The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice

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SUMMARY

In the developing vertebrate CNS, members of the Wnt gene family are characteristically expressed at signaling centers that pattern adjacent parts of the neural tube. To identify candidate signaling centers in the telencephalon, we isolated Wnt gene fragments from cDNA derived from embryonic mouse telencephalon. In situ hybridization experiments demonstrate that one of the isolated Wnt genes, Wnt7a, is broadly expressed in the embryonic telencephalon. By contrast, three others, Wnt3a, 5a and a novel mouse Wnt gene, Wnt2b, are expressed only at the medial edge of the telencephalon, defining the hem of the cerebral cortex.

The Wnt-rich cortical hem is a transient, neuron-containing, neuroepithelial structure that forms a boundary between the hippocampus and the telencephalic choroid plexus epithelium (CPe) throughout their embryonic development. Indicating a close developmental relationship between the cortical hem and the CPe, Wnt gene expression is upregulated in the cortical hem both before and just as the CPe begins to form, and persists until birth. In addition, although the cortical hem does not show features of differentiated CPe, such as expression of transthyretin mRNA, the CPe and cortical hem are linked by shared expression of members of the Bmp and Msx gene families.

In the extra-toes (XtJ) mouse mutant, telencephalic CPe fails to develop. We show that Wnt gene expression is deficient at the cortical hem in XtJ/XtJ mice, but that the expression of other telencephalic developmental control genes, including Wnt7a, is maintained. The XtJ mutant carries a deletion in Gli3, a vertebrate homolog of the Drosophila gene cubitus interruptus (ci), which encodes a transcriptional regulator of the Drosophila Wnt gene, wingless. Our observations indicate that Gli3 participates in Wnt gene regulation in the vertebrate telencephalon, and suggest that the loss of telencephalic choroid plexus in XtJ mice is due to defects in the cortical hem that include Wnt gene misregulation.

Key words: Choroid plexus, Cortical hem, Extra-toes, Gli3, Telencephalon, Wnt2b, Mouse

INTRODUCTION

The telencephalon is the largest, most complex part of the mammalian CNS (Nauta and Feirtag, 1986), and must be patterned during development into numerous functional subdivisions. Patterning of the developing telencephalon requires the division of the cerebral cortex into different types of cortex, such as archicortex and neocortex, and the subdivision of these large cortical regions into many functionally specialized areas. Broader divisions include those between cerebral cortex and subcortical nuclei, and between two strikingly different types of tissue that develop in the medial wall of the telencephalon, the medial cerebral cortex and the non-neuronal, secretory epithelium of the choroid plexus (CPe). What are the mechanisms by which these divisions are set up in the developing telencephalon? One approach to this question is to isolate from the embryonic telencephalon members of developmental control gene families implicated in patternning elsewhere in the embryo.

The Wnt family of developmental control genes encodes secreted proteins that participate in tissue patterning and morphogenesis (Parr and McMahon, 1994). The extent to which Wnt proteins operate as morphogens themselves, directly acting to pattern adjacent tissues, or serve to establish local signaling centers from which other morphogens act, remains uncertain (Zecca et al., 1996). Nonetheless, in both vertebrate and invertebrate species, Wnt gene expression marks sites of morphogenetic signaling, by appearing at boundaries between developmental compartments and at the edges of morphogenetic fields (Lawrence and Struhl, 1996; Parr et al., 1993). In Drosophila development, expression of wingless, which is the canonical member of the Wnt gene family, distinguishes the boundaries between parasegments, the border between dorsal and ventral compartments in the wing imaginal disc, and the dorsal and ventral perimeters of the developing optic lobe (Diaz-Benjumea and Cohen, 1995; Kaphingst and Kunes, 1994). In vertebrate development, Wnt gene expression marks the sites of neuroectoderm signaling centers that control
dorsal-ventral patterning in the spinal cord, and rostral-caudal patterning at the junction of the midbrain and hindbrain (Bally-Cuif et al., 1995; Parr et al., 1993).

To gain insight into how the telencephalon is patterned, we searched for members of the Wnt gene family whose expression might mark signaling centers within the embryonic telencephalon. A well-established PCR procedure (Gavin et al., 1990) was used to isolate several members of the Wnt gene family, including the previously unreported mouse ortholog of the human WNT13 gene (Katoh et al., 1996), from embryonic day 12.5 (E12.5) mouse telencephalon. E12.5 is early in the growth and development of the telencephalon, when macroscopic patterning is likely to be still underway. In situ hybridization was employed to determine the patterns of expression of the isolated Wnt genes within the embryonic telencephalon. Three Wnt genes, including the novel mouse Wnt gene, were found to be expressed selectively at the medial margin of the telencephalon, defining a zone that we term the Wnt-rich ‘cortical hem’.

The Wnt-rich cortical hem forms a boundary between two major components of the medial telencephalon throughout their embryonic development. The hippocampus, a part of the medial cerebral cortex, develops dorsal to the cortical hem, and the CPe differentiates ventral to the cortical hem. By birth, much of the growth and basic patterning of both the CPe and hippocampus is complete (Sturrock, 1979; Tole et al., 1997), and the cortical hem, as defined by multiple Wnt gene expression, disappears. The cortical hem is therefore positioned to provide patterning signals to both the developing hippocampus and the choroid plexus. In the present study, we have focused on the developmental relationship of the cortical hem to the telencephalic CPe.

Previous histological studies indicate that the CPe is generated from a specialized part of the neuroepithelium of the medial telencephalon that is distinguished from the rest of the telencephalic neuroepithelium by becoming progressively effaced (MacKenzie et al., 1991; Maruyama and D’Agostino, 1967; Nicholson-Flynn et al., 1996; Sturrock, 1979; Zaki, 1981). The presumptive CPe, or ‘choroid plaque’, first appears at the dorsal midline of the telencephalic vesicle as the midline invaginates to form the medial walls of the two telencephalic hemispheres (Sturrock, 1979). At E10.5 in the mouse, the choroid plaque is a small zone of thinning neuroepithelium at the midline, identifiable by the presence of many pyknotic cells (Sturrock, 1979; Zaki, 1981). As the medial walls continue to invaginate, differentiated CPe appears in the position of the choroid plaque. On either side of the plaque, a part of the medial wall of each hemisphere also begins to show evidence of cell death and to thin (Furuta et al., 1997; Sturrock, 1979). CPe continues to ramify ventral to the region of thinning neuroepithelium, until, by birth, the CPe is histologically mature (Sturrock, 1979), and the region of thinning neuroepithelium has disappeared.

In the present study, we show that the region of thinning neuroepithelium in the medial telencephalon is identical with the Wnt-rich cortical hem. Although several other genes and gene products, such as members of the Bmp and Msx gene families, as well as high molecular mass tropomysins (Furuta et al., 1997; Nicholson-Flynn et al., 1996; MacKenzie et al., 1991, 1992; present study), are expressed in the cortical hem, they are each expressed elsewhere in the medial telencephalic wall as well. To date, only the expression pattern of multiple Wnt genes uniquely distinguishes the cortical hem – the part of the neuroepithelium suggested by histological studies to participate in generating the CPe. These observations suggest that Wnt signaling could play a specific role in the initial division of the medial wall neuroepithelium into a part that generates the medial cerebral cortex, and another part that forms the CPe.

Franz (1994) has reported that the extra-toes mutant mouse does not develop telencephalic choroid plexus, and that this appears to be due directly to the extra-toes mutation rather than indirectly to general forebrain dysmorphology. Intriguingly, the extra-toes mutant carries an intragenic deletion of a gene, Gli3 (Hui and Joyner, 1993; Schimmang et al., 1992), that is homologous to a Drosophila gene implicated in the regulation of wingless. In Drosophila, the zinc-finger transcription factor, cubitus interruptus (ci), is a transcriptional regulator of wingless, mediating response to a hedgehog signal (Von Ohlen et al., 1997). In vertebrates, three ci homologs, Gli, Gli2 and Gli3, have been identified (Hui et al., 1994; Orenic et al., 1990; Ruppert et al., 1990), one of which, Gli, has been shown to be a target of sonic hedgehog signaling (Lee et al., 1997). In an analysis of the extra-toes mutant mouse, we have tested the hypotheses that Gli3 may participate in Wnt gene regulation in the vertebrate telencephalon, and that the defect in telencephalic choroid plexus in extra-toes mice is accompanied by Wnt gene misregulation in the cortical hem.

MATERIALS AND METHODS

Mice

Outbred CD-1 timed pregnant mice were obtained from the University of Chicago Cancer Research Center Transgenic Facility. Xl+/+ mutant mice in a C3HeB/FeJ background were obtained as homozygotes from the Jackson Laboratory (Bar Harbor, ME), and were interbred. Midday of the day of vaginal plug discovery was considered embryonic (E) day 0.5. Homozygous Xl+/+ embryos and their littermates were recovered for gene expression analysis from Xl+/+ intercrosses at E10.5, E12.5 or E16.5. homozygote embryos were readily distinguished from homozygote and wild-type embryos by their appearance (Hui and Joyner, 1993; Johnson, 1967). We checked our classifications by processing selected litters for whole-mount in situ hybridization demonstrating Gli3 gene expression, which proved to be undetectable in phenotypically homozygote embryos and present in heterozygote and wild-type animals (data not shown). 20% of the embryos recovered at E12.5, and 16% of those recovered at E16.5, were classified as homozygote (Table 1). That these percentages are slightly lower than 25% may be due to early lethality of the Xl+ mutation; consistent with this interpretation, many embryos (21 in 32 litters) appeared to be in the process of being resorbed at the time of killing (these embryos were not included in the total number recovered).

Isolation of telencephalic Wnt gene fragments

cDNA was prepared from E12.5 telencephalon total RNA isolated by guanidinium-acid phenol extraction and employed as substrate for the isolation of telencephalic Wnt gene fragments. In the present study, we show that the region of thinning neuroepithelium in the medial telencephalon is identical with the Wnt-rich cortical hem. Although several other genes and gene products, such as members of the Bmp and Msx gene families, as well as high molecular mass tropomysins (Furuta et al., 1997; Nicholson-Flynn et al., 1996; MacKenzie et al., 1991, 1992; present study), are expressed in the cortical hem, they are each expressed elsewhere in the medial telencephalic wall as well. To date, only the expression pattern of multiple Wnt genes uniquely distinguishes the cortical hem – the part of the neuroepithelium suggested by histological studies to participate in generating the CPe. These observations suggest that Wnt signaling could play a specific role in the initial division of the medial wall neuroepithelium into a part that generates the medial cerebral cortex, and another part that forms the CPe.

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Wnt gene fragment PCR amplification scheme of Gavin et al. (1990). Gel-purified PCR products were subcloned into EcoRI-XbaI-digested pBluescript II KS(+) plasmid (Stratagene). 45 recombinant clones were sorted by HaeIII, HindIII and RsaI fingerprinting, and representatives of each group were sequenced. Five distinct mouse Wnt genes were identified, expression of one of which (Wnt4) was not reproducibly detected with in situ hybridization experiments on E12.5 telencephalon. Wnt7b, which is expressed in the early embryonic telencephalon (Parr et al., 1993), was not among the Wnt genes recovered, suggesting that our screen of telencephalic Wnt genes was not exhaustive.

One of the recovered fragments identified a novel mouse Wnt gene, Wnt2b. This fragment was employed in high-stringency cDNA library screens (E12.5 mouse λEX10 library, Novagen; E11.5 mouse λgt10 library, Clontech) as previously described (Ragsdale et al., 1989). Nucleotide sequencing was done by the dideoxynucleotide method (Sequenase kit, US Biochemical) and with the Applied Biosystems Prism 377 and 377XL DNA sequencers (University of Chicago Cancer Research Center), and was analyzed with GeneWorks software.

**Histology**

Harvested mouse embryos were immersed in 4% paraformaldehyde in phosphate-buffered saline and processed for two-color wholemount non-radioactive in situ hybridization with a modification of the method of Nieto et al. (1996). Significant changes in the protocol include replacement of proteinase K digestion with detergent treatment (Rosen and Beddington, 1993) and use of the chromagens nitroblue tetrazolium (Boehringer; 350 μg/ml) and tetranitroblue tetrazolium (Sigma; 350 μg/ml), which were selected from a range of chromagens tested for sensitivity and color separation (T. A. Sanders and C. W. Ragsdale, unpublished data). Some embryo brains were cryoprotected after fixation, sectioned into 40 μm coronal sections using a sledge microtome (Leica), and processed for in situ hybridization using a method described previously (Tole et al., 1997; Tole and Patterson, 1995).

Riboprobes incorporating digoxigenin- or fluorescein-labeled nucleotides were synthesized from linearized plasmids with T7 or SP6 polymerase (Boehringer). Probes for Wnt3a, Wnt5a and Wnt7a were derived from the subcloned PCR fragments. Wnt2b gene expression was demonstrated with cDNA clone pRK (1 kb insert in pEX10 vector; linearization by EcoRI digestion, antisense riboprobe transcription with SP6 polymerase). Class III β-tubulin gene expression was detected with cDNA clone p82-2, in which a 321 bp insert derived from a 3’ untranslated region of the mouse β-tubulin gene (Burgoyne et al., 1988) was subcloned in the pBluescript II SK(+) vector (BanHI digestion, T7 transcription). Other probes employed were derived from a 1 kb mouse Gli2 cDNA, a 0.8 kb mouse Gli3 cDNA (Hui et al., 1994), a 0.6 kb rat transferrin cDNA (Duan et al., 1989), a 2.2 kb mouse neurogenin2 genomic fragment (Sommer et al., 1996), a 0.7 kb mouse Mx1 cDNA, a 0.85 kb mouse Mx2 cDNA (MacKenzie et al., 1991, 1992), a 1.2 kb mouse Bmp2 cDNA, a 2 kb mouse Bmp6 cDNA, a 0.8 kb mouse Bmp7 cDNA (Furuta et al., 1997), a mouse Bmp4 cDNA (IMAGE Consortium, GenBank number AA473799), and a 0.8 kb mouse Fgfr8 cDNA (Crossley and Martin, 1995).

Dividing cells in mouse embryos were labeled with 5-bromo-2′-deoxyuridine (BrDU) (100 mg/kg) delivered intraperitoneally to pregnant mice 2 hours before killing. Nucleotide incorporation in tissue sections was detected with antibody M0744 (DAKO) followed by dianimobenzidine peroxidase immunohistochemistry.

**RESULTS**

**Multiple Wnt genes define a boundary zone between the developing hippocampus and the choroid plexus**

To identify members of the Wnt gene family that are specifically expressed in the developing telencephalon, a PCR procedure (Gavin et al., 1990) was employed to isolate Wnt gene fragments from cDNA derived from the cerebral hemispheres of E12.5 CD-1 mice. Sequencing the PCR products showed that cDNA fragments from five different Wnt genes had been isolated: Wnt3a, 4, 5a, 7a and a previously unreported mouse Wnt gene. cDNA library screens employing the novel Wnt gene fragment yielded overlapping cDNA clones providing 711 base pairs of coding sequence (GenBank database accession number AF038384). Sequence comparisons establish that this novel mouse Wnt gene is the ortholog of the recently described human WNT13 gene (Katoh et al., 1996), with 98% identity over the 236 amino acid C-terminal fragment for which we have sequence data. Like human WNT13, this mouse Wnt gene is closely related to the Wnt2 subfamily, with amino acid identity scores of 69% to mouse Wnt2 (McMahon and McMahon, 1989) and 79% to Xenopus Wnt2b (Landesman and Sokol, 1997). Following the Wnt gene nomenclature suggestions of Cadigan and Nusse (1997) we identify this new mouse Wnt gene as mouse Wnt2b.

In situ hybridization experiments showed that four of the isolated Wnt genes, Wnt2b, 3a, 5a and 7a, are strongly expressed in E12.5 telencephalon. Moreover, three Wnt genes, Wnt2b, 3a and 5a, show a striking, similar pattern of expression in the telencephalon. The expression of Wnt2b, representative of the three, is shown in Fig. 1. Wnt2b is strongly expressed in a band of tissue along the medial telencephalic wall, adjacent to the lateral ventricle (Fig. 1A). Views of the medial face of the telencephalic hemisphere (Fig. 1B) or coronal sections through the telencephalon (Fig. 1D-F) show the close association of the Wnt2b expressing zone with the newly forming CPe of the lateral ventricle (Fig. 1B,D). The band of Wnt2b expression is dorsal to the forming CPe at rostral levels (Fig. 1B,D), and curves ventrally at caudal levels to surround the caudal end of the CPe (Fig. 1B,E,F). Expression of Wnt3a surrounding the developing telencephalic CP has been reported previously (Roelink and Nusse, 1991); we found that both Wnt3a and 5a show the same characteristic curved band of expression along the medial face of the telencephalon as Wnt2b (Fig. 3A,H). Multiple Wnt genes are therefore expressed at the continuous, curving line of attachment between the differentiating CPe and adjacent telencephalic neuroepithelium.

The same zone of tissue that is delineated by the expression of Wnt2b, 3a and 5a is also distinguished by the absence of detectable expression of a fourth isolated Wnt gene, Wnt7a. At E12.5, Wnt7a is strongly expressed in the lateral and dorsal cerebral cortex (Fig. 1C), but not at the medial margin of the cerebral hemisphere (Fig. 1G). Expression of Wnt7a thus appears complementary to that of Wnt2b, 3a and 5a.

**The Wnt-rich boundary tissue is neuron-containing neuroepithelium and forms the cortical hem**

What type of tissue makes up the Wnt-rich boundary zone between the differentiating CPe and adjacent neuroepithelium? Is it CPe at an early stage of differentiation, cortical neuroepithelium or a third type of tissue? Differentiating CPe forms a simple columnar epithelium, then matures into cuboidal epithelium (Sturrock, 1979). Cortical neuroepithelium is a pseudostratified epithelium in which the nuclei of dividing cells translocate within the ventricular zone.
Fig. 1. The cortical hem is marked by the complementary expression of Wnt2b and 7a. (A) Dorsal view of a CD-1 mouse forebrain at E12.5. Rostral is to the left. Wnt2b is strongly expressed along the caudal two-thirds of the medial wall of the telencephalon (t), next to the lateral ventricle. In the diencephalon (d), Wnt2b expression marks the dorsal midline, and two patches on either side of the midline. (B-D,F) A medial view of a telencephalic hemisphere at E12.5 (B, rostral is to the left) and coronal sections through a similar hemisphere (D, midline is to the left). Wnt2b expression defines the hem of the embryonic cerebral cortex (B), and abuts the developing choroid plexus epithelium (cpe) dorsally (B,E), caudally (B,F), and caudoventrally (B,E). (C,G) Dorsal view of a mouse forebrain at E12.5 (C, midline to the left). Wnt7a is expressed in most of the embryonic cerebral cortex (C), but not in the cortical hem (hem, arrows in G). Bar in A, 550 μm (A); 420 μm (B); 700 μm (C); 130 μm (D); 110 μm (E); 170 μm (F); 130 μm (G).

At E12.5, cells expressing Wnt2b, 3a and 5a at the boundary between the cortex and CPe do not form a simple columnar or cuboidal epithelium, nor do they express TTR (Fig. 2A,C). Like adjacent cortical neuroepithelium, the Wnt-rich tissue is organized as a pseudostratified epithelium, in which dividing cells labeled with BrdU form a broad VZ (Fig. 2G). At E12.5, moreover, the Wnt-rich tissue contains a preplate-like layer of neurons that express class III β-tubulin (Fig. 2F). Finally, expression of the putative neuronal determination factor, neurogenin2 (Ngn2)/MATH4A (Gradwohl et al., 1996; Sommer et al., 1996) distinguishes the VZ of the entire cerebral cortical neuroepithelium, including the Wnt-rich tissue, but avoids the CPe (Fig. 2B,D). The Wnt-rich tissue is therefore embryonic cortical neuroepithelium, rather than CPe, and thus represents the hem of the developing cerebral cortex.

Between the Wnt-rich cortical hem and the CPe, a junctional epithelium, a few cells wide, can be identified. Junctional epithelium does not express, or expresses weakly, TTR, Wnt2b, 3a, 5a and Ngn2 (Fig. 2B,C, and data not shown), and does not contain a VZ or preplate (data not shown). Thus, at E12.5, four tissues can be identified in the medial wall of the telencephalon.
with respect to the morphological and molecular features indicated in Fig. 2. Moving from dorsal to ventral, these are: embryonic cerebral cortex (Ngn2+, Wnt2b/3a/5a−, TTR−, neuron-containing neuroepithelium), the cortical hem (Ngn2+, Wnt2b/3a/5a+, TTR−, neuron-containing neuroepithelium), junctional epithelium (Ngn2−, Wnt2b/3a/5a−, TTR− epithelium) and CPe (Ngn2−, Wnt2b/3a/5a−, TTR+, columnar epithelium).

The Wnt-rich cortical hem shows subtle differences with neighboring embryonic cortex. The cortical hem becomes progressively thinner than adjacent neuroepithelium (Fig. 5A), as described previously for the neuroepithelium that gives rise to CPe (Sturrock, 1979). Suggesting that fewer neurons are generated in the cortical hem at E12.5 compared with adjacent cortex, or that neurons are being removed by cell death, class III β-tubulin expression is weaker in the cortical hem than in adjacent cortex (data not shown), and cells immunoreactive for MAP2, another neuronal marker, could not be detected in the same region in the rat at a comparable embryonic age (Nicholson-Flynn et al., 1996). Perhaps the most dramatic difference between the cortical hem and adjacent cortex, however, is that the former shares with the developing CPe the expression of several members of the Msx and Bmp gene families.

**The cortical hem expresses genes implicated in epithelial/ mesenchymal inductive interactions**

Early in choroid plexus morphogenesis, head mesenchyme cells invade the developing CPe to form the second, mesenchymal or stromal layer of the choroid plexus (CPm) (Birge, 1961; Sturrock, 1979). The invasion by mesenchyme appears to be one of the motive forces that pushes the CPe out

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**Fig. 3.** The cortical hem and choroid plexus epithelium share strong expression of Bmp and Msx genes, but not of Wnt genes. Medial views of telencephalic hemispheres at E12.5 (A-D,H), rostral to the left, and coronal sections through E12.5 hemispheres (E-G,I, medial to the left).

(A-C,H) Expression of Wnts3a, Msx2, Bmp6 and Wnts5a marks the same curved band of tissue in the medial telencephalon, the cortical hem.

(B,F) Msx2 is additionally expressed in the choroid plexus epithelium (cpe, arrow in B).

(D,E,G) Bmp7 and Msx1 are expressed strongly in the cpe (arrow in D), and in the ventral part of the cortical hem. (H,I) Wnts5a is additionally expressed in the mesenchymal cells (arrows) that are invading the medial wall of the telencephalon to form the stromal layer of the choroid plexus. Bar in A, 550 μm (A-D,H); 140 μm (E,F); 70 μm (G,I).

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**Fig. 4.** Wnt gene expression is upregulated in the cortical hem both before, and just as, choroid plexus begins to form. Dorsal views of forebrain at E10.5 (A-C) and E11.5 (D-F). Coronal section through midline of telencephalon at E10.5 (G). E10.5 whole embryo (H).

(A-H) In the medial wall of the telencephalon at E10.5, Wnts3a is already expressed strongly in the cortical hem (A,G), but no TTR-expressing choroid plexus epithelium is detectable (B), and no Wnt2b is expressed (C,H). Wnt2b is already expressed at the dorsal midline of the diencephalon and midbrain (C,H). The choroid plaque is evident at the midline of the telencephalon (between arrowheads in G), and expresses Wnts3a only weakly. A day later, at E11.5, TTR expression marks differentiating telencephalic choroid plexus epithelium (E), and Wnts5a and 2b expression has been upregulated in the hem (D,F). (H) Sites of strong Wnt2b expression at E10.5 include the optic cup (oc), and the otic vesicle (ov). Bar in G, 380 μm (A-C); 540 μm (D-F); 70 μm (G); 900 μm (H).
into the ventricles (Birge, 1961, 1962). Further, inductive interactions between neuroepithelium and invading head mesenchyme appear to be required for at least some aspects of CPe differentiation (Birge, 1961, 1962; Cavallaro et al., 1993). We accordingly sought evidence that the Wnt-rich cortical hem is involved in such interactions. Members of the Msx and Bmp gene families are characteristically expressed at sites of epithelial/mesenchymal interactions elsewhere in the embryo, including the developing kidney and tooth (MacKenzie et al., 1991, 1992; Thesleff et al., 1995). Moreover, expression of both gene families has been previously reported in the dorsal and medial telencephalon between E9.5 and E13.5 (Furuta et al., 1997; MacKenzie et al., 1991). We therefore examined the expression of Msx1 and 2, and Bmp4, 6 and 7 with respect to the Wnt-rich cortical hem.

At E12.5, Msx2 and Bmp6 show the same characteristic curved band of strong expression along the medial face of the telencephalon as Wnt2b, 3a and 5a (Figs 1B, 3A-C,H), neatly distinguishing the cortical hem from adjacent embryonic cortex. Msx1 and Bmp4 and 7 are also expressed in the cortical hem, but strong expression is restricted to the ventral part of the hem (Fig. 3D,E,G, and data not shown). At E12.5, Msx1 and 2, and Bmp4, 6 and 7 are also expressed in the CPe itself (Fig. 3B-G, and data not shown), the junctional epithelium (Fig. 3E-G, and data not shown), and the head mesenchyme that invades the CPe to form the CPm (MacKenzie et al., 1991, 1992; Furuta et al., 1997; data not shown). By contrast, among the Wnt genes examined, only Wnt5a was detected in the CP. Wnt5a expression appears in the CPm, but not the CPe (Fig. 3H,I). Thus, whereas the expression of multiple Wnt genes uniquely distinguishes the cortical hem, the more extensive expression of Bmp and Msx genes in the cortical hem and CPe suggests a close relationship between the two tissues, and implicates the cortical hem in the inductive interactions that shape development of the choroid plexus.

The cortical hem is detectable by Wnt gene expression before telencephalic CPe appears, and persists throughout CPe morphogenesis

The temporal pattern of Wnt gene expression in the medial telencephalon supports the involvement of Wnt genes in the formation of two different tissue districts in the medial wall. At E10.5, the dorsal midline of the telencephalon has just begun to invaginate, creating the medial walls of the two telencephalic hemispheres (Fig. 4). No differentiated CPe can be detected by TTR expression in the medial wall at E10.5 (Fig. 4B), but Wnt3a is already strongly expressed in a medial band marking the cortical hem (Fig. 4A,G). The choroid plate at the telencephalic midline (between arrowheads in Fig. 4G) expresses Wnt3a, but weakly compared with the cortical hem. By E11.5, telencephalic CPe has begun to express TTR (Fig. 4E), and the cortical hem is now delineated by the expression of the three Wnt genes, Wnt2b, 3a and 5a (Fig. 4D,F). A previous study reported the expression of Wnt3a along the dorsal midline of the telencephalon as early as E9.5 (Parr et al., 1993). In the present study, however, we relate the expression of Wnt3a and two other Wnt genes to the onset of differentiation of the CPe. We find that both before, and just as the CPe begins to differentiate in the telencephalon, Wnt gene expression is upregulated in the immediately adjacent cortical hem.

Comparisons of gene expression patterns at E10.5 and E12.5 (Figs 3 and 4) refine the subdivisions that can be identified in the medial wall of the developing telencephalon. First, differentiating CPe is identifiable by its strong expression of TTR, and is thereby distinguished from the choroid plaque and its probable continuation, the junctional epithelium. The latter two divisions are likely to contain precursor cells that directly generate the CPe (Maruyama and D’Agostino, 1967; Sturrock, 1979; Zaki, 1981). Second, strong expression of Wnt genes uniquely distinguishes the cortical hem from adjacent cortical neuroepithelium, CPe, junctional epithelium and the choroid plaque. Third, there may be subdivisions within the cortical hem itself. Although Bmp6 and Msx2 are expressed throughout the cortical hem, Bmp4, 7 and Msx1 are strongly expressed only in the ventral part that adjoins the CPe, suggesting a difference between ventral and dorsal parts of the cortical hem.

As choroid plexus morphogenesis continues, the cortical hem, as defined by the expression of multiple Wnt genes, maintains its position relative to the developing CPe, but shrinks. Thus, by E15.5-16.5, overlapping expression of Wnt2b, 3a and 5a marks a few cells along the lateral margin of the hippocampal fimbria-fornix (Fig. 5B,D), which remains the dorsal point of attachment of the CPe to the neuroepithelium. At birth, when CPe is histologically mature (Sturrock, 1979), intense expression of multiple Wnt genes next to the CPe has disappeared (data not shown). Likewise, the territory of expression of Msx2 and Bmp6, two other markers of the cortical hem, shrinks as CPe matures (data not shown).

The expression in the medial telencephalon of Wnt3a and several Bmp and Msx genes has previously been described as marking prospective archicortex, or hippocampus, as well as choroid plexus (Furuta et al., 1997; Roelink and Nusse, 1991; Yoshida et al., 1997). However, by the age at which a hippocampal anlage is identifiable by morphology (about E14.5) the Wnt-rich cortical hem is clearly separate from regions of the neuroepithelium thought to generate hippocampal neurons (Fig. 5A) (Altman and Bayer, 1990).

As an exception to the circumscribed expression of multiple Wnt genes in the cortical hem, Wnt3a is newly expressed outside the cortical hem as the medial telencephalon matures (Fig. 5C,D). At E13.5, Wnt5a is expressed in the cortical plate of the entire medial cerebral cortex, which includes the hippocampus and adjacent limbic cortical areas (Fig. 5C). Subsequently, Wnt5a expression retreats back along the medial telencephalic wall, and by E16.5 is largely confined to the hippocampal dentate gyrus (Fig. 5D). Expression of Wnt5a in these neuronal cell layers implies that Wnt5a is expressed in postmitotic neurons. Telencephalic Wnt5a expression is, therefore, broader than that of Wnt2b and 3a, marking not only the cortical hem, but also the CPm, and developing neurons of the medial cortex. Similar to Wnt3, which is implicated in several stages of cerebellar development (Salinas et al., 1994), Wnt5a may play a variety of roles in the development and differentiation of the medial telencephalon.

Expression of Wnt2b at other sites in the embryonic nervous system

Choroid plexus also develops in the hindbrain, where CPe differentiates by E10.5 (Thomas and Dziadek, 1993). Wnt1 and Wnt3a are expressed next to the hindbrain site of choroid plexus generation from E9.5 onwards (Parr et al., 1993), and
Wnt2b is more weakly expressed in the same region by E11.5 (data not shown). Expression of Wnt2b elsewhere in part resembles that of Wnt3a (Roelink and Nusse, 1991). At E10.5, Wnt2b, like Wnt3a, is expressed at the dorsal midline of the neural tube and in the otic vesicle (Fig. 4H). However, at E10.5, Wnt2b expression at the dorsal midline of the neural tube does not continue caudal to the isthmus (Fig. 4H), whereas Wnt3a expression extends into the spinal cord (Roelink and Nusse, 1991). Further, Wnt2b, unlike Wnt3a, appears to function in the developing eye. By E10.5 Wnt2b is expressed in the pigmented epithelium of the retina (Fig. 4H). Other sites of Wnt2b expression include the nasal epithelium, and a part of the diencephalon (Fig. 1A, and data not shown.)

**Wnt2b, 3a and 5a expression is deficient in the telencephalon of the extra-toes mutant, and telencephalic CPe fails to form**

Homozygous XtJ embryos and their littermates were recovered from XtJ/+ intercrosses at E10.5, E12.5 or E16.5, ages that span the period of normal telencephalic choroid plexus development. Consistent with a previous report (Hui and Joyner, 1993), no Gli3 expression was detectable by in situ hybridization in XtJ/XtJ embryos. By contrast, in wild-type CD-1 or C3H mice between E9.5 and E12.5, Gli3 is readily detected throughout telencephalic neuroepithelium (Fig. 6A,B and data not shown), including the cortical hem, but not the choroid plexus epithelium (Fig. 6B and data not shown). Thus, Gli3 is expressed appropriately to affect the development of the cortical hem.

Consistent with previous descriptions of the Harwell strain of extra-toes mice, many XtJ/XtJ embryos (28/43 XtJ/XtJ embryos recovered) showed an exencephaly, probably due to delayed closure of the anterior neural tube (Franz, 1994; Johnson, 1967). In exencephalic embryos, a massive overgrowth of the midbrain partially enveloped the forebrain, and the morphology of the telencephalon was severely disrupted. Also consistent with previous descriptions (Franz, 1994), however, about one third of XtJ/XtJ embryos (15/43 recovered) showed no exencephaly, and no marked overgrowth of the midbrain.

At E12.5, the telencephalon appeared smaller than normal in non-exencephalic XtJ/XtJ mice (Fig. 7A-D), but showed several normal features of morphology and gene expression. For example, the embryonic cerebral cortex of non-exencephalic XtJ/XtJ mice was defined, as in wild-type animals, by the strong expression of Ngn2 (data not shown) and class III β-tubulin (Fig. 7E), and the formation of a neuronal preplate (Fig. 7E). Further, the dorsal midline had begun to invaginate to form the medial walls of the telencephalic hemispheres (Fig. 7E). Invagination at E12.5 appeared less complete than in wild-type mice, so that in most XtJ/XtJ embryos, the two medial walls of the telencephalon did not appose one another at the dorsal midline (compare Figs 1A,C and 7B,D). Instead, the original roof of the telencephalon formed a broad ‘bridge’ region between the two hemispheres (marked ‘b’ in Fig. 7B; see also Fig. 7D). A somewhat similar morphology has been described in mice deficient in Emx2 expression (Yoshida et al., 1997). To control for the effects of grossly abnormal brain morphology on telencephalic development, we compared exencephalic and non-exencephalic XtJ/XtJ mice, and present a detailed analysis of non-exencephalic XtJ/XtJ embryos, and 35 littermate controls were assayed with in situ hybridization for expression of Wnt1, 2b, 3a, 5a, 7a, Fgf8 and TTR.

At E12.5, the medial telencephalic wall in non-exencephalic XtJ/XtJ mice was composed of a curved, cortical structure (Fig. 7E), ending in a small wedge of tissue (Fig. 7E-G) that resembled the junctional epithelium at the base of the CPe in wild-type mice. However, no TTR-expressing, cuboidal epithelium extruded from this wedge in any XtJ/XtJ embryo examined (Fig. 7F). Nor could expression of Wnt2b, 3a or 5a be detected in adjacent tissue that might correspond to the cortical hem (Fig. 7G). Expression of Wnt2b, 3a or 5a elsewhere in the telencephalon was either undetectable (Fig. 7A), or weak and diffuse (Fig. 7B). Nonetheless, Wnt2b, 3a and 5a were strongly expressed at other appropriate embryonic sites, such as the otic vesicle (Fig. 7H), or nasal epithelium (data not shown). Wnt1, 2b and 3a were additionally expressed next to the fourth ventricle where TTR-expressing choroid plexus did form in XtJ/XtJ mice (Fig. 7A, and data not shown). TTR, Wnt2b and 3a were also expressed in some mutant mice at the dorsal midline of the diencephalon (Fig. 7A, and data not shown). Thus, although Wnt gene expression was downregulated in the medial telencephalon of XtJ/XtJ mice, it was strikingly maintained at other sites, such as the hindbrain, at which choroid plexus was successfully generated.

The deficiency of Wnt gene expression and the absence of CPe in the medial telencephalon of XtJ/XtJ mice at E12.5 did not appear to represent a simple developmental delay. Five non-exencephalic XtJ/XtJ embryos examined at E16.5 still showed neither CPe, assayed by TTR expression, nor detectable expression of Wnt2b, 3a and 5a in the medial telencephalon (data not shown).

Observations of XtJ/XtJ mice at E12.5 and E16.5 indicate that Wnt gene expression is deficient in the medial telencephalon of the mutant mice during the normal period of CPe formation. In wild-type mice, Wnt3a is expressed earlier, before the onset of CPe differentiation. In XtJ/XtJ mice at E10.5, Wnt3a expression in the medial telencephalon was weak or undetectable (Fig. 7K), indicating that Wnt gene expression is compromised by the time the choroid plaque is forming and the medial telencephalon begins to invaginate.

Finally, observations of exencephalic XtJ/XtJ embryos were consistent with those of non-exencephalic mutants. In 27 exencephalic brains, no telencephalic choroid plexus formed at either E12.5 or E16.5, as assessed by morphology or TTR expression, and no telencephalic expression of Wnt2b, 3a or 5a was detected (data not shown). Exencephalic XtJ homozygotes can show severe malformations of the brainstem as well as forebrain, yet choroid plexus develops in the fourth ventricle and in the diencephalon, and Wnt2b and 3a are expressed at these sites (data not shown).

**Telencephalic expression of Fgf8 and Wnt7a persists in the extra-toes mutant**

The deficiency of Wnt gene expression in the medial telencephalon does not reflect a general failure of developmental control gene expression in the telencephalon of XtJ/XtJ mice. For example, expression of Fgf8, which may be involved in directing regionalization in the forebrain (Shimamura and Rubenstein, 1997), was at least partially maintained in XtJ/XtJ embryos (Fig. 7D). In wild-type mice at E12.5, Fgf8 is expressed in the medial wall of the
The Wnt-rich cortical hem and its relationship to the CPe

In vertebrate development, Wnt gene expression marks signaling centers that regulate patterning in the spinal cord and brainstem (Parr et al., 1993; Bally-Cuif et al., 1995; McMahon and Bradley, 1990). In the present study, we have drawn on this observation to identify a potential source of patterning signals within the embryonic telencephalon. We find that the expression of multiple Wnt genes, including a previously unreported mouse Wnt gene, Wnt2b, marks out a longitudinal strip of neuroepithelium in the medial telencephalon, which we term the cortical hem. The Wnt-rich cortical hem forms the boundary between the developing hippocampus, the most medial part of the cerebral cortex, and the telencephalic choroid plexus. We show that, in the Xl/ mouse mutant, a defect in the cortical hem that includes downregulation of Wnt gene expression is associated with the loss of at least one of these adjacent structures, the choroid plexus. Determining if the hippocampus is also missing or mispatterned in the Xl/ mouse mutant remains for a future study that will employ molecular markers of the hippocampal subfields (Tole et al., 1997).

The Wnt-rich cortical hem is a transient structure, in that it appears to shrink as development proceeds, and cannot be identified by Wnt gene expression in the postnatal animal. The shrinkage of the cortical hem, as defined by gene expression, is likely to be due at least in part to progressive cell loss. Apoptotic cell death is increased, and cell proliferation is decreased in the region of the Wnt-rich cortical hem compared with adjacent cortical neuroepithelium (Furuta et al., 1997; Maruyama and D’Agostino, 1967; Sturrock, 1979; Zaki, 1981). Additionally,
Figure 7. In the medial telencephalon of Xl^+/Xl^+ mice, Wnt gene expression is deficient, and TTR-expressing choroid plexus epithelium does not form. (A-H,J) Brains of non-exencephalic Xl^+/Xl^+ mice at E12.5. (I) Section through the cortical hem and choroid plexus epithelium (cpe) of a wild-type CD-1 mouse brain at E12.5. (K) A wild-type mouse embryo at E10.5 (left), and an Xl^+/Xl^+ littermate (right). (A) Medial views of the two telencephalic hemispheres (t), and lateral view of the brainstem and diencephalon from the same mouse. Rostral is to the left. Wnt2b (purple) and TTR (brown) are expressed in the hindbrain (arrowhead), but not in the telencephalon. TTR expression appears in the diencephalon (d) as well. (B-D) Forebrain in dorsal (B,D) or lateral (C) views, rostral to the left. Wnt5a is severely downregulated in the medial telencephalon (B), but expression of Wnt2a is maintained in the lateral and dorsal telencephalon (C), and Fgf8 expression is maintained in the medial telencephalon (D). Note that in some non-exencephalic Xl^+/Xl^+ brains (D), the partially invaginated roof of the telencephalon forms a broad ‘bridge’ (b) between the two hemispheres. (E-H) Coronal sections from a single Xl^+/Xl^+ mouse; dorsal is up. A curving cortical structure has developed in the medial wall of the telencephalon (E), ending in a wedge-shape (arrowhead, F) similar to the base of the cpe in wild-type mice. Differentiating neurons have formed a preplate and express the class III β-tubulin (purple in E). Neither TTR (F) nor Wnt2b (G) are expressed at the margin of the medial telencephalic wall. Arrows in G indicate the site that may correspond to the cortical hem in Xl^+/Xl^+ embryos. (H) Wnt2b expression is barely detectable (arrowhead) at the base of the medial telencephalic wall. (K) At E10.5, an Xl^+/Xl^+ embryo (right) shows relatively normal Wnt3a expression in the spinal cord, hindbrain and diencephalon, compared with a littermate control embryo (left). The control embryo shows strong expression of Wnt3a in the cortical hem (arrowhead), but in the comparable region in the Xl^+/Xl^+ embryo, Wnt3a expression is barely detectable (arrowhead). Bar in A, 1.4 mm (A); 900 µm (B,D); 700 µm (C); 220 µm (E); 110 µm (F-H); 55 µm (I); 150 µm (J); 1.2 mm (K).

However, the Wnt-rich cortical hem may shrink by progressively contributing cells to adjacent structures. Modern techniques of fate mapping will be required to determine whether the Wnt-rich cortical hem contributes cells to the CPe, the hippocampus, or both. However, classical morphological studies suggest that at least some cells from the cortical hem are recruited into the developing CPe (Maruyama and D’Agostino, 1967; Sturrock, 1979; Zaki, 1981). The cortical hem appears to be the thinning neuroepithelium described in these studies as giving rise to the CPe, and gene expression patterns support a close developmental relationship between the CPe and the cortical hem. The entire stretch of the medial wall that includes the CPe, junctional epithelium and the cortical hem expresses Bmp and Msx genes (Furuta et al., 1997; MacKenzie et al., 1991; present study), as well as high molecular mass tropomyosins, which may regulate the cell shape changes and cell movements of choroid plexus morphogenesis (Nicholson-Flann et al., 1996).

Wnt gene downregulation and loss of telencephalic CPe in the Xl^+ mouse mutant

In non-exencephalic Xl^+/Xl^+ mice, we observed some thinning of the neuroepithelium in the medial telencephalon, and an accumulation of cells, perhaps by proliferation, at the medial edge of the telencephalon. Wnt signaling therefore may not be required for these processes. However, cells with a specific CPe identity fail to develop. Could the absence of Wnt signaling at the cortical hem underlie this failure? Several observations from the present study implicate Wnt gene function in CPe development: the expression of multiple Wnt genes in the cortical hem surrounding the developing CPe; the cumulative expression of Wnt genes in the cortical hem before and just as the CPe begins to appear; and the tight correlation in the Xl^+ mutant between Wnt gene expression and CPe generation at different sites. However, the Xl^+ mutant is not equivalent to a mouse line generated in a gene targeting experiment in which Wnt2b, 3a and 5a expression is selectively depleted in the medial telencephalon. That is, the loss of CPe in the telencephalon of Xl^+/Xl^+ mice could be due to the Gli3 deficiency directly, or to a consequence of the Gli3 deficiency other than the loss of Wnt signaling in the cortical hem.

Gli3 has not been detected in the embryonic CPe itself (Hui et al., 1994; present study), therefore the development of telencephalic CPe appears unlikely to depend on Gli3 expression within that tissue. However, Gli3 is expressed in embryonic head mesenchyme (Hui et al., 1994), as is Wnt5a (present study). Therefore, a Gli3 deficiency could disrupt inductive interactions between the developing CPe and head mesenchyme. Suggesting that this is not the primary cause of the loss of telencephalic CPe in the Xl^+/Xl^+ mouse, the CPe can form a broad ‘bridge’ (b) between the two hemispheres. (E-H) Coronal sections from a single Xl^+/Xl^+ mouse; dorsal is up. A curving cortical structure has developed in the medial wall of the telencephalon (E), ending in a wedge-shape (arrowhead, F) similar to the base of the cpe in wild-type mice. Differentiating neurons have formed a preplate and express the class III β-tubulin (purple in E). Neither TTR (F) nor Wnt2b (G) are expressed at the margin of the medial telencephalic wall. Arrows in G indicate the site that may correspond to the cortical hem in Xl^+/Xl^+ embryos. (H) Wnt2b expression is barely detectable (arrowhead) at the base of the medial telencephalic wall. (K) At E10.5, an Xl^+/Xl^+ embryo (right) shows relatively normal Wnt3a expression in the spinal cord, hindbrain and diencephalon, compared with a littermate control embryo (left). The control embryo shows strong expression of Wnt3a in the cortical hem (arrowhead), but in the comparable region in the Xl^+/Xl^+ embryo, Wnt3a expression is barely detectable (arrowhead). Bar in A, 1.4 mm (A); 900 µm (B,D); 700 µm (C); 220 µm (E); 110 µm (F-H); 55 µm (I); 150 µm (J); 1.2 mm (K).
develop into a TTR-expressing, cuboidal epithelium, although not a convoluted plexus, in the absence of mesenchymal interactions (Birge, 1962; Thomas and Dziadek, 1993). In the Xtl/XTl mouse, CPE development appears to have stalled at an early stage, before cuboidal, TTR-expressing epithelium is detected, and therefore perhaps before signals from the mesenchyme become important.

Due to the low yield of non-exencephalic Xtl/XTl mice (15 out of 225 embryos recovered), we have not explored other possible gene expression defects at the cortical hem that might follow from the Gli3 deficiency. For example, the expression of Bmp and Msx genes remains to be examined in Xtl/XTl mice. Given the links between Wnt and Bmp signaling in other systems, the two gene families appear likely to interact in the development of the medial telencephalon too. In Drosophila, wingless is implicated in patterning the optic lobe, operating at least in part through regulation of the expression of the Drosophila Bmp family member, dpp (Kapthingst and Kunes, 1994). In the vertebrate neural tube, signaling from the ectoderm overlying the dorsal spinal cord, probably mediated by Bmp proteins, induces Wnt1 expression, as well as other markers of dorsal cell identity (Dickinson et al., 1995; Liem et al., 1995; Marcelle et al., 1997). If expression of Bmp family members is disrupted in Xtl/XTl mutants too, it will be important to test whether Wnt proteins are required to regulate Bmp expression in the cortical hem, or vice versa, and where Gli3 might operate in this pathway.

Gli3 regulation of cortical hem Wnt gene expression

A parsimonious explanation for the deficiency of Wnt gene expression in the Xtl/XTl mouse is that it is due to a direct action of the Xtl mutation. In Drosophila parasegment development, ci activates wingless expression in response to a hedgehog signal (Alexandre et al., 1996; Dominguez et al., 1996; Von Ohlen et al., 1997). Could Gli3 similarly respond to a hedgehog signal to regulate Wnt gene expression in the cortical hem? Of the three identified vertebrate hedgehog genes, only sonic hedgehog has been reported to be expressed near the cortical hem, but its expression in the choroid plexus appears after the Wnt-rich cortical hem has been established (Bitgood and McMahon, 1995). The present study therefore raises the possibility that a Gli family member is required for Wnt gene expression in the absence of hedgehog signaling. Consistent with this possibility, several wingless expression boundaries in Drosophila appear not to be established in response to hedgehog signaling (Kapthingst and Kunes, 1994; Lawrence and Struhl, 1996). Further, a genetic analysis of wingless autoregulation during segment polarity determination suggests a hedgehog-independent requirement for ci function in wingless expression (Hooper, 1994).

Why is the expression of Wnt2b, 3a and 5a downregulated in the telencephalon of Xtl/XTl mice, but not at other sites? Hui and colleagues (1994) have shown that some regions that express high levels of Gli3, such as the spinal cord, appear not to be morphologically affected in Xtl mutants, and suggest that Gli2, which shares an almost identical expression pattern with Gli3, might functionally substitute for Gli3 in these unaffected regions. The expression of Gli2 appears slightly weaker in the cortical hem than in immediately adjacent cortical neuroepithelium (data not shown). In the Xtl/mutant, therefore, Gli2 expression levels might be insufficient to maintain the expression of Wnt genes at the cortical hem.

Conclusion

Because the medial walls of the telencephalic hemispheres are formed by the invagination of the telencephalic vesicle, the cortical hem arises from the dorsal midline of the telencephalic vesicle. The dorsalmost cells of the telencephalon, the CPE and the hippocampus, are generated on either side of the cortical hem. In the developing spinal cord and brainstem, the roofplate, which also lies at the dorsal midline of the neural tube, directs development of adjacent dorsal cell groups via secreted peptides encoded by members of the Wnt and Bmp gene families (Ikeya et al., 1997; Liem et al., 1995). Findings from the present study suggest that the cortical hem should be investigated as a potential, analogous source of midline cues that direct development of the dorsal telencephalon.

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