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# A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordia in the early mouse embryo

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## SUMMARY

**Sonic hedgehog (Shh) is a key signal in the specification of ventral cell identities along the length of the developing vertebrate neural tube. In the presumptive hindbrain and spinal cord, dorsal development is largely Shh independent. By contrast, we show that *Shh* is required for cyclin D1 expression and the subsequent growth of both ventral and dorsal regions of the diencephalon and midbrain in early somite-stage mouse embryos. We propose that a *Shh*-dependent signaling relay regulates proliferation and survival of dorsal cell populations in the diencephalon and midbrain. We present evidence that *Fgf15* shows *Shh*-dependent expression in the diencephalon and may participate in this interaction, at least in part, by regulating the ability of dorsal neural precursors to respond to dorsally secreted Wnt mitogens.**

Key words: Sonic hedgehog, Mouse, Growth, CNS

## INTRODUCTION

The vertebrate central nervous system (CNS) undergoes a complex morphogenesis during which the various terminally differentiated cell types (neurons and glia) are generated at precise positions in appropriate numbers to establish the blueprint of the neural circuitry. The controls of cell fate specification and cell proliferation are key aspects of the developmental program, and both these processes are regulated by local signaling centers. Because of its relatively simple and conserved structure, the spinal cord has been the principal focus of study (reviewed by Tanabe and Jessell, 1996; Jessell, 2000).

Dorsal signaling is initiated by the surface ectoderm at neural plate stages and continued by roof plate cells, which occupy the dorsal midline of the neural tube after neural tube closure. A number of TGFβ family members are expressed in one or both of these two signaling regions. Several lines of evidence indicate that their individual or combinatorial actions specify distinct dorsal neural fates (reviewed by Lee and Jessell, 1999). The roof plate also expresses several members of the Wnt-family, including *Wnt1* and *Wnt3a* (Parr et al., 1993). Both, analysis of *Wnt1/3a* compound mutants, and the results of ectopic activation of Wnt signaling within the neural tube, suggest that these Wnt signals regulate cell proliferation (Dickinson et al., 1994; Ikeya et al., 1997; Lee et al., 2000; Megason and McMahon, 2002) (S. M. Lee, M. I., S. Megason, S. Takada. and A. P. M., unpublished). Thus, the roof plate coordinates growth and pattern by the production of two distinct classes of signal.

Accumulating evidence indicates that sonic hedgehog (Shh), a glycoprotein secreted by the notochord and floor plate, acts directly as a morphogen to specify distinct ventral cell identities (reviewed by Briscoe and Ericson, 1999; Jessell, 2000). The ventral half of the spinal cord is missing in *Shh* mutants, while the dorsal half remains (Chiang et al., 1996), consistent with notochord ablation experiments (Placzek et al., 1990; Van Straaten and Heckking, 1991; Yamada et al., 1991). Shh may not be the exclusive ventralizing factor, for example, retinoid signaling is implicated in induction of v0 and v1 populations of ventral interneurons in the presumptive spinal cord (Pierani et al., 1999). The notochord has also been shown to regulate cell proliferation in neural plate explants, consistent with the possibility that Shh secreted from this source acts as a mitogen (Van Straaten et al., 1989; Placzek et al., 1993). Indeed, ectopic expression of *Shh* in the dorsal neural tube (Rowitch et al., 1999), or ectopic activation of the Shh pathway through the removal of patched 1 (*Ptch1*) activity, results in dramatic hyper-proliferative phenotypes (Goodrich et al., 1997). Together these studies on the control of growth and pattern within presumptive spinal cord regions demonstrate that dorsal and ventral halves are largely regulated independently of one another.

The brain is considerably more complex. Several studies indicate that its organization is based upon an early segmental scaffold of repeating metameric units termed neuromeres. This is most obvious in the rhombomeres, the neuromeres of the hindbrain, where rhombomeric boundaries are barriers to cell mixing maintaining the clonal restriction of cell populations (reviewed by Lumsden and Krumlauf, 1996). In addition, appropriate expression of Hox genes and other regulatory factors within subsets of rhombomeres is critical for their

patterning (reviewed by Krumlauf et al., 1993; Wilkinson, 1993). Whereas the midbrain is thought to arise from a single neuromere (reviewed by Lumsden and Krumlauf, 1996), Puelles and colleagues have argued that the forebrain can be subdivided into six prosomeric units, three that generate the diencephalon excluding the hypothalamus (P1 to P3, caudal to rostral), and three that make up the telencephalon (Puelles and Rubenstein, 1993; Rubenstein and Puelles, 1994). Whether prosomeres exist in the same developmental and functional sense as rhombomeres is debatable; however, prosomere boundaries serve as a useful set of coordinates for the description of forebrain development.

As in presumptive spinal cord regions, signaling by Wnt, Hedgehog and TGFβ-family members has been shown to regulate the growth and pattern of brain regions. For example, *Shh* is expressed in the ventral forebrain, midbrain and hindbrain and its ventralizing properties extend into these regions (reviewed by Briscoe and Ericson, 1999; Jessell, 2000). Thus, Shh is a general ventralizing factor along the entire anteroposterior (AP) axis of the neural tube. How, then, does the same signal specify distinct cell types within different regions? Part of the answer appears to lie in the combinatorial action of Shh and other signaling factors, as well as intrinsic differences in the regional response to Shh that result from earlier patterning events (Dale et al., 1997; Ye et al., 1998). In addition to its role in cell fate specification, Shh has been proposed to act as a mitogen in the expansion of granule cell precursors in the external granule layer of the cerebellum, a relatively late event in CNS development (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Furthermore, misregulation of hedgehog signaling is implicated in the development of medullablastomas, a granule cell tumor (Vorechovsky et al., 1997; Raffel et al., 1997).

Although, Shh is expressed predominantly in the ventral neural tube at early stages, by 10.5 days post-coitum (dpc) there is a prominent dorsal extension at the zona limitans intrathalamica (ZLI), which lies at the boundary between P2 and P3 (Echelard et al., 1993; Shimamura et al., 1995). This raises the possibility that Shh signaling in the diencephalon may play a broader role in its development. Consistent with this view, *Shh* mutants have a disproportionate reduction in the size of the diencephalon relative to the hindbrain region at 11.5 dpc (Chiang et al., 1996).

We demonstrate that Shh signaling is critical for the proliferation and survival of neural precursors in the diencephalon and anterior midbrain, prior to the initiation of expression in the ZLI. Unlike other regions of the neural tube, Shh signaling is required for the normal development of both dorsal and ventral regions of the diencephalon and anterior midbrain, though analysis of the expression of Shh targets suggests that Shh does not signal directly within dorsal regions. Our data indicate a Shh-dependent signaling relay between ventral and dorsal regions that coordinates their growth. We suggest that fibroblast growth factor (FGF) and Wnt signaling may mediate these mitogenic and survival effects.

## MATERIALS AND METHODS

### Mice

Generation of *Shh* mutant mice has been described previously (St-Jacques et al., 1998). *Shh* heterozygous mutants were maintained on a Black Swiss-Webster background. Mating was assumed to have occurred at midnight, and embryonic stages are represented in days post-coitum (dpc). To score embryonic stages more precisely, somite numbers were counted.

### Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization of embryos was performed as previously described (Parr et al., 1993). Digoxigenin probes were synthesized using the Digoxigenin RNA labeling Kit (Roche).

### BrdU incorporation analysis

Dissected embryos were incubated in DMEM with BrdU for 30 minutes (S. Hayashi and A. P. M., unpublished) and processed for frozen sections. BrdU incorporation into newly replicated DNA was detected immunochemically as previously described (Dickinson et al., 1994) using anti-BrdU antibody (Pharmingen) and Alexa 568-conjugated anti-mouse IgG antibody (Molecular Probes). Labeled sections were counterstained with YoPro1 (Molecular Probes) and analyzed under the confocal microscope (Zeiss). The boundary between the telencephalon, diencephalon and midbrain were determined by morphological criteria and nuclei in each region were counted. Five wild-type and five *Shh* mutant embryos were examined and the statistical significance was calculated using Student's *t*-test.

### TUNEL assay

Frozen sections of embryos were treated with proteinase K and processed for TUNEL assay using ApopTag Red In Situ Apoptosis Detection Kit (Intergen Company).

### Plasmid construction

The entire coding region of an *Fgf15* cDNA (kindly provided by Dr Murre) was subcloned into the expression vector, pCIG (Megason and McMahon, 2002). This base vector contains a constitutive promoter, multiple cloning site and an internal ribosomal entry signal (IRES) that is followed by a cDNA encoding green fluorescent protein (GFP). *pCIG-F15*-transfected cells produce both FGF15 and GFP.

### Explant culture and electroporation

Mouse embryonic brains were dissected between the 14- and 16-somite stages and placed in DNA solution (1 mg/ml). Electrodes were placed 4 mm apart at both sides of the explants, then rectangular pulses (22 V, 50 mseconds, three times) were given by a T820 electroporator and a BTX500 optimizer (BTX). The explants were cultured in collagen matrix with the medium which contained 50% DMEM (Gibco), 10% fetal bovine serum (Hyclone) and 40% rat serum (Harlan). Collagen gels were prepared as previously described (Tessier-Lavigne et al., 1987; Artinger and Bronner-Fraser, 1993). After 40 hours, the explants were fixed in 4% paraformaldehyde and processed for whole-mount RNA in situ hybridization.

## RESULTS

### The dorsal parts of the *Shh* mutant brain are reduced in size between 8.5 dpc and 9.5 dpc

Although dorsal and ventral structures appear to be absent in the diencephalon and anterior midbrain of *Shh* mutants at 11.5 dpc (Chiang et al., 1996), the cellular and molecular mechanisms underlying this phenotype have not been addressed. To this end, we examined the development of these regions at early somite stages. In wild-type embryos at the five-somite stage (8.5 dpc), the diencephalic region (P1-3) occupies a narrow strip of cells between the much larger presumptive midbrain and telencephalic regions (Inoue et al., 2000). By

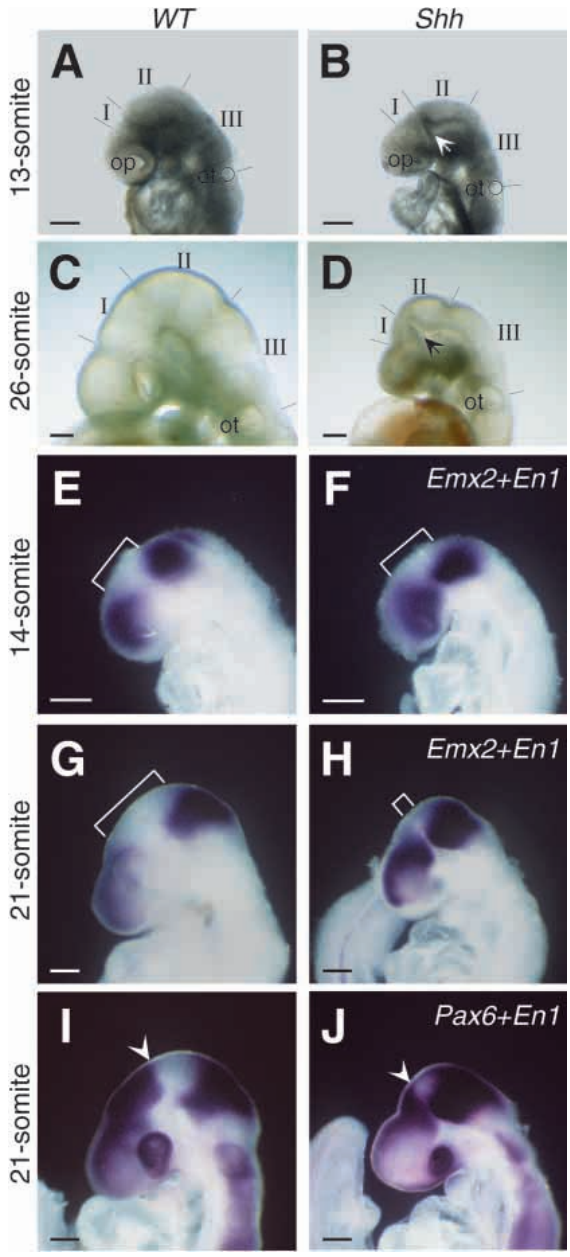


**Fig. 1.** A failure in growth of diencephalic and midbrain primordia in *Shh* mutants. Morphology of wild-type (A,C) and *Shh* mutant (B,D) brains at 8.5 dpc (13 somite; A,B) and 9.5 dpc (26 somite; C,D). At 8.5 dpc, the presumptive diencephalon is recognized as a narrow region between two constrictions (Region I). Region II corresponds to the midbrain and Region III to the anterior hindbrain between the isthmus and otic vesicle (ot). A sharp ventral constriction was observed in *Shh* mutants (arrows in B,D). At 9.5 dpc, regions I and II of *Shh* mutant were smaller than those of wild type, but Region III of the mutants was still comparable with that of wild type (see text, Table1). (E-J) Expression patterns of *Emx2*, *En1* and *Pax6*. A gap between the *Emx2* and *En1* expression domains represents the anterior midbrain and P1/2 (brackets in E-H). No difference was observed in this region at the 14-somite stage (E,F) but by the 21-somite stage, the gap was greatly reduced in *Shh* mutants (G H). The anterior midbrain, a gap between the *Pax6* and *En1* expression domains, was also reduced in *Shh* mutants (I,J). Arrowheads in I,J indicate the forebrain-midbrain boundary. Scale bars: 200 μm.

10.5 dpc, the diencephalon has undergone a disproportionate growth relative to other regions within the developing brain (Warren and Price, 1997). *Shh* mutant embryos became morphologically distinguishable from wild type after the seven-somite stage due to obvious midline defects (data not shown). At the 13-somite stage, ventral structures are absent along the length of the neural tube in *Shh* mutants (Chiang et al., 1996) (Fig. 1B). The lack of the ventral midline hinge enhances the ventral contour of the neural tube upon lateral view (Fig. 1B). The ventral constriction between the diencephalon and midbrain was much deeper than that of wild-type embryos (arrow in Fig. 1B). However, dorsal structures appeared relatively normal in size.

To perform a quantitative analysis, we divided the brain into three regions and measured the length of each of these along the AP axis at the dorsal midline. Region I corresponds to the diencephalon, Region II to the midbrain and Region III represents the anterior hindbrain between the isthmus and otic vesicle (see Fig. 1A). No significant difference was measurable in the length of each region when wild-type and *Shh* mutants were compared at the 13-somite stage (Fig. 1A,B) (Table 1). However, by the 26-somite stage (9.5 dpc), Region I and II were significantly reduced in *Shh* mutant embryos, while the length of Region III was unaffected (compare Fig. 1C with 1D; Table 1). At 10.5 dpc, mutant embryos were generally smaller in size than wild-type littermates. However, the diencephalon and midbrain were still disproportionately smaller in *Shh* mutants (data not shown). These data point to a requirement for *Shh* in normal growth of the dorsal diencephalon and midbrain between the 13- to 26-somite stages.

To confirm the above results using regional molecular markers, we performed RNA in situ hybridization with probes for three homeobox containing regulatory factors (*Emx2*, *En1* and *Pax6*) that demarcate different brain regions. Using the coordinates of the prosomere model (Puelles and Rubenstein, 1993; Rubenstein and Puelles, 1994), *Emx2* is expressed in the alar plate (dorsal region) of the presumptive telencephalon (P4) and anterior diencephalon (P3) (Shimamura et al., 1995). *En1* is expressed throughout the presumptive midbrain at early stages and is downregulated in the anterior midbrain as development proceeds (Davis and Joyner, 1988; Davis et al., 1991; McMahon et al., 1992). Thus, the gap between *Emx2* and *En1* expression domains corresponds to P1/2 and the anterior midbrain. At the 14-somite stage, the individual domains of

**Table 1. Analysis of regional growth in brain primordial in wild-type and *Shh* mutant embryos**

| Region | I | II | III |
|---|---|---|---|
| 8.5 dpc | | | |
| wild type | 161.37±12.77 | 437.77±26.35 | 637.80±32.37 |
| *Shh* | 172.95±10.06 | 440.65±20.97 | 635.16±28.65 |
| 9.5 dpc | | | |
| wild type | 564.86±21.06 | 865.06±33.39 | 943.04±27.60 |
| *Shh* | 231.34±3.26* | 540.66±21.65** | 944.92±64.05 |

The brain was divided into three parts: I, II and III (see text and Fig. 1 for definitions). The dorsal midline length along the AP axis was measured. At the 13-somite stage (8.5 dpc), there was no significant difference in size of each region between wild type and *Shh* mutants. At the 26-somite stage (9.5 dpc), Regions I and II of *Shh* mutants were significantly smaller than those of wild type. *$P$=0.00022; **$P$=0.00004. All values are shown in μm.

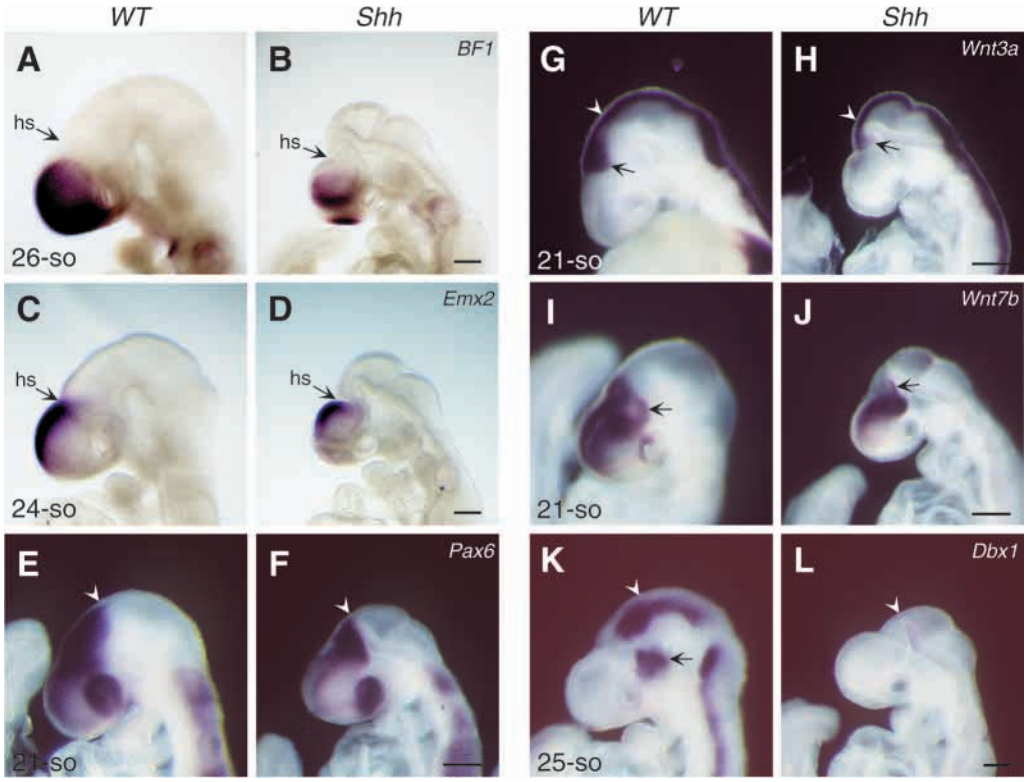


**Fig. 2.** Expression of region-specific markers in the forebrain and midbrain at 9.5 dpc. *Bf1* (A,B), *Emx2* (C,D), *Pax6* (E,F), *Wnt3a* (G,H), *Wnt7b* (I,J) and *Dbx1* (K,L) were examined in wild-type (A,C,E,G,I,K) and *Shh* mutants (B,D,F,H,J,L). Expression of *Bf1* was weaker in *Shh* mutants than in wild type. *Emx2* expression was essentially the same between wild-type and *Shh* mutants, although the telencephalon of *Shh* mutants was smaller than that of wild-type embryos at this stage. *Pax6* expression in the forebrain terminates at the forebrain-midbrain boundary (arrowhead) in both wild type and *Shh* mutants. *Wnt3a* expression undergoes a wedge-shaped lateral expansion in P2 of wild-type embryos at this stage (G, arrow). This expansion was detected in the mutant, although expression was reduced (H, arrow). *Wnt7b* was expressed in the anterior diencephalon (I, arrow) as well as the telencephalon of wild type. *Wnt7b* expression in the anterior diencephalon was also detectable in *Shh* mutants (J, arrow). *Dbx1* was expressed in the basal plate of P3 (K, arrow) and alar plates of P1/2 and midbrain (K). *Dbx1* expression was almost at background levels in *Shh* mutants (L). All arrowheads indicate the boundary between the diencephalon and midbrain. hs, hemispheric sulcus (the boundary between the telencephalon and diencephalon). Scale bars: 200 μm.

*Emx2* and *En1* expression were similar in size between *Shh* mutant and wild-type embryos and importantly, the region between their expression domains was comparable in length (bracket in Fig. 1E,F). By contrast, although there was no difference in the size of either expression domain at the 21-somite stage, the region in between was greatly reduced (bracket in Fig. 1G,H). A reduction in the growth of this region was evident as early as the 16-somite stage (data not shown).

*Pax6* expression provides a third useful landmark. *Pax6* is expressed throughout the entire alar plate of the forebrain, the sharp posterior boundary demarcates the diencephalic-midbrain junction (Warren and Price, 1997; Mastick et al., 1997; Gringley et al., 1997) (Fig. 2E). Thus, the gap between *Pax6* and *En1* expression domains at mid-somite stages corresponds to the anterior midbrain. This region was also greatly reduced at the 21-somite stage in *Shh* mutants (Fig. 1I,J). Although the size of the *En1* expression domain was not altered, there was a marked decrease in the size of the *Pax6* expression domain in the forebrain, most likely reflecting the truncated development of P1 and P2 regions (Fig. 1I,J). Thus, the analysis of regional markers was consistent with the results of the morphological analysis: the dorsal parts of P1/2 and the anterior midbrain developed normally in *Shh* mutants until the 14-somite stage but shortly thereafter their growth was retarded.

To determine whether the growth deficiency reflected a failure in regional specification of the alar plate, we analyzed expression of brain factor 1 (*Bf1*), *Emx2*, *Pax6*, *Wnt3a*, *Wnt7b*, *Dbx1* and *Pax7* at 21- to 26-somite stages (~9.5 dpc). BF1 is a winged-helix type transcription factor essential for telencephalic development (Xuan et al., 1995). *Bf1* expression was appropriately restricted to the telencephalon, although the level of expression was somewhat reduced, as was the size of its expression domain in the smaller telencephalic vesicle of the *Shh* mutant (Fig. 2A,B). Next, we analyzed *Emx2* and *Pax6* expression individually. In *Shh* mutants, both genes were expressed in a similar pattern and at a comparable level with wild type (Fig. 2C-F). Note that the posterior boundary of the *Pax6* expression domain remains sharp and is positioned at the physical constriction that reflects the diencephalic-midbrain boundary in wild-type embryos (arrowhead in Fig. 2E,F), although this constriction is positioned more dorsally in *Shh* mutants. *Wnt3a*, which encodes a secreted glycoprotein, is expressed at the dorsal midline of the developing neural tube. After the 17-somite stage, a wedge-shaped ventral extension of *Wnt3a* expression is initiated in the diencephalon (Parr et al., 1993) (arrow in Fig. 2G). This P2-specific characteristic was present in *Shh* mutants, although the size of this domain and the levels of *Wnt3a* expression were both greatly reduced (arrow in Fig. 2H). A second member of the Wnt family, *Wnt7b*, is expressed in the dorsal telencephalon and anterior diencephalon (Parr et al., 1993) (Fig. 2I). *Wnt7b* was expressed in the same pattern in *Shh* mutants, though its diencephalic expression domain was also diminished (Fig. 2J). Together, these data suggest that the dorsal telencephalon and diencephalon were correctly specified in *Shh* mutants and the dorsal identity of these regions were maintained until at least 9.5 dpc.

*Dbx1*, which encodes a homeodomain protein, is expressed in the basal plate of P3 and the alar plates of both P1/2 and the entire midbrain. *Dbx1* is also expressed in an intermediate zone where the sulcus limitans forms at hindbrain and spinal cord levels (Shoji et al., 1996) (Fig. 2K). *Dbx1* expression was almost completely absent in *Shh* mutant brains at the 25-somite stage (Fig. 2L), while spinal cord expression was maintained (Pierani et al., 1999) (data not shown). Expression was observed in the alar plate of *Shh* mutants at the 13-somite stage (data not shown). Thus, in contrast to the aforementioned markers, both dorsal and ventral expression of *Dbx1* in the diencephalon and midbrain is dependent on *Shh*.

*Pax7* encodes a paired-type homeodomain protein that is broadly expressed in the alar plate along most of the length of the early neural tube (Jostes et al., 1990). Pax7 extends into ventral regions of the presumptive spinal cord in the absence of Shh signaling (Litingtung and Chiang, 2000; Briscoe et al., 2001), consistent with the loss of ventral cell identities and dorsalization of the neural tube. In the forebrain and midbrain, *Pax7* shows a similar ventral extension indicating that ventral cell fates were lost but dorsal cell fates were maintained in *Shh* mutants (data not shown). Thus, the observed loss of *Dbx1* expression does not reflect a general failure in dorsal specification within these brain regions of *Shh* mutant embryos.

## Cell proliferation is decreased in alar plates of the diencephalon and midbrain of *Shh* mutants

The failure of diencephalic/midbrain development could reflect altered proliferation of neural precursors within these primordia. To examine this possibility directly, we performed an analysis of BrdU incorporation at 15- to 16-somite stages (Fig. 3A,B). In wild-type embryos, the entire telencephalon, diencephalon and midbrain showed a BrdU incorporation rate of ~60% using our labeling protocol (Fig. 3C-E,G). In *Shh* mutants, incorporation in the telencephalon was not significantly different from wild type (66.36±1.82%; Fig. 3F,G). By contrast, the diencephalon and midbrain showed significantly fewer S-phase cells (41.46±0.65% and 50.08±1.82%, respectively; Fig. 3F,G). Thus, a reduced rate of proliferation in diencephalic and midbrain precursors at 15- to 16-somite stages most probably contributes to the observed reduction in diencephalic and midbrain regions at later stages.

To address the molecular mechanism underlying this regional proliferative deficiency, we examined the expression of cyclin D1 (*Ccnd1*). Cyclin D1 regulates the G1 phase of the cell cycle, through the control of cyclin-dependent kinases (Sherr and Roberts, 1999). In wild-type embryos at the 14- (data not shown) and 17-somite stages (Fig. 3H), *Ccnd1* was expressed broadly in the developing brain with highest levels in the anterior midbrain and lower levels in the diencephalon. In *Shh* mutants, *Ccnd1* expression was undetectable throughout the diencephalic region, while expression in the anterior midbrain was greatly reduced at both stages (data not shown and Fig. 3I). Thus, the cell proliferation defects in the diencephalon and midbrain are likely to result, at least in part, from reduced levels of *Ccnd1*.

## Wnt signaling is perturbed in P1 and P2 alar plates of *Shh* mutants

Recent studies indicate that *Ccnd1* is a direct target of Wnt signaling. In the presence of a Wnt signal, β-catenin forms a complex with TCF/LEF proteins, which are HMG-box containing transcription factors, and this complex activates transcription of target genes (Molenaar et al., 1996; Korinek et al., 1997; Morin et al., 1997), one of which is *Ccnd1* (Shtutman et al., 1999; Tetsu and McCormick, 1999). *Tcf4* is expressed in the ventral telencephalon, the alar plates of P1/2 and rhombomere 5 (r5) of the hindbrain at 8.5 dpc (Cho and Dressler, 1998) (Fig. 4A,B). *Wnt1* and *Wnt3a* are expressed in the roof plate of the diencephalon and in the absence of both signals there is a failure of diencephalic development (S. M. Lee, M. I., S. Megason, S. Takada. and A. P. M., unpublished). Thus, *Tcf4* is well placed to respond to these Wnt signals.

To determine whether a disruption in dorsal Wnt-signaling may contribute to the reduced proliferation observed in *Shh* mutants, we analyzed *Tcf4*, *Wnt1* and *Wnt3a* expression. Robust localized expression of *Tcf4* was first observed in the

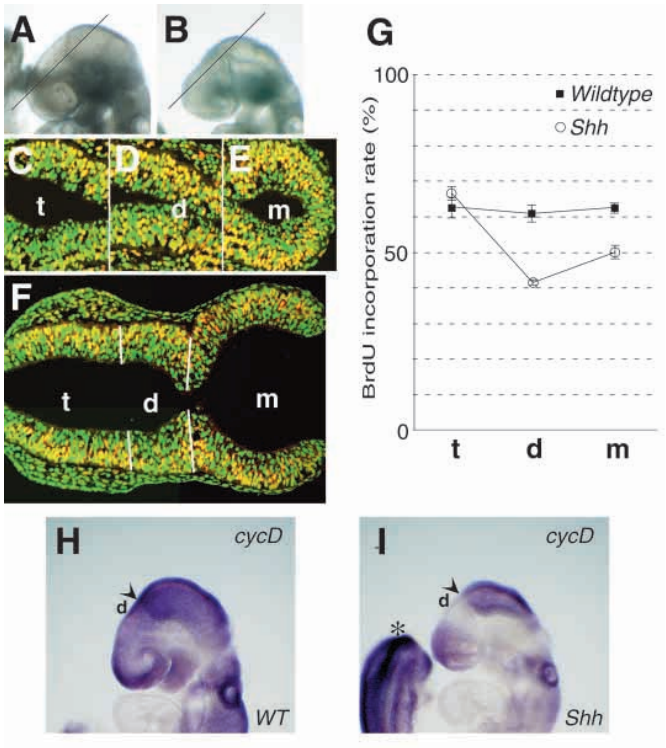


**Fig. 3.** Cell proliferation defects in *Shh* mutants. (A-G) BrdU incorporation analysis at the 15- to 16-somite stages. (A,B) The plane of sections examined. BrdU was detected by immunostaining with Alexa 568-conjugated secondary antibody [yellow because of overlap with YoPro1 (green) labeled nuclei]. In wild type, the telencephalon (C), diencephalon (D) and midbrain (E) showed similar incorporation rates (G, black square). In *Shh* mutants (F), only the telencephalon showed a comparable rate with that of wild-type embryos, while the diencephalon and midbrain showed significantly decreased rates of BrdU incorporation (G, circles). (H,I) *Ccnd1* (cyclin D1) expression at the 17-somite stage. In wild type (H), *Ccnd1* expression was detected in all three brain regions. The anterior midbrain showed a higher level of expression than others. In *Shh* mutants (I), its expression was absent from the diencephalon and weaker in the anterior midbrain. Note that the strong expression in the tail was maintained in *Shh* mutants (I, asterisk). Arrowhead in H and I indicates the diencephalon-midbrain boundary. t, telencephalon; d, diencephalon; m, midbrain.

alar plate of the P1/2 region in wild-type embryos at the 14-somite stage (arrowhead in Fig. 4A,B), and expression levels increased by the 16-somite stage (arrowhead in Fig. 4D). Surprisingly, *Tcf4* expression was barely detectable in the dorsal diencephalic region of *Shh* mutants at 14-somite (arrowhead in Fig. 4C) or later stages (arrowhead in Fig. 4E and data not shown). By contrast, *Tcf4* expression was unaltered in r5 of *Shh* mutants (arrow in Fig. 4C). *Wnt1* and *Wnt3a* continue to be expressed at the dorsal midline of the diencephalon in *Shh* mutants, although their expression levels were slightly reduced compared with wild type (Fig. 4F-I). Thus, there is a close temporal and spatial correlation between the failure of *Tcf4* activation, decreased *Ccnd1* expression and −a pronounced reduction in proliferation in the dorsal diencephalic primordium of *Shh* mutants. These data are consistent with a model in which Shh signaling is required to upregulate *Tcf4* expression in the alar plate of P1/2 thereby promoting the Wnt-mediated proliferation of dorsal diencephalic precursors.

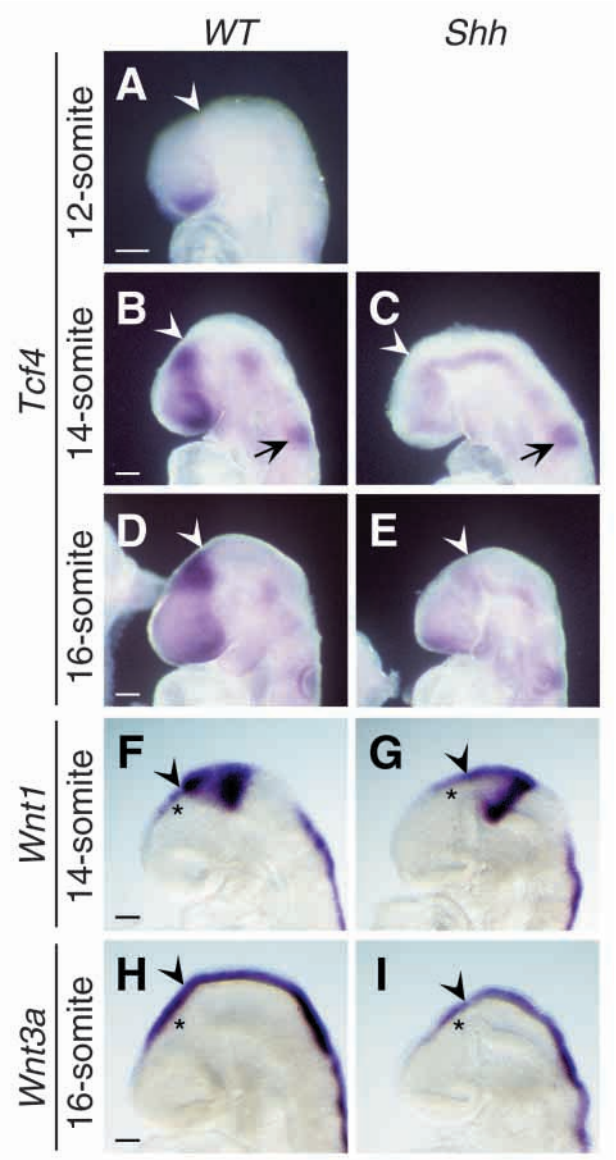


**Fig. 4.** Expression analysis of Wnt signaling components. *Tcf4* expression was examined at the 12- (A), 14- (B,C) and 16-somite (D,E) stages. At the 12-somite stage, *Tcf4* expression was not detectable in the diencephalon (A). Expression here was first observed at the 14-somite stage in wild type (B), but not in *Shh* mutants (C). r5 expression of *Tcf4* was observed in both wild-type and *Shh* mutants at this stage (B,C, arrow). At the 16-somite stage, *Tcf4* expression became stronger in the wild-type diencephalon (D), but was still not detectable in the diencephalon of *Shh* mutants (E). *Wnt1* (F,G) and *Wnt3a* (H,I) were also examined at the 14- and 16-somite stages, respectively. Their expression in the dorsal midline of the diencephalon (*) was maintained in *Shh* mutants (G,I). Arrowhead indicates the diencephalon-midbrain boundary. Scale bars: 100 μm.

### Cell death is increased in alar plates of the diencephalon and midbrain of *Shh* mutants

The finding of ectopic cell death in the developing spinal cord and somites of *Shh* mutants suggests that Shh may promote the survival of certain cell types (Chiang et al., 1996; Borycki et al., 1999; Litingtung and Chiang, 2000). To determine whether cell death contributes to the diencephalic/midbrain phenotype, we performed a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay on *Shh* mutants. In wild-type brains, there were very few TUNEL-positive cells at the 15- and 17-somite stages (Fig. 5A) (data not shown). By contrast, a large number of TUNEL-positive cells were observed in *Shh* mutants (Fig. 5B) (data not shown), suggesting that ectopic cell death contributes to the dorsal brain phenotype of *Shh* mutant embryos.

*Bmp4* expression in the developing forebrain is associated with local programmed cell death (Furuta et al., 1997) and BMP4 induces cell death when ectopically applied in the embryonic brain (Golden et al., 1999). In the developing mouse brain, *Bmp4* is expressed at low levels at the dorsal midline of the telencephalon and anterior diencephalon (P3 and anterior P2), but not in the caudal diencephalon (posterior P2 and P1) and midbrain at these early somite stages (Furuta et al., 1997). *Shh* mutants and wild-type embryos showed a similar expression pattern for *Bmp4* at the 15-somite stage (Fig. 5C,D) but there was a significant upregulation of *Bmp4* expression in the diencephalic region and midbrain of *Shh* mutants by the 19-somite stage (Fig. 5E,F). Thus, *Shh* activity appears to be required to repress *Bmp4* expression at the dorsal midline of the diencephalon and midbrain. Although the ectopic expression of *Bmp4* may contribute to later cell death, these data cannot explain the significant increase in cell death at earlier somite stages.

### *Tcf4* and *Bmp4* are indirect targets of Shh signaling

The altered dorsal expression of *Tcf4* and *Bmp4* together with the correlated changes in cell proliferation and cell death, raises the issue of whether Shh signals directly to dorsal regions of the diencephalon and midbrain at the 14- to 25-somite stages. To address this issue, we examined expression of *Shh* and *Ptch*. *Ptch* encodes the Shh receptor, its upregulation in response to Hedgehog signaling is a highly conserved transcriptional response that serves to limit the range of Hedgehog signaling (reviewed by Ingham and McMahon, 2001). As demonstrated in previous studies (Echelard et al., 1993), *Shh* was expressed only in ventral regions at the 14-somite and earlier stages (Fig. 6A; data not shown). The first evidence of a dorsal expansion, at the presumptive ZLI, was not observed until the 24-somite

stage (arrow in Fig. 6C; data not shown) (Shimamura et al., 1995). Upregulation of *Ptch* was also confined to ventral regions corresponding approximately to the basal plate (Fig. 6B,D). Thus, the analysis of *Shh* expression and *Shh* target gene response indicates that Shh signaling is restricted to the basal plate; consequently, *Tcf4* and *Bmp4* are unlikely to be direct targets of Shh signaling.

### FGF signaling is also affected in *Shh* mutants

As the effects on the alar plate in the absence of Shh signaling appear to be indirect, our data suggest the presence of a Shh-dependent signaling relay that regulates expansion of the dorsal diencephalic/anterior midbrain primordia. One such relay in the vertebrate limb connects Shh with FGFs to coordinate outgrowth and patterning of the limb bud (Laufer et al., 1994; Niswander et al., 1994; Yang and Niswander, 1995; Zuniga et al., 1999; Lewis et al., 2001) (reviewed by Martin, 1998; Caruccio et al., 1999; Kraus et al., 2001). To explore the possibility of a similar relay in the brain, we analyzed expression of all available Fgf family members (*Fgf1* to *Fgf23*). One of these, *Fgf15* (McWhirter et al., 1997), showed an interesting, dynamic pattern with regard to diencephalic/midbrain development.

In 12-somite stage wild-type embryos, *Fgf15* was strongly expressed in the ventral and intermediate parts of P1/2 and the anterior midbrain just dorsal to the Shh expression domain (Fig. 7A,B). By the 14-somite stage, *Fgf15* expression extended dorsally overlapping the *Dbx1* and *Tcf4* expression domains (Fig. 7D), dorsal expression of *Fgf15* intensified by the 16-somite stage (Fig. 7F). *Fgf15* was also strongly expressed just posterior to the midbrain/hindbrain isthmus (Fig. 7B,D,F). In *Shh* mutants, no *Fgf15* expression was observed within the forebrain or midbrain between the 12- and 16-somite stages (arrowhead in Fig. 7C,E,G). By contrast, *Fgf15* expression was detected caudal to the midbrain/hindbrain isthmus (arrow in Fig. 7C,E,G). Thus, *Fgf15* expression is *Shh* dependent and, given its temporal and spatial expression, *Fgf15* is well placed to participate in a signaling relay that connects ventral and dorsal regions of the diencephalon and midbrain.

All FGF signaling is thought to be mediated by four receptor tyrosine kinases (Reid et al., 1990; Mansukhani et al., 1992; Avivi et al., 1991; Stark et al., 1991) (reviewed by Ornitz and Itoh, 2001). Two of these, *Fgfr2* and *Fgfr3*, localize to the caudal diencephalon and anterior midbrain in the chick (Walshe and Mason, 2000). We therefore examined the expression of these receptors in the developing brain of wild-type and *Shh* mutant embryos. At the 13-somite stage, just prior to the upregulation of *Tcf4*, both receptors were detected in the alar plates of the caudal diencephalon and anterior midbrain, overlapping the normal *Tcf4* and *Dbx1* expression domains (Fig. 7H,L). In *Shh* mutants at this time, *Fgfr2* showed

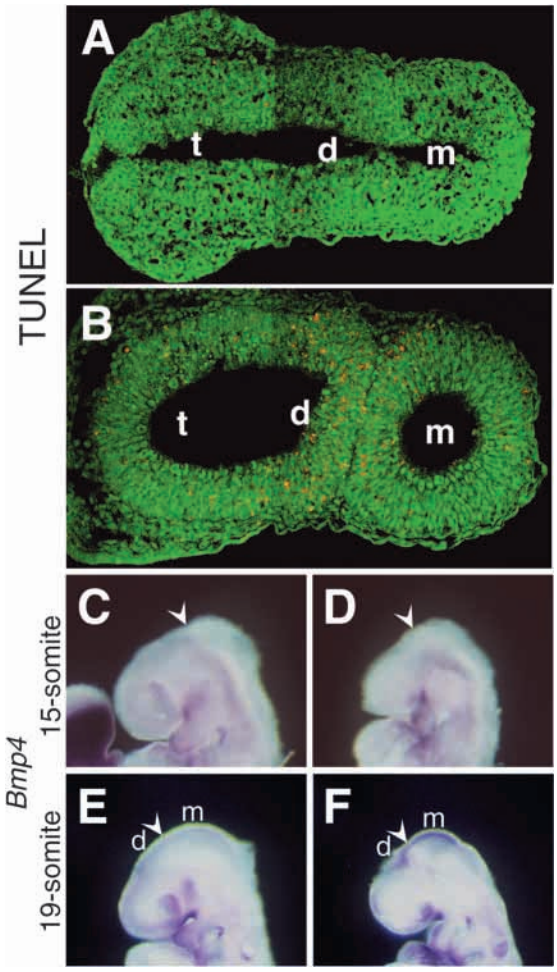


**Fig. 5.** Increased cell death in *Shh* mutant brains. TUNEL assay was performed on wild-type (A) and *Shh* mutant (B) sections at the 15-somite stage. The plane of sections is shown in Fig. 4A,B. Few cells were positive for TUNEL (orange dot) in wild-type embryos, while *Shh* mutants exhibited increased cell death especially in the diencephalon and anterior midbrain. *Bmp4* expression was examined at the 15-somite (C,D) and 19-somite (E,F) stages. At the 15-somite stage, there was no *Bmp4* expression in the dorsal midline of the diencephalon and midbrain. At the 19-somite stage, robust expression of *Bmp4* was observed in this region in *Shh* mutants (F). Arrowhead indicates the diencephalon-midbrain boundary. t, telencephalon; d, diencephalon; m, midbrain.

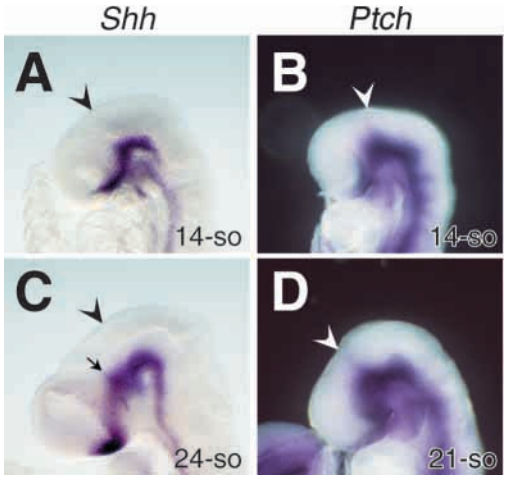


**Fig. 6.** Shh signaling is confined to ventral brain regions. *Shh* (A,C) and *Ptch* (B,D) expression were examined at the 14- (A, B), 24- (C) and 21-somite (D) stages. *Ptch* was not detectable in any dorsal parts of the brain. Note that lateral expansion of *Shh* expression in the ZLI initiates at the 24-somite stage (C, arrow). Arrowhead indicates the diencephalon-midbrain boundary.

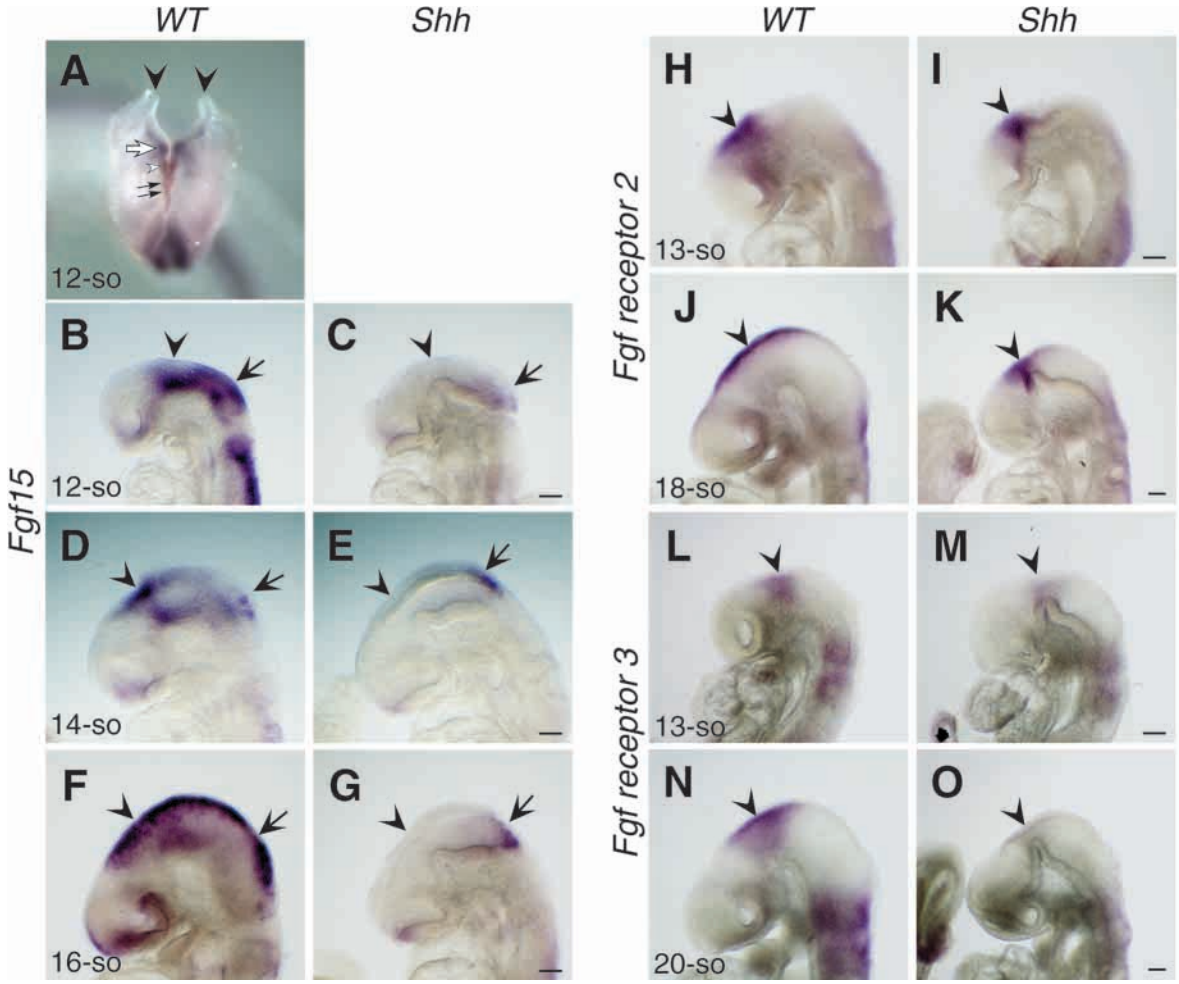


**Fig. 7.** *Fgf15* and Fgf receptor expression in the diencephalon and midbrain of wild-type (A,B,D,F,H,J,L,N) and *Shh* (C,E,G,I,K,M,O) mutant embryos. (A) *Fgf15* and *Shh* expression. (B-G) *Fgf15*, (H-K) *Fgfr2* and (L-O) *Fgfr3* expression. (A) At the 12-somite stage, *Fgf15* (purple) was strongly expressed in the ventral regions of the diencephalon (white arrow), dorsal to the *Shh* expression domain (white arrowhead, brown). Black arrows indicate the ventral *Shh* expression domain in the midbrain. (B) A lateral view reveals the extent of ventral expression of *Fgf15* in the brain. Note that *Fgf15* expression was undetectable in dorsal regions of the diencephalon and midbrain at this stage. (D,F), *Fgf15* shows a ventral to dorsal expansion from the 12- to 16-somite stage in diencephalic and midbrain primordia of wild-type embryos. (C,E,G), *Shh* mutants showed no expression of *Fgf15* in the caudal diencephalon and anterior midbrain at all stages examined, whereas expression at the midbrain/hindbrain isthmus (arrow) remains in *Shh* mutants. Expression of *Fgfr2* in the diencephalon and anterior midbrain in wild type (H,J) extends ventrally in *Shh* mutants (I,K). Downregulation in the expression of *Fgfr3* (compare L,N with M,O) correlates with the growth defect in these regions of *Shh* mutants. Arrowhead indicates the diencephalon-midbrain boundary. Scale bars: 100 μm.

a similar expression pattern to that of wild-type embryos (Fig. 7I) while expression of *Fgfr3* was downregulated (Fig. 7M). By the 18-somite stage, expression of *Fgfr2* was restricted to the dorsal-most parts of the diencephalon and anterior midbrain in wild-type embryos (Fig. 7J). In *Shh* mutants, its expression domain was expanded ventrally but reduced along the anteroposterior axis (Fig. 7K), reflecting both the dorsalization and smaller size of these brain regions. By contrast, expression of *Fgfr3*, which continues to be expressed strongly in the alar plate of the caudal diencephalon and anterior midbrain in wild-type embryos at the 20-somite stage (Fig. 7N), was downregulated to almost background levels in *Shh* mutant embryos (Fig. 7O). Thus, expression of both FGF-ligand and FGF receptors is *Shh* dependent in the diencephalic/midbrain region.

### Overexpression of *Fgf15* expands the *Tcf4* expression domain

These results suggest that Shh may regulate dorsal cell types in these regions through an *Fgf15* relay. To test this model, we used electroporation of an *Fgf15* expression construct into brain explants isolated from 14- to 16-somite stage mouse embryos to determine whether ectopic expression of *Fgf15* would modulate *Tcf4* expression. Electroporation was restricted to one side of the brain explant, providing an internal control for the ectopic expression of *Fgf15* (Fig. 8A,B). Electroporation of a control vector expressing GFP into the diencephalic/midbrain region did not alter the *Tcf4* expression domain (Fig. 8C), whereas overexpression of *Fgf15* resulted in a robust expansion of the *Tcf4* expression domain that was limited to the electroporated side of the explants (Fig. 8D).

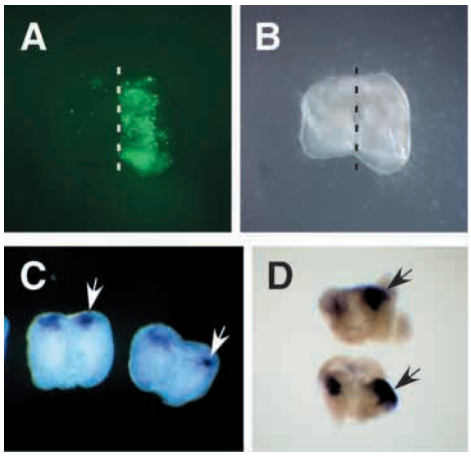


**Fig. 8.** *Fgf15* induces an expansion of *Tcf4* expression in brain explants. (A) GFP activity in the right side of the brain explant shown in B after electroporation with pCIG-F15. The broken line in both panels indicates the ventral midline. Explants were cultured for 40 hours after electroporation and examined for *Tcf4* expression. In the case of the control vector expressing only GFP (C), there was no significant difference in *Tcf4* expression on the electroporated (right, indicated by arrow) and non-electroporated sides (left). By contrast, explants electroporated with the *Fgf15*-expressing vector (pCIG-F15) showed an expansion of the *Tcf4* expression domain on the electroporated side (D, arrow). Rostral is towards the top in all panels.

Furthermore, the hybridization signal was more intense on the electroporated side, suggesting that transcription of *Tcf4* was also upregulated by *Fgf15* (Fig. 8D). These results are consistent with the idea that *Fgf15* regulates *Tcf4* expression. Next, we examined whether expression of *Fgf15* induces ectopic expression of *Tcf4* by examining *Bf1*, *Tcf4* and *En1* expression. As described above, *Bf1* is expressed in the telencephalic alar plates, *Tcf4* in P1/2 alar plates and *En1* in the middle/posterior midbrain. Thus, a gap between *Bf1* and *Tcf4* expression domains represents the P3 alar plate, and a gap between *Tcf4* and *En1* expression domains corresponds to the anterior midbrain. Comparable gaps were observed between these expression domains on the electroporated and control sides, suggesting that ectopic expression of *Fgf15* leads to an expansion of the endogenous *Tcf4* domain, rather than de novo activation of *Fg15* in other brain regions (data not shown). Finally, to determine whether *Fgf15* is sufficient to rescue *Tcf4* expression we electroporated brain explants from *Shh* mutant embryos at the 10-somite stage. Although, GFP activity was visible within 8 hours post electroporation, we failed to observe any activation of *Tcf4* or rescue of the diencephalic growth defect (data not shown).

## DISCUSSION

Shh has been intensively studied for its role in the dose-dependent induction of distinct ventral cell identities along the length of the developing vertebrate neural tube (reviewed by Jessell, 2000). Removal of either the notochord, which supplies the initial Shh signal (Placzek et al., 1990; Van Straaten and Heckking, 1991; Yamada et al., 1991), or Shh itself (Chiang et al., 1996) leads to a failure in the specification of ventral cell types in both the presumptive hindbrain and spinal cord, but dorsal development appears grossly normal. By contrast, broader DV deficiencies in forebrain and midbrain regions are evident in *Shh* mutants at early somite stages (prior to 9.5 dpc of mouse development), suggesting that in these regions dorsal development is in someway Shh dependent. We have addressed this mechanism by focusing on the presumptive diencephalic and anterior midbrain regions. Our data support a model in which Shh is required for the proliferative expansion of both dorsal and ventral neural precursors, at least in part through the regulation of *Ccnd1* expression. Furthermore, our evidence suggests that a Shh-dependent FGF15 signaling relay may be one component that coordinates the expansion of dorsal and ventral neural precursors.

### Shh signaling controls cell proliferation

Our morphological and marker analyses indicate that Shh is clearly essential for the dramatic growth of both dorsal and ventral regions of the diencephalic and anterior midbrain that occurs between the 14- and 25-somite stages. Furthermore, analysis of regional markers suggests that the primary patterning of these regions is not altered in *Shh* mutants, rather it is the subsequent failure of expansion of dorsal and ventral neural precursors that leads to a gross reduction in these brain regions. Consistent with this view, the diencephalon and anterior midbrain of *Shh* mutants showed a decreased BrdU incorporation rate at the 15- to 16-somite stages in comparison with adjacent telencephalic regions. In wild-type embryos, the anterior midbrain showed the highest level of *Ccnd1* expression in the developing brain, while the diencephalon showed modest levels that correlate with the initiation of growth. The dramatic downregulation of *Ccnd1* in the anterior midbrain and absence of activity in the diencephalic primordium of *Shh* mutants suggest that regulation of G1 cyclin activity is at least one mechanism by which Shh regulates growth of these brain primordia.

Ectopic expression studies have demonstrated that Shh can have a mitogenic role in the developing CNS (Rowitch et al., 1999). In particular in the cerebellum there is good evidence that Purkinje cell-supplied SHH is the principal mitogen for proliferation of cerebellar granule cell precursors (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Moreover, several G1 cyclins, including *Ccnd1*, are transcriptional targets of this Shh-mediated mitogenic response (Kenney and Rowitch, 2000). Thus, it is reasonable to postulate that Shh may play a relatively direct role in regulating neural precursor proliferation in the diencephalic and midbrain regions. However, whereas this might be true in the basal plate in ventral regions, it is unlikely to be true for dorsally located precursors in the alar plate. Although recent evidence indicates that Shh may act directly over a distance of up to 300 μm (Lewis et al., 2001), analysis of Shh target gene expression in diencephalic and midbrain anlagen provides no evidence of active Shh signaling in the dorsal half of these brain primordia. Thus, the evidence is more consistent with a Shh-dependent signaling relay controlling proliferation in dorsal regions.

### Shh regulates Wnt and FGF signaling

Two other families of signaling factors have been implicated

in the expansion of CNS precursor populations at early neural plate/neural tube stages: the Wnt and FGF families. Wnt1 and Wnt3a, either alone or in combination, are both necessary and sufficient for the expansion of CNS precursors in several regions of the developing CNS (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992; Dickinson et al., 1994; Ikeya et al., 1997; Megason and McMahon, 2002). Interestingly, in the absence of both *Wnt1* and *Wnt3a* activities, there is a broad deficiency in both the diencephalon and midbrain that appears to result from a growth defect at early somite stages (S. M. Lee, M. I., S. Megason, S. Takada. and A. P. M., unpublished). Thus, whereas development of the midbrain is *Wnt1* dependent (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), diencephalic development is co-regulated by Wnt1 and Wnt3a, both of which are most likely secreted by cells at the dorsal midline (Parr et al., 1993). Furthermore, characterization of the canonical Wnt-signaling pathway mediated by the Wnt1/Wnt3a class of ligand indicates that a transcriptional complex between β-catenin and LEF/TCF factors is responsible for the activation of Wnt targets, one such target appears to be *Ccnd1* (Tetsu and McCormick, 1999; Shtutman et al., 1999).

One member of the Lef/Tcf family, *Tcf4* is expressed specifically in the alar plates of P1/2 at the 14-somite stage in wild-type embryos. This timing in expression correlates with the first appearance of a phenotype in the dorsal diencephalic region of *Shh* mutants. Interestingly, our results indicate that upregulation of *Tcf4* is itself dependent on Shh signaling. Thus, the loss of *Tcf4* activity might downregulate the response to dorsal Wnt1/3a signals, thereby contributing to a deficiency in the proliferation of dorsal diencephalic precursors. However, the absence of *Tcf4* expression cannot by itself explain the diencephalic phenotype, as *Tcf4* mutants do not exhibit a brain phenotype (Korinek et al., 1998). A possible functional redundancy amongst Lef/Tcf members that are more broadly expressed in the neural tube at this time could be a complicating factor (Galceran et al., 1999). Furthermore, *Tcf4* expression is restricted to the diencephalon but a similar phenotype is observed in the anterior midbrain. Signaling between P1 and the anterior midbrain has been proposed as a possible regulatory mechanism; however, the nature of this signaling is unclear. Interestingly, we observe that *Dbx1* expression is absent in both the dorsal diencephalon and anterior midbrain of *Shh* mutants, suggesting that *Dbx1* may act in some way to co-ordinate development of these brain regions, although the exact activity of *Dbx1* has not been determined.

A key issue in our study is the molecular link between Shh ventrally and *Tcf4* dorsally. Our data suggest that FGF15 could be one factor. *Fgf15* is expressed ventrally in the diencephalon and midbrain at the appropriate time adjacent to the *Shh* expression domain. *Fgf15* expression is clearly Shh dependent, although determining whether this regulation is direct or indirect will require a detailed analysis of the *Fgf15 cis*-regulatory regions. Given that *Fgf15* shows differential expression at distinct anteroposterior positions of the developing neural tube, it is apparent that other Shh-independent regulatory controls must govern its precise spatial expression. FGF/Shh interactions have been demonstrated in other aspects of embryonic development, notably in the limb and lung. Shh is required for the maintenance of expression of several Fgfs in the apical ectodermal ridge of the developing limb bud (Laufer et al., 1994; Niswander et al., 1994; Yang and Niswander, 1995; Zuniga et al., 1999; Lewis et al., 2001) (reviewed by Martin, 1998; Caruccio et al., 1999; Kraus et al., 2001) and for localization of *Fgf10* in the lung bud (Pepicelli et al., 1998). In the former, this regulation is mediated indirectly through a signaling relay (Zuniga et al., 1999); in the latter, it is not clear whether regulation is direct or indirect.

Our data indicate that overexpression of *Fgf15* leads to an apparent upregulation and expansion of the endogenous *Tcf4* expression domain in wild-type embryos, but is insufficient to activate *Tcf4* or rescue the proliferative deficiency in the diencephalon of *Shh* mutants when brain explants are electroporated at the 10-somite stage, prior to a visible diencephalic phenotype. Thus, while basal activation of *Tcf4* is not Shh/FGF15-dependent (Fig. 4E), FGF15 may mediate a Shh signaling relay to dorsal regions to upregulate *Tcf4* expression: however, additional factors are likely to be required for a wild-type response. The nature of these additional factors remains to be determined. Alternatively, there may be a narrow time window of FGF15 responsiveness that is not mimicked in the current set of experiments. Given that there is a dramatic visible reduction in the size of the diencephalic region that occurs over a seven-somite (~12 hour) interval (Fig. 1F,H), this remains a possibility.

The molecular mechanism by which FGF15 signals is currently unclear. Mouse FGF15 is reported to be the ortholog of human FGF19, although the amino acid identity between them is significantly less (51%) than that observed between most human and mouse FGF orthologs (more than 90%) (Nishimura et al., 1999). Human FGF19 has been reported to bind exclusively to FGF receptor 4 in vitro (Xie et al., 1999). However, *Fgfr4* is not expressed in the brain at early somite stages. By contrast, *Fgfr2* and *Fgfr3* both localize to the diencephalic and midbrain primordia and display altered expression in *Shh* mutants. Given the high divergence between the mouse FGF15 and human FGF19, one possibility is that mouse FGF15 has a distinct receptor specificity from its suggested human counterpart. Signaling through these spatially restricted receptors might also explain the spatially restricted response to ectopic *Fgf15* expression that was observed on electroporation of *Fgf15* into brain explants.

FGFs have been known for sometime to act as both mitogens (Reynolds and Weiss, 1996; Gritti et al., 1996; Lee et al., 1997) and survival factors (Desire et al., 1998; Learish et al., 2000) for specific types of neural stem/precursor cells. However, the role of FGF signaling in the developing neural tube has only recently been investigated. *Fgf8* is expressed specifically at the midbrain/hindbrain junction and expression is essential for the expansion of midbrain precursors (Crossley and Martin, 1995; Crossley et al., 1996; Meyers et al., 1998). Although it is not clear how this expansion is effected, it is remarkable that this is the same target population that requires a Wnt1 input, pointing to additional links between FGF and Wnt signaling in the growth of specific brain primordia. FGF signaling is directed through tyrosine kinase receptors that are thought to activate various pathways, including mitogen-activated protein (MAP) kinase (reviewed by Boilly et al., 2000), protein kinase C (Logan and Logan, 1991; Hurley et al., 1996) and signal transducers and activators of transcription (Su et al., 1997). As both *Myc* and *Ccnd1* are activated by a MAP-kinase cascade (Lavoie et al., 1996; Aziz et al., 1999), FGF signaling may

independently regulate the cell cycle in the diencephalic/midbrain region. In this case, FGF15 and Wnt1/3a signaling may act cooperatively to regulate cell proliferation through the regulation of *Ccnd1* and presumably other factors. With respect to this possibility, Wnt1 and MEK1 have been demonstrated to act cooperatively to inhibit glycogen synthase kinase-3β activity leading to the accumulation of cyclin D1 (Rimerman et al., 2000).

If FGF15 acts exclusively as a mitogen, an FGF15-mediated expansion of *Tcf4*-expressing cells, rather than a transcriptional expansion of the *Tcf4* expression domain, could explain the observed increase in *Tcf4* expression in wild-type brain explants ectopically expressing *Fgf15*. However, this model could not account for the observed upregulation of *Tcf4* expression levels within its normal diencephalic domain in response to FGF15. Furthermore, the loss of *Fgf15* expression and downregulation of *Fgfr3* expression in *Shh* mutants occurs concomitant with a failure to elevate *Tcf4* expression to normal levels prior to a detectable diencephalic growth defect.

In addition to decreased cell proliferation in the diencephalon and midbrain, we also observed an increased rate of cell death. This observation could reflect a link between mitogen activity and cell survival, or an alternative mechanism by which Shh enhances survival of neural precursors. Cell death in the neural tube has been associated with BMP4 signaling (Trousse et al., 2001; Graham et al., 1994; Golden et al., 1999). We observed ectopic upregulation of *Bmp4* in the dorsal diencephalon and midbrain of *Shh* mutants but only after the 15-somite stage. Thus, ectopic *Bmp4* expression is too late to account for the initial increase in cell death in *Shh* mutants but could play a role at later stages. Interestingly, BMP4 has been shown to suppress *Dbx1* expression in vitro (Pierani et al., 1999), thus, an upregulation in *Bmp4* expression could account for the downregulation in *Dbx1* expression observed at the 25-somite stage.

In summary, our data provide evidence that the proliferative activity of neural precursors within dorsal regions of the diencephalon and anterior midbrain is regulated in response to a signaling relay governed by the ventral activity of Shh. This result contrasts with the ventrally restricted actions of Shh in more caudal areas of the developing CNS. Interestingly, *Shh* mutants display a marked reduction in the development of dorsal telencephalic regions at later stages (26-somite, 9.5 dpc) that correlates with the downregulation of *Bf1*, a factor known to regulate the expansion of telencephalic precursors (Xuan et al., 1995). Thus, other regions of the forebrain might also rely upon a Shh-regulated relay to co-ordinate their growth.

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