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Lamination of the cerebral cortex is disturbed in Gli3 mutant mice

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Abstract

The layered organization of the cerebral cortex develops in an inside-out pattern, a process which is controlled by the secreted protein reelin. Here we report on cortical lamination in the Gli3 hypomorphic mouse mutant Xt/Pdn which lacks the cortical hem, a major source of reelin+ Cajal Retzius cells in the cerebral cortex. Unlike other previously described mouse mutants with hem defects, cortical lamination is disturbed in Xt/Pdn animals. Surprisingly, these layering defects occur in the presence of reelin+ cells which are probably derived from an expanded Dbx1+ progenitor pool in the mutant. However, while these reelin+ neurons and also Calretinin+ cells are initially evenly distributed over the cortical surface they form clusters later during development suggesting a novel role for Gli3 in maintaining the proper arrangement of these cells in the marginal zone. Moreover, the radial glial network is disturbed in the regions of these clusters. In addition, the differentiation of subplate cells is affected which serve as a framework for developing a properly laminated cortex.

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Introduction

The cerebral cortex as the main centre for all higher cognitive functions develops a layered structure which is essential for its function. This lamination develops in an inside-out fashion and requires the secreted glycoprotein reelin (Tissir and Goffinet, 2003). Lack of functional reelin, as in the reeler mouse mutant (Curran and D’Arcangelo, 1998; D’Arcangelo et al., 1995; Ogawa et al., 1995) and in human congenital lissencephaly patients (Hong et al., 2000) or interference with reelin signalling (Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999) result in inversion of cortical layers and in abnormally dispersed cells.

In contrast to the well understood role of reelin signalling, relatively little is known about the identity of the reelin+ cell population directing cortical lamination. Reelin is expressed at high levels in CR cells, a major cell population in the MZ (D’Arcangelo et al., 1995; Meyer and Wahle, 1999; Ogawa et al., 1995) and in human congenital lissencephaly patients (Hong et al., 2000) or interference with reelin signalling (Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999) result in inversion of cortical layers and in abnormally dispersed cells.

In contrast to the well understood role of reelin signalling, relatively little is known about the identity of the reelin+ cell population directing cortical lamination. Reelin is expressed at high levels in CR cells, a major cell population in the MZ (D’Arcangelo et al., 1995; Meyer and Wahle, 1999; Ogawa et al., 1995). However, only indirect evidence supports a role for CR cells in controlling cortical layering (Ringstedt et al., 1998; Super et al., 2000). The understanding of such a role is further complicated by the existence of several reelin+ cell populations with different sites of origins, migration routes, destination and molecular profiles. Some CR cells are generated from Dbx1+ progenitor cells in the septum and in the ventral pallium at the pallial/subpallial boundary (PSB) which express reelin but not p73 and preferentially populate the rostroventral and ventral cortex (Bielke et al., 2005). Consistently, ablation of Dbx1+ progenitor cells predominantly leads to cytoarchitectural defects in the lateral cortex (Bielke et al., 2005). In addition, the caudomedial wall of the telencephalon, including the cortical hem, is a major source of a CR cell population which is characterized by the expression of reelin, p73, Calretinin and glutamate (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004). The role of these CR cells in cortical layering has recently been tested by genetic inactivation of p73 and by ablation of the hem (Meyer et al., 2004; Yoshida et al., 2006). Surprisingly, both mutants show normal cortical lamination except for the caudal cortex, suggesting that hem derived CR cells are not required to control cortical layering and that other sources of reelin, particularly cortical plate (CP) interneurons, are sufficient to allow lamination to proceed (Yoshida et al., 2006).

The Gli3 mouse mutant extra-toes (Xt) represents another mouse mutant with defective cortical hem development. This mutant shows severe defects in patterning the dorsal telencephalon (Fotaki et al., 2006; Grove et al., 1998; Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000a) and in preplate differentiation (Theil, 2005). In particular early cortical layering, i.e. the formation of the subplate (SP) and the marginal zone (MZ) is severely disrupted in these animals making it difficult to study cortical lamination. To circumvent these difficulties, we made use of the compound Gli3 hypomorphic mouse mutant Xt/Pdn which shows much milder regionalization defects. This mutant lacks the cortical hem and consequently contains few cortical reelin+/p73+ CR cells but has
numerous reelin+/p73− CR cells probably arising from an expanded Dbx1+ progenitor pool. Despite the presence of these reelin+ cells, however, cortical lamination is disturbed in the mutant. These reelin+ cells form dense cell clusters and the radial glial scaffold is severely disturbed at the sites of these clusters. Our analysis therefore suggests a role for Gli3 in regulating cortical lamination by maintaining an even distribution of CR neurons over the cortical surface.

**Materials and methods**

**Mice**

\(Xt^J\) and \(Pdn\) heterozygous animals were kept on a mixed C57Bl6/J and C3H/He background, respectively, and were interbred. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. \(Xt^J/Pdn\) embryos were readily distinguished from heterozygous and wild-type embryos by forebrain and/or limb morphology (Kuschel et al., 2003). For each marker and each stage, 3–5 different, non-exencephalic embryos were analysed at rostral, medial and caudal levels of the developing cortex.

**Explant culture of telencephalic tissue**

The dorsal telencephalon or the neocortex of E10.5 wildtype and Gli3 mutant embryos was dissected in HBSS and the surface ectoderm removed manually. Explants were cultured on Millicell-CM culture plate inserts (Millipore, #P1C03K50) in organ culture dishes. Culture medium was Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 20% fetal calf serum, 1× non-essential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO) and 1× streptomycin/penicillin (GIBCO). Tissue pieces were maintained under in vitro conditions for 48 h and then processed for in situ hybridization.

**In situ hybridization and immunohistochemistry**

Antisense RNA probes for Bmp4 (Jones et al., 1991), Conductin (Lustig et al., 2002), Cux2 (Zimmer et al., 2004), Dbx1 (Yun et al., 2001), Dlx2 (Bulfone et al., 1993), ER81

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**Fig. 1.** Dorsal midline defects in \(Xt^J/Pdn\) embryos. In situ hybridization analysis on coronal sections through the brains of wild-type (A–D, I–L), and \(Xt^J/Pdn\) (E–H, M–P) embryos. (A–C, E–G) Cortical hem expression of Wnt2b, Wnt3a is absent in \(Xt^J/Pdn\) embryos while Wnt8b expression remains in the highly abnormal dorsomedial region. (D, H) \(Xt^J/Pdn\) mutants lack Conductin expression in the dorsomedial telencephalon. (I, J, M, N) Bmp4 expression and signalling as indicated by Msx1 expression is reduced in the dorsal telencephalon. (K, L, O, P) Delay of choroid plexus development in \(Xt^J/Pdn\) embryos. While only few cells express Ttr at reduced levels at E12.5, Ttr expression has recovered at E14.5. Note the disorganized structure of the choroid plexus. dm: dorsomedial telencephalon, Th: thalamus.
Results

Regionalization defects in the telencephalon of \textit{Xt}/\textit{Pdn} embryos

Previously, we reported that the early cortical layering, i.e. the formation of the preplate and its derivatives, the MZ and the SP, is severely affected in extra-toes (\textit{Xt}) embryos (\textit{Theil}, 2005) in which a deletion removes all \textit{Gli3} sequences 3’ of the second zinc finger (\textit{Büschler et al.}, 1998). Due to the severity of the phenotype, however, it is difficult to analyze the proper lamination process in these animals. We therefore focussed on the \textit{Gli3} hypomorphic mouse \textit{Xt}/\textit{Pdn} (\textit{Kuschel et al.}, 2003; \textit{Schimmang et al.}, 1994) in which the \textit{Gli3} transcript levels are reduced due to the integration of a retrotransposon (\textit{Thien and Rüther}, 1999). Before analyzing cortical lamination we started to de

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Dorsal telencephalon and diencephalon are morphologically separated in \textit{Xt}/\textit{Pdn} embryos. Immunohistochemical and in situ hybridization analysis on E12.5 wildtype (A–E) and \textit{Xt}/\textit{Pdn} (F–J) using the indicated markers. (A, F) Foxg1 is expressed in the telencephalon except for the cortical hem (h) in wildtype and the highly abnormal dorsomedial region of the \textit{Xt}/\textit{Pdn} mutant embryo. (B, G) Calbindin stains interneurons originating in the LGE and migrating into the neocortex in both genotypes. \textit{Ngn2} (C, H) and \textit{Dlx2} (D, I) staining reveals the dorso/ventral subdivisions of the telencephalon and diencephalon. (E, J) \textit{Shh} expression marks the zona limitans intrathalamicz (zli).}
\end{figure}
by all telencephalic cells except for the cortical hem and for CR neurons (Hanashima et al., 2002; Tao and Lai, 1992). This expression pattern is maintained in the mutant where only the dorsomedial region is negative for Foxg1 (Figs. 2A, F). In contrast to Xt/J embryos, we did not notice the presence of Foxg1− cells within the Xt/Pdn neocortex. Similarly, Calbindin immunostaining did not reveal cell clusters in the neocortex (Figs. 2B, G) as described for Xt/J embryos (Fotaki et al., 2006). Finally, in situ hybridization for Ngn2, Dlx2 and Shh indicated the presence of the major subdivisions of the Xt/Pdn telencephalon and diencephalon (Figs. 2C–E, H–J). These data suggest that the development of the dorsomedial telencephalon is severely affected in both, Xt/J and Xt/J/Pdn embryos, but that the latter show a morphological and molecular separation of telencephalon and diencephalon.

The Xt/Pdn cortex shows a severe reduction in hem derived CR cells

Our previous analysis showed that preplate development and differentiation are severely affected in Xt/J embryos (Theil, 2005). We therefore analyzed this process in Xt/J/Pdn embryos. The transcription factor Tbr1 is expressed in the preplate and is essential for its differentiation (Hevner et al., 2001). Immunohistochemical analysis revealed a continuous band of Tbr1+ cells at the outer part of the developing cortex in E12.5 and E14.5 wild-type and in Xt/J/Pdn embryos although the mutant preplate appeared undulated dorsomedially (Figs. 3A, F, K, P). Similarly, the expression of MAP2 which labels preplate neurons and the SP/MZ at E12.5 and E14.5, was unaffected in the E12.5 Xt/J/Pdn cortex but the mutant SP and MZ appeared more diffuse at E14.5. In addition, several MAP2+ cell clusters were detected in the medial neocortex (Figs. 3B, G, L, Q).

We next used in situ hybridization analysis to gain insights into the cellular composition of the preplate and its derivatives. Hem derived CR cells express reelin, p73 and Calretinin (Alcantara et al., 1998; del Rio et al., 1995; Hevner et al., 2003b; Meyer et al., 2004, 1999, 2002; Ogawa et al., 1995; Soda et al., 2003). This analysis showed a single layer of reelin expressing cells at the entire wild-type E12.5 and E14.5 cortical surface (Figs. 3C, M). While in E12.5 Xt/J/Pdn embryos the layer of reelin expressing cells appeared relatively normal (Fig. 3H), reelin expressing cells formed small aggregates in the dorsomedial E14.5 mutant neocortex (Fig. 3R) which are reminiscent of the large clusters of reelin expressing cells in the Xt/J cortex (Theil, 2005). In addition, reelin expression was detected at low levels in two rows of cells within the wild-type E14.5 neocortex which correspond to migrating interneurons and which have recently been suggested to play an important role in cortical lamination (Alcantara et al., 2006; Yoshida et al., 2006). Distinct rows of reelin expressing neurons were only detected in the mutant lateral neocortex while more medial regions showed an...
uneven distribution of reelin in the CP. In contrast to the less severely affected reelin expression, p73 expression was drastically altered in XttJ/Pdn embryos. Like reelin+ cells, p73 expressing cells covered the cortical surface of wild-type embryos (Figs. 3D, N) whereas 12.5 XttJ/Pdn embryos had only a few p73 expressing cells in the neocortical MZ and a cluster of cells in the dorsomedial telencephalon. A similar distribution of p73 expressing cells was observed in E14.5 XttJ/Pdn embryos (Fig. 3S). Taken together with the altered reelin expression this finding suggests a severe reduction in the hem derived reelin+p73+ CR subpopulation and a concomitant increase in a reelin−/p73− CR cell population similar to our previous observations in Xtt/XT embryos (Theil, 2005). Finally, immunohistochemical analysis of Calretinin expression, a marker for CR and pioneer neurons, showed fewer abnormalities in E12.5 XttJ/Pdn embryos. As in wild-type embryos, Calretinin+ neurons formed a single layer except for the dorsomedial telencephalon where several layers were detected (Figs. 3E, J). In contrast, at E14.5, Calretinin+ neurons formed dense clusters dorsomedially but were absent in the lateral MZ similar to p73 expression (Figs. 3O, T). This analysis suggests that the formation of the preplate is not affected in XttJ/Pdn embryos, but that the MZ shows a marked reduction of hem derived reelin+/p73+ Calretinin+ CR neurons and a clustering of reelin− and Calretinin+ cells.

Interestingly, similar alterations in the cellular composition of the MZ were observed in newborn mutants. In wild-type newborn animals, strong reelin expression was detected in Cajal Retzius cells within the MZ (Fig. 4A). An additional weak expression was detected in the lower cortical plate at the level of layer IV/V (Yoshida et al., 2006). In XttJ/Pdn animals we could identify fewer reelin expressing cells in the MZ (Fig. 4B). Also, a band of reelin expressing cells was observed in the lateral CP but not in the medial neocortex where these cells showed a strong dispersal (Fig. 4B). In contrast to the numerous reelin expressing cells in the MZ, few p73+ cells were found in the XttJ/Pdn neocortical MZ while groups of p73 expressing cells were found in the highly dysmorphic dorsomedial cortex (Figs. 4C, D). Finally, Calretinin+ cells were detected in the wildtype MZ and SP while Calretinin expression was absent from the mutant MZ and SP (Figs. 4E, F). The ectopic Calretinin expression in the lateral cortical MZ corresponds to an ectopic nerve bundle (T.T., unpublished data). Taken together these data indicate that hem derived CR cells (p73−; reelin+; Calretinin+) are nearly absent while reelin+/p73− cells are found in the medial and lateral cortex.

Neocortical lamination in XttJ/Pdn embryos

Given these alterations in the MZ and its importance for the neocortical layers in XttJ/Pdn animals. As these pups die shortly after birth this analysis was confined to the P0 stage. The transcription factors Cux2, RORβ, and ER81 distinguish emerging layers II/III, IV and V, respectively (Figs. 5A, C, E). In the XttJ/Pdn mutants, we could identify regions in the medial (Cux2) or lateral neocortex (RORβ) and ER81 which show a layered expression of these markers, though cells expressing these genes are more dispersed within these domains. In addition, we also identified regions where the cortical layering is severely disturbed. In these areas, the Cux2, RORβ and ER81 expression domains are undulated and discontinuous. Cells expressing these genes were even found close to the ventricular surface (Figs. 5B, D, F). Finally, the Tbr1 transcription factor shows high level expression in the SP, layer VI and CR neurons and weaker expression levels in layer II/III neurons.

Fig. 4. Development of the MZ and subplate is affected in XttJ/Pdn embryos. Coronal sections through the brains of newborn wild-type (A, C, E) and XttJ/Pdn mutant animals (B, D, F) (A′, B′, C′, D′, E′ and F′) Higher magnifications of the boxed areas in A, B, C, D, E and F. (A, B) Fewer reelin expressing cells in the MZ are found in the XttJ/Pdn brains. Reelin+ cells are not so densely packed as in wild-type embryos. Also note the more dispersed distribution of weakly reelin expressing cells in layer IV/V (arrowheads). (C, D) p73 expression is strongly reduced in the mutant MZ except for the dorsomedial most area (arrowheads). (E, F) The XttJ/Pdn neocortex lacks Calretinin staining in the MZ. The arrow in F marks an ectopic cluster of Calretinin positive fibers.
Fig. 5. Cortical lamination defects in Xtl/Pdn embryos. In situ hybridization and immunofluorescence analysis on coronal sections of newborn wild-type (A, C, E, G) and Xtl/Pdn animals (B, D, F, H). (A, C, E) Cux2, RORβ, and ER81 expression mark cortical laminae II/III, IV, and V of wild-type embryos, respectively. (B, D, F) In Xtl/Pdn embryos, the expression of these markers is more diffuse and even occurs close to the ventricular surface (see insets B’, D’ and F`). (G, H) Tbr1 is strongly expressed in layer VI, SP and CR neurons and at weaker levels in layer II/III of wildtype brains. In the mutant, Tbr1* cells are positioned close to the ventricular surface (arrowheads). Ectopic Tbr1 staining was also found in the MZ (arrow).

Fig. 6. BrdU birthdating analysis of cortical neurons. (A–F) Coronal sections at P0 stained with an anti-BrdU antibody after BrdU administration at E11.5 (A, D), E13.5 (B, E) and E15.5 (C, F). (A, D) In wild-type embryos, administration of BrdU at E11.5 labels MZ and SP neurons (arrowheads), whereas only few or no neurons are BrdU labelled in the MZ (arrowheads) and SP of Xtl/Pdn animals, respectively. (B, C, E, F) BrdU* neurons are found in the lower and upper cortex of wild-type animals after labelling at E13.5 (B) and E15.5 (C), respectively. (E, F) In Xtl/Pdn newborns, BrdU labelled neurons occupy all cortical layers.
(Fig. 5G). Similar to the other markers, Tbr1+ cells were also found in an abnormal position close to the ventricular surface (Fig. 5H). In addition, we did not observe Tbr1 staining in the MZ characteristic of CR neurons. These results suggest that the layered neocortical organization is disturbed in the mutant.

To further analyze cortical laminar we performed a birthdating analysis of cortical neurons. To this end, we injected pregnant mice with BrdU at E11.5, E13.5 and E15.5 and examined the distribution of labeled neurons at P0 to determine the migration of cortical neurons. After labeling preplate neurons at E11.5, we detected BrdU+ cells in the MZ and the SP of wild-type pups (Fig. 6A). In contrast, only few neurons were labeled in the MZ of Xt/J/Pdn newborns consistent with the lack of Calretinin+ neurons and the reduced numbers of p73 and reelin expressing cells. Also, no labeling was observed in the SP (Fig. 6D). Furthermore, wild-type neurons labeled by injection at E13.5 and E15.5 predominately migrated to layers IV/V and II/III of the P0 cortex, respectively (Figs. 6B, C). Similar to our findings on lamina specific gene expression, however, BrdU+ neurons showed a more dispersed distribution and in some regions did not migrate to their prospective layers but settled throughout the entire CP in the newborn Xt/J/Pdn neocortex (Figs. 6E, F). Thus, the BrdU birthdating analysis further confirms the lamination defects in the Gli3 mutant cortex.

Clusters of reelin+ neurons disrupt CP organization

Next, we started to analyze causes for the cortical lamination defects in Xt/J/Pdn embryos. As reelin is required for neocortical organization (D’Arcangelo et al., 1995; Ogawa et al., 1995; Rice and Curran, 2001), the altered distribution of reelin+ cells and in Fig. 7. Ectopic reelin+ clusters disrupt the organization of the CP. Coronal sections through the brains of E14.5 wild-type (A–D, I–K) and Xt/J/Pdn (E–H, L–N) embryos. (A, E) The Sox5 expression domain exhibits gaps in the mutant CP (arrows). Also note the reduced expression levels in the medial neocortex and the complete absence of Sox5 expression in the lateral neocortex. (B, F) In situ hybridization (Sox5) combined with immunohistochemical analysis (reelin) shows that clusters of reelin+ neurons are located within the Sox5 negative areas. (C, G) Immunostaining for RC2 reveals a shortening of radial glial fibers at the sites of reelin+ clusters. Between the two reelin+ clusters, radial glial cells extend their processes to the pial surface. (D, H) CS56 staining reveals the MZ and SP. The arrow in (H) marks the SP underneath a cluster, arrowheads CS56+ cells in the CP. (I, J, K) Dlx2 and Calbindin expression mark migrating interneurons in the wildtype neocortex. (L, M, N) The interneuron migratory routes appear more diffuse in Xt/J/Pdn embryos. Note the absence of Dlx2 or Calretinin clusters.
particular the clustering of these cells in the $Xt^f/Pdn$ mutant may interfere with proper cortical layering. To examine this possibility we first analyzed the generation of the CP in the developing mutant cortex. Sox5 marks CP neurons in the E14.5 medial and lateral neocortex of wild-type embryos (Fig. 7A). While Sox5 expression is nearly completely abolished in the lateral neocortex of $Xt^f/Pdn$ embryos the Sox5 expression domain shows several gaps in more medial cortical areas (Fig. 7E) which correspond to the ectopic reelin clusters in the mutant CP (Fig. 7F). Moreover, even single ectopic reelin+ cells in the CP are surrounded by a ring of Sox5 expressing cells (data not shown). This complementary expression patterns suggests an exclusion of Sox5 expressing CP neurons from the reelin+ territories.

To further analyze whether the reelin+ cell aggregates might interfere with the cortical lamination process we examined the formation of the radial glial scaffold which is essential for guiding migrating cortical neurons (Rakic, 2003). In wild-type embryos and in most parts of the mutant cortex, radial glial cells extend fibers from the ventricular to the pial cortical surface (Figs. 7C, G). However, in regions immediately underlying the reelin+ cell clusters, the radial glial scaffold appears to be disrupted in the mutant (Fig. 7G). In these areas, radial glial fibers are severely shortened and do not reach the pial surface but end within the CP suggesting that the exclusion of Sox5 expressing CP neurons from the reelin clusters is caused by this shortening of the radial glial fibers.

As the reelin+ aggregates appear to be smaller than the actual gaps in the CP (Fig. 7F) we investigated the possibility that SP cells might be part of these clusters. The SP as well as the MZ is labelled by MAP2 and CS56. Interestingly, MAP2+ cells form clusters in the E14.5 $Xt^f/Pdn$ neocortex (Fig. 3Q). In contrast, immunofluorescence analysis with CS56 revealed two separate rows of cells corresponding to the MZ and the SP (Figs. 7D, H). Interestingly, the SP cells surround the lower end of a bulge located in the medial neocortex. However, the CS56 staining appeared more diffuse and we occasionally observed groups of CS56+ cells in the CP.

A clustering of reelin+ cells has been observed in transgenic mice overexpressing BDNF under the control of the nestin enhancer (Ringstedt et al., 1998). These reelin+ aggregates form as a consequence of a segregation from clusters of GABAergic interneurons (Alcantara et al., 2006). This analysis prompted us to investigate the distribution of interneurons which are derived from the ventral telencephalon, enter the cortex by tangential migration and are marked by Dbx2, Gad67 and Calbindin expression (Anderson et al., 2001; Ang et al., 2003; Nery et al., 2002). In situ hybridization for Dbx2 and Gad67 revealed migrating interneurons on their migratory routes in the MZ and in the intermediate zone (IZ) of the E14.5 wild-type neocortex (Fig. 7I and data not shown). In the $Xt^f/Pdn$ neocortex, these interneurons are more diffusely distributed, but do not cluster in the MZ (Fig. 7L). Immunofluorescence analysis for Calbindin showed a similar pattern (Figs. 7J, K, M, N) suggesting that the reelin+ aggregates form independently of potential defects in interneuron development.

As the radial glia scaffold is disrupted at the sites of reelin+ clusters we finally investigated whether this disruption might cause the lamination defects in $Xt^f/Pdn$ embryos. To this end, we performed double immunofluorescence staining for Nestin which marks the radial glia scaffold and for Tbr1 to reveal lamina organization in newborn animals. In wild-type P0 pups, the layer specific distribution of Tbr1+ neurons coincides with radial glial extensions from the ventricular to the pial surface (Figs. 8A, C). In the Gli3 mutant P0

![Fig. 8. A disorganized radial glial network correlates with lamination defects in the Gli3 mutant neocortex. Immunofluorescence analysis on coronal sections through the brains of wildtype (A–C) and $Xt^f/Pdn$ (D–I) P0 animals with the indicated antibodies. (A–C) Radial glia scaffold and distribution of Tbr1+ neurons in layer VI (high level expression) and in layer II/III (low level expression). (D–F) The layer specific distribution of Tbr1+ neurons in layer VI neurons is severely disturbed in regions with a disorganized radial glial network. (G–I) In regions where radial glia extensions reach the pial surface positioning of Tbr1+ neurons is slightly more diffuse than in wild-type.](image-url)
neocortex, however, regions where the laminar organization of Tbr1+ layer VI neurons is severely disturbed correspond to areas with a dramatic disorganization of the glial scaffold (Figs. 8D–F). In contrast, in regions where the radial glia reach the pial surface Tbr1+ layer VI neurons show a layered though more diffuse organization than in the wild-type cortex (Figs. 8G–I). This analysis therefore suggests that the disruption of the radial glial scaffold which is present already early in development causes at least some of the lamination defects in the Gli3 mutant.

Expansion of Dbx1 expression in Gli3 mutants

Despite the absence of the cortical hem reelin expressing cells are present in the Gli3 mutant cortex (Fig. 3R and Theil, 2005). To address the potential origin of these cells, we analyzed Dbx1 expression in Gli3 mutants. Recently, reelin+ cells have been reported to originate from the potential origin of these cells, we analyzed expression of mutation. However, we observed a widespread, though patchy Gli3 investigation whether marker genes (Kuschel et al., 2003; Tole et al., 2000b) we could not the rostral most dorsal telencephalon expresses ventral telencephalic expression is con Dbx1 in the septum and in the ventral pallium (VP) (Bielle et al., 2005) raising the possibility that the Dbx1+ progenitor cells in the septum and in the ventral pallium (VP) we performed in situ hybridization for Dbx1+ progenitor cells residing immediately at the PSB of E12.5 wild-type embryos (Figs. 9A–C). To investigate whether this ectopic Dbx1 expression represents an expansion of the VP we performed in situ hybridization for Sfrp2 and Tgfα which are co-expressed with Dbx1 in the VP (Assimacopoulos et al., 2003; Kim et al., 2001). Interestingly, Sfrp2 is ectopically expressed in groups of cells within the Xtl/Xtl neocortex while its expression remains confined to the VP region of Xtl/Pdn embryos (Figs. 9D–F). In contrast, Tgfα expression expands into the neocortex of both mutants (Figs. 9G–I) suggesting that several VP markers are ectopically expressed in the Gli3 mutant neocortex though to different extents.

The ectopic Dbx1 expression might also suggest that an increased Dbx1+ progenitor pool may give rise to the reelin+ but p73− cells. To begin to address this hypothesis we employed an explant culture assay using wildtype E10.5 telencephalic tissue. This time point corresponds to the start of CR cell emigration from the cortical hem when only few CR cells have reached the neocortex (Muzio and Mallamaci, 2005). In a control experiment, we first tested whether the complete dorsal telencephalon of wildtype embryos can give rise to CR neurons under these conditions using in situ hybridization for reelin and p73. Indeed, we could detect two stripes of reelin expression in the centre of the explants and strong reelin expression at the lateral margins of the explants but only a few reelin expressing cells in the centres of the two telencephalic hemispheres which corresponds to neocortical tissue (n=4) (Fig. 10A). Also, p73 expression is confined to the midline regions of the explant (n=4) (Fig. 10E). In the next set of experiments, we dissected just neocortical tissue excluding dorsal midline and VP tissue and analyzed the formation of reelin expressing cells after 48 h in vitro culture. In line with a previous report (Muzio and Mallamaci, 2005), neocortical tissue from wildtype embryos did not give rise to reelin or p73 expressing cells (n=8 for both markers) (Figs. 10B, E). However, when we cultivated neocortical tissue from either Xtl/Xtl or Xtl/Pdn embryos which, in contrast to the wildtype explant, expresses Dbx1 we observed strong reelin but not p73 expression in the explants (n=6 for both mutants and for both markers) (Figs. 10C, D, G, H). In combination with the ectopic Dbx1

Fig. 9. Ectopic Dbx1 expression in the Gli3 mutant neocortex. Coronal sections through the brains of E12.5 wild-type (A, D, G), Xtl/Xtl (B, E, H), and Xtl/Pdn (C, F, I) embryos. (A, D, G) Dbx1, Sfrp2 and Tgfα expression in wild-type neocortex are confined to the VP area at the dorsal/ventral telencephalic boundary. (B, C) Dbx1 is ectopically expressed in the VZ of the Gli3 mutant neocortex. (E, F) Sfrp2 is ectopically expressed in the Xtl/Xtl neocortex but not in the Xtl/Pdn mutant. (H, I) Expanded Tgfα expression domain in the Gli3 mutant neocortex as indicated by arrows.
expression, this result suggests that the ectopic Dbx1⁺ progenitors may give rise to reelin⁺ neurons in the Gli3 mutants.

Discussion

Regionalization defects in the Gli3 compound heterozygous mutant Xf/Pdn

Xf/Xf embryos were previously shown to have severe defects in the regionalization of the telencephalon which are also present in Xf/Pdn embryos but in a milder form. In the latter mutant, the expression of ventral telencephalic markers in dorsal locations occurs in a smaller domain and is restricted to the rostral most telencephalon (Kuschel et al., 2003). Dorsomedial structures are highly defective showing a morphological absence of the hippocampus and an overgrowth of choroid plexus tissue consistent with an altered balance between Bmp and Wnt signalling. In contrast to Xf/Xf embryos (Fotaki et al., 2006) the telencephalon and diencephalon are not fused and we could not find evidence for mixing of cells from both tissues. In addition to these findings, our analysis revealed a novel regionalization defect in Gli3 mutants, namely an expansion of the VP into the lateral and dorsal pallium. This expansion may result from a lack of Emx1 expression (Kuschel et al., 2003; Theil et al., 1999) as has been suggested previously (Medina et al., 2004; Puelles et al., 2000) or from a reduced Lhx2 expression (Mangale et al., 2008). Alternatively, Gli3 could play a general role in controlling Dbx1 expression as ectopic Dbx1 transcription was also found in the Xf/Xf spinal cord (Persson et al., 2002). Irrespective of the exact mechanism, these data indicate that the Xf/Pdn telencephalon has similar but milder regionalization defects than Xf/Xf embryos consistent with Pdn being a hypomorphic Gli3 allele. Our analysis also shows that except for this VP expansion the Xf/Pdn neocortex is largely unaffected allowing us to investigate Gli3 functions in layering.

Xf/Pdn mice show cortical lamination defects

Our analysis of cortical layering indicates a lamination phenotype in the Xf/Pdn neocortex. The expression of several layer specific markers including the layer IV/V expression of reelin indicate a stronger dispersal of cortical neurons throughout the neocortex. This analysis also revealed areas with strong layering defects where cortical neurons were even positioned close to the ventricular surface. Given the mildly affected cortical lamination in hem ablated animals the finding of layering defects in the Xf/Pdn neocortex comes as a surprise especially as significant numbers of reelin⁺ cells are present in the Gli3 mutant MZ. Their molecular profile (reelin⁺ Calretinin⁻) and the fact that, unlike wildtype neocortical tissue, explants from mutant neocortex give rise to reelin⁺ but not p73⁺ cells in an in vitro culture assay strongly suggests that these cells derive from the expanded Dbx1⁺ progenitor pool in the mutant. The generation of these reelin⁺ cells in the Xf/Pdn neocortex but not in the hem ablated animals is likely to reflect differences in the timing of hem loss and concomitant changes in Wnt mediated patterning of the dorsal telencephalon and/or differences in patterning the VP (see above). Irrespective of the mechanism, these additional reelin⁺ cells are not sufficient to drive radial migration of cortical neurons. Therefore, additional signalling pathways and their interaction with reelin signalling may underlie cortical lamination (Meyer et al., 2004; Yoshida et al., 2006). Furthermore, the reelin⁺/p73⁻/Calretinin⁻ cell population might functionally differ from hem derived reelin⁺ CR cells and might not be able to fully compensate for the loss of the latter cells (Bielle et al., 2005; Meyer et al., 2004). Collaboratively, these findings point at intrinsic functional differences between CR cell subpopulations. Such differences may be important for the establishment of different lamination patterns in distinct cortical regions.

Gli3 functions in cortical lamination

Except for a difference in timing, the Gli3 mutation and hem ablation both lead to a loss of the cortical hem but have strikingly different effects on layering suggesting hitherto unknown roles for Gli3 in lamination. The most striking observation of this manuscript relates to the rearrangement of reelin⁺ and Calretinin⁺ cells which initially show an even distribution over the cortical surface but cluster later in Xf/Pdn embryos. These clusters could mechanically block access to the upper CP. Also, migrating neurons could be differentially exposed to reelin signals consistent with a recent report linking regular spaced clusters of CR cells in the immature presubicular cortex with the formation of vertical arrays of CP neurons (Nishikawa et al., 2002). More importantly, however, the radial glial network which serves as a guidance structure for migrating cortical neurons (Rakic, 2003) is severely disturbed in the vicinity of the reelin⁺ cell clusters. A severe shortening of these processes and their detachment from the pial surface may lead to a failure to guide migrating neurons to the upper CP. Indeed, regions in the P0 Xf/Pdn neocortex with the most severe lamination defects correlate with sites where the radial glial scaffold is severely disturbed. At present it is unknown whether the clustering of neurons in the MZ precedes the disorganization of the
radial glial scaffold or vice versa. However, as the disturbance of the radial glial network is only found locally while Gli3 is expressed throughout the VZ it seems more likely that the formation of MZ clusters is the primary cause of the lamination phenotype. The formation of these clusters could involve a role for Gli3 in controlling the adhesive properties of neurons which are an important determinant in establishing cortical layers and are known to affect the spreading and distribution of reelin and other MZ cell types (Borrell and Marin, 2006; Paredes et al., 2006). The formation of these clusters could also involve changes in adhesion as CR cells express specific cell adhesion molecules (Seki and Arai, 1991; Tsuru et al., 1996). Similarly, loss of Gli function in the spinal cord results in neuronal dispersal in the developing spinal cord (Bai et al., 2004; Lei et al., 2004; Wijgerde et al., 2002). As the molecules which control the adhesion of CP neurons and/or CR cells are currently unknown future work will have to address the identity of such factors.

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