfere with the growth of callosal axons and cause the formation of Probst bundles (Magnani et al., in press). Moreover, an ectopic expression of the chemorepellent axon guidance molecule Slit2 in the cortical midline also inhibits the migration of callosal axons and of guidepost neurons to their correct position at the CSB (Magnani et al., in press). However, while these findings clearly indicate an important role of *Gli3* in positioning of callosal guidepost cells at the midline, it remains unclear exactly when and where *Gli3* controls this process since it is expressed in both cortical and septal progenitor cells, i.e., on either side of the CSB, as well as in progenitors of the medial ganglionic eminence from which the GABAergic guidepost neurons are derived (Niquille et al., 2009). Moreover, *Gli3* is expressed in the forebrain from its induction at E8.5 till the end of cortical neurogenesis at E18.5 (Hui et al., 1994). This widespread and prolonged expression raises the possibility that *Gli3* could be required in dorsal and/or ventral telencephalic progenitor cells to control CC formation.

To address the spatial dependence of CC formation on *Gli3* expression, we employed a conditional knock-out approach to determine the effects of specific inactivation of *Gli3* in the ventral and dorsal telencephalon on the positioning of midline guideposts and on callosal development. We show that deletion of *Gli3* in progenitors of the septum and of the medial ganglionic eminence has no obvious defects on callosal development. In contrast, loss of *Gli3* function in the cortex using an *Emx1Cre* driver line results in severe disorganization of guidepost cells and in the formation of a severely hypoplastic CC. Examination of early developmental stages further showed that early changes in Wnt/β-catenin and Fgf8 signalling lead to the premature formation of ectopic glial fibres and to ectopic *Slit1/2* expression in the septum and that these alterations in the development of midline guideposts interfere with midline crossing of callosal axons. Collectively, these findings suggest that *Gli3* acts in $Emx1^{+}$ progenitors to control development of midline guidance cues and CC formation.

Materials and methods

Mice

Emx1Cre (Gorski et al., 2002), *Zic4Cre* (Rubin et al., 2010), *Nkx2.1Cre* (Kessaris et al., 2006), $Gli3^{fl/fl}$ (Blaess et al., 2008) and *ROSA26CAG dual stop EGFP reporter* (*RCE*) (Sousa et al., 2009) mice were kept on a mixed background, and were interbred. $Emx1Cre;Gli3^{fl/+}$, $Zic4Cre;Gli3^{fl/+}$ and $Nkx2.1Cre;Gli3^{fl/+}$ mice were mated with $Gli3^{fl/fl}$ mice to obtain $Emx1Cre;Gli3^{fl/fl}$ and $ZicCre;Gli3^{fl/fl}$ and $Nkx2.1Cre;Gli3^{fl/fl}$ conditional mutant embryos. Likewise, $Nkx2.1Cre;Gli3^{fl/+}$ mice were mated with $Gli3^{fl/fl}$;*RCE* females to obtain $Nkx2.1Cre;Gli3^{fl/fl}$;*RCE* conditional mutant embryos. $Emx1Cre;Gli3^{fl/+}$, $Zic4Cre;Gli3^{fl/+}$ and $Nkx2.1Cre;Gli3^{fl/+}$ embryos were used as controls. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. For each marker and each stage, 3–5 embryos were analysed.

In situ hybridization and immunohistochemistry

Antisense RNA probes for *Axin2* (Lustig et al., 2002), *Emx1* (Simeone et al., 1992), *Fabp7* (Genepaint. RNA probe 653), *Fgf8* (Crossley and Martin, 1995), *Gli3* (NM_008130, Genbank, 132–5113 bp), *Robo1* (Erskine et al., 2000), *Six3* (Oliver et al., 1995), *Slit1/2* (Erskine et al., 2000), *Sprouty2* (Minowada et al., 1999), *Wnt7b* (Parr et al., 1993) and *Wnt8b* (Richardson et al., 1999) were labelled with digoxigenin. In situ hybridisation on 10 μm serial paraffin sections of mouse brains were performed as described (Theil, 2005).

Immunohistochemical analysis was performed as described previously (Theil, 2005) using antibodies against the following antigens: Calbindin (CB) (1:1000, Swant); Calretinin (CR) (1:1000, CHEMICON); Glia Fibrillary Acidic Protein (GFAP) (1:1000, Dako-Cytomation); GFP (1:500, Abcam) Nf1a (1:1000, Active Motif); neural cell adhesion molecule L1 (1:1000, CHEMICON); Satb2 (1:50, Abcam); Tbr1 (1:2500, CHEMICON). Primary antibodies for immunohistochemistry were detected with Alexa- or Cy2/3-conjugated fluorescent secondary antibodies. For counter staining TOPRO-3 (1:2000, Invitrogen) was used.

Carbocyanine dye injection and analysis

P7 pups were perfused transcardially with 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS). For callosal labelling, single crystals of the lipophilic tracer DiI were placed into the cortex of whole brains using pulled glass capillaries. Dyes were allowed to diffuse at 37 °C for 5–6 weeks in 4% (w/v) PFA in PBS. Brains were rinsed in PBS, embedded in agarose and sectioned coronally on a vibratome at 100 μm. Sections were cleared in 9:1 glycerol:PBS solution containing the nuclear counter-stain TOPRO3 (0.2 μM) overnight at 4 °C.

Western blotting

Protein was extracted from the dorsal telencephalon of E12.5 $Gli3^{fl/+}$ (control) and $Emx1Cre;Gli3^{fl/fl}$ embryos as described previously (Fotaki et al., 2006). Equivalent amounts of protein were subjected to gel electrophoresis on a 3–8% gradient Tris-acetate gel (Invitrogen), and protein was transferred to a nitrocellulose membrane, which was incubated with rabbit polyclonal anti-Gli3 antibody (1:500; Abcam). After incubating with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2000; Dako), signal was detected using an ECL Plus detection kit (Amersham GE healthcare).

Statistical analysis

Analysis was performed on data collected from brains of at least 3 embryos of each genotype. Mann–Whitney test was used to compare the proportion of $Satb2^{+}/Dapi^{+}$ cells. To compare the density of $Satb2^{+}$ cells a normality test (Shapiro–Wilk) was performed first for 2-way analysis of variance and if failed statistical comparisons were made by Holm–Sidak (for more than 2-group comparisons). Independent *t*-test analysis (2 sample) was performed for cortical thickness measurements. For all statistical analyses SPSS software was used. Asterisks indicate $P < 0.05$.