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Gli3 is required for *Emx* gene expression during dorsal telencephalon development

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SUMMARY

Dentate gyrus and hippocampus as centers for spatial learning, memory and emotional behaviour have been the focus of much interest in recent years. The molecular information on its development, however, has been relatively poor. To date, only *Emx* genes were known to be required for dorsal telencephalon development. Here, we report on forebrain development in the *extra toes* (*Xt^J*) mouse mutant which carries a null mutation of the *Gli3* gene. This defect leads to a failure to establish the dorsal di-telencephalic junction and finally results in a severe size reduction of the neocortex. In addition, *Xt^J*/*Xt^J* mice show absence of the hippocampus (Ammon's horn plus dentate gyrus) and the choroid plexus in the lateral ventricle. The medial wall of the telencephalon, which gives rise to these structures, fails to invaginate during embryonic development.

On a molecular level, disruption of dorsal telencephalon development in *Xt^J*/*Xt^J* embryos correlates with a loss of *Emx1* and *Emx2* expression. Furthermore, the expression of *Fgf8* and *Bmp4* in the dorsal midline of the telencephalon is altered. However, expression of *Shh*, which is negatively regulated by *Gli3* in the spinal cord, is not affected in the *Xt^J*/*Xt^J* forebrain. This study therefore implicates *Gli3* as a key regulator for the development of the dorsal telencephalon and implies *Gli3* to be upstream of *Emx* genes in a genetic cascade controlling dorsal telencephalic development.

Key words: Mouse, *Gli3*, *Shh*, *Emx*, Forebrain development

INTRODUCTION

Patterning of the vertebrate forebrain involves a regionalisation process that subdivides the forebrain primordium into distinct dorsoventral (D/V) and anteroposterior (A/P) domains (for review see Fishell, 1997; Rubenstein and Beachy, 1998). The molecular and genetic cascades leading to the establishment of this A/P and D/V polarity have only begun to be elucidated. For example, the mechanisms that delineate dorsal telencephalon from dorsal diencephalon are only poorly understood and could involve homeobox-containing genes of the *Emx* gene family (Boncinelli et al., 1993; Morita et al., 1995). These genes are homologues of the *Drosophila empty spiracles* (*ems*) gene, which controls anterior head segmentation in the fly (Dalton et al., 1989; Cohen and Jürgens, 1990; Walldorf and Gehring, 1992). Consistent with a function in A/P patterning, *Emx1* and *Emx2* show nested and overlapping expression patterns in the vertebrate forebrain (Simeone et al., 1992a,b, 1993). The phenotypes of mice in which *Emx* genes have been inactivated have, however, not been informative on this aspect. Rather, the effects of *Emx* mutations seem to be confined to dorsal telencephalon development. While *Emx1*$^{-/-}$ mice lack the corpus callosum (Qiu et al., 1996; Yoshida et al., 1997), *Emx2* mutants mainly display defects in medial cortical structures (Pellegrini et al., 1996; Yoshida et al., 1997). The dentate gyrus is missing and the hippocampus and medial limbic cortex are greatly reduced in size. Therefore, a function of *Emx* genes in establishing the di-telencephalic junction still remains to be shown.

D/V patterning of the anterior neural plate is controlled by several signalling centers (Shimamura and Rubenstein, 1997). Signals from the anterior neural ridge (ANR) regulate expression of *Brain factor1* (*Bf1*) (Shimamura and Rubenstein, 1997), which is required for growth and patterning of the telencephalon (Xuan et al., 1995). Fgf8 represents an important component of this signal as Fgf8 applied to the prosencephalic neural plate mimics the effects of the ANR (Shimamura and Rubenstein, 1997). In addition, the anterior non-neural ectoderm, the ANR and later the roof of the forebrain produce several secreted factors of the bone morphogenetic protein (Bmp) family (Furuta et al., 1997; Shimamura and Rubenstein, 1997). Bmps have been shown to induce the expression of *Msx1* in the dorsal midline of the forebrain and to repress the expression of *Bf1* (Furuta et al., 1997; Shimamura and Rubenstein, 1997). Also, *noggin*, encoding a secreted protein that binds to Bmps and prevents the latter from interacting with its receptor, is expressed in the telencephalic roof plate (trp) (Shimamura et al., 1995),

suggesting that Bmp activity is under stringent control during dorsal forebrain development.

The prechordal mesendoderm represents a key determinant in the specification of the ventral forebrain and produces Sonic hedgehog protein (Shh) (for review see Rubenstein and Beachy, 1998). *Shh* is expressed throughout the axial mesendoderm (Echelard et al., 1993) and has been implicated in ventral patterning throughout the neuraxis (Ericson et al., 1995; Hynes et al., 1995; Dale et al., 1997; Shimamura and Rubenstein, 1997). Mice mutant for *Shh* are cyclopic and exhibit disruptions of ventral forebrain formation (Chiang et al., 1996). Mutations of the human *SHH* gene have also been identified in patients with holoprosencephaly (Belloni et al., 1996; Roessler et al., 1996). These studies therefore implicate Shh as an essential mediator of the inductive effects of the prechordal mesendoderm.

Gli1, *Gli2* and *Gli3* encode highly conserved zinc finger transcriptional regulators which have been implicated in certain aspects of *Shh* signal transduction (Kalderon, 1997; Ruiz i Altaba, 1997). All three *Gli* genes show dorsoventrally restricted expression domains throughout the whole neural tube (Hui et al, 1994; Lee et al., 1997; Platt et al., 1997; Sasaki et al., 1997). *Gli1* transcription is induced upon Shh signalling in the ventral neural tube adjacent to the *Shh* domain, while *Gli2* and *Gli3* expression occurs in progressively more dorsal regions. *Gli3* was also shown to act as a repressor of *Shh* expression in the dorsal spinal cord as well as in the anterior limb bud (Büscher et al., 1997; Masuya et al., 1997; Ruiz i Altaba, 1998) and to interfere with Shh-mediated patterning of the ventral neural tube (Ruiz i Altaba, 1998). While these expression patterns and the involvement in the Shh signal transduction pathway suggest important roles for *Gli* genes in forebrain patterning, relatively little is known about their function during this process. Although $Gli2^{-/-}$ and also $Gli1^{-/-}$; $Gli2^{-/-}$ mice show multiple defects during development of the caudal neural tube, they do not develop obvious forebrain defects (Ding et al., 1998; Matise et al., 1998). In contrast, dorsal forebrain development is disrupted in *extra-toes* (*Xt*) mutant mice (Johnson, 1967) in which a deletion removes all *Gli3* sequences 3′ of the second zinc finger (Büscher et al., 1998). A preliminary analysis suggested that Xt^J/Xt^J mice lack the choroid plexus of the lateral ventricle and the olfactory bulbs while the cerebral cortex shows abnormal lamination (Franz, 1994). The role of *Gli3* in forebrain patterning, however, has not been analysed further. Here, we show that *Gli3* is essential for formation of the dorsal telencephalic boundary and for the development of the pallium. Interestingly, Xt^J/Xt^J embryos are characterised by a loss of *Emx1/Emx2* expression and display altered *Fgf8* and *Bmp4* expression patterns. This study therefore implicates *Gli3* as a key regulator of dorsal forebrain development and provides insights into a genetic cascade controlling specification of the dorsal telencephalon.

MATERIALS AND METHODS

Mice

Xt^J mutant mice were kept as heterozygous animals in a mixed C57Bl6/C3H background and were interbred. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. Xt^J/Xt^J embryos older than E9.5 could readily be distinguished from heterozygous and wild-type embryos by forebrain morphology (Johnson, 1967). Embryos younger than E9.5 were classified either as heterozygous/wild-type or as homozygous by PCR reactions on yolk sac DNA (Büscher et al., 1998).

Whole-mount in situ hybridisation

In situ hybridisations on whole-mount mouse embryos were performed as described (Xu and Wilkinson, 1998) using the following riboprobes: *Gli1* (Hui et al., 1994), *Shh* (Echelard et al., 1993), *Pax6* (Walther and Gruss, 1991), *Ptc* (Goodrich et al., 1996), *Bmp4* (Jones et al., 1991), *Msx1* (Hill et al., 1989), *Bf1* (Tao and Lai, 1992), *Fgf8* (Crossley and Martin, 1995), *Emx1*, *Emx2* (Simeone et al., 1992b), *Six3* (Oliver et al., 1995), *Hesx1* (Thomas et al., 1995), *noggin* (Shimamura et al., 1995), *Wnt1* (Shimamura et al., 1994), *Dlx2* (Bulfone et al., 1993), *Otx1*, *Otx2* (Simeone et al., 1993) and *Nkx2.1* (Lazzaro et al., 1991).

Histology and BrdU incorporation

Harvested embryos were fixed in 4% paraformaldehyde in PBS, dehydrated in ethanol and embedded in 100% Paraplast. 4 μm sections were stained with Hematoxylin/eosin and Cresyl violet according to standard procedures. For BrdU incorporation experiments, pregnant females were injected intraperitoneally with 100 μg of BrdU per g body weight and killed after 1 hour. Sections of BrdU-labeled embryos were processed for immunohistochemical analysis using an anti-BrdU monoclonal antibody (Bio-Science, #010198).

RESULTS

Xt^J/Xt^J embryos were classified into two groups based on overall brain morphology (Johnson, 1967; Franz, 1994). Some embryos (n=13/117) showed a severe exencephaly, probably as a result of a delayed neural tube closure. Due to potential interferences with forebrain development exencephalic embryos were excluded from this study. In contrast, the remaining, non-exencephalic Xt^J/Xt^J embryos showed no marked overgrowth of the midbrain but the telencephalic vesicles were drastically reduced in size as described previously (Johnson, 1967; Franz, 1994). A morphological and molecular analysis of forebrain development in these non-exencephalic embryos is the subject of this study.

Progressive differentiation defects in the cortex and telencephalic roof plate of Xt^J/Xt^J mutants

Detailed morphological inspection of Xt^J/Xt^J and wild-type embryonic brains at different stages documents a gradual differentiation failure and final disappearance of the dorsal pallium. Alterations in the forebrain of Xt^J/Xt^J embryos became apparent by E9.5 (Johnson, 1967). By this stage, the mutant telencephalon seemed already retarded in growth, while dorsal midline structures typical of the diencephalon and midbrain appear normally (Fig. 1A,B). At E11.5 the cortical primordium is present, although its morphogenesis is defective (Fig. 1C-F). Medial cortical tissues are absent, and the trp is abnormally growing outward. As a consequence, the paraphysial arch (ppa) does not form. At E13.5 (Fig. 1G,H), the ventricular layer of the pallium is folded and medial pallial structures fail to form. The progression of the pallial defect is dramatically evident by E14.5, when the pallium has almost completely disappeared rostrally (Fig. 2A), the only remnant is a mass of tissue without cytoarchitectonic differentiation

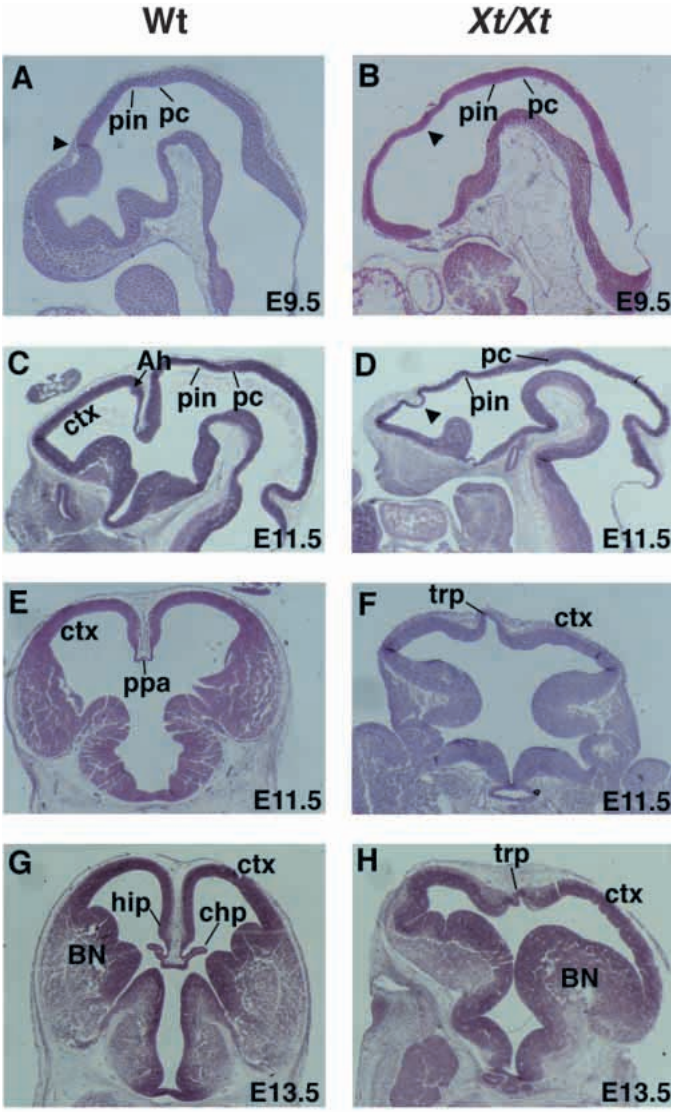

Fig. 1. Defects in the dorsal telencephalon of midgestational Xt^J/Xt^J embryos. (A,C,E,G) Wild-type; (B,D,F,H) Xt^J/Xt^J embryos. Sagittal sections of E9.5 (A,B) and E11.5 (C,D) brains. In Xt^J/Xt^J embryos, the dorsal portion of the telencephalon is reduced in size and the di-telencephalic junction (arrowhead) does not form. Development of Ammon's horn (Ah) was never seen. Dorsal diencephalic structures, the pineal gland (pin) and the posterior commissure (pc), develop normally. Transverse sections of E11.5 (E,F) and E13.5 (G,H) brains. In Xt^J/Xt^J embryos, the telencephalic roof plate (trp) and adjacent medium pallium do not invaginate. Medial structures of the pallium, hippocampus (hip), medial cortex and choriod plexus of the lateral ventricle (chp), do not form. BN, basal nuclei; ppa, paraphysial arch.

(Fig. 2A, double asterisk). In the midline, the trp seems to show a degree of differentiation in what is perhaps the ppa (Fig. 2A). Caudally, however, the rough shape of the pallium and a remnant of the lateral ventricle can still be seen (Fig. 2B). The folding of the pallial neuroepithelium is extensive and a few finger-shaped prolongations of the ventricular layer invade the mantle (asterisk in Fig. 2B).

By E18.5, the normal appearance of the pallium has completely disappeared (Fig. 2C,D). In addition, all olfactory structures are absent (compare Fig. 2E-G). Instead, the basal nuclei primordia are displaced dorsally to meet the dorsal-anterior diencephalon. In the midline, a part of the basal ganglia, which probably represents the lamina terminalis and later its derivative, the septum, is dorsally and caudally displaced to meet the diencephalic roof plate (arrow in Fig. 2E). Consequently, the basal ganglia occupy a dorsal position in the telencephalon and are followed immediately by the thalamus.

The ventricular layer, lining the lateral ventricles, seems to grow into the mantle forming finger-shaped, radial structures (asterisk in Fig. 2B). In transverse section, these structures appear as round islands of cells with a tiny central lumen (asterisk in Fig. 2C). Also, a large number of fibers can be seen in the basal telencephalic mantle layer (Fig. 2B,F), which could correspond to the internal capsule.

To characterise further the affected region in the Xt^J/Xt^J forebrain, we performed in situ hybridisation analysis of several molecular markers with characteristic forebrain expression patterns. At E10.5 brain, expression of *Bf*1 occurs in almost the entire telencephalon except for the medial regions adjacent to the roof in both wild-type and Xt^J/Xt^J embryos (Fig. 3A,B). Consistent with this unaltered *Bf1* expression, the proliferative characteristics of the E10.5 and E12.5 Xt^J/Xt^J dorsal telencephalon appeared indistinguishable from the wild-type (data not shown and Fig. 3C-F). In both wild-type and mutant brains, comparable numbers of BrdU pulse-labelled nuclei form a front migrating away from the ventricular surface and towards the incipient mantle layer suggesting that impaired proliferation does not underlie the disruption of dorsal forebrain development. In the E11.5 wild-type telencephalon, the paired box transcription factor *Pax6* and the *Dlx2* homeobox gene are expressed in a complementary pattern in the cortex and the basal ganglia, respectively (Stoykova et al., 1996; Fig. 3G,I). Expression of both genes appears unaltered in the *Gli3* mutant forebrain (Fig. 3H,J). Taken together, this morphological and molecular data on the Xt^J/Xt^J forebrain indicate failure to form the dorsal di-telencephalic boundary and to specify the dorsomedial region of the telencephalon. In contrast, the subdivision of the telencephalon into pallium and striatum appears initially unaffected by the *Gli3* mutation.

Gli3 is required for the expression of *Emx* genes in the dorsal forebrain

To analyze forebrain patterning in Xt^J/Xt^J mutants, the expression of some diagnostic genetic markers expressed in distinct domains of the brain was examined. At E8.5, *Hesx1* and *Six3* are expressed in the anterior neural plate (Oliver et al., 1995; Hermesz et al., 1996; Thomas et al., 1995; Fig. 4A,C). Xt^J/Xt^J mutants show an identical expression pattern (Fig. 4B,D) suggesting that specification of the anterior neural ectoderm occurs normally in the mutant. *Emx2* expression starts in the lateral neural plate of E8.5 wild-type embryos. Expression gradually increases with development and, by E9.0, transcripts were detected in the dorsal forebrain covering the presumptive dorsal di-telencephalic boundary (Simeone et al., 1992a,b; Fig. 4E). In Xt^J/Xt^J embryos, however, *Emx2* expression was absent from rostral forebrain while expression in the presumptive nephrogenic cord was unaffected by the *Gli3* mutation (Fig. 4F). This loss of *Emx2* expression does not simply reflect a delay in the onset of expression, as we were never able to detect *Emx2*

transcription above background levels in E8.5 to E9.5 Xt^J homozygous embryos (n=10 embryos). Similarly, *Emx2* expression was specifically lost from the telencephalic vesicles of E11.0 Xt^J/Xt^J embryos (compare Fig. 4G,H). Mutation of the *Gli3* gene also affected expression of *Emx1*. At E10.5, *Emx*1 transcripts were confined to the telencephalic vesicles in a domain that was contained within the *Emx2*-positive area (Fig. 4I). In Xt^J/Xt^J embryos, however, no *Emx1* transcripts were detectable (n=5 embryos) (Fig. 4J). Taken together, these data strongly suggest a requirement for *Gli3*

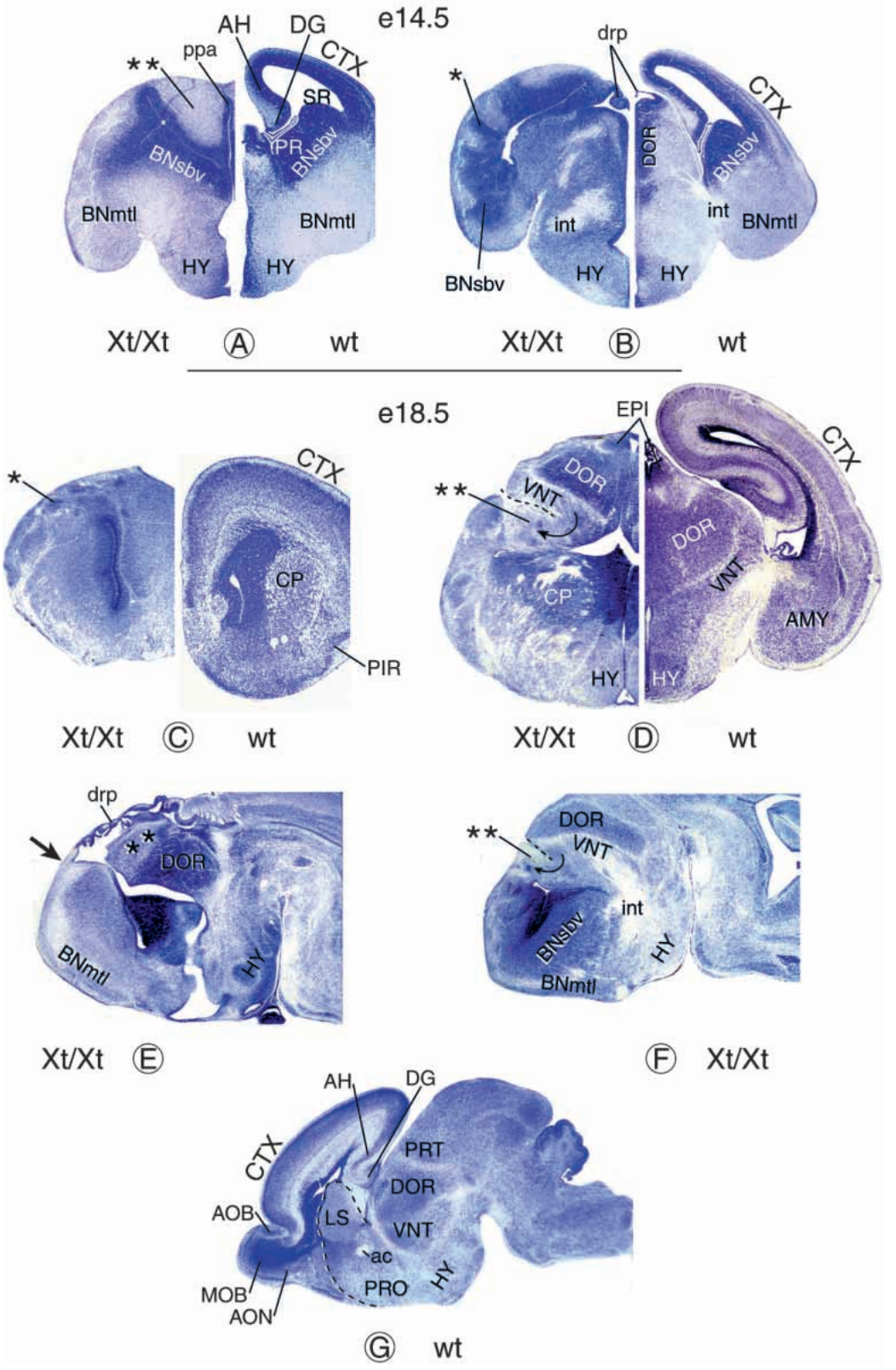

Fig. 2. (A,B) Transverse sections through the brains of E14.5 Xt^J/Xt^J and wild-type embryos. Sections in A are rostral to those in B. Asterisk (*), 'islands' of neuroepithelium; double asterisk (**), putative cortical tissue. (C,D) Transverse sections through the brains of E18.5 Xt^J/Xt^J and wild-type fetuses. Sections in C are rostral to those in D. (E-G) Sagittal sections through the brains of E18.5 Xt^J/Xt^J (E,F) and wild-type (G) fetuses. (E) Sagittal and (F,G) parasagittal planes of section. Arrow in E shows the meeting point of lamina terminalis region the diencephalic roof plate. The dotted line and the bent arrow in E and G mark the partial separation and the medial fusion between telencephalon and diencephalon. The dotted line in G indicates the boundary between the missing or altered tissues (pallium and olfactory structures) and the tissues that are left intact. ac, anterior commissure; AH, Ammon's horn; AMY, amigdala; AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; BN, basal nuclei; BNmtl, basal nuclei mantle layer; BNsbv, basal nuclei subventricular layer; CP, caudate putamen; CTX, cortex; DG, dentate gyrus; DOR, dorsal thalamus; drp, diencephalic roofplate; EPI, epithalamus (habenula); HY, hypothalamus; int, internal capsule; LS, lateral septum; MOB, main olfactory bulb; PIR, piriform cortex; ppa, paraphysial arch; PR, pallidal ridge; PRO, preoptic area of hypothalamus; PRT, pretectum; SR, striatal ridge; VNT, ventral thalamus.

in the induction and/or maintenance of *Emx* gene expression in the dorsal forebrain.

We next analysed *Otx1* and *Otx2* expression both of which are involved in A/P patterning of the rostral brain (Simeone et al., 1993). In E8.5-10.5 Xt^J/Xt^J embryos, both genes were expressed within their correct expression domain at normal expression levels (data not shown). However, in the forebrain of E11.5 Xt^J/Xt^J embryos, the expression levels of *Otx1* and *Otx2* appeared reduced in the mutant forebrain (Fig. 4K-N).

Fgf8 and *Bmp4* expression is altered in the dorsal midline of Xt^J/Xt^J embryos

Gli3 mutant embryos show a failure of the telencephalic roof and of the medial pallium to invaginate. We therefore tried to identify altered gene expression patterns in the dorsal midline.

At E9.5, *Fgf8* expression was localised to a restricted region of the telencephalon corresponding to the commissural plate (Fig. 5A). While we could not observe any change in the *Fgf8* expression pattern in Xt^J mutants before the 10- to 12-somite stage, *Fgf8* transcription began to spread at E9.5 into more caudal regions of the roof in a gradient-like fashion (Fig. 5B). The extent of this effect appeared to be variable and became stronger later in development. At E11.5, *Fgf8* expression was still restricted to the commissural plate of wild-type embryos (Fig. 5C). In contrast, Xt^J/Xt^J embryos showed a considerable extension of this expression domain into more caudal regions. In the extreme case presented in Fig. 5D, this domain covered the complete telencephalic roof.

At E9.0, *Bmp4* begins to be expressed in the wild-type dorsal neuroectoderm around the junction between the prospective telencephalon and diencephalon and in the anteriormost dorsal roof of the telencephalon (Furuta et al., 1997; Fig. 5E). While in E9.5 Xt^J/Xt^J embryos, this latter expression domain remained unchanged, *Bmp4* expression was not detected at the di-telencephalic boundary (Fig. 5F). This defect was also observed at E10.5 (data not shown) arguing against the possibility that the onset of *Bmp4* expression is only delayed in homozygous Xt^J embryos.

In the dorsal midline of the wild-type telencephalon, transcription of the *Msx1* homeobox gene, which can be induced by Bmp2/Bmp4 (Furuta et al., 1997), starts at E9.5 and increased to higher levels at E11.0 (Hill et al., 1989 and Fig. 5G). In addition, *Msx1* is highly expressed in the surface ectoderm and head mesenchyme. Consistent with the loss of *Bmp4* expression from the dorsal midline of the telencephalon in Xt^J/Xt^J embryos, *Msx1* transcripts were specifically lost from the telencephalic roof of *Gli3* mutant embryos (Fig. 5H). In the dorsal midline of the more posterior neural tube and in the surface ectoderm, however, *Msx1* expression was unaffected.

Noggin, a negative regulator of the Bmp signalling pathway, is expressed in the dorsal midline throughout the entire neuraxis of E9.5 wild-type embryos with highest expression levels in the telencephalon (Shimamura et al., 1995; Fig. 5I). In age-matched homozygous Xt^J embryos, *noggin* expression appeared unaltered (Fig. 5J).

Wnt genes encode secreted proteins that participate in tissue patterning and morphogenesis (for review, see Cadigan and Nusse, 1997). In E10.5, wild-type *Wnt1* is expressed in the dorsal midline extending from the posterior diencephalon caudally through the entire body axis (Wilkinson et al., 1987; Fig. 5K). Similar to the wild-type situation, *Wnt1* transcripts were not detected in the roof of the telencephalon of Xt^J homozygous embryos, on the contrary, the rostral border of *Wnt1* expression domain seemed to lie more caudally (Fig. 5L). Taken together, the alterations in *Fgf*8 and *Bmp4* expression patterns suggest a caudal expansion of the rostral midline region in the absence of dorsomedial regional specification.

The Shh signal transduction pathway is not ectopically activated in the dorsal telencephalon of Xt^J homozygous embryos

Gli genes have been reported to play a role in transducing the Shh

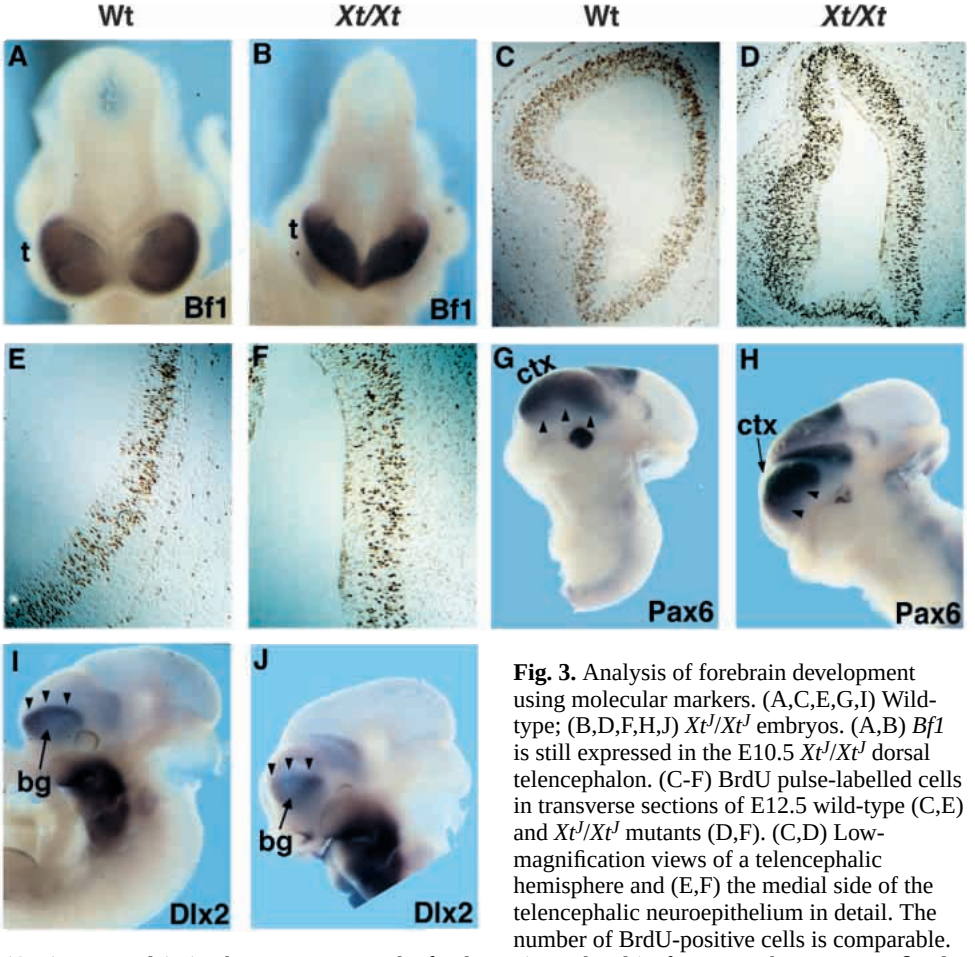

Fig. 3. Analysis of forebrain development using molecular markers. (A,C,E,G,I) Wild-type; (B,D,F,H,J) Xt^J/Xt^J embryos. (A,B) *Bf1* is still expressed in the E10.5 Xt^J/Xt^J dorsal telencephalon. (C-F) BrdU pulse-labelled cells in transverse sections of E12.5 wild-type (C,E) and Xt^J/Xt^J mutants (D,F). (C,D) Low-magnification views of a telencephalic hemisphere and (E,F) the medial side of the telencephalic neuroepithelium in detail. The number of BrdU-positive cells is comparable. (G,H) *Pax6* and (I,J) *Dlx2* expression in the forebrain (arrowheads) of E11.5 embryos are confined to the dorsal and ventral telencephalon, respectively. bg, basal ganglia; ctx, cortex; t, telencephalon.

signal, and *Gli3* was also shown to repress the expression of *Shh* in anterior limb regions of the mouse (Büscher et al., 1997; Masuya et al., 1997) and in the dorsal spinal cord (Ruiz i Altaba, 1998). These findings raised the possibility that the forebrain phenotype of homozygous Xt^J embryos might have been caused by an ectopic expression of *Shh* or an ectopic activation of the Shh signal transduction pathway in the dorsal telencephalon. To test for this, we first compared the *Shh* expression pattern in wild-type and Xt^J/Xt^J embryos. At E9.5, *Shh* expression can normally be found throughout the ventral neuroaxis (Echelard et al., 1993; Fig. 6A). Xt^J/Xt^J embryos show an identical expression pattern. *Shh* transcripts were exclusively confined to the ventral neural tube at all axial levels (Fig. 6E). Similarly, we were never able to detect any *Shh* expression in the dorsal forebrain of E8.5 or E10.5 *Gli3* mutant embryos (data not shown). Also, in both wild-type and Xt^J/Xt^J E9.5 embryos, expression of the Shh target genes *Patched* (*Ptc*) and *Gli1* (Goodrich et al., 1996; Lee et al., 1997; Platt et al., 1997) is confined to the ventral region of the neural tube and excluded from the dorsal forebrain (compare Fig. 6B,C with 6F,G). Furthermore, expression of the *Nkx*2.1 homeobox gene, which is induced by Shh in explant cultures of the anterior neural plate (Pera and Kessel, 1997), remained restricted to ventral structures in the telencephalon and diencephalon (Fig. 6D,H). These results indicate that the anomalies observed in the dorsal forebrain of *Gli3* mutant embryos are neither caused by an ectopic expression of *Shh* nor by an ectopic activation of the Shh signal transduction pathway.

DISCUSSION

Differentiation defects in the dorsal telencephalon of Xt^J/Xt^J mutants develop progressively

In this paper, we demonstrate that *Gli3* is involved in the specification of the dorsal telencephalon and the development of all structures that derive from it (cortex, hippocampus, dentate gyrus, choroid plexus). Morphologically, alterations of the Xt^J/Xt^J forebrain became first evident by E9.5 with a failure to establish the di-telencephalic junction. Although the developmental function of this boundary has not been analysed experimentally, several of the early defects might be attributed to this defect. First, boundary regions have specialised properties that often have an organisational influence on adjacent domains (Lumsden and Krumlauf, 1996). The loss of such a function might explain the size reduction of the pallium in Xt^J/Xt^J mice. Secondly, fusion of the telencephalon with the diencephalon might indicate a mixing

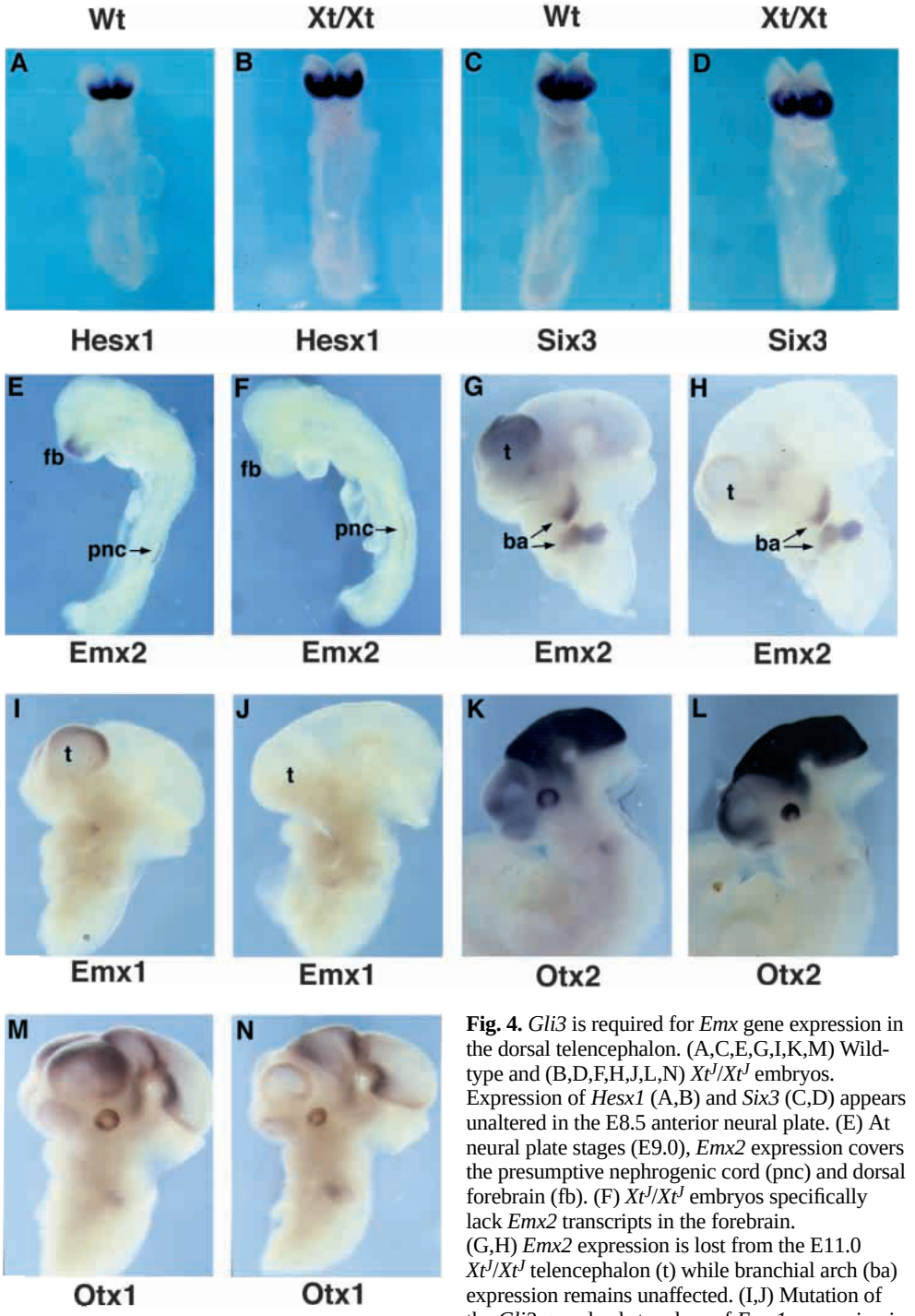

Fig. 4. *Gli3* is required for *Emx* gene expression in the dorsal telencephalon. (A,C,E,G,I,K,M) Wild-type and (B,D,F,H,J,L,N) Xt^J/Xt^J embryos. Expression of *Hesx1* (A,B) and *Six3* (C,D) appears unaltered in the E8.5 anterior neural plate. (E) At neural plate stages (E9.0), *Emx2* expression covers the presumptive nephrogenic cord (pnc) and dorsal forebrain (fb). (F) Xt^J/Xt^J embryos specifically lack *Emx2* transcripts in the forebrain. (G,H) *Emx2* expression is lost from the E11.0 Xt^J/Xt^J telencephalon (t) while branchial arch (ba) expression remains unaffected. (I,J) Mutation of the *Gli3* gene leads to a loss of *Emx1* expression in E10.0 embryos. (K,L) *Otx2* and (M,N) *Otx1* expression in E11.5 embryos appears unaltered except for a lower level expression in the telencephalon.

of cells from both territories. Boundary regions in the hindbrain restrict the movement of cells between neighbouring segments (Guthrie and Lumsden, 1991; Guthrie et al., 1993), thereby maintaining separate identities within these domains. An enlargement of the *Pax*6 and *Otx*1 diencephalic expression domains (Fig. 3E-H) and a caudal shift of the *Wnt1* dorsal midline expression might therefore indicate that, in the absence of *Gli3*, cells, which would normally be specified as telencephalic, now succumb to dorsalising influences and contribute to the diencephalon.

Despite the severe size reduction of the pallium and its poor differentiation at later stages, early patterning of the telencephalon does occur to a certain extent in Xt^J/Xt^J embryos. The subdivision of the telencephalon into pallium and basal ganglia is not affected as judged by morphological criteria and by the existence of distinct expression domains of *Pax*6 and *Dlx*2. Cortical neurons are capable of extending axons towards the internal capsule which, however, appears disorganised. The internal capsule normally carries two major groups of fibers: the cortico-thalamic, cortico-pontine and cortico-spinal tracts originating in the cortex and the thalamo-cortical tract deriving from the thalamus (De Carlos and O'Leary, 1992). In Xt^J/Xt^J mutants, the telencephalon is still able to produce the descending axons and also to attract thalamo-cortical axons. Upon reaching the internal capsule, these bundles become disorganised suggestive of an axon guidance or of an axon fasciculation defect.

At E14.5, the cortical neuroepithelium begins to show indentations of the ventricular layer. These alterations could lead to a failure to generate a properly laminated cortex. Cortical lamination has been reported to be determined to a large extent in the neuroepithelium, by a mechanism related to the mitosis of stem cells (McConnell, 1989). Since *Gli3* is expressed in the cortical neuroepithelium at these stages (Schimmang et al., 1992; Hui et al., 1994), *Gli3* might directly control cortical cytoarchitecture.

The severity of the morphological defects in Xt^J/Xt^J embryos progressively increases, a fact probably related to the growth of the basal ganglia and dorsal diencephalon primordia. These occupy the room left by the dorsal pallium and ultimately fuse with the pallium. The difference in relative growth of the affected and unaffected regions of the brain, together with the failure to establish the dorsal di-telencephalic boundary, might explain the progressivity of the phenotype.

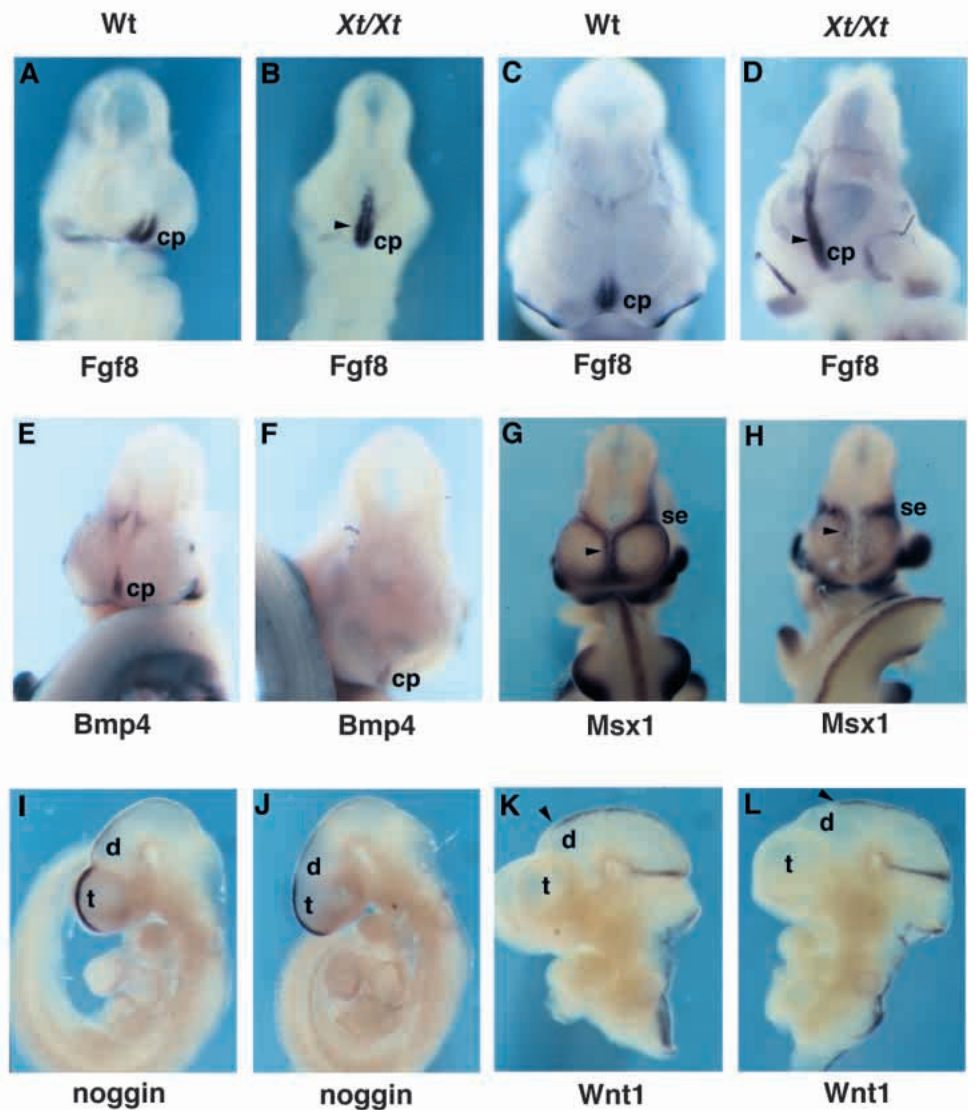

Fig. 5. Altered gene expression patterns in the telencephalic roof plate of Xt^J/Xt^J embryos. (A,C,E,G,I,K) Wild-type; (B,D,F,H,J,L) Xt^J/Xt^J embryos. *Fgf8* expression in the E9.5 (A,B) and E11.5 (C,D) telencephalon. (A,C) In wild-type embryos, *Fgf8* transcripts are confined to the commissural plate (cp). At E9.5, *Fgf8* expression starts to extend into more caudal regions of the telencephalic roof plate of Xt^J/Xt^J embryos (B) finally covering the complete telencephalic roof (D). The arrowhead marks the dorsal end of the commissural plate. (E,F) E9.5 Xt^J/Xt^J embryos lack *Bmp4* expression at the di-telencephalic boundary while expression in the commissural plate remains unaltered. (G,H) Similarly, loss of *Msx1* expression from the telencephalic roof (arrowhead) of E11.0 Xt^J/Xt^J embryos is observed, while expression in the surface ectoderm (se) is still present. (I,J) Xt^J/Xt^J embryos show normal *noggin* expression in the dorsal midline of the neural tube of E9.5 embryos with higher expression levels in the telencephalon. (K,L) *Wnt1* expression at E10.5. Note the caudal shift of the anterior expression limit (arrowhead) in the mutant. d, diencephalon.

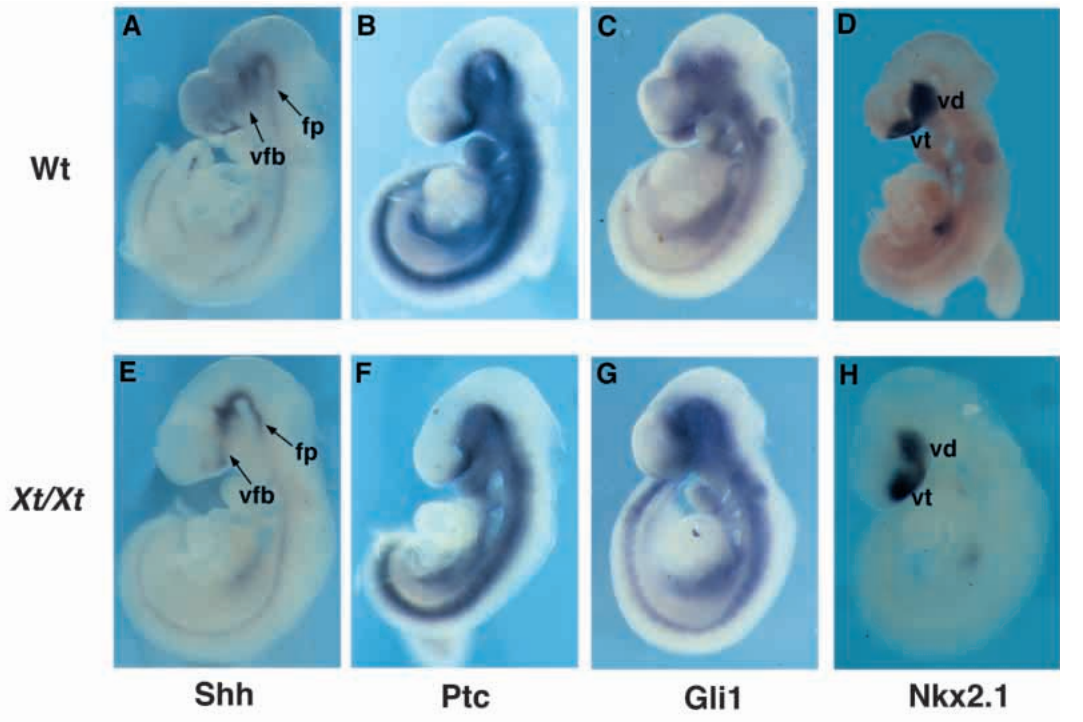

Fig. 6. Shh signalling pathway is not ectopically activated in the dorsal forebrain of Xt^J/Xt^J embryos. (A-D) E9.5 wild-type; (E-H) Xt^J/Xt^J embryos. (A,E) *Shh* expression is found in the ventral neural tube throughout the whole neuraxis. *Shh* transcripts were not detected in the dorsal forebrain of Xt^J/Xt^J embryos (E). *Ptc* (B,F) and *Gli1* (C,G) expression is induced upon Shh signalling in the neural tube in a ventral domain adjacent to the Shh expressing region of both, wild-type and Xt^J/Xt^J embryos. (D,H) *Nkx2.1* expression remains confined to the ventral diencephalon (vd) and telencephalon (vt) of Xt^J/Xt^J embryos. Fp, floor plate; vfb, ventral forebrain.

Failure of di-telencephalic boundary formation and impairment to specify dorsal telencephalon identity correlate with loss of *Emx* gene expression

Based upon their expression pattern, *Emx* genes are candidates for playing a role in subdividing the prosencephalon. Furthermore, their *Drosophila* homologue, *empty spiracles*, functions as a gap gene as well as a segment identity gene during head segmentation (Dalton et al., 1989; Cohen and Jürgens, 1990; Walldorf and Gehring, 1992) suggesting that *Emx* genes might also be involved in specifying dorsal telencephalon identity. However, the phenotypes of mice in which *Emx* genes have been inactivated have not provided clues on these potential roles. Analysis of the forebrain phenotype of Xt^J/Xt^J mice therefore provides the first evidence for a gene controlling formation of the di-telencephalic boundary and specification of dorsal telencephalon identity.

Defects in $Emx2^{-/-}$ mice and Xt^J/Xt^J mice affect the same tissues (Pellegrini et al., 1996; Yoshida et al., 1997) suggesting that *Emx2* and *Gli3* might act within a genetic cascade. Indeed, we show that expression of both *Emx1* and *Emx2* is specifically lost from the dorsal forebrain of Xt^J/Xt^J embryos. As we might have missed initial low level expression of *Emx2*, these data indicate that *Gli3* is required at least for the maintenance of *Emx* gene expression in the forebrain. A potential role of *Gli3* in the establishment of *Emx* expression remains unclear at present. Furthermore, this genetic analysis does not provide evidence for a direct transcriptional link as an early block in the establishment of dorsal telencephalic identity could have indirectly affected expression of later genes.

The similarity in phenotypes and the lack of *Emx* expression in the forebrain suggest that the defects in the dorsal forebrain of Xt^J/Xt^J embryos mainly arise through a failure to activate *Emx* gene expression. As formation of the di-telencephalic boundary and of Ammon's horn principally occurs in $Emx2^{-/-}$ mice (Yoshida et al., 1997), disruption of forebrain development in these mice appears less severe than in Xt^J/Xt^J animals. In this regard, *Emx* genes might functionally compensate for the loss of the other and Xt^J/Xt^J embryos might already anticipate an *Emx1*/*Emx2* double knock-out. Alternatively, *Gli3* regulates the expression of other, yet to be identified factors important for dorsal forebrain development. Interestingly, mice carrying a reduced *Otx* gene dosage display a reduction of Ammon's horn probably due to an establishment of an isthmic-like structure in the caudal diencephalon and a subsequent repatterning of the dorsal telencephalon (Acampora et al., 1997; Suda et al., 1997). However, isthmic development occurs normally in Xt^J/Xt^J embryos (data not shown), suggesting that the reduced *Otx1*/*Otx2* expression levels observed at E11.5 might be secondary to loss of *Emx1*/*Emx2* expression. Regardless of the exact mechanism, the analysis of forebrain development in Xt^J/Xt^J embryos places the *Gli3* gene upstream of *Emx* genes in a genetic cascade controlling patterning of the dorsal forebrain.

Defects in choroid plexus and medium pallium development in Xt^J/Xt^J mice correlate with alteration in *Fgf8* and *Bmp4* expression

The *Gli3* mutation also affects development of the telencephalic roof and the juxtaposed medium pallium. Moreover, formation of the choroid plexus is disrupted in the lateral ventricle while its development occurs normally in the 4th ventricle (Franz, 1994 and data not shown). The expression patterns of several regulatory genes were found to be altered in the telencephalic dorsal midline of Xt^J/Xt^J embryos. While *Fgf8* is ectopically activated in the trp, Bmp signalling is negatively affected by the *Gli3* mutation as judged by the loss of *Bmp4* and *Msx1* expression and by the maintenance of *noggin* expression. Interestingly, Fgf8 and Bmp2/Bmp4 were

shown to act antagonistically on cell proliferation and differentiation in the dorsal forebrain (Furuta et al., 1997). Ectopic *Fgf8* expression and loss of Bmp signalling in the roof plate as observed in Xt^J/Xt^J embryos might therefore disrupt the balance between these two processes. The maintenance of *Bf1* expression and of the proliferative characteristics of the telencephalon as well as the disorganisation of trp in Xt^J/Xt^J embryos at later stages are consistent with this idea. Although this consideration suggests these alterations to be important for development of the trp support for this proposed role cannot be obtained from a loss-of-function approach. The $Fgf8^{-/-}$ mutant dies during gastrulation (Meyers et al., 1998) and *Bmp4* homozygous mutants only survive to E9.5 (Winnier et al., 1995) Therefore, the possibility remains that the altered *Fgf8* and *Bmp4* expression patterns might be secondary to changes in dorsal telencephalon development. In Xt^J/Xt^J embryos, the trp might not be specified so that their derivatives, the medial pallium plus choroid plexus, would never develop. Accordingly, *Fgf8* expression would expand caudally following an expansion of ventral midline tissue while the lack of *Bmp4* expression might be due to a failure to specify dorsal telencephalic cells. To distinguish between these alternatives, it will be necessary to generate *Fgf8* and *Bmp4* mutants with a specific deletion of their forebrain expression domains.

In addition to Bmps, several *Wnt* genes have been implicated in the control of telencephalic choroid plexus formation. *Wnt2b*, *Wnt3a* and *Wnt5a* are expressed in the boundary region between the developing hippocampus and the choroid plexus and this expression domain is specifically lost in E10.5 Xt^J/Xt^J embryos (Grove et al., 1998). Since earlier *Wnt* gene expression patterns are still to be analysed, the possibility remains that this loss of *Wnt* gene expression is secondary to alterations in Bmp signalling that we already observe at E9.5. Nevertheless, interactions between *Bmp* and *Wnt* genes are likely to be important for dorsal telencephalon development. The identification of potential *Gli3*-dependent interactions between *Wnt* and *Bmp* genes during medial telencephalon development requires a detailed time-course analysis of their expression patterns in Xt^J/Xt^J embryos.

Emx$2^{-/-}$ mice also lack the choroid plexus in the lateral ventricle due to a lateral shift of the pallio-choroidal boundary towards the *Emx*1 expression domain and a corresponding expansion of the roof (Yoshida et al., 1997). Altered *Wnt1* and *noggin* expression patterns are suggestive of a diencephalisation of the trp. These changes in choroid plexus development of *Emx*$2^{-/-}$ mice appear to be different from the alterations in Xt^J/Xt^J embryos. Although it is difficult to morphologically define the boundaries of the roof in *Gli3* mutant embryos, expression of *Fgf8* and *noggin* remain dorsoventrally restricted suggesting that the roof has not expanded. Furthermore, *Wnt1* and *noggin* expression remain unaltered in Xt^J/Xt^J embryos. The alterations in the *Fgf8* and *Bmp4* expression patterns rather suggest an anteriorisation of the trp. Taken together, this data indicates that *Gli3* controls other pathways than *Emx*2 during development of the choroid plexus.

A novel, *Shh*-independent function of *Gli3* in dorsal forebrain development

Despite its expression in the dorsal neural tube along the whole neuraxis, mutation of the *Gli3* gene only results in dorsal forebrain alterations. This discrepancy between *Gli3* expression and spatially restricted defects might be explained by functional redundancy with other *Gli* family members (Mo et al., 1997). *Gli2* and *Gli3* expression overlap within the dorsal neural tube suggesting that *Gli2* may compensate for the loss of *Gli3* at midbrain, hindbrain and spinal cord levels but not in the forebrain. Differences in the relative temporal and spatial expression patterns of both genes may account for the regionally restricted alterations. Alternatively, *Gli3* function in the forebrain might require interaction with forebrain-specific factors. The identification of such partners will shed further light on the molecular mechanisms by which *Gli3* controls forebrain development.

Gli3 was previously shown to act as a negative regulator of Shh-mediated patterning processes by repressing the expression of *Shh* itself and of Shh target genes suggesting that the forebrain alterations in Xt^J/Xt^J mice might result from a ventralisation of the dorsal forebrain. However, several observations contradict this interpretation. Morphological defects were only observed in the dorsal telencephalon while the ventral telencephalon appeared unaffected. Furthermore, neither *Shh* nor known Shh target genes were ectopically activated in the dorsal forebrain indicating that *Gli2* and/or other yet unknown factors might compensate for the loss of a *Gli3* repressor function in the forebrain. Nevertheless, these factors cannot substitute for a loss of *Gli3* in dorsal forebrain patterning. Therefore, *Gli3* fulfills a novel, *Shh*-independent function.

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