Abnormal Positioning of Diencephalic Cell Types in Neocortical Tissue in the Dorsal Telencephalon of Mice Lacking Functional Gli3

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Introduction
Gli3 (glioma-associated oncogene homolog), a zinc finger transcription factor (Ruppert et al., 1990), is an important component of the Sonic hedgehog (Shh) signaling pathway in mammals that resembles the hedgehog (Hh) signaling pathway in Drosophila (Ingham and McMahon, 2001). In the absence of Hh signal, cubitus interrupts (Ci), the fly homolog of mammalian Gli proteins (Hui et al., 1994), is cleaved to yield a transcriptional repressor, whereas in the presence of Hh, cleavage is repressed and the full-length isoform of Ci acts as a transcriptional activator (Aza-Blanc et al., 1997; Methot and Basler, 1999). It has been shown that Shh can similarly regulate Gli3 (von Mering and Basler, 1999; Aza-Blanc et al., 2000). Insight into the function of Gli3 in vivo has been gained with the study of the extratoes (Xt) mouse mutant, which has a 51.5 kb deletion in the Gli3 gene that includes the zinc-finger domain and is presumed to render it nonfunctional (Hui and Joyner, 1993; Maynard et al., 2002). Mice homozygous for the Xt′ mutation (Gli3<sup>xtox</sup> mice) die perinatally with multiple phenotypic defects, including polydactyly and a high incidence of exencephaly, whereas non-exencephalic embryos display severe telencephalic abnormalities (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000; Kuschel et al., 2003; Theil, 2005).

The telencephalic phenotype of the Gli3<sup>xtox</sup> mutant includes a reduction in the size of the dorsal telencephalon, absence of olfactory bulbs, failure of the medial wall of the dorsal telencephalon to invaginate, and absence of the choroid plexus in the lateral ventricles (Hui and Joyner, 1993; Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). Recently, Gli3 has been implicated in the maintenance of a proper laminar organization of the neocortex, as well as the apical/basal cell polarity of cortical precursors (Theil, 2005).

Several studies have reported ectopic expression of ventral telencephalic markers, such as Islet1, Dlx2 (distal-less homeobox), and Mash1 (mammalian achaete-schute homolog), in the dorsal telencephalon of the Gli3<sup>xtox</sup> mutant (Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003). However, the lack of...
dorsomedial telencephalon in these mice would result in an abnormal
joining of the remaining dorsal telencephalon (neocortex) to the
diencephalon, and previous studies might not have taken this abnormal forebrain anatomy into account when inter-
preting alterations in gene expression.

In this study, we performed a detailed analysis of the embry-
onic day 12.5 (E12.5) Gli3Xt/Xt forebrain and propose that some of
the previously described ectopic dorsal expression of ventral
markers in the Gli3Xt/Xt telencephalon actually reflects relatively
normal gene expression in the diencephalon. We then focused on
the development of the mutant neocortex. We present evidence
that the telencephalic–diencephalic border in the E12.5 Gli3Xt/Xt
mutants is compromised and that neocortical progenitors inter-
spersed with diencephalic cells subsequently segregate into well
organized rosettes. Finally, we analyzed Gli3Xt/Xt forebrain
younger than E12.5 and traced the likely origin of the clusters of
misplaced diencephalic cells in the mutant neocortex to the pres-
ence of occasional cells of diencephalic character in the mutant
dorsal telencephalon at E10.5.

Materials and Methods

**Animals.** Animal care was according to institutional guidelines. Gli3Xt/+ CBA mice were mated, and the morning of the vaginal plug was defined as E0.5. Embryos were genotyped by PCR, as described previously (May-
nard et al., 2002), fixed in 4% paraformaldehyde, and processed into paraffin blocks.

**Bromodeoxyuridine injections, immunohistochemistry, and immunoflu-
orescence.** A 30 min pulse of bromodeoxyuridine (BrdU) (70
μg/g body weight, i.p.) was administered to pregnant dams, and E13.5 embryos were collected.

Sections were cut serially at 10 μm and reacted using standard proto-
cols. Antigen retrieval was achieved by microwaving sections in 10 mM
sodium citrate buffer. Mouse monoclonal antibodies were against BrdU
(1:200; BD Biosciences, Oxford, UK), Mash1 (1:100; BD Biosciences),
β-tubulin III (1:500; Sigma, Poole, UK), reelin (1:1000; Chemicon, Har-
row, UK), Ise1 (1:50; Developmental Studies Hybridoma Bank, Uni-
v ersity of Iowa, Iowa City, IA), Lim1 and Lim2 (Lim is the three gene
products Lin-11/Isf-1/Mec-3) (1:200; Developmental Studies Hybrid-
oma Bank), nestin (1:100; Developmental Studies Hybridoma Bank),
and Pax6 (paired box gene) (1:400; Developmental Studies Hybridoma
Bank). Rabbit polyclonals were against calretinin and calbindin (1:2000;
Swant, Bellizona, Switzerland), Pax2 (1:200; Covance via Cambridge
BioScience, Cambridge, UK), FoxGl (forkhead box gene) (1:200; BD Biosciences, Oxford, UK), signal was detected using ECL Plus detection (Amer-
sham, Little Chalfont, UK) according to the instructions of
the manufacturer. Equivalent amounts of protein were subjected to SDS-
PAGE on a 3–8% gradient Tris-acetate gel (Invitrogen), and protein was
transferred to a nitrocellulose membrane, which was incubated with rab-
bit polyclonal anti-Gli3 antibody (1:100; Santa Cruz Biotechnology,
Heidel-
berg, Germany). After incubating with an HRP-conjugated anti-
rabbit IgG secondary antibody (1:2000; DakoCytomation, High
Wycombe, UK), signal was detected using ECL Plus detection (Amer-
sham Biosciences, Little Chalfont, UK) according to the instructions of
the manufacturer. Band intensity was measured using a densitometer
and the Quantity One-4.0.3 software (Bio-Rad, Hemel Hempstead, UK).

**Results**

**Gli3 expression in wild-type forebrain**

We examined the expression of Gli3 mRNA in the mouse fore-
brain at midgestational stages. In *in situ* hybridization showed that,
in the telencephalon, Gli3 was expressed in the ventricular zone of
the developing neocortex and dorsomedial telencephalon (Fig.
1A, B). No expression was detected in the choroid plexus (Fig.
1A, B), in agreement with previous studies (Grove et al., 1998). In

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Gli3 expression in E12.5 forebrain. A, B, *In situ* hybridization in coronal sections of the diencephalon shows expression of Gli3 mRNA in the epithalamus (ET) and dorsal (DT) and ventral (VT) thalamus and in the optic chiasm (op). In the telencephalon, Gli3 is expressed in the neocortex (nctx), dorsomedial telencephalon (dmT), and the lateral (LGE) and medial (MGE) ganglionic eminences and is absent from the choroid plexus (ChP), indicated by an arrowhead. Scale bars, 250 μm. C, Western blot analysis of Gli3 protein in E12.5 wild-type and Gli3Xt/Xt tissue. In dorsal (D) and ventral (V) wild-type telencephalic tissue, both the long (170 kDa) and short (80 kDa) Gli3 isoforms are present, although their relative amounts differ. Both isoforms are absent in Gli3Xt/Xt whole-head protein extracts. The arrowhead indicates a nonspecific band that is used as an internal loading control.
the ventral telencephalon, there was a high lateral-to-medial gradient of expression of Gli3 through the ventricular zone of the lateral ganglionic eminence and medial ganglionic eminence (Fig. 1B). Gli3 was also expressed in the diencephalic ventricular zone, in a high-to-low gradient from epithalamus through dorsal thalamus to ventral thalamus (Fig. 1A) and in the hypothalamus at the level of the optic chiasm (Fig. 1B). Gli3 protein exists in two forms, a long 170 kDa full-length isofrom and an 80 kDa isofrom formed by cleavage of the full-length product (Aza-Blanc et al., 1997; Dai et al., 1999; Wang et al., 2000). To examine the spatial distribution of these two isofroms, we performed Western blots using an antibody against the N-terminus of Gli3. The results showed bands at \( \sim 170 \) and 80 kDa, in extracts from wild-type dorsal and ventral telencephalon that were absent in Gli3\(^{Xt/Xt}\) tissue (Fig. 1C) and correspond to the previously described full-length and cleaved isofroms, respectively (Aza-Blanc et al., 1997; Dai et al., 1999; Wang et al., 2000). The antibody also detected non-specific bands of intermediate size and unknown identity, whose intensities varied in proportion to the total amount of protein loaded in each lane (Fig. 1C, arrow). Total levels of Gli3 protein were approximately twofold higher in the dorsal compared with the ventral telencephalon, reflecting the pattern of Gli3 mRNA expression (Fig. 1C). We then quantified the ratio between the full-length and cleaved forms in these tissues. Dorsally, the cleaved form was present at 2.75 \( \pm \) 0.45 times (mean \( \pm \) SD; \( n = 7 \)) the concentration of the full-length form, whereas ventrally the ratio was lower, at 1.33 \( \pm \) 0.50 (mean \( \pm \) SD; \( n = 8 \)) and the difference between these two ratios was significant (Student’s \( t \) test; \( p < 0.0005 \)). Overall, the highest concentration of Gli3 was of the cleaved form in the dorsal telencephalon (Fig. 1C).

**Identification of the limits of the dorsal telencephalon in Gli3\(^{Xt/Xt}\) mutants**

We performed a detailed rostrocaudal morphological comparison of E12.5 wild-type and Gli3\(^{Xt/Xt}\) forebrain sections immunolabeled with Pax6, which is highly expressed in the dorsal telencephalon and diencephalon (Walther and Gruss, 1991; Stoykova and Gruss, 1994; Mastick et al., 1997), and Mash1, which is expressed in the ventral telencephalon and diencephalon (Lo et al., 1991; Guillelmet and Joyner, 1993; Porteus et al., 1994). Figure 2 depicts representative sets of wild-type and mutant forebrain sections in a caudal-to-rostral order. The most caudal diencephalic parts of the mutant forebrain (epithalamus, dorsal and ventral thalamus) appear relatively normal both anatomically and in their expression of Pax6 and Mash1 (Fig. 2A, A’, B, B’, E, E’, F, F’), although the ventricular space appears slightly enlarged in mutants. At a level in which caudal telencephalic lobes are clearly visible in wild-type sections (Fig. 2B, F), only the most caudal tips of the telencephalic lobes are observed in corresponding Gli3\(^{Xt/Xt}\) sections (Fig. 2B’, F’). This may be because of the diminished size of the Gli3\(^{Xt/Xt}\) lobes compared with wild type and/or their altered position. Moving rostrally, the dorsomedial telencephalon and choroid plexus, seen in wild types in Figure 2, C, D, G, and H, are absent in Gli3\(^{Xt/Xt}\) embryos, and the connection between the third and the lateral ventricle is greatly enlarged (Fig. 2C’, D’, G’, H’). The intense labeling for Pax6 in the ventral thalamus of mutant embryos (Fig. 2B’) is continuous with strong staining for Pax6 in the neocortex (Fig. 2C’), because Pax6 low-expressing tissue that
would normally intervene (the dorsomedial telencephalon) (Fig. 2 C) is missing in the mutant. In wild-type sections, Mash1 is expressed in the ganglionic eminences (Fig. 2 G, H) and in the ventral thalamus (Fig. 2 F, G) but is not observed in the neocortex (Fig. 2 G, H, area of tissue between the lines). In comparable sections in the Gli3Xt/Xt mutant, Mash1 immunoreactivity is also present in the ganglionic eminences (GE) and absent from the adjacent Pax6-positive area (Fig. 2, compare C, G). This suggests that the Mash1-negative region (Fig. 2 G, H, area between the lines) is neocortical tissue, and the adjacent dorsal region of intense Mash1 staining most likely corresponds to diencephalic tissue [ventral thalamus and/or eminentia thalami (Fig. 2 G, H, labeled VT&EmT)].

To confirm that Mash1-expressing tissue adjacent to the neocortex is diencephalic and not telencephalic in origin in Gli3Xt/Xt embryos, we performed immunohistochemistry using an antibody against Foxg1. Foxg1 is expressed in the dorsal and ventral telencephalon and is absent from the dorsal and ventral diencephalon (Tao and Lai, 1992; Hanashima et al., 2002). As shown in Figure 3, A and A’, Foxg1 expression is found in the telencephalon of both wild-type and Gli3Xt/Xt embryos and is absent from the adjacent Mash1-positive tissue in the mutant (Fig. 2 G’, labeled VT&EmT), confirming that this tissue is not telencephalic. These results clearly delineate the neocortical region and confirm that the Mash1-positive region dorsal to the Foxg1-positive neocortical region is diencephalic.

Figure 3. Forebrain marker analysis in wild-type (Gli3+/+ (A–D) and Gli3 mutant (Gli3Xt/xt) (A’–D’). E12.5 coronal sections. Foxg1 protein expression delineates telencephalic tissue in wild type (A) and mutant (A’). The area between the lines corresponds to Mash1-negative neocortex (nctx) in the mutant (see Fig. 2G’, H’). Islet1 and Dlx proteins are both found in the ventral telencephalon (ganglionic eminences (GE)) in both wild type (B, C) and mutant (B’, C’). They are also observed in the ventral thalamus (VT) in wild type and within a region in the mutant that corresponds to the ventral thalamus and eminentia thalami (EmT). Note that neither Islet1 nor Dlx proteins are expressed in neocortex of the mutant (area between the lines in B’, C’). Shh mRNA is expressed in the ZLI in wild type (D) and mutant (D’), defining the border between the dorsal thalamus (DT) and ventral thalamus. Note that the orientation of the Shh-positive ZLI is perpendicular to the main axis in the wild types but not in the mutants. Shh mRNA expression is also observed in the hypothalamus (HT) in both wild type and mutant. Scale bars, 250 μm.

Figure 4. Expression patterns of Pax6 and Mash1 in E13.5 coronal wild-type (A, B) and Gli3Xt/Xt (A’, B’) sections. In wild type, Pax6 expression is observed in the neocortex (nctx), dorsomedial telencephalon (dmT), epithalamus (ET), dorsal (DT) and ventral (VT) thalamus, and the ventricular zone of the eminentia thalami (EmT) (A). In Gli3Xt/Xt mutants, expression is found in similar areas, except the dorsomedial telencephalon, which is not present (A’). Mash1 expression is observed in the ventral telencephalon (ganglionic eminences (GE)) and in the ventral thalamus and hypothalamus (HT) in wild type (B). At a comparable level, Mash1 expression is found in a similar region in Gli3Xt/Xt mutants (B’). Scale bars, 500 μm.
To provide additional evidence for this, we examined the pattern of expression of Islet1 and Dlx in Gli3<sup>X<sup>−/−</sup></sup> E12.5 embryos (Fig. 3B′, C′). These genes are normally expressed in the ventral telencephalon and the ventral thalamus (Fig. 3, B, C), and previous reports have suggested that they are ectopically expressed in the dorsal telencephalon of the Gli3<sup>−/−</sup> mutant (Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003). As in wild types, the expression of Islet1 and Dlx is confined to (1) the ventral telencephalon and (2) the ventral thalamus and/or eminentia thalami region in Gli3<sup>−/−</sup> embryos (Fig. 3B′, C′). That this latter region corresponds to the mutant equivalent of ventral thalamus and/or eminentia thalami is further supported by the expression pattern of Shh transcript in the zona limitans intrathalamica (ZLI) (Echelard et al., 1993; Marti et al., 1995), which defines the border between the dorsal and ventral thalamus (Figdor and Stern, 1993; Rubenstein et al., 1994; Kiecker and Lumsden, 2004). Shh mRNA expression is present in both wild-type and Gli3<sup>−/−</sup> sections (Fig. 3, D, D′), showing clearly that the Islet1, Dlx-positive region just below the ZLI corresponds to ventral thalamus in both wild types and mutants. Furthermore, the region that corresponds to the neocortex (Fig. 3B′, C′, area of tissue between the lines) does not express either Islet1 or Dlx. Altogether, our results show that the previously described ectopic expression of Mash1, Islet1, and Dlx in the dorsal telencephalon of Gli3<sup>−/−</sup> mutants (Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003) is actually expression in the diencephalon (ventral thalamus and/or eminentia thalami).

We examined Pax6 and Mash1 expression patterns during later stages of Gli3<sup>−/−</sup> forebrain development by immunohistochemistry on caudal-to-rostral sets of sections from E13.5 to E15.5. At most levels, Pax6 immunoreactivity was confined to the ventral telencephalon and ventral thalamus, and Mash1 immunoreactivity was confined to the ventral telencephalon and ventral thalamus in both wild types and Gli3<sup>−/−</sup> mutants (Fig. 4, A, A′, B, B′), similar to results at E12.5. Results for both Mash1 and Pax6 expression in E14.5 and E15.5 wild-type and Gli3<sup>−/−</sup> tissue were also similar (data not shown), although their interpretation at these stages became more complex because of the high degree of disorganization of the putative Gli3<sup>−/−</sup> neocortex (see below).

The Gli3<sup>−/−</sup> neocortex contains clusters of cells with characteristics of the eminentia thalami

High-power images of Foxg1 immunostaining at E12.5 revealed patchy expression of Foxg1 in the Gli3<sup>−/−</sup> neocortex (Fig. 5A′), which was not observed in the wild type (Fig. 5A). A similar patchy staining was also observed with an antibody against Lhx2 (Fig. 5B′), which is normally expressed throughout the neocortex in a high-dorsal-to-low-lateral gradient (Fig. 5B) (Monuki et al., 2001).

We examined the nature of the Foxg1-negative cells observed in the Gli3<sup>−/−</sup> neocortical area. Results described above suggested their possible identity. They are immunonegative for Mash1, Islet1, and Dlx (Figs. 2, G′, H′, 3B′, C′) (supplemental data, available at www.jneurosci.org as supplemental material), indicating that they do not share properties with neuronal progenitors or postmitotic cells from the ventral telencephalon and/or ventral thalamus. However, the tissue negative for Foxg1, Mash1, Islet1, and Dlx is positive for Pax6 (Fig. 2C′, D′) (supplemental data, available at www.jneurosci.org as supplemental material). Pax6 is expressed not only in the dorsal telencephalon and ventral thalamus but also in the ventricular zone of the eminentia thalami (Fig. 2C) (Puelles et al., 2000), which forms part of the rostral boundary between the diencephalon and the telencephalon (Rubenstein et al., 1994; Puelles and Rubenstein, 2003). We examined whether the Foxg1-immunonegative patches could have an eminentia thalami identity and used as a marker calretinin, which labels eminentia thalami postmitotic cell somata and fibers in wild-type mice (Fig. 5C–D) (supplemental data, available at www.jneurosci.org as supplemental material) (Abbott and Jaco-
In Gli3<sup>KO/Xt</sup> mutants, the eminentia thalami region, as revealed by intense calretinin staining, lies above the dorsal limit of the neocortical area and displays a thinner postmitotic layer compared with the wild type (Fig. 5C<sup>D</sup><sup>'</sup>/D<sup>'</sup>) (supplemental data, available at www.jneurosci.org as supplemental material). Adjacent to this area, small, calretinin-positive cell clusters were observed in the mutant neocortex (Fig. 5C<sup>D</sup><sup>'</sup>/D<sup>'</sup>).

To provide additional evidence about the eminentia thalami nature of these clusters, we examined the expression pattern of the transcription factor Lim2, also known as Lhx5, which specifically labels the eminentia thalami and ventral thalamus in E12.5 wild types (Fig. 5E) (Sheng et al., 1997). The antibody used recognizes both Lim1 (Lhx1) and Lim2 (Lhx5) proteins, but Lim1 mRNA expression is very weak in these tissues at this age (Sheng et al., 1997). In the Gli3<sup>KO/Xt</sup> mutant neocortex, Lim2 immunostaining presented a patchy expression (Fig. 5E'), similar to that observed with calretinin and complementary to the Foxg1-negative patches (Fig. 5, compare A', C', E'). To confirm this, we performed double immunofluorescence for Foxg1 and Lim2 in sagittal E12.5 wild-type and Gli3<sup>KO/Xt</sup> sections. In wild types, Lim2 immunostaining was confined in the eminentia thalami, whereas Foxg1 specifically labeled the dorsal and ventral telencephalon (Fig. 5F'). In Gli3<sup>KO/Xt</sup> mutant, Lim2 immunostaining was found in the Foxg1-positive region in the form of patches, and the two markers did not colocalize (Fig. 5F'). Tbr1, another marker of the eminentia thalami (Puelles et al., 2000), also revealed the presence of clusters in the vicinity of the Foxg1-immunonegative patches (data not shown).

Finally, we observed that Pax2, a well described marker of the hindbrain, optic chiasm, and optic stalk (Nornes et al., 1990; Puschel et al., 1992), labels a distinct population of eminentia thalami cells, found in close proximity to the choroid plexus in wild-type E12.5 sections (Fig. 5G). In the Gli3<sup>KO/Xt</sup> mutants, Pax2 immunostaining revealed dispersed patch-like expression (Fig. 5G') that was similar to that revealed with the calretinin and Lim1/2 antibodies. Similar results were also obtained in a sagittal plane for all of the eminentia thalami markers examined (calretinin, Tbr1, and Pax2) (data not shown).

The above results strongly support an eminentia thalami identity for the cell clusters observed in the neocortical region of the Gli3<sup>KO/Xt</sup> mutants. However, because calretinin, Lim2, and Tbr1 also label Cajal-Retzius cells in the marginal zone (del Río et al., 1995; Super et al., 1998; Hevner et al., 2001, 2003; Yamazaki et al., 2004), it was possible that the calretinin-positive, Lim2-positive, and Tbr1-positive clusters in the Gli3<sup>KO/Xt</sup> neocortex comprised this cell type. To examine this possibility, we used immunostaining with reelin, which is also found in Cajal-Retzius cells (Alcantara et al., 1998), and calbindin, which is not normally observed in this cell population (Hevner et al., 2003; Jimenez et al., 2003). We did not observe any reelin-positive cell clusters in the Gli3<sup>KO/Xt</sup> mutant (Fig. 6A<sup>A</sup>, B<sup>B</sup>). In fact, the number of reelin-positive cells was significantly lower than in wild type (Fig. 6A<sup>A</sup>, A<sup>'</sup>, B<sup>B</sup>, B<sup>'</sup>), which is in agreement with recently published data (Theil, 2005). Calbindin immunostaining was detected in the ventral telencephalon in both wild type and mutant (Fig. 6C<sup>C</sup>'), as described previously (Davila et al., 2005). However, it also labeled lightly eminentia thalami neurites in the wild type and mutant (Fig. 6D<sup>D</sup>, D<sup>'</sup>) and was also detected in the vicinity of calretinin-positive clusters in the neocortex (Fig. 6C<sup>C</sup>', D<sup>D</sup>'), (compare Figs. 6D<sup>'</sup>, 5C'). These results indicate that the calretinin-positive, Lim2-positive, and Tbr1-positive clusters observed among the Foxg1-negative patches are not Cajal-Retzius cells.

A few dispersed reelin-positive cells in the Gli3<sup>KO/Xt</sup> neocortex (Fig. 6B', arrows) were found in the same region as the calretinin/calbindin-positive ectopic clusters (data not shown), suggesting that a few Cajal-Retzius cells (Fig. 6B) may contribute to these clusters of displaced eminentia thalami cells. However, it is likely that these reelin-positive cells are derived from the eminentia thalami region.
thalamis, because reelin also labels a population of eminentia thalami cells (Fig. 6A).

Altogether, our results clearly show that the neocortex of Gli3-/- mutant embryos contains not only neocortical cells but also cells of eminentia thalami identity, indicating that the border between the telencephalon and diencephalon is compromised in this mutant.

Eminentia thalami clusters are first observed in the Gli3-/- mice at E11.5

To gain insight into the developmental stage at which the eminentia thalami clusters start to form in the neocortical region of Gli3-/- embryos, we studied the expression of eminentia thalami markers in sagittal sections at E11.5 (Fig. 7). Sections through wild types (Fig. 7A–C) are at, or close to, the plane marked (I) in supplemental data A (available at www.jneurosci.org as supplemental material); sections through mutants are at the planes marked in supplemental data A’ (available at www.jneurosci.org as supplemental material) as either (II) (Fig. 7A’–C’) or (III) (Fig. 7A”–C”). E11.5 is the stage at which wild-type calretinin expression is first observed in the eminentia thalami (Abbott and Jacobowitz, 1999). In wild types, the eminentia thalami lacks Foxg1 (Fig. 7A), expresses calretinin in its differentiating cells (Fig. 7B) and Pax2 in a collection of cells close to the choroid plexus (Fig. 7C), and is located caudal to the dorsomedial telencephalon, which does not express Foxg1 (Fig. 7A) (Dou et al., 1999). In mutants, the Foxg1 nonexpressing eminentia thalami is directly caudal to the dorsal telencephalon, which is immunopositive for Foxg1 (Fig. 7A, A’, A’a). In wild types, its differentiating cells are detected by their expression of calretinin (Fig. 7B, B’). Pax2 is expressed mainly in dorsal eminentia thalami of mutants (Fig. 7C, C’). In Gli3-/- mutants, calretinin-positive and Pax2-positive cell clusters were observed in close proximity to the eminentia thalami and within the Foxg1-positive telencephalic region (Fig. 7B, B’, B”, B”, B’’, B’’, B’’). Clusters that are positive for both calretinin and Pax2 were more numerous in the most lateral parasagittal sections of the Gli3-/- mutants (Fig. 7B, B”, C”, c), in which the Foxg1-immunopositive dorsal telencephalic patches are also more abundant (Fig. 7A’, a). These results show that, at E11.5, eminentia thalami cells are already present in the Gli3-/- neocortex in the form of clusters.

At E10.5, the choroid plexus and eminentia thalami have not differentiated yet into distinct morphological structures (Sturrock, 1979; Abbott and Jacobowitz, 1999). In addition, markers that specifically label the eminentia thalami at later developmental stages are either not expressed yet (calretinin and Tbr1) or label a broader region (Lim2) (Abbott and Jacobowitz, 1999). The only informative marker for the study of the developing eminentia thalami at E10.5 was Pax2.

Sagittal sections through the telencephalic–diencephalic boundary region of wild-type E10.5 embryos showed Pax2-positive cells in a similar relative position to that at E11.5 (Fig. 8A, a). They are within a region identified as diencephalic on the basis of morphology, intense Pax6 immunoreactivity (Fig. 8A, a) (Mastick et al., 1997), and absence of Foxg1 immunoreactivity (data not shown). These data indicate that, as at E11.5, the Pax2-expressing cells are on the diencephalic side of the diencephalic–telencephalic boundary at E10.5, in close proximity to the hippocampal primordium, which is immunonegative for Foxg1 (Dou et al., 1999).

In the E10.5 mutant, Pax2-positive cells are mainly within the diencephalic region, which presents intense Pax6 immunostaining similar to that observed in the wild type (Fig. 8A, a’). This tissue is joined directly to Foxg1/Pax6-immunopositive tissue corresponding to the Gli3-/- dorsal telencephalon (Fig. 8A, a’), because the intervening Foxg1-immunonegative hippocampal primordium is absent in the Gli3-/- embryos, in accordance with published data (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). A few, isolated Pax2-positive cells were observed in the Foxg1/Pax6-positive dorsal telencephalon (Fig. 8A, a’, a”, a”, a”, arrowheads, B”, asterisk). These results show that, at E10.5, individual Pax2-immunopositive cells from the Gli3-/- diencephalon are present ectopically in the adjacent dorsal telencephalon of the mutants.

In the diencephalon, we observe a mixed population of diencephalic cells that either express Pax2 but not Pax6 (Fig. 8B, B’),...
Gli3Xt/Xt

Rosette-like structures form in the residual dorsal telencephalon strengthens the possibility that the Pax2-cells that do not express Pax2 express Pax6. The Pax6 expression was still found among neocortical cells (Fig. 9, compare A, B with D, E). At E16.5, no calretinin–positive clusters were detected, in agreement with the reduced calretinin expression observed in the wild-type eminentia thalami at this stage (data not shown).

At E13.5, Foxg1-positive tissue in the residual neocortex had formed rosette-like structures (Fig. 9A). Although the number and size of these structures were variable, they all consistently contained a central lumen and were formed close to the ventricular zone. There was no obvious correlation between the frequency of their appearance and position within the neocortex. The fact that these rosettes were Foxg1 positive (Fig. 9A–G) and Mash1 negative (data not shown) indicated that they comprised neocortical tissue and did not have a ventral telencephalic or diencephalic character. They were immunopositive for markers of neural progenitors, such as Pax6 (Fig. 9H1), BrdU (Fig. 9I), and nestin (Fig. 9J), and negative for the postmitotic neuronal marker β-tubulin III (Fig. 9K). Tbr2 staining, which is normally detected in the cortical intermediate zone and early postmitotic neurons (Englund et al., 2005), was found in the outermost cells of the rosettes (Fig. 9L). At later stages, rosettes became more numerous and were also found close to the pial surface (Fig. 9B, C, F). At E16.5, the lumen of the center of the rosettes was not as clearly distinguishable as in previous stages (Fig. 9F′). These findings indicate that the rosettes contain cells with a neocortical identity that continue to proliferate in an organized manner, near the lumen.

Discussion

The severe reduction in size of the dorsal telencephalon and the lack of dorsomedial telencephalon in the Gli3Xt/Xt mouse have been reported (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). However, previous studies neither precisely defined the extent of the remaining dorsal telencephalon nor showed how this disrupted structure is joined to its neighboring region, the diencephalon. Our study allowed us to identify the area of the Gli3Xt/Xt forebrain corresponding to neocortical tissue (summarized in supplemental data, available at www.jneurosci.org as supplemental material). By studying the expression patterns of Foxg1, which is expressed in developing telencephalon but not diencephalon (Tao and Lai, 1992; Hanashima et al., 2002), Pax6, a well characterized marker of the dorsal telencephalon and diencephalon (Walther and Gruss, 1991; Stoykova and Gruss, 1994; Mastick et al., 1997), and Mash1, which is expressed in both the ventral telencephalon and ventral thalamus (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994), we were able to distinguish the dorsal and ventral limits of the residual Gli3Xt/Xt dorsal telencephalon. Based on this analysis, we demonstrated that the Mash1-, Dlx-, and Islet1-immunopositive regions found cases, these two cell types seemed to segregate from the Foxg1-positive region (Fig. 9, compare A, B with D, E). At E16.5, no calretinin–positive clusters were detected, in agreement with the reduced calretinin expression observed in the wild-type eminentia thalami at this stage (data not shown).

Figure 8. Pax2, Pax6, and Foxg1 immunoreactivity in wild-type (Gli3+/+)(A, a, B) and Gli3 mutant (Gli3Xt/Xt)(A′, a′, A′′, A′′′, B′, B′′) sagittal sections at E10.5. Pax2-immunopositive cells are located within the Pax6-positive diencephalon (D) in both wild type (A, a) and mutant (A′, a′). A few dispersed Pax2-positive cells are located in the Pax6-positive dorsal telencephalon (dTel) in the mutant (arrowheads in A′ and a′) but are never observed in the wild-type dorsal telencephalon. The big arrowhead in A′ indicates the Pax2-immunopositive optic cup. Foxg1-immunolabeling shows the limits of the dorsal telencephalon in the mutants (A′, a′). Double immunofluorescence with Pax6 (green) and Pax2 (red) reveals the presence of diencephalic cells that are either Pax2-positive and Pax6-negative (arrows) or Pax2 positive and Pax6 negative (asterisks) in both wild type (B) and mutant (B′). Some of the displaced cells found in the dorsal telencephalic region in the mutants are Pax2 positive and Pax6 negative (asterisk in B′), further supporting their eminentia thalami origin. Note that A′ and A′′ are serial sections from the same specimen. a, a′, and a′′ are magnifications of the boxed areas in A, A′, and A′′, respectively. B′ and B′′ are magnifications of the boxed areas of the inset in B′. Scale bars: A, A′, A′′, 100 μm; a, a′, a′′, B, B′, B′′, 50 μm.

Rosette-like structures form in the residual Gli3Xt/Xt neocortex

To study the development of the Gli3Xt/Xt neocortex and the behavior of the eminentia thalami clusters at later developmental stages, we immunostained consecutive mutant sections for calretinin and Foxg1 from E13.5 to E16.5. Foxg1-negative areas were observed in the E13.5 Gli3Xt/Xt neocortical region (Fig. 9A). At later stages of development (E14.5 and E16.5), the Gli3Xt/Xt neocortical area became highly disorganized and large Foxg1-negative patches were found in the midst of Foxg1-positive tissue (Fig. 9B, C). At E13.5 and E14.5, calretinin–positive clusters were still found among neocortical cells (Fig. 9D, E), and, in most

Figure 9. Calretinin immunoreactivity in wild-type (Gli3+/+)(A, D) and Gli3 mutant (Gli3Xt/Xt)(B, E) sagittal sections at E13.5. Calretinin-positive clusters were detected in the neocortical region (Fig. 9, compare A, B with D, E). At E16.5, no calretinin–positive clusters were detected, in agreement with the reduced calretinin expression observed in the wild-type eminentia thalami at this stage (data not shown).

Figure 9. Calretinin immunoreactivity in wild-type (Gli3+/+)(A, D) and Gli3 mutant (Gli3Xt/Xt)(B, E) sagittal sections at E13.5. Calretinin-positive clusters were detected in the neocortical region (Fig. 9, compare A, B with D, E). At E16.5, no calretinin–positive clusters were detected, in agreement with the reduced calretinin expression observed in the wild-type eminentia thalami at this stage (data not shown).
at the dorsal end of the Gli3\(^{Xt/Xt}\) neocortex correspond to the ventral thalamus. This is in contrast to previous studies that have reported that these ventral telencephalic markers are ectopically expressed in the dorsal telencephalon of Gli3\(^{Xt/Xt}\) mice (Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003). This discrepancy can be attributed to the fact that previous studies did not define the Gli3\(^{Xt/Xt}\) telencephalic limits relative to the diencephalon and did not consider that many ventral telencephalic markers are also expressed in the developing diencephalon.

Correspondence between the severity of the forebrain defects in Gli3\(^{Xt/Xt}\) forebrain and the expression of Gli3

Our expression analysis of Gli3 mRNA in the developing mouse telencephalon reveals high expression dorsally and a high lateral-to-low medial gradient ventrally, as described previously (Grove et al., 1999; Nery et al., 2001) may account for the high levels of the processed Gli3 isoform, which might act as a repressor of the Shh signaling pathway. (Basler, 1999). In the dorsal telencephalon, absence of Shh (Sussel et al., 2004; Litingtung et al., 2002) is significant more of the short than the long isoforms ventrally. Furthermore, it agrees with our observation that levels of Gli3 protein are higher in the dorsal than in the ventral telencephalon.

Estimates of the relative amounts of the long and short isoforms of Gli3 in the developing telencephalon showed that there is significantly more of the short than the long isoform in the dorsal telencephalon, whereas there are almost equivalent levels of both isoforms ventrally. The ratio of the cleaved to the full-length isoform of Gli3 in the dorsal and ventral telencephalon are analogous to those described in the anterior and posterior limb bud, respectively (Wang et al., 2000; Litingtung et al., 2002; Chen et al., 2004). Litingtung et al. (2002) proposed that the ratio of the two Gli3 forms is crucial for digit number and identity. Similarly, regional differences in this ratio may be important for dorsoventral patterning of the telencephalon. Relatively high expression of the cleaved Gli3 repressor form in the anterior limb domain has been correlated with absence of Shh signaling (Wang et al., 2000), in a similar manner to that described in the Drosophila anterior wing bud (Methot and Basler, 1999). In the dorsal telencephalon, absence of Shh (Sussel et al., 1999; Nery et al., 2001) may account for the high levels of the processed Gli3 isoform, which might act as a repressor of the Shh signaling pathway.

Neocortical cells form rosettes intermingled with eminentia thalami cells in Gli3\(^{Xt/Xt}\) mutants

Having defined the limits of the presumptive neocortex in the E12.5 Gli3\(^{Xt/Xt}\) mutant, we evaluated its development at later stages. Our analyses led to the observation of two striking features. First, starting at E13.5, we noticed the formation of rosettes, which consisted of cells surrounding a lumen. These became more numerous as development proceeded. They were composed of neocortical neural progenitors, and the relative position of S-phase cells around the lumen was similar to that of S-phase cells around the ventricle in wild types. Tbr2 expression in cells surrounding the outermost surface of the rosettes resembled that observed overlying the ventricular zone in the neocortex of wild types (Englund et al., 2005). It appears that the rosettes resemble well-organized neocortical progenitors that segregate from surrounding cells, many of which express markers of the eminentia thalami.

Previous work described the formation of aberrant structures in the Gli3\(^{Xt/Xt}\) dorsal telencephalon but did not characterize the cell types involved (Theil et al., 1999). Rosette-like structures forming close to the ventricle have also been described in mice with loss-of-function mutations in the genes for the membrane-associated protein Lgl1 (lethal giant larvae homolog 1) (Klezovitch et al., 2004) and the myosin II-B heavy chain (Tullio et al., 2000; Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003). This discrepancy can be attributed to the fact that previous studies did not define the Gli3\(^{Xt/Xt}\) telencephalic limits relative to the diencephalon and did not consider that many ventral telencephalic markers are also expressed in the developing diencephalon.

Figure 9. The development of the Gli3\(^{Xt/Xt}\) neocortex after E12.5. As for E12.5, Foxg1-negative patches are also observed at E13.5 (A) and E14.5 (B) Gli3\(^{Xt/Xt}\) sections and are positive for calretinin (D, E). At E16.5, Foxg1-negative patches persist (G) but are no longer labeled with calretinin. Note that A, D and B, E are serial sections from the same specimens. After E12.5, rosettes start to form in the Gli3\(^{Xt/Xt}\) neocortex. Rosettes are positive for Foxg1 (F), indicating that this tissue is telencephalic and not diencephalic. These rosettes are immunopositive for Pax6 (H), BrdU (J), and nestin (K) and immunonegative for β-tubulin III (Tuj1) (L), demonstrating that these clusters comprise neural progenitors. Tbr2 is present in the outermost cells of the rosettes (K); Cresyl violet staining at E16.5 reveals a greater number of rosettes with a less visible lumen (F, F'). F' is a high magnification of the boxed area in F. Scale bars: A–F, 500 μm; F', 100 μm; G–L, 50 μm.
2001). Their formation has been attributed to alterations in the adhesive properties of the neuroepithelial cells and in their apical–basal cell polarity, defects that have been described recently in Glis3−/− neocortical tissue (Theil, 2005) and may be the primary cause for the rosette formation in this mutant.

The second important observation in the E12.5 Glis3−/− dorsal telencephalon was the presence of patches of Foxg1-negative cells that expressed markers of the neighboring eminentia thalami among the Foxg1-positive neocortical cells. Calretinin, which labels postmitotic cells and fibers found in the eminentia thalami (Abbott and Jacobowitz, 1999), was expressed by cells in these patches that were located near the marginal zone. Additional evidence for the origin of these clusters was their expression of Lim2 and Tbr1, known markers of eminentia thalami (Sheng et al., 1997; Puelles et al., 2000), and of Pax2, a newly described marker of an eminentia thalami population found close to the choroid plexus in wild types.

In mammals, the eminentia thalami is a transitional developmental structure (Keyser, 1972), is proposed to act as an organizer for the diencephalon, and appears at approximately E11 (Abbott and Jacobowitz, 1999). It is found in the rostralmost diencephalic area (prosomere 3) (Puelles and Rubenstein, 2003) and forms part of the rostral boundary between the diencephalon and the telencephalon (Trujillo et al., 2005). We found that eminentia thalamic clusters first appear among the dorsal Glis3−/− telencephalon at E11.5. At E10.5, when eminentia thalami cells have not yet started to differentiate and the medial walls of the dorsal telencephalon have not invaginated, we observed a few dispersed Pax2-positive cells within the Foxg1/Pax6-positive dorsal telencephalic area, close to the presumptive mutant diencephalocerebral boundary. These Pax2-positive cells are most likely the precursors of the Pax2-positive clusters observed 1 d later. The most straightforward hypothesis to explain the aberrant presence of cells of eminentia thalami identity in neocortical tissue in Glis3−/− mutants is that the medial wall of the dorsal telencephalon normally prevents eminentia thalami cells reaching the neocortex. If the dorsomedial telencephalon is lost, as in Glis3−/− mutants, the lifting of this restriction on the movement of eminentia thalami cells might allow them to mix with neocortical cells. The cortical hem, a Bmp/Wnt (bone morphogenetic protein/wingless-type MMTV integration site family)-rich tissue found in the dorsomedial telencephalon, is suggested to act as a signaling center (Grove et al., 1998; Grove and Tole, 1999) and is missing in Glis3−/− mutants (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). This tissue might be the source of molecular signals that prevent mislocation of diencephalic cells in the telencephalon. It is interesting that cells characteristic of eminentia thalami mislocated in the dorsal telencephalon at E10.5 are not respecified to the fate of the majority of their neighbors, suggesting a strong commitment to an eminentia thalami fate. The fact that some of these Pax2-mislocated cells do not express Pax6 provides additional evidence as to their eminentia thalami origin, because Pax6 is expressed by all dorsal telencephalic precursors at this developmental stage (Walther and Gruss, 1991).

Other factors might contribute to the abnormalities described here. For example, a well studied diencephalic boundary, the ZLI, which separates the dorsal and ventral thalamus (Figdor and Stern, 1993; Rubenstein et al., 1994; Kiecker and Lumsden, 2004), is a source of Shh (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004) and is considered a secondary organizer of the diencephalon (Echevarria et al., 2003; Vieira et al., 2005). Although Shh expression in the presumptive ZLI is maintained in the Glis3−/− mutants, the characteristic wild-type shape of the ZLI, perpendicular to the main axis of the neural tube (Kiecker and Lumsden, 2004) (Fig. 3D), is altered. This change in ZLI orientation might affect the signaling properties that the ZLI exports on the developing diencephalon and might directly or indirectly influence the formation of an intact diencephalo–telencephalic boundary.

In summary, the present study challenges the concept of widespread ventralization in the dorsal telencephalon of Glis3−/− mutants and shows that the absence of functional Gli3 results in abnormal localization of cells of diencephalic identity in the mutant neocortical region. Our results highlight the importance of considering the relative position of the diencephalon to the telencephalon when analyzing the telencephalic defects of this mutant.

References


