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Gli3 controls corpus callosum formation by positioning midline guideposts during telencephalic patterning.

Abbreviated title: Corpus callosum development requires *Gli3*

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ABSTRACT

The corpus callosum (CC) is the principal axonal tract responsible for the communication between the two cortical hemispheres and its malformation has been associated with a wide range of cognitive, behavioural and neurological defects. During callosal development, several guide post cells including the midline glia cells and callosal sling neurons are crucial in guiding callosal across the dorsal midline but it remains unknown how these cues acquire their correct position at the corticoseptal boundary (CSB) where callosal axons cross the midline. The *Gli3* hypomorphic mouse mutant *Polydactyly Nagoja* (*Pdn*) shows early patterning defects and lacks the corpus callosum. Transplantation experiments and analysis of a *Gli3* conditional mutant demonstrated that midline abnormalities are primarily responsible for the absence of the CC. In fact, the cingulate cortex contains ectopic glial cells which block the path of callosal axons and shows an up-regulation of the axon guidance molecule *Slit2*. Positioning of the guidance cues is largely rescued in *Pdn/Pdn*; *Slit2*$^{-/-}$ double mutants. We further show that these midline abnormalities are caused by an imbalance in Fgf8 and Wnt7b/Wnt8b signalling at the CSB and that up-regulation of Fgf signalling in the rostromedial telencephalon is sufficient to induce agenesis of the CC. Hence, Gli3 controlled patterning processes have an important role in CC formation by organizing midline structures that guide callosal axons across the midline.

INTRODUCTION

The corpus callosum is the largest fibre tract in the brain connecting neurons of the two cerebral hemispheres. Its principal cognitive function is to coordinate the information between the left and right cortex. Malformation of the corpus callosum can manifest as partial agenesis, hypoplasia across the entire structure or complete agenesis (ACC) and has been associated with mental retardation having a wide range of cognitive, behavioural and neurological consequences (Richards et al., 2004; Paul et al., 2007). ACC has been identified in over 50 different human congenital syndromes (Jeret et al., 1987; Richards et al., 2004). This large number of syndromes affecting the corpus callosum is likely to reflect the complexity of the processes controlling callosal development.

The cellular and molecular mechanisms regulating the formation of the corpus callosum have been extensively studied with a strong emphasis on the processes which control midline crossing of callosal axons. These analyses revealed several guidance events critical for callosal development. The midline zipper glia (MZG) has been suggested to be required to initiate the fusion of the dorsal midline producing the substrate on which callosal axons navigate (Silver et al., 1993). In addition, a number of guide-post cues are located along the path of callosal axons and in particular at the cortical septal boundary (CSB). These cues include the midline glial cell populations composed of the indusium griseum glia (IGG) and the glial wedge (GW) (Lindwall et al., 2007; Paul et al., 2007), and the callosal sling which consists of GABAergic or glutamatergic neuronal populations expressing Calbindin (CB) and Calretinin (CR)/Tbr1, respectively (Niquille et al., 2009). Finally, axons from the cingulate cortex pioneer the corpus callosum and function as a scaffold for later arriving neocortical axons (Koester and O'Leary, 1994; Rash and Richards, 2001; Piper et al., 2009). In addition, several axon guidance molecules including Slit2 and Sema3c which are produced by midline glial cells and by callosal sling neurons, respectively, have been identified with essential roles in callosal development (Bagri et al., 2002; Niquille et al., 2009). Taken together, these studies reveal complex interactions between callosal axons and their environment, however, it remains largely unknown how these guide posts acquire their correct position along the path of callosal axons and how the expression of these guidance molecules is regulated which is a prerequisite for their function.

Gli3 encodes a zinc finger transcription factor with key roles in forebrain development. The work of several laboratories has shown that *Gli3* is essential for early patterning of the telencephalon (Theil et al., 1999; Tole et al., 2000b; Kuschel et al., 2003; Fotaki et al., 2006; Yu et al., 2009; Magnani et al., 2010). In particular, it is crucial for the development of midline structures including the choroid plexus, the cortical hem and the hippocampus. *Gli3* exerts its function both cell autonomously

(Quinn et al., 2009) by directly regulating transcription as well as cell non-autonomously by establishing and maintaining the expression of signaling molecules crucial for telencephalic development since *Gli3* mutants show an up-regulation of *Fgf8* expression and a down-regulation of *Wnt* and *Bmp* gene expression in the dorsomedial telencephalon (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000b; Aoto et al., 2002). In addition, we recently showed an important *Gli3* function in controlling axon pathfinding in the forebrain. The *Gli3* hypomorphic mouse mutant *Polydactyly Nagoya* (*Pdn*) has reduced levels of wild-type *Gli3* transcript (Thien and Rüther, 1999; Ueta et al., 2002) and Gli3 protein (Magnani et al., 2010) and shows severe pathfinding defects of corticothalamic and thalamocortical axons (Magnani et al., 2010). Moreover, *Pdn* mutants lack the corpus callosum (Naruse et al., 1990) but the mechanisms underlying these defects were not identified. Here, we present a detailed characterisation of *Gli3's* role in callosal development. Transplant experiments and conditional inactivation of *Gli3* demonstrate that it is required for correct midline development. Midline glia populations and callosal sling neurons are severely disorganized in *Pdn* mutants. In the dorsomedial telencephalon and later in the cingulate cortex, *Pdn* mutants show an up-regulation of *Slit2* expression. Interestingly, the distribution of callosal axons and of callosal sling neurons is greatly improved in *Pdn/Pdn*;*Slit2-/-* double mutants. Moreover, glial cells are formed ectopically in the *Pdn* cingulate cortex and extend processes from the ventricular to the pial surface thereby blocking the migration of callosal axons. A disorganization of radial glia and *Slit2* up-regulation were already detected in the E12.5 cortical midline well before callosal axons approach the midline. These changes correlate with an upregulated *Fgf8* expression and a downregulation of *Wnt7b/8b* expression in the septum and rostromedial cortex, respectively, and lead to altered expression of the Emx1, Six3 and Nfi transcription factors at the CSB which have essential functions in CC formation. Finally, *sprouty1/2* mutants have an up-regulation of Fgf signalling in the rostromedial telencephalon similar to *Pdn* mutants and display ACC combined with a severe disorganization of the midline glia populations. Taken together, our data demonstrate a hitherto uncharacterized link between early patterning and the positioning and expression of midline guidance cues essential for callosal development.

RESULTS

Pdn/Pdn mutants lack all major forebrain commissures

A previous histological analysis showed that the *Gli3* hypomorphic mouse mutant *Polydactyly Nagoja (Pdn)* displays agenesis of the corpus callosum (CC) (Naruse 1990). Here, we confirm this result with neurofilament (NF) staining and cortical DiI labelling (Fig. 1). Coronal sections of P0 control brains immunostained for NF revealed the major forebrain axon tracts including the CC, the anterior and hippocampal commissure, the fornix and the ipsilateral perforant pathway (PFP) connecting the cortex with the septum (Fig. 1A-D). In contrast, *Pdn*/*Pdn* mutant cortical axons are detected in the cingulate cortex although their path is disrupted at several positions (Fig. 1E-H). Those axons approaching the midline fail to cross and form Probst bundles (Fig. 1H). In addition, the anterior and the hippocampal commissure are absent while the PFP is formed (Fig. 1E-F and data not shown). To analyze CC formation more specifically, we injected DiI crystals into one cortical hemisphere. This analysis revealed callosal axons projecting towards the contralateral hemisphere of control brains but not in *Pdn* mutant brains in which callosal axons project caudally within dorsomedial regions of the cerebral cortex (Fig. 1I-K). Taken together, these analyses show severe axon pathfinding defects in *Pdn*/*Pdn* mutants with a complete absence of all major forebrain commissures.

CC midline guidance are severely disorganized in *Pdn/Pdn* brains

To gain insights into the origins of the ACC in *Pdn* mutants, we analyzed the callosal guidance cues which guide callosal axons across the midline. These cues are comprised of pioneer axons of the cingulate cortex, midline glia cells and the callosal sling neurons each of which are essential for callosal development (Piper et al., 2009). The cingulate pioneer axons are immunopositive for Neuropilin-1 (Npn-1) and occupy the dorsalmost part of the CC (Fig. 2 A,B). In *Pdn*/*Pdn* mutants, Npn-1+ axons are present but fail to project to the contralateral hemisphere and form dense bundles (Fig. 2C, D). Glutaminergic neurons of the callosal sling express either Calretinin or Calbindin and occupy strategic positions at the CSB. Wild-type CR+ and CB+ neurons are both located in the IG region and CR+ neurons are also found within the corpus callosum where they delineate its ventral and dorsal parts (Fig. 2A,B,E,F). In *Pdn/Pdn* mutant brains, CR+ neurons are dramatically disorganized but maintain their spatial organization with callosal axons, with clusters of CR+ neurons surrounding the Probst bundles (Fig. 2C,D). CB+ neurons remain concentrated in the medial cortex although they are more diffusely distributed and clusters of CB+ neurons abnormally

intermingle with callosal axons (Fig. 2 G, H). Finally, GFAP immunostaining on control P0 coronal brain sections labels the glial wedge (GW), the indusium griseum glia (IGG) and the midline zipper glia (MZG) (Fig. 2I, J). In *Pdn/Pdn* brains, GFAP+ cells are severely disorganized. Several GFAP+ fascicles are formed in ectopic positions in the cingulate cortex (Fig. 2K, L). Some fascicles span the whole cortical width and transect the path of callosal axons which are unable to further progress along their route (Fig. 2L). The IGG could not be identified and the midline zipper glia expands into more ventral regions of the septum (Fig. 2K). Taken together, these data show a dramatic disorganization of glial and neuronal guide post cells in the P0 *Pdn* mutant brain.

CC midline defects cause agenesis of the corpus callosum in *Pdn* mutants

The ACC and the severe disorganization of the CC midline in *Pdn* mutants might be caused by a failure of callosal axons to navigate in the cortical midline region leading to the formation of Probst bundles and to the redistribution of the midline guidance cues. Alternatively, the disorganization of guidance cues might cause the pathfinding defects and lead to axonal bundling. The next experiments aimed at distinguishing between these possibilities. Our previous analyses showed that Satb2+ callosal neurons are formed correctly in *Pdn*/*Pdn* mutants and that they acquire their correct lamina position (Magnani et al., 2010) suggesting that lamination defects are unlikely to cause the ACC. To test more directly whether *Pdn* mutant callosal axons are capable of following midline guidance cues we used an in vitro transplantation assay. *Pdn* mutants were crossed with mice ubiquitously expressing a tau-GFP fusion protein (Pratt et al., 2000). Tissue from the frontal cortex of E17.5 GFP+ embryos was homotopically transplanted into cortical sections of age-matched GFP- embryos and the migration pattern of callosal axons was monitored using GFP immunofluorescence. In control transplants, outgrowing callosal axons crossed the midline region (n=8 out of 9) (Fig. 3A). After transplantation of *Pdn/Pdn*; GFP+ cortex into control cortex, *Pdn/Pdn* axons also migrated across the midline and reached the contralateral cortex (n=7 out of 8) (Fig. 3B). However, when we transplanted control; GFP+ cortical tissue into *Pdn/Pdn*; GFP- frontal cortex, callosal axons did not grow into the mutant dorsomedial cortex (n=0 out of 7) (Fig. 3C) and only a few axons projected along the surface of the mutant host tissue, (n=4 out of 7) (Fig. 3C). This failure in callosal axon outgrowth is in stark contrast to corticofugal axons which project into the lateral cortex and into the striatum under these conditions (Magnani et al., 2010). These results show that *Pdn/Pdn* callosal axons are able to cross the cortical midline in a wild-type environment and suggest that the *Pdn/Pdn* dorsomedial cortex is not as permissive to callosal axon outgrowth as wild type cortex.

To further study whether *Gli3* mutant callosal axons have the ability to cross the midline, we analyzed *Emx1Cre;Gli3* conditional mutants (Gorski et al., 2002; Blaess et al., 2008). In these

animals, inactivation of *Gli3* expression is restricted to the dorsal telencephalon starting in the dorsomedial telencephalon from E10.5 onwards. By E12.5, before the birth of callosal neurons, Gli3 protein is absent from cortical tissue while *Gli3* expression remains unaffected in the ventral telencephalon including the septum (Supplementary Fig. 1). In *Emx1Cre;Gli3* conditional mutants, Satb2+ callosal neurons are specified and acquire their correct lamina position (Supplementary Fig. 2). Interestingly, these mutants form a small but discernible corpus callosum at P0 which greatly enlarges by P7 although some Probst bundles were still detected at both ages (Fig. 4D-F). The formation of a hypoplastic CC coincides with less severe defects in the organization of the cortical midline at P0. Some Tbr1+ and CB+ callosal sling neurons are scattered in the dorsomedial cortex and associate with the Probst bundles but at least one stream of callosal sling neurons delineates the ventral path of the CC (Fig. 4D,E). Moreover, the IGG is located in its correct position dorsal to the hypoplastic CC although it is expanded (Fig. 4F). Most notably, the GW does not form the ectopic glial fibers characteristic of *Pdn* mutants (Fig. 4F). These data indicate that *Gli3* mutant callosal axons are able to cross the cortical midline. Taken together with the results of the transplantation experiments, these data strongly suggest that the severe disorganization of the *Pdn/Pdn* cortical midline is the major cause of the ACC.

Pdn midline defects are already present at patterning stages of telencephalic development

To study the origins of these midline defects, we investigated the formation of the midline guideposts at earlier embryonic stages. At E16.5, the cingulate pioneer axons approach the midline in wild-type embryos (Fig. 5A, B). In *Pdn*/*Pdn* mutants, these axons have reached the CSB but form abnormal structures reminiscent of the Probst bundles found at P0 (Fig. 5C, D). Moreover, Tbr1+, CR+ and CB+ glutaminergic neurons fail to form a well organized band of neurons at the CSB and there are less CB+ neurons in the future IG region (Fig. 5E-L). In the cingulate cortex, the cortical plate is disrupted in several positions and callosal axons stop their migration at these positions (Fig. 5G, H, K, L). Finally, in wildtype embryos, radial glial cells at the CSB have started to differentiate into GW cells, to translocate to the pial surface to form the IGG and to express GFAP and Glast Smith2006 (Fig. 5M,N). In *Pdn* mutants, GFAP+;GLAST+ cells are not confined to the CSB but are also present ectopically in the cingulate cortex and extend projections from the ventricular to the pial surface (Fig. 5O,P). Since this glial translocation is controlled by Fgf signalling (Smith et al., 2006) we investigated *Fgf8* expression and that of its target gene *sprouty2*. In the rostral cortex of control embryos, both genes are expressed in the IGG and in the glial wedge and *sprouty2* expression expands into the cingulate cortex (Supplementary Fig. 3A,B). In *Pdn* embryos, however, *Fgf8* expression is confined to the septum and the cingulate cortex which is connected to the septum only

by a thin tissue stripe lacks *sprouty2* expression (Supplementary Fig. 3C,D). Taken together with our E18.5 findings, these data strongly suggest that glial cells are formed at ectopic positions in the cingulate cortex and fail to retract their processes due to defective Fgf signalling.

We also analyzed the expression patterns of axons guidance molecules important for midline crossing of callosal axons. Callosal sling and cingulate neurons express *Sema3c* thereby attracting callosal axons towards the midline (Niquille et al., 2009; Piper et al., 2009) (Fig. 5Q). In the *Pdn* cingulate cortex, however, *Sema3c* expression is reduced (Fig. 5S). Moreover, *Slit2* which prevents callosal axons from projecting into the septum and channels these axons to the contralateral cortex (Bagri et al., 2002) is expressed in the septum, the GW and in the cingulate cortex in a graded manner (Fig. 5R). Interestingly, *Pdn*/*Pdn* mutants show a severe up-regulation of *Slit2* expression in the cingulate cortex, while its expression in the septum is not affected (Fig. 5T). Thus, the distribution of the guide post cells and their expression profile are severely altered in the E16.5 *Pdn*/*Pdn* cortex.

From these analyses, the ectopic glial differentiation and the up-regulation of *Slit2* represent the most prominent changes at the cortical midline. Therefore, we further analysed the development of these alterations. Up-regulation of *Slit2* expression in the *Pdn*/*Pdn* dorsomedial cortex and in the septum can already be observed at E14.5 and at E12.5 (Fig. 6A-D). In addition, the E14.5 dorsomedial cortex already contains RGC clusters expressing high levels of the RGC marker Blbp (Fig. 6E,F). Moreover, in E12.5 wild-type embryos, the expression of *Fabp7* which encodes the Blbp antigen is up-regulated in neurogenic RGCs in the dorsomedial cortex of wild-type embryos with a sharp expression boundary at the CSB (Fig. 6G). In contrast, the *Pdn* dorsomedial cortex lacks this *Fabp7* high level expression domain, but shows clusters of RGCs with high levels of *Fabp7* expression next to cells having little or no *Fabp7* transcripts (Fig. 6F,H). Taken together, these data show that the *Pdn* mutant dorsomedial telencephalon is already defective at E12.5 well before callosal axons approach the dorsomedial cortex further emphasizing that midline defects are responsible for the ACC.

Midline defects are partially rescued in *Pdn*/*Slit2* double mutants

To test for a role of the *Slit2* up-regulation in the development of the *Pdn* callosal phenotype we crossed *Pdn* with *Slit2* mutant mice and analyzed the formation of the CC in E18.5 *Pdn*/*Slit2* double mutants. In these animals, the formation of the corpus callosum is not rescued but the overall organization of callosal axons and of the midline structures is dramatically improved (Fig. 7C, D, H, I, M, N). In the *Pdn* cingulate cortex, callosal axons form a thin intermediate zone disrupted by several, large Probst bundles (Fig. 7B,G,L). In *Pdn*/*Pdn*;*Slit2*$^{+/-}$ and in *Pdn*/*Pdn*;*Slit2*$^{-/-}$ embryos,

callosal axons migrate to the CSB without forming Probst bundles (Fig. 7C, D, H, I, M, N). In addition, the organization and positioning of the midline guidance cues is partially rescued in the *Pdn*/*Slit2* double mutants (Fig. 7C, D, H, I, M, N). CB+ neurons are located in the IG region of the double mutants similar to control embryos and *Slit2*-/- single mutants but are scattered in the *Pdn*/*Pdn* cortex (Fig. 7A-E). CR+ neurons are located in the dorsomedial cortex of the double mutants while these cells are mostly associated with the Probst bundles in *Pdn*/*Pdn* mutants (Fig. 7F-I). Interestingly, in *Slit2*-/- mutant mice, the CR+ cell population is largely absent from the dorsomedial cortex but large numbers of CR+ neurons are located ventrally to the CC in close association with the ectopic fibre bundles (Fig. 7J). In contrast, the midline glia shows several abnormalities in *Pdn*/*Slit2* double mutants (Fig. 7K-N). The IGG is missing and ectopic glial fascicles are still formed although they are confined to the ventral most part of the cortex and do not inhibit the migration of callosal axons (Fig. 7M, N). Taken together, these analyses show a remarkable recovery of midline morphology and the organization of callosal sling neurons in *Pdn*/*Slit2* double mutants.

The *Pdn* mutation affects patterning of the dorsomedial telencephalon

While midline morphology is largely improved in *Pdn*/*Slit2* double mutants, callosal axons fail to cross the midline suggesting that additional factors are affected in *Pdn* mutants. Previously, we and others have shown that *Gli3* plays a crucial role in establishing and maintaining the expression of several signalling molecules in the telencephalon. In particular, a whole mount in situ hybridization analyses showed an ectopic *Fgf8* expression and a *Bmp4*/*Wnt3a* downregulation in the dorsomedial telencephalon of E12.5 *Pdn*/*Pdn* embryos (Kuschel et al., 2003). To focus more specifically on the corticoseptal region, we confirmed and extended these previous findings by in situ hybridisation on coronal sections. In wild-type embryos, *Fgf8* expression is confined to the commissural plate, but is detected more dorsally and at higher levels in the *Pdn*/*Pdn* corticoseptal region (Fig. 8A, F). Similarly, *sprouty2* expression is up-regulated and extends dorsally into the ventralmost cortex indicating that Fgf signalling is ectopically activated in *Pdn* mutants (Fig. 8B, G). In addition, we identified two *Wnt* genes, *Wnt*7b and *Wnt*8b, whose expression is confined to the dorsomedial telencephalon with a sharp expression boundary at the CSB (Fig. 8C, D). In *Pdn* mutants, *Wnt*7b and *Wnt*8b expression is strongly reduced in the dorsomedial telencephalon but *Wnt*7b is expressed at higher levels in the septum (Fig. 8H, I). Similarly, expression of the *Wnt* target gene *Axin2* is severely reduced in *Pdn* mutants (Fig. 8I, J). These analyses therefore indicate severe changes in Fgf and Wnt signalling in the rostromedial telencephalon of *Pdn* mutants.

We next analyzed whether these changes could affect the patterning of the corticoseptal region. Recently, the expression domains of several transcription factors, including Six3, Emx1 and Nfia, were shown to delineate the regions where the corpus callosum, the hippocampal commissure and the anterior commissure cross the midline at E16.5 (Moldrich et al., 2010). These factors are important regulators of early forebrain development and *Emx1* and *Nfia* mutant mice display defective callosal development. We therefore analyzed their expression in the early rostromedial telencephalon. In E12.5 wild-type embryos, *Six3* and *Emx1* are expressed in the septum and cortex, respectively (Fig. 8K). However, in *Pdn* mutants, *Six3* expression expands into dorsal cortical areas whereas *Emx1* expression is completely lost (Fig. 8O) and (Kuschel et al., 2003). Furthermore, Nfia and two other members of the *Nfi* gene family, *Nfi*b and *Nfix*, are expressed at high levels in the cortex and at lower levels in the dorsalmost septum (Fig. 8L-N). In *Pdn* mutants, the high level expression domains of all three factors in the cortex is lost while low level expression in the septum remains except for Nfia which is up-regulated in the septum (Fig. 8P-R). These data strongly suggest that the patterning of the rostromedial telencephalon is severely affected in *Pdn* mutants and that the CSB where callosal axons will later cross the midline is poorly defined.

We also analyzed Fgf and Wnt signalling and the expression of cortical/septal markers in the *Emx1*Cre;*Gli3* conditional mutants which show a milder callosal phenotype (Fig. 9). Interestingly, these mutants, show a slight up-regulation of *Fgf8* and *sprouty2* expression whereas *Wnt*8b and *Axin2* expression are slightly reduced (Fig. 9F,G,I,J). Only the high level *Wnt*7b expression domain is ventrally shifted from the cortex into the septum (Fig. 9H). Moreover, *Six3* and *Emx1* expression remain confined to the septum and cortex, respectively (Fig. 9P,Q). Nfia expression was slightly shifted ventrally into the septum, but still showed high expression levels in the dorsomedial cortex (Fig. 9R). Finally, Fabp7 and Slit2 are slightly down-regulated (Fig. 9N,O,S,T). Thus, *Emx1*Cre;*Gli3* conditional mutants display more subtle patterning defects in the rostromedial telencephalon than *Pdn* mutants and the severity of patterning abnormalities in *Gli3* mutants correlates with the severity of the callosal phenotype.

Sprouty1-2 **mutants display agenesis of the corpus callosum**

To directly test whether changes in Fgf and *Wnt* signalling could lead to defective callosal development, we started to analyse CC formation in *sprouty1/2* double mutants. These embryos show an up-regulation of Fgf signalling and a reduction in Wnt signalling in the rostromedial telencephalon (Faedo et al., 2010) similar to *Pdn* mutants. Since the effect of these mutations on callosal development has not been analyzed previously, we performed immunostainings on E18.5 *sprouty1/2* double mutant brains. We first confirmed that the formation of Satb2+ callosal

projection neurons and their positioning in the upper cortical layers is not affected in these mutants (Fig. 10A,B). In contrast, Neurofilament and Tuj1 staining revealed agenesis of the CC in *sprouty1-2* mutants (Fig. 10A-H). Callosal fibres project towards the midline, but fail to cross it and form Probst bundles. The analysis of the midline guidance cues showed no obvious differences in the distribution of CB+ neurons in the dorsomedial cortex (Fig. 10X, Y). Also, CR+ sling neurons intermingle with callosal axons and delineate their ventral and dorsal organization (Fig. 10G, H). However, the midline glia populations are severely affected. Several GFAP+ glia fibres abnormally cluster at the corticoseptal boundary transecting the path of callosal axons at several locations while the IGG could not be identified (Fig. 10I,J). Taken together, these data show that up-regulation of Fgf signalling during patterning of the rostromedial telencephalon is sufficient to induce callosal malformations.

DISCUSSION

Telencephalic patterning controls callosal development

Callosal axons cross the cortical midline at the CSB which plays a crucial role in the midline crossing event. Several guide post cells including the midline glia populations and the callosal sling are organized in strategic positions to closely interact with callosal axons and to guide them to the contralateral cortical hemisphere. While the work of several labs has shown the importance of complex interactions between the axons and these guidance cues, it remains largely unknown how the guide posts acquire their position at the CSB. The *Gli3* hypomorphic mutant *Pdn* provides an interesting model to address this question. *Gli3* is known to have crucial roles in patterning the telencephalon (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000a; Theil, 2005; Friedrichs et al., 2008; Fotaki et al., 2009; Quinn et al., 2009; Yu et al., 2009) and in axon guidance (Magnani et al., 2010) and we here show that the normal distribution of several guide posts is severely affected in *Pdn* embryos. Most importantly, the cortical midline region contains ectopic glial fibers which transect the path of callosal axons and shows an up-regulation of the *Slit2* guidance molecule. Several lines of evidence strongly suggest that the ACC in *Pdn* mutants is caused by defective development of the guidance cues rather than by defects in callosal axons. Cortical layering and the expression of the callosal determinant Satb2 are not affected in *Pdn* embryos (Magnani et al., 2010). Moreover, *Pdn* mutant callosal axons are capable of midline crossing in a wild-type environment and the CC forms in *Emx1Cre;Gli3* conditional mutants although its development is delayed. Finally, our time course analyses revealed that molecular changes in the cortical midline relevant to the callosal malformation occur as early as E12.5 well before callosal axons arrive at the midline region. This latter finding strongly hints to the origin of the callosal defects as early patterning defects. Indeed, the corticoseptal region is severely mispatterned in *Pdn* mutants. Consistent with previous findings on the role of *Gli3* in establishing/maintaining telencephalic signalling centres, *Pdn* mutants have reduced *Wnt7b*/*Wnt8b* expression and show an up-regulation of *Fgf8* expression and signalling in the rostromedial telencephalon. These changes in major signalling pathways coincide with altered expression patterns of several transcription factors including Six3, Emx1 and the Nfi factors which have important functions in forebrain development and control callosal development (Qiu et al., 1996; Lagutin et al., 2003; Shu et al., 2003; Campbell et al., 2008; Plachez et al., 2008). Unlike *Pdn* mutants, however, the expression of these factors is only mildly affected in the *Gli3* conditional mutants. Taken together, these findings strongly suggest that *Gli3* controlled early patterning processes play a crucial role in setting up the spatial organization of midline

guidance cues and provide a hitherto uncharacterized link between early patterning and callosal development.

Early and late role of Fgf signalling in callosal development

What are the mechanisms underlying this connection between patterning and callosal development? *Gli3* has a crucial role in controlling the expression of signalling molecules including Fgf8 in the telencephalon and Fgf signalling is essential for callosal development. Previous analyses on the role of Fgf signalling have focussed on the formation of guidance cues at the cortical midline in conditional Fgf8 or Fgf receptor mutants (Tole et al., 2006; Moldrich et al., 2010) and have revealed an important function for Fgf signalling in controlling the translocation of glial cells from the ventricular to the pial surface to form the IGG (Smith et al., 2006). Due to its altered midline morphology, *Pdn* mutants lack Fgf signalling in the E16.5 cingulate cortex and consistent with the above findings, *Pdn* mutant glial cells form fibres spanning the whole width of the cortex thereby blocking the migration of callsoal axons. In addition to this late role, we here identify a novel, earlier role of *Fgf8* in callosal development. In contrast to our findings at E16.5, *Fgf8* expression is up-regulated in the commissural plate of E12.5 *Pdn* mutants coinciding with a mispatterning of the corticoseptal region. Moreover, *sprouty1/2* double mutants in which Fgf signalling is increased in the rostromedial cortex (Faedo et al., 2010) partially mimic the callosal phenotype of *Pdn* mutants showing the formation of ectopic glial wedge fibres and agenesis of the corpus callosum. Therefore, up-regulation of Fgf signalling during patterning of the rostromedial telencephalon is sufficient to induce callosal malformation. In light of these findings, it will be interesting to characterize patterning of the CSB in the Fgf receptor mutants and the *Fgf8* conditional mouse mutant. Taken together, these data show that a loss of as well as an increase in Fgf signalling can cause ACC strongly suggesting that regulating *Fgf8* expression levels represents a crucial step in callosal development.

An interesting question therefore concerns the mechanisms regulating *Fgf8* expression levels in the commissural plate. Gli3 acts as a repressor of *Shh* expression and Shh signalling in the ventral telencephalon (Magnani et al., 2010) but this increase in Shh signalling remains confined to the ventral most septum of *Pdn* embryos and is therefore unlikely to affect callosal development directly. It could also up-regulate *Fgf8* expression in the commissural plate indirectly via the *Shh*/*Fgf8* positive feedback loop (Ohkubo et al., 2002), however, a similar but more severe ectopic *Fgf8* expression in the *Gli3* null mutant *extra-toes* (Xt^J) (Theil et al., 1999; Aoto et al., 2002; Kuschel et al., 2003) persists in Xt^J/*Shh* double mutants (Rash and Grove, 2007). To distinguish between these possibilities, it will be interesting to analyze callosal development in *Shh*/*Pdn* double

mutants. Alternatively, *Wnt7b*/*8b* expression is already down-regulated before the onset of ectopic *Fgf8* expression in the E9.0 *Pdn* telencephalon (Ueta et al., 2008). This and the reduced Wnt signalling in the *sprouty1/2* double mutants (Faedo et al., 2010) suggest an antagonistic interaction between Fgf and Wnt signalling in controlling patterning of the CSB, potentially in parallel to the negative regulatory relationship between Fgf8 and Bmp4 (Theil et al., 1999; Shimogori et al., 2004). These interactions might be part of an integral system to control *Fgf8* expression levels in the commissural plate which are important in patterning the rostromedial cortex and in positioning midline guidance cues.

Downstream effectors of Fgf signalling

Our data indicate that *Gli3* controlled Fgf8/Wnt signalling plays key roles in the patterning of midline tissues in early stages of development which is crucial in organizing the later midline region to allow midline crossing of callosal axons. Characterization of the *Pdn* mutant also helped to identify potential downstream effectors of Fgf signalling. One striking finding of our analyses is the formation of ectopic glial fibers which originate from clusters of RGCs in the rostromedial telencephalon of *Pdn* mutants. Several studies have implicated Fgf signalling in the regulation of RGC development (Kang et al., 2009; Sahara and O'Leary, 2009) and sprouty1/2 double mutants show an up-regulation of *Fabp7* expression throughout the rostromedial cortex (Faedo et al., 2010). Compared to the patchy *Fabp7* expression in *Pdn* mutants, this finding indicates the existence of an additional, unknown genetic pathway which restricts high level *Fabp7* expression to a few cells in *Pdn* mutants. In addition, several transcription factors with key functions in early forebrain and callosal development show altered expression patterns in the corticoseptal region of *Pdn* embryos. Mutations of the human *SIX3* gene lead to holoprosencephaly (Rosenfeld et al., ; Wallis et al., 1999), while inactivation of murine *Six3* gene results in severe truncations of the prosencephalon due to increased Wnt signalling (Lagutin et al., 2003). While the severity of these phenotypes is indicative of important functions in forebrain development it obscures potential role(s) for *Six3* in callosal formation. In contrast, *Emx1* mutants lack the corpus callosum though for unknown reasons (Qiu et al., 1996) and *Emx1* expression is repressed by Fgf8 (Crossley et al., 2001; Garel et al., 2003; Kuschel et al., 2003; Storm et al., 2003; Storm et al., 2006). Furthermore, the Nfi transcription factors, Nfia, Nfib and Nfix, have high expression level domains dorsally to the CSB (Campbell et al., 2008; Plachez et al., 2008) overlapping with the domains of *Wnt7b/8b* expression suggesting regulatory relationships between these genes. Mutations in the *Nfi* genes lead to mild but consistent callosal defects (Shu et al., 2003). The lack of the CC in these mutants has been attributed to a control of glial specific gene expression but our data suggest that these factors might

have an earlier patterning role which might be obscured by redundancy between these factors. Taken together, these findings suggest complex interactions between these transcription factors and signalling molecules to pattern the CSB which is prerequisite for setting up the proper organization of midline guidance cues.

Finally, several aspects of the *Pdn* callosal phenotype are the result of an up-regulation of *Slit2* expression. By reducing the *Slit2* gene dosage in a *Pdn* mutant background, callosal axons are able to approach the cortical midline without forming Probst bundles suggesting a function for *Slit2* in controlling the permissiveness of the cingulate cortex for the growth of callosal axons. This idea is consistent with the temporal expression profile of *Slit2* in wild-type embryos which becomes down-regulated in the cingulate cortex after E14.5 (compare Fig. 5 and 6) and with the observation that *Slit2* mutant mice show huge ectopic axon bundles at the cortical midline next to the CC. Moreover, the disorganization of the callosal sling neurons is largely rescued in the *Pdn*/*Slit2* double mutants which could be secondary to the axonal rescue. Alternatively, *Slit2* could also regulate the migration of the callosal sling neurons into the cortical midline (Niquille et al., 2009) similar to its effect on the migration of LGE guidepost cells to form a permissive corridor for thalamocortical axons (Bielle et al.). Surprisingly, we observed the increased *Slit2* expression as early as E12.5 suggesting that early patterning defects affect *Slit2* expression. In this respect, Gli3 might repress *Slit2* expression in the dorsomedial cortex or *Slit2* could be a downstream target of Fgf signalling given its co-expression with sprouty2 throughout midline development (see Figures 6, 8 and Supplementary Figure 3) and its down-regulation in the septum of *Fgfr1* mutant mice (Tole et al., 2006). Irrespective of the exact mechanism, the up-regulation of *Slit2* provides a direct link between early patterning processes and the coordination of midline development to allow midline crossing of callosal axons.

Conclusion

Our analyses provide for the first time strong evidence that early patterning of the cortical midline has a pivotal role in callosal development and provide detailed insights into the processes which control the formation of midline guidance cues at their correct position. In these processes, *Gli3* takes centre stage by controlling Fgf and Wnt signalling at the rostral midline which in turn regulates the expression of several transcription factors and of the *Slit2* axon guidance molecule. Interestingly, the human GLI3 gene is mutated in Acrocallosal Syndrome patients who lack the CC (Elson et al., 2002). CC malformations are also a frequent hallmark of ciliopathies in which the function of the primary cilium and hence Gli3 processing is affected (Tobin and Beales, 2009).

Therefore, our findings will provide a framework for understanding the defective processes which underlie the ACC in these patients.

MATERIALS AND METHODS

Mice. *Pdn* heterozygous animals were kept on a C3H/He background and were interbred (Ueta et al., 2002). *Emx1Cre; Gli3* $^{flox/+}$ males and *Gli3* $^{flox/flox}$ females (Gorski et al., 2002; Blaess et al., 2008) were kept on a mixed background. *Sprouty1* and *2* single mutants were kept on a CD1 background and interbred (Basson et al., 2005; Shim et al., 2005). τGFP (Pratt et al., 2000) and *Slit2* (Plump et al., 2002) mice were bred into the *Pdn* line. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. In analyses of *Pdn* mutant phenotypes, heterozygous and wild type embryos did not show qualitative differences and both were used as control embryos. For *Emx1Cre Gli3* conditional embryos *Gli3* $^{flox/flox}$, *Gli3* $^{flox/+}$, *Emx1Cre; Gli3* $^{flox/+}$ were used as controls. For each marker and each stage, 3-5 embryos were analysed.

In situ hybridization and immunohistochemistry. Antisense RNA probes for *Gli3, Sema3C (Bagnard et al., 2000), Slit2 (Erskine et al., 2000), Fabp7, Fgf8 (Crossley and Martin, 1995), Sprouty2 (Minowada et al., 1999), Axin2 (Lustig et al., 2002), Wnt7b (Parr et al., 1993), Wnt8b (Richardson et al., 1999), Nf1b, Nf1x, Emx1 (Simeone et al., 1992)* and *Six3* (Oliver et al., 1995) were labelled with digoxigenin. In situ hybridisation on 12 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 molecules: β-III-tubulin (Tuj1 antibody; 1:1000, Sigma); brain lipid-binding protein (Blbp) (1:500, CHEMICON); calbindin (CB) (1:1000, Swant); calretinin (CR) (1:1000, CHEMICON); Glast (1:5000, CHEMICON); glia fibrillary acidic protein (GFAP) (1:1000, DakoCytomation); green fluorescent protein (GFP) (1:1000, Abcam); Nf1a (1:1000, Active Motif); neural cell adhesion molecule L1 (1:1000, CHEMICON); Neurofilament (2H3; 1:5, DSHB); Neuropilin-1 (Npn-1) (1:1000, R&D Systems); Satb2 (1:50, Abcam); Tbr1 (1:2500, CHEMICON).

Carbocyanine dye injection and analysis. Brains were fixed overnight in 4% (w/v) paraformaldehyde (PFA) at 4°C. For callosal labelling, single crystals of the lipophilic tracer DiI were injected into the cortex of whole brains using pulled glass capillaries. Dyes were allowed to diffuse at 37^{o}C for 10-12 days in 4% (w/v) PFA in phosphate buffered saline (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.

Explant culture. Organotypic slice cultures of rostral levels of the embryonic mouse telencephalon were prepared as previously described (Lopez-Bendito et al., 2006). Brain slices were cultured on polycarbonate culture membranes (8 μm pore size; Corning Costar) in organ tissue dishes containing 1 ml of medium (Neurobasal/B-27 [Gibco] supplemented with glutamine, glucose,

penicillin and streptomycin). Slices were cultured for 72hrs, fixed with 4% PFA and processed for anti-GFP immunofluorescence as described above.

Western blotting. Protein was extracted from dorsal telencephalon of E12.5 *Gli3* $^{flox/+}$ (control) and *Emx1Cre; Gli3* $^{flox/flox}$ 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 ECL Plus detection (Amersham GE healthcare).

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FIGURE LEGENDS

Figure 1: Absence of the corpus callosum (CC) in P0 *Pdn/Pdn* mutants. **(A-D)** Neurofilament (NF) staining reveals the CC in control animals. The anterior commissure (ac) and fornix (f) form at rostral levels (A,B) while the hippocampal commissure (HC) is detectable caudally (C, D). **(E,F)** *Pdn/Pdn* mutants lack the CC. Cortical axons migrate towards the midline, but do not project to the contralateral hemisphere (F, H) and form Probst bundles (arrows in H). AC and HC are missing (E-H). **(I-K)** DiI injections in the cortex (asterisks in I, J) confirms ACC in *Pdn/Pdn* brains. Callosal axons project towards the midline, but are unable to cross it instead projecting into dorso-medial regions of the cerebral cortex and forming Probst bundles (arrowheads in K).

Figure 2: Disorganization of midline structures in P0 *Pdn/Pdn* brains. **(A-D)** Neuropilin-1 (Npn-1) stains the pioneer axons of the cingulate cortex (CiC) in control and *Pdn/Pdn* mutants. *Pdn*/*Pdn* Npn-1+ axons fail to reach the contralateral hemisphere forming Probst bundles (C, D). **(A, B)** Calretinin (CR) labels neurons of the callosal sling. **(C, D)** In *Pdn/Pdn* brains, CR+ neurons are disorganized and clusters of CR+ neurons are associated with Probst bundles (arrowhead in D). **(E, F)** Calbindin (CB) labels callosal sling neurons located in the indusium griseum (IG). **(G, H)** CB+ neurons abnormally cluster with Tuj1+ callosal axons in the *Pdn/Pdn* cortex (arrowheads in H). **(I-J)** GFAP immunofluorescence labels the glial wedge (GW), the indusium griseum glia (IGG) and the midline zipper glia (MZG) in control brains. **(K, L)** In *Pdn/Pdn* brains, GFAP+ fascicles are ectopically formed within the CiC (arrowhead in L) and transect the path of callosal axons. The midline zipper glia expands into more ventral regions of the septum.

Figure 3: *Pdn/Pdn* callosal axons are able to cross the cortical midline in a wild-type environment. **(A)** Transplantation of E17.5 control GFP+ frontal cortex leads to migration of GFP+ callosal axons across the midline (n=8 out of 9). **(B)** *Pdn/Pdn*; GFP+ cortex transplantion into control cortex also shows crossing callosal axons (n=7 out of 8). **(C)** After transplantation of control; GFP+ cortical tissue into *Pdn/Pdn*; GFP- frontal cortex callosal axons do not project into the intermediate zone (n=0 out of 7), only few axons project along the surface of the mutant tissue (arrowhead) (n=4 out of 7).

Figure 4: CC formation is delayed in *Emx1Cre;Gli3* conditional mutants. **(A-C, F-H)** L1 staining shows callosal axons crossing the midline in P0 *Emx1Cre;Gli3*$^{fl/fl}$ brains. Some axons form Probst bundles (arrowheads in F, G and H). **(A, B, F, G)** Some Tbr1+ and CB+ callosal sling neurons are abnormally scattered in the *Emx1Cre;Gl* $^{fl/fl}$ cingulate cortex and associate with axon fascicles, others

are normally organized at the midline along the hypoplastic CC. **(C, H)** GFAP immunostaining labels the GW and the IGG in both control and *Emx1Cre; Gli*$^{fl/fl}$ brains but the IGG is expanded (F). (D,E,I,J) Cresylviolet and neurofilament staining reveal the CC in P7 control and Emx1Cre;Gli3 conditional mutants. Note the formation of Probst bundles in the mutant (E,J).

Figure 5: The *Pdn* midline is disorganized at early stages of callosal development. E16.5 coronal sections were stained with the indicated antibodies (A-P) and probes (Q-T). **(A-D)** Npn-1+ pioneer axons approach the midline in both wild-type and *Pdn* mutant embryos but *Pdn*/*Pdn* pioneer axons form abnormal structures in the cingulate cortex (arrowheads in D). **(A-L)** Tbr1+, CR+ and CB+ neurons fail to form the callosal sling at the mutant CSB. The *Pdn/Pdn* cortical plate is disrupted in several positions, where L1+ callosal axons stop their migration (arrowhead in H). **(M-P)** GLAST+ radial cells located at the CSB start to differentiate in GW and up-regulate GFAP. In *Pdn* mutants, GFAP+;GLAST+ cells are ectopically formed in the CiC (arrowheads in P). **(Q, S)** *Sema3c* is expressed by callosal sling and cingulate neurons in both control and mutant E16.5 brains with slightl reduced levels in the CiC of *Pdn* mutants. **(R, T)** In control embryos, *Slit2* is expressed in septum, the GW and in the cingulate cortex in a graded manner (arrowhead in R). *Pdn*/*Pdn* mutants show a severe up-regulation of *Slit2* expression in the cingulate cortex (arrowheads in T).

Figure 6: *Pdn* midline defects already occur at patterning stages of telencephalic development. **(A-D)** Up-regulation and dorsal expansion of *Slit2* expression in the *Pdn*/*Pdn* dorsomedial cortex and septum is observed at E14.5 and E12.5 (arrowheads in B and D). **(E, F)** The E14.5 dorsomedial cortex displays ectopic Blbp+ radial glia clusters (arrowheads in F). **(G, H)** *Fabp7* encoding the Blbp antigen is expressed at high levels in the dorsomedial cortex of control embryos with a sharp expression boundary at the CSB. The *Pdn* dorsomedial cortex lacks this *Fabp7* high level expression domain but shows clusters of cells expressing high levels of *Fabp7* (arrowheads in H).

Figure 7: Midline defects are partially rescued in *Pdn*/*Slit2* double mutants. **(A, F, K)** Immunostaining on control brain sections revealing L1+ callosal axons, CR+ and CB+ cortical sling neurons and GFAP+ midline glia cells. **(B, G, L)** The *Pdn* cingulate cortex displays the formation of large L1+ Probst bundles and disorganization of midline glia populations and callosal sling neurons. **(C, D, H, I, M, N)** In *Pdn*/*Pdn*;*Slit2*$^{+/-}$ and in *Pdn*/*Pdn*;*Slit2*$^{-/-}$ embryos, callosal axons reach the CSB without forming Probst bundles, but do not cross the midline . Also, organization and positioning of midline guidance cues is partially rescued in the *Pdn*/*Slit2* double mutants. **(C, D)** In *Pdn*/*Pdn*;*Slit2*$^{+/-}$ and in *Pdn*/*Pdn*;*Slit2*$^{-/-}$ embryos, CB+ neurons are normally located in the IG region similar to control embryos. **(H, I)** *Pdn*/*Pdn*;*Slit2*$^{+/-}$ and in *Pdn*/*Pdn*;*Slit2*$^{-/-}$ CR+ sling nerons neurons

are normally localized in the cingulate cortex. **(M, N)** In *Pdn*/*Slit2* double mutants, the IGG is absent and ectopic glial fascicles are formed at the CSB. **(E, J, O)** In *Slit2*$^{-/-}$ embryos, huge L1+ axon bundles are ectopically detected in the septum resulting in dense Probst bundles. CR+ neurons abnormally locate in the septum tightly associated with the Probst bundles (arrows in J).

Figure 8: Patterning defects in the E12.5 *Pdn* rostromedial telencephalon. **(A, B, F, G)** *Fgf8* and *Sprouty2* expression are normally confined to the commissural plate (cp) and septum respectively, but are shifted dorsally and expressed at higher levels at the *Pdn*/*Pdn* corticoseptal boundary (arrows). **(C, D, H, I)** *Wnt7b* and *Wnt8b* are expressed in the dorsomedial telencephalon with a sharp expression boundary at the CSB. In *Pdn* mutants, cortical *Wnt7b* and *Wnt8b* expression are strongly reduced (arrows) and *Wnt7b* expression is shifted ventrally into the septum. **(E, J)** *Axin2* expression is severely reduced in the *Pdn* dorsomedial cortex (arrow). **(K, O)** *Six3* is expressed in the septum of control brains, while in *Pdn* mutants its expression expands into dorsalmedial cortical areas (arrows). **(L, M, N, P, Q, R)** Nfia, *Nfi*b and *Nfix* are expressed at high levels in the dorsomedial cortex and at lower levels in the control septum. In *Pdn* mutants, the high expression level domains of these factors is lost, Nfia expression is up-regulated in the septum (arrow in P).

Figure 9: *Emx1Cre;Gli3* conditional mutants display subtle patterning defects in the E12.5 rostromedial telencephalon. **(A, B, F, G)** *Emx1Cre; Gli3*$^{fl/fl}$ embryos display no obvious changes in the expression pattern of *Fgf8* and *Sprouty2*. **(C-E, H-J)** The high level *Wnt7b* expression domain is shifted ventrally into the septum of *Emx1Cre;Gli3*$^{fl/fl}$ mutants (arrow in H) and *Wnt*8b and *Axin2* expression are slightly reduced in the *Emx1Cre;Gli3*$^{fl/fl}$ dorsomedial cortex. **(K, L, P, Q)** *Six3* and *Emx1* expression remain confined to the septum and cortex, respectively, of control and conditional mutant. **(M, R)** *Emx1Cre; Gli3*$^{fl/fl}$ displays high levels of Nfia expression in the dorsomedial cortex and in the dorsal septum. **(N, S)** In *Emx1Cre; Gli3*$^{fl/fl}$ mutants, *Fabp7* expression is reduced in the dorsomedial cortex (arrow in S). **(O T)** *Slit2* expression pattern is not severely affected in *Emx1Cre; Gli3*$^{fl/fl}$ mutants though mildly down-regulated.

Figure 10: *Sprouty1-2* double mutants lack the CC. **(A, B)** Satb2+ callosal neurons are normally positioned in the upper cortical layers II/III and IV above the Tbr1+ neurons in layer VI. **(C-J)** Neurofilament and Tuj1 staining reveal agenesis of the CC in *sprouty1-2* mutants. Callosal fibres fail to cross the midline and form Probst bundles. **(E-H)** No obvious differences in the distribution of CB+ and CR+ callosal sling neurons are detected in the dorsomedial cortex of *Sprouty1-2* double mutants. **(I, J)** GFAP immunofluorescence reveals abnormally formed midline glia populations.

Several GFAP+ glia fibres abnormally cluster at the CSB (arrowheads in J), while the IGG can not be identified.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: *Gli3* is inactived in the dorsal telencephalon of *Emx1Cre;Gli3* conditional mutants **(A-F)** *Gli3* in situ hybridization using a full length Gli3 hybridization probe on coronal sections of E10.5, E11.5 and E12.5 control and *Emx1Cre;Gli3*$^{fl/fl}$ embryos. **(A-C)** *Gli3* is expressed at high levels in the dorsal telencephalon and LGE and at lower levels in the MGE. **(D-F)** *Gli3* expression is gradually reduced from medial to lateral in the dorsal telencephalon of *Emx1Cre;Gli3*$^{fl/fl}$ embryos (arrowheads). By E12.5, *Gli3* mRNA is strongly reduced throughout the cortex while *Gli3* expression remains unaffected in the ventral telencephalon and the septum (F). **(G)** Gli3 Western blot analysis with E12.5 dorsal telencephalic extracts. In *Emx1Cre;Gli3*$^{fl/fl}$ mutant cortex, Gli3 activator (Gli3A) and repressor forms (Gli3R) are absent.

Supplementary Figure 2: Cortical lamination is not affected in *Gli3* cortical conditional mutants **(A-D)** *Emx1Cre;Gli3*$^{fl/fl}$ and control coronal brains sections immunostained with Satb2 and Tbr1. Satb2+ callosal neurons are normally positioned in the upper cortical layers II/III and IV above the Tbr1+ neurons in layer VI in both conditional mutants and control embryos.

Supplementary Figure 3: Defective Fgf signalling in the dorsomedial cortex of E16.5 *Pdn* mutants. **(A**) In control brains, *Fgf8* expression is confined to the GW and the IGG. **(C)** In *Pdn* brains, *Fgf8* expression, is restricted to the septum. **(B)** S*prouty2* is highly expressed in the GW and IGG of control brains and at lower levels in the cortical plate of the cingulate cortex. **(D)** In *Pdn* mutants, *Sprouty2* expression is only detected in the septum but absent from the cingulate cortex. Note the abnormal, thin strip of tissue which connects cortex and septum (asterisks in C and D).

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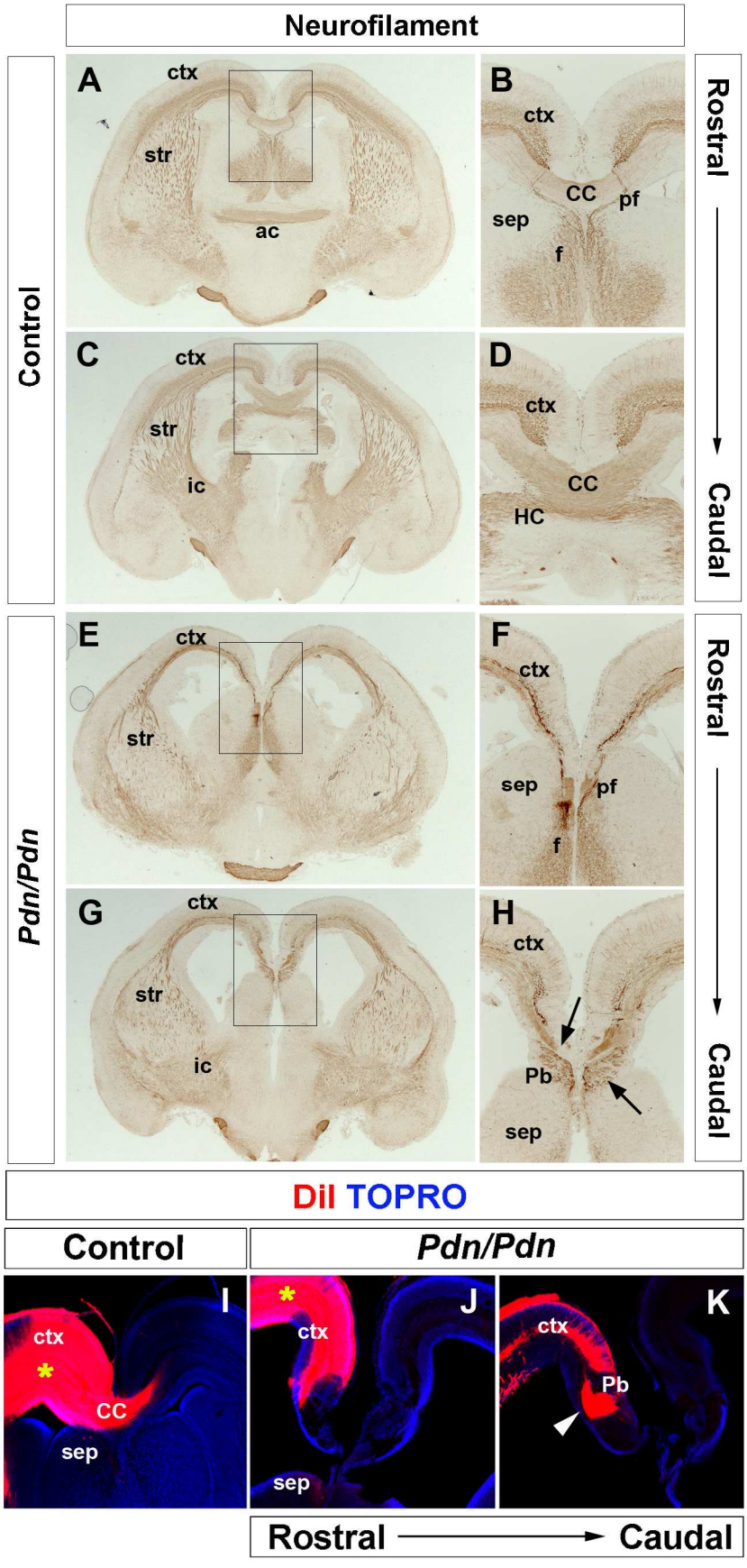
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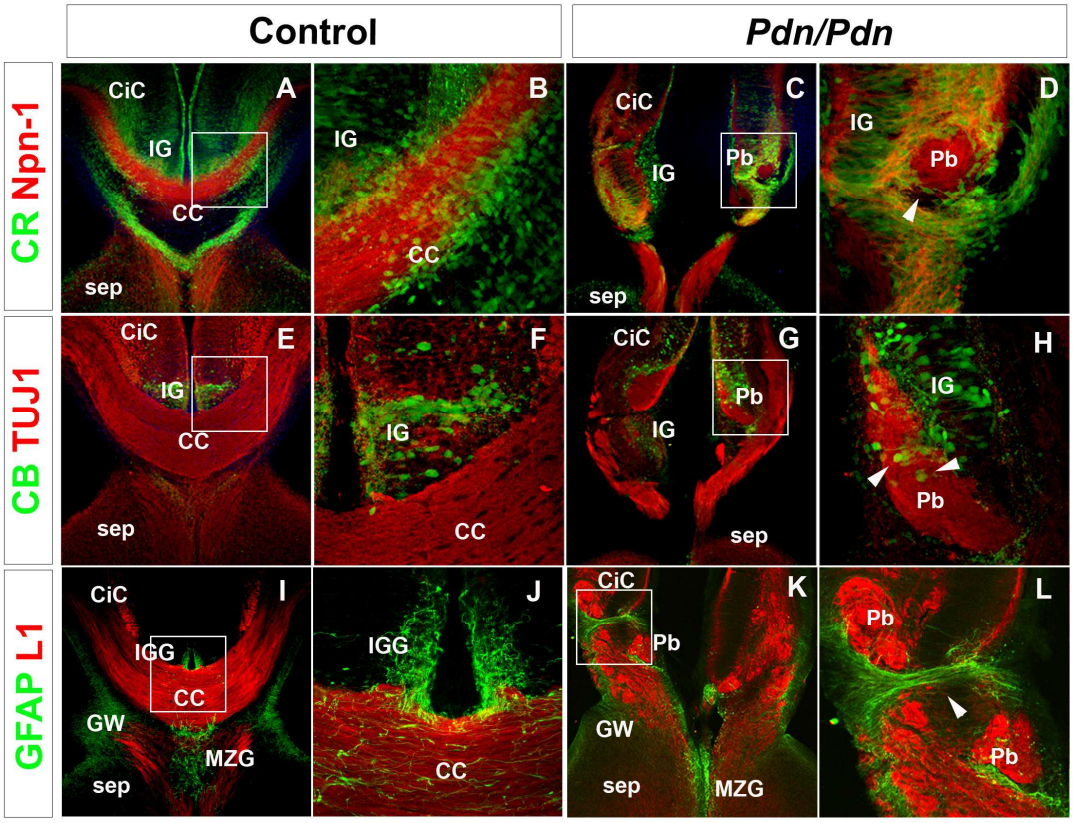
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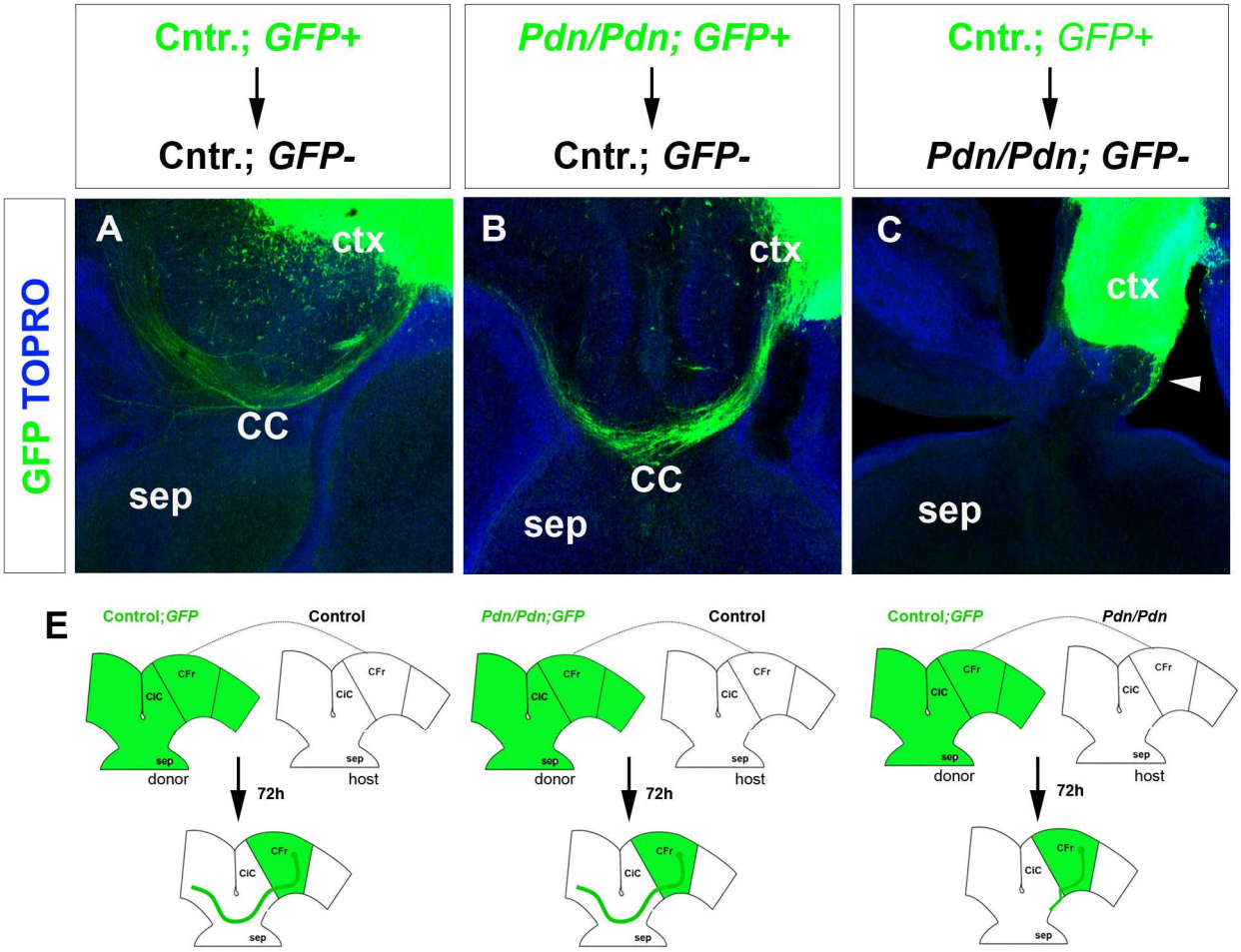

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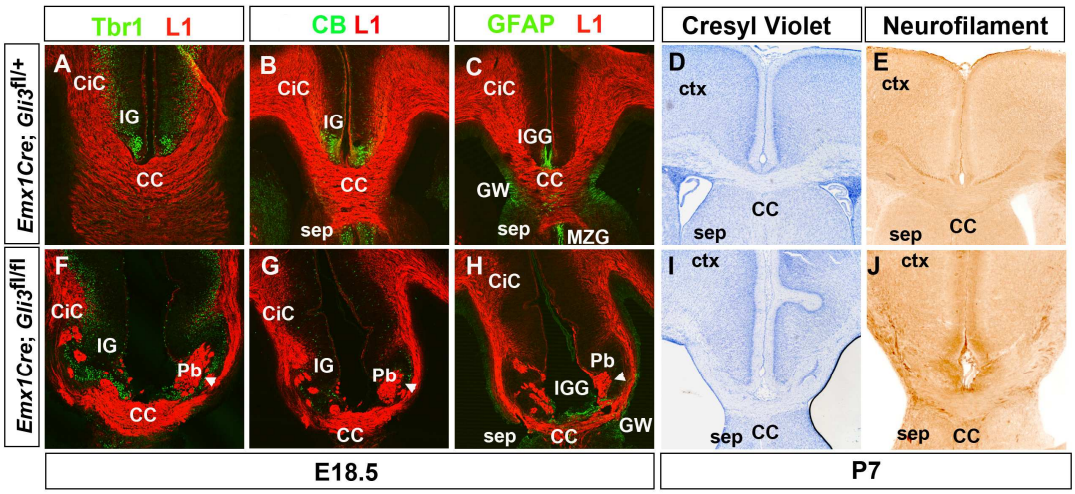

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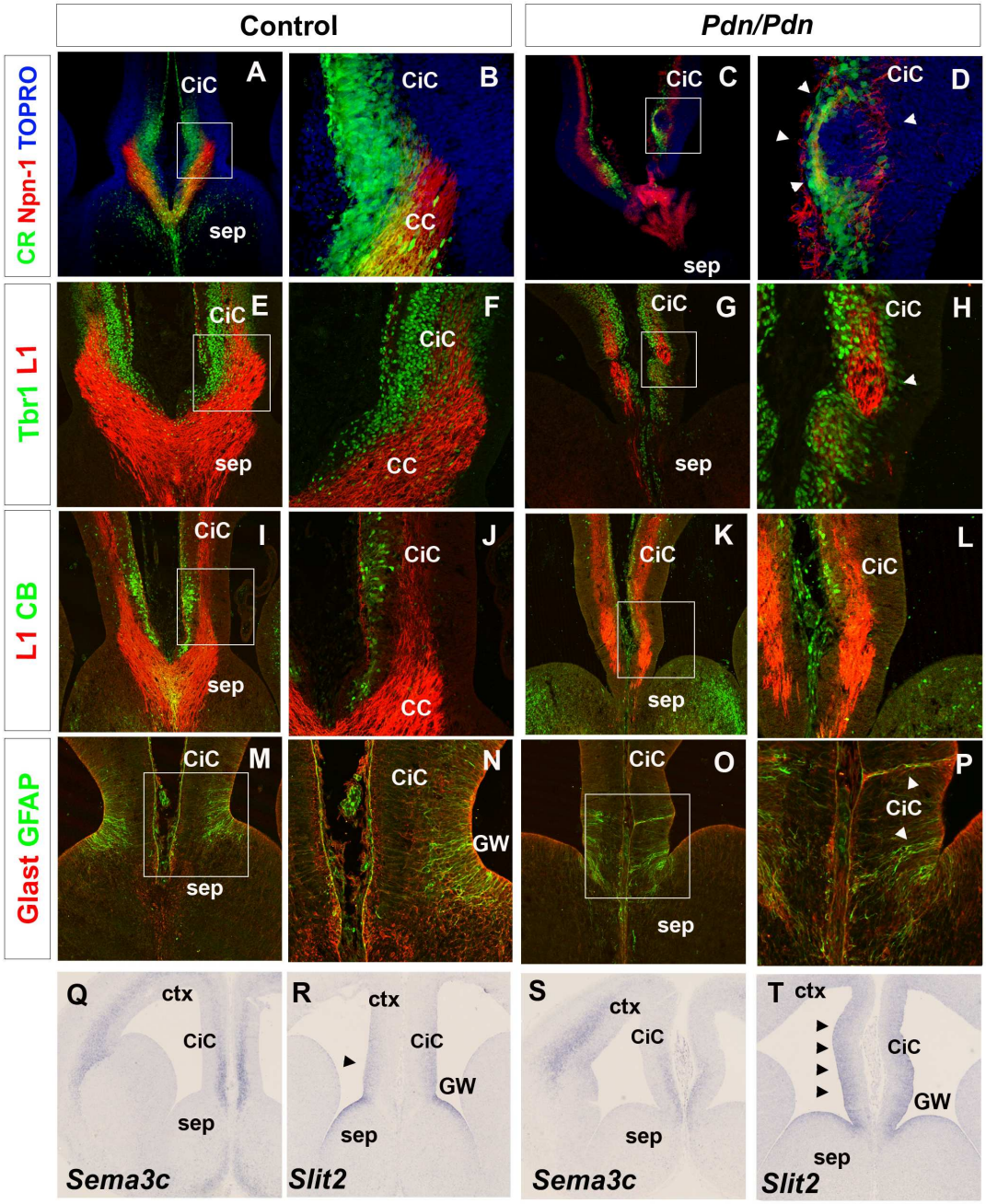
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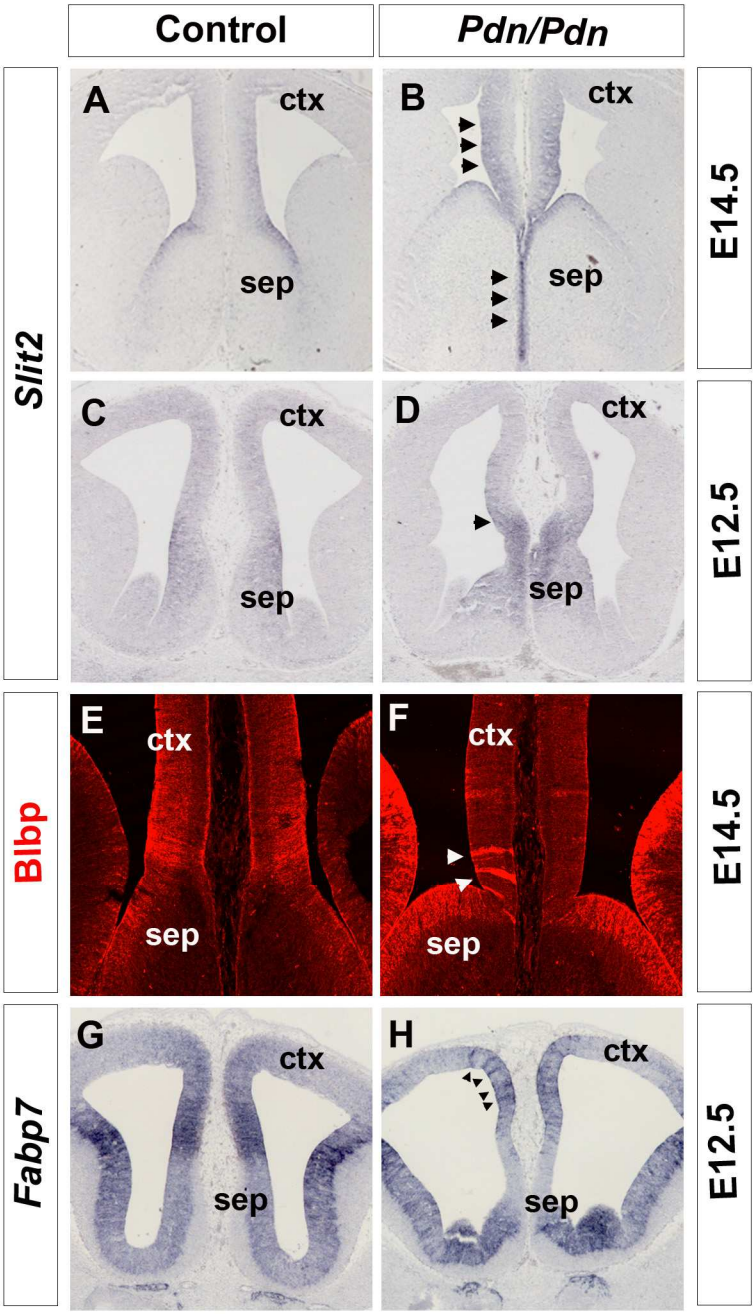
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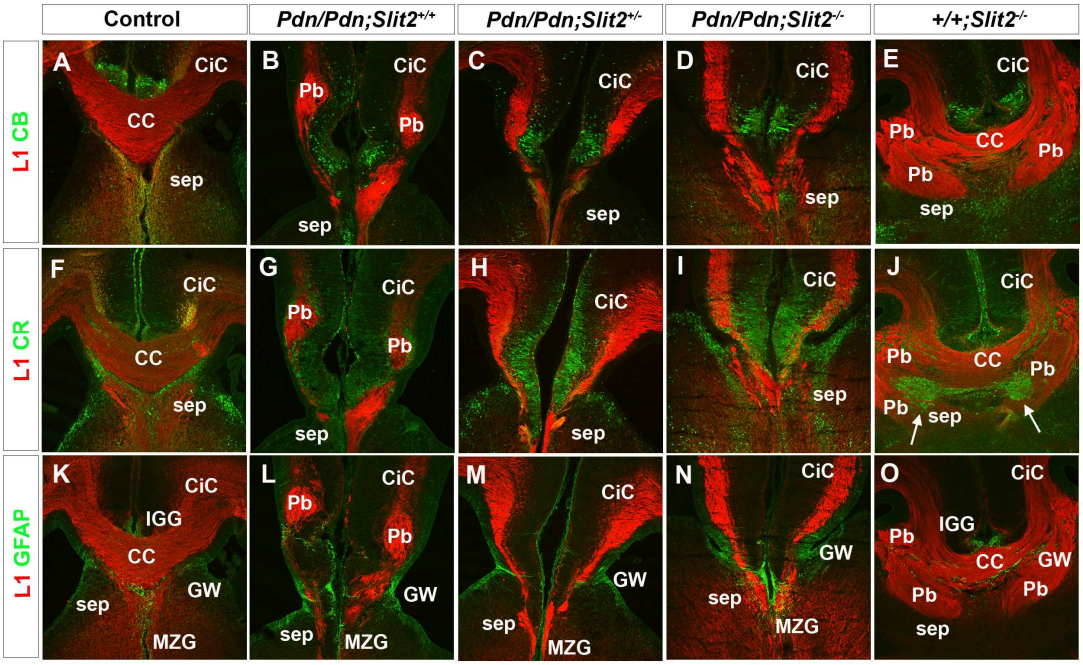
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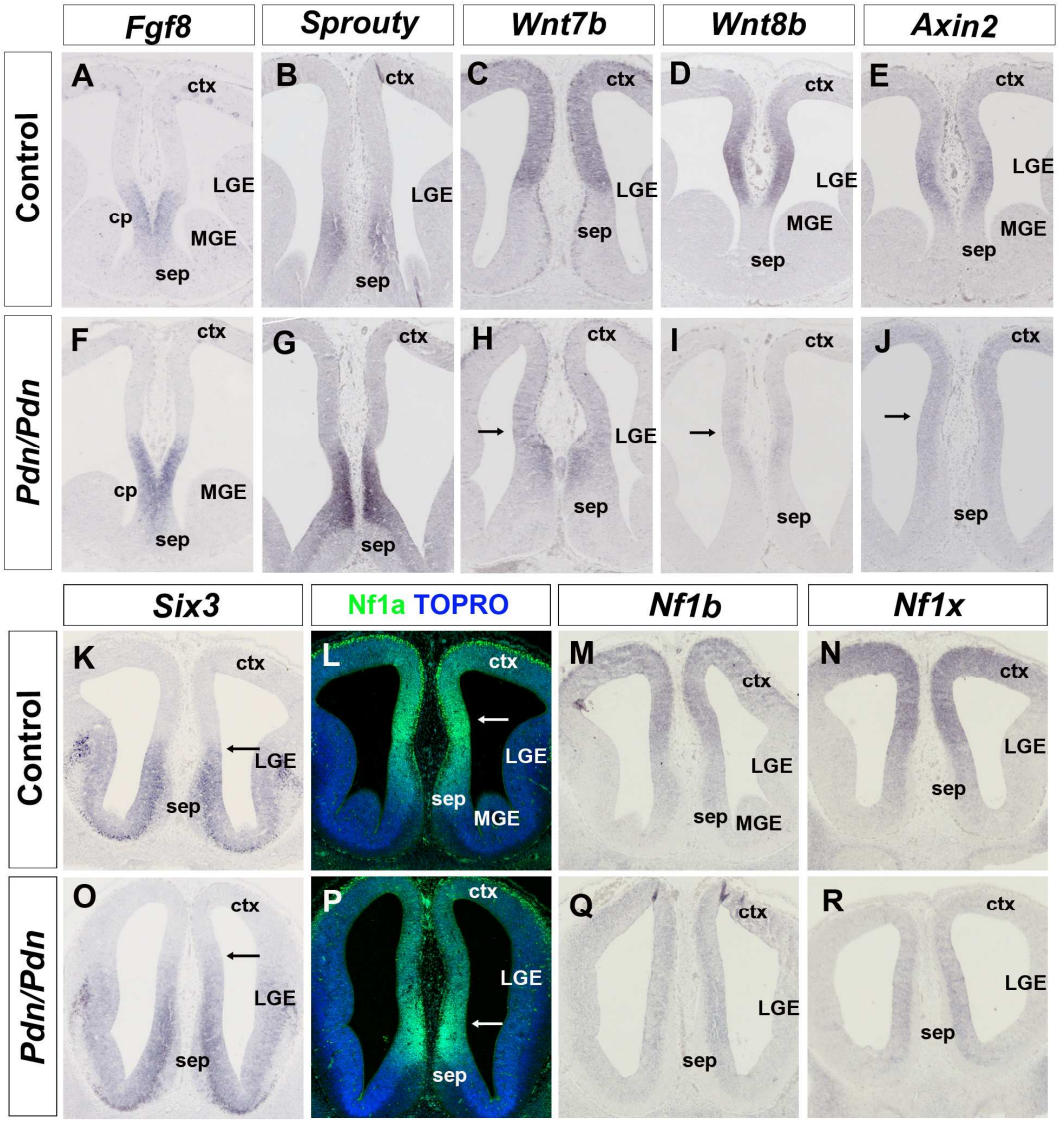
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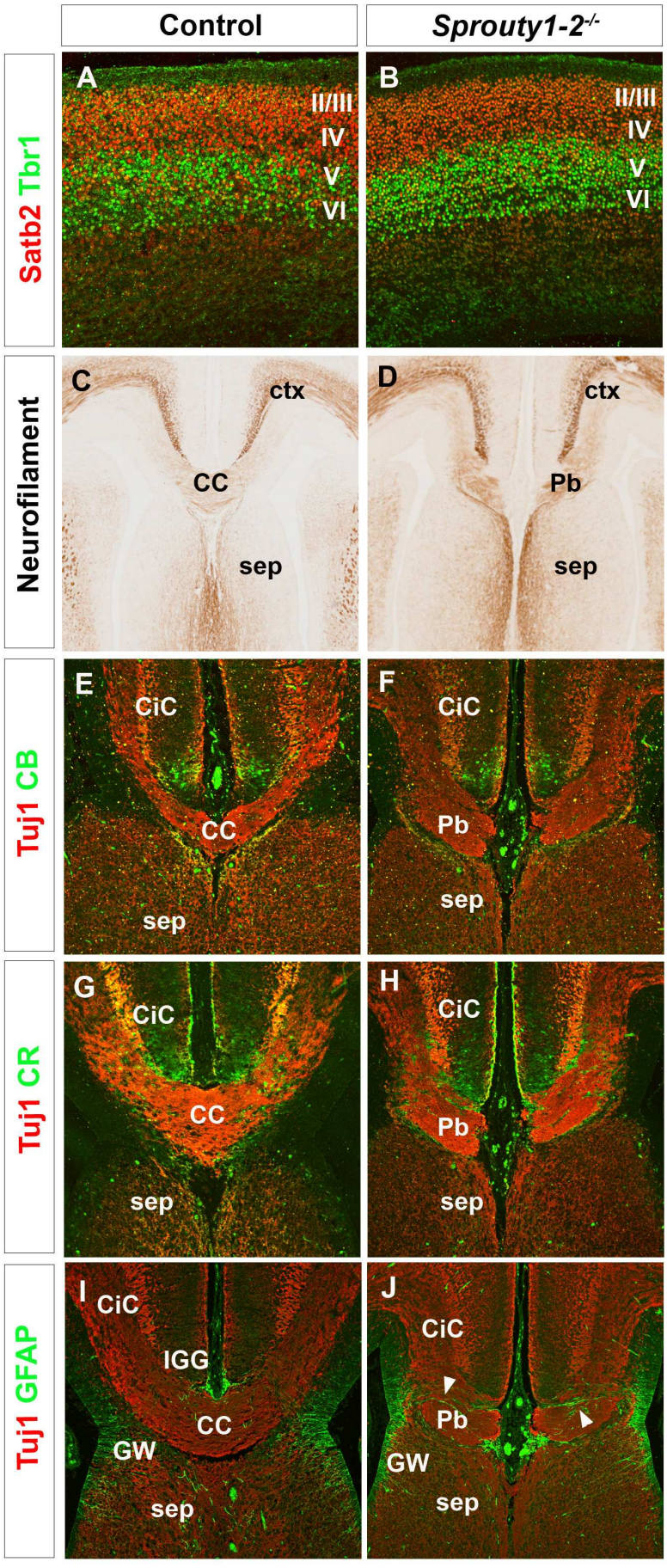
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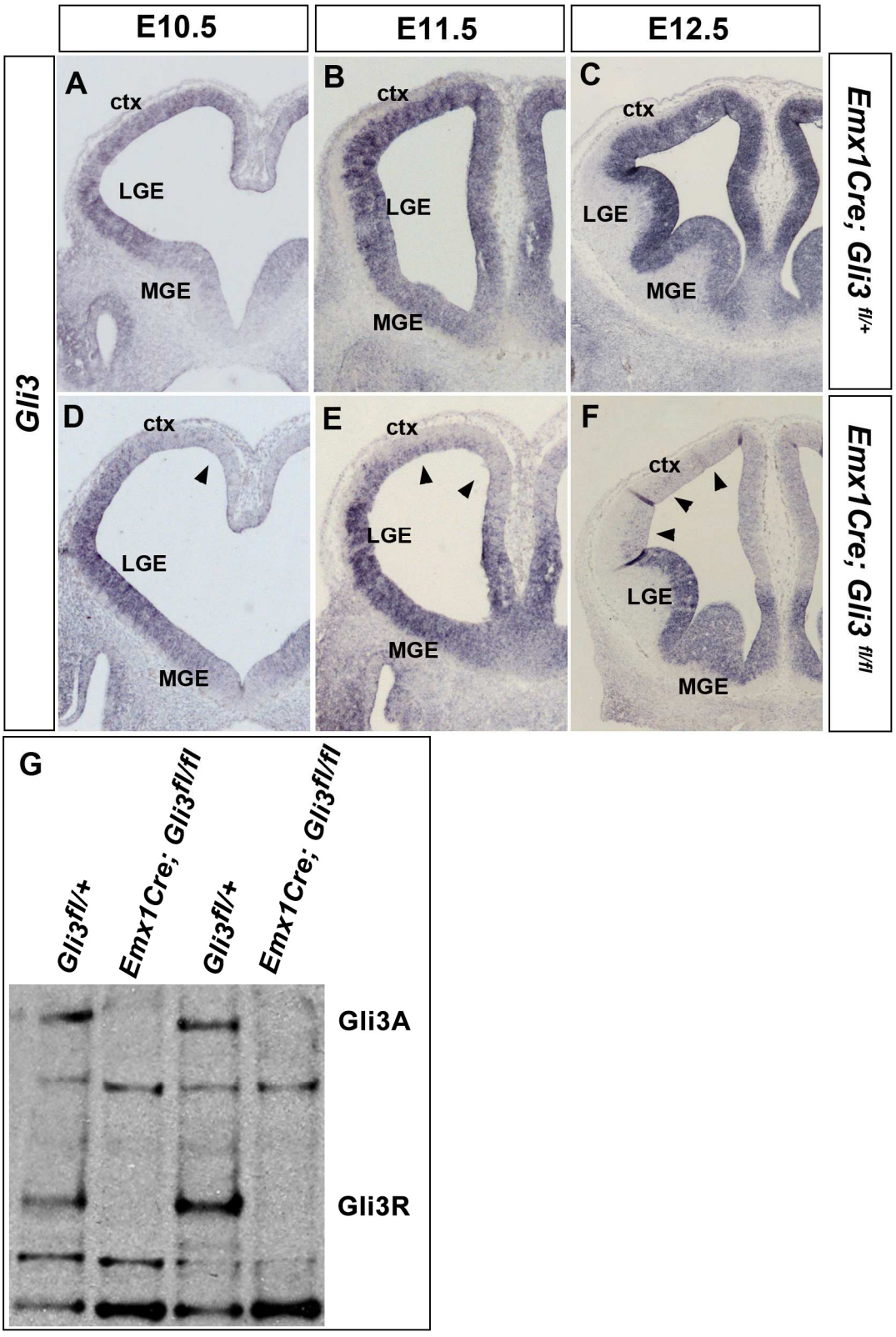
Supplementary Figure 1:



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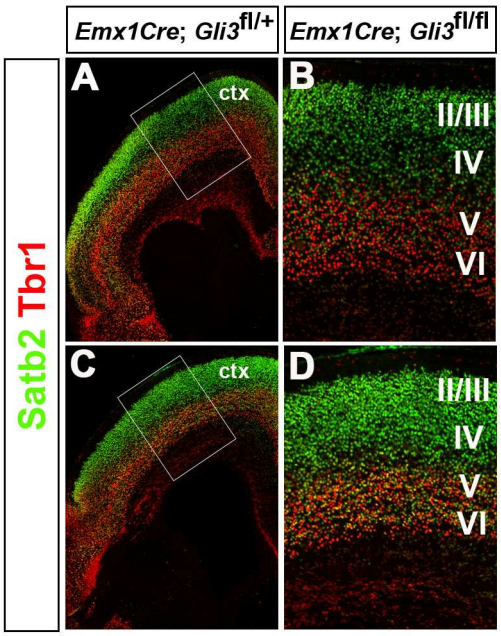

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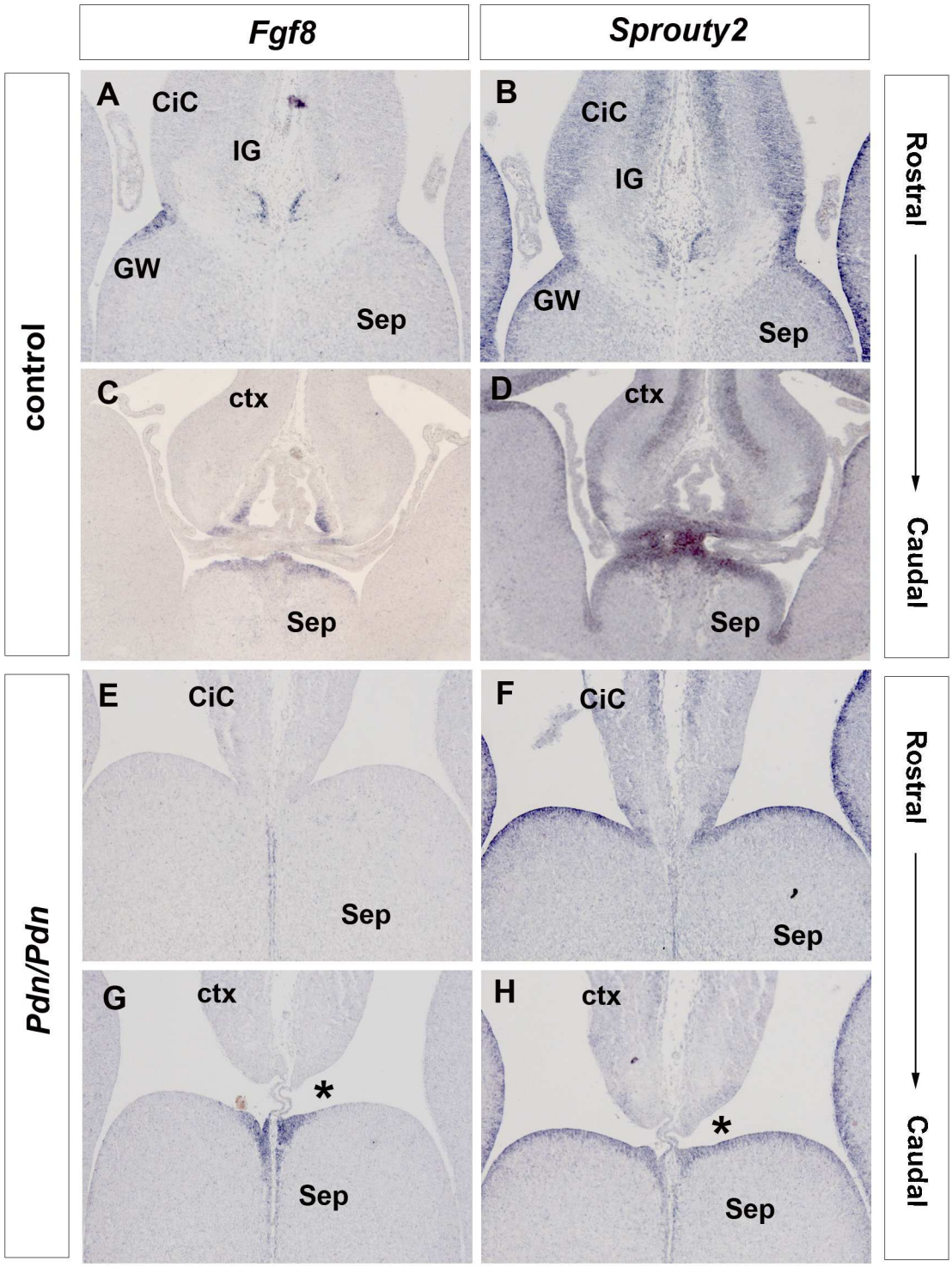

Supplementary Figure 4: