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Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog

Jhumku D. Kohtz[1], Darren P. Baker[2], Giorgio Corte[3] and Gord Fishell[1,*]

[1]Developmental Genetics Program and the Department of Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10016, USA
[2]Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142, USA
[3]Laboratory of Immunobiology, IST-CBA and Department of Clinical and Experimental Oncology, University of Genoa, Genoa, Italy
*Author for correspondence (e-mail: fishell@saturn.med.nyu.edu)

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SUMMARY

The cortex and basal ganglia are the major structures of the adult brain derived from the embryonic telencephalon. Two morphologically distinct regions of the basal ganglia are evident within the mature ventral telencephalon, the globus pallidus medially, and the striatum, which is positioned between the globus pallidus and the cortex. Deletion of the Sonic Hedgehog gene in mice indicates that this secreted signaling molecule is vital for the generation of both these ventral telencephalic regions. Previous experiments showed that Sonic Hedgehog induces differentiation of ventral neurons characteristic of the medial ganglionic eminence, the embryonic structure which gives rise to the globus pallidus. In this paper, we show that later in development, Sonic Hedgehog induces ventral neurons with patterns of gene expression characteristic of the lateral ganglionic eminence. This is the embryonic structure from which the striatum is derived. These results suggest that temporally regulated changes in Sonic Hedgehog responsiveness are integral in the sequential induction of basal telencephalic structures.

Key words: Telencephalon, Sonic Hedgehog, Rat, Neurogenesis

INTRODUCTION

The mechanisms by which regional territories are specified within the vertebrate telencephalon are not well understood (Allman, 1990; Northcutt and Kaas, 1995; reviewed in Rubenstein et al., 1994; and Fishell, 1997). Given the apparent dorsal origin of telencephalic tissue in higher vertebrates (Couly and Le Dourain, 1987), it is interesting to consider how the basal ganglia, which constitute the ventral regions of the telencephalon, are generated. Initially, the entire telencephalon is made up of a uniform sheet of pseudostratified neuroepithelium. Within the dorsal-most telencephalon this sheet-like morphology is maintained throughout development. In contrast, proliferation within the ventral aspects of the telencephalon generates a number of eminences in this region prior to the initiation of neurogenesis.

These eminences are named according to their position: the medial ganglionic eminence (MGE) and the lateral ganglionic eminence (LGE). Together, they make-up the anlage of the telencephalic basal ganglia. In the mature animal, the MGE appears to develop into the globus pallidus, whereas the LGE likely gives rise to the striatum, the largest nucleus within the basal ganglia (Smart and Sturrock, 1979). These structures arise sequentially during development, with the MGE appearing immediately after anterior neuropore closure, followed later by the appearance of the LGE. At present, we know little about the molecular mechanisms by which these developmental steps are mediated.

It has been demonstrated that the ventral axis of the nervous system is specified positionally by extrinsic cues emanating from the axial mesoderm (van Straaten et al., 1988; Placzek et al., 1990; Yamada et al., 1991; Placzek et al., 1993). For instance, in the spinal cord, ventral cell types in the neural tube are generated due to their proximity to inductive signals from the notochord. The cue shown to be both necessary and sufficient to drive this process is the protein Sonic Hedgehog (Shh) (Echelard et al., 1993; Kraus et al., 1993; Yamada et al., 1993; Roelink et al., 1994). Within the spinal cord, *Shh* is first expressed in the mesoderm and later in ventral regions of the neural tube. Shh induces ventral structures in a concentration-dependent manner, with the highest levels inducing the most ventral structures, such as floorplate, and lower levels inducing progressively more dorsal cell types, such as motor neurons. Shh appears to be essential for establishing ventral identity throughout most of the CNS (Echelard et al., 1993; Kraus et al., 1993; Roelink et al., 1994; Hynes et al., 1995), including the forebrain (Ericson et al., 1995; Dale et al., 1997; Shimamura and Rubenstein, 1997).

Recent work by a number of groups (Barth and Wilson, 1995; Macdonald et al., 1995; Ericson et al., 1995; Dale et al.,

1997; Hauptmann and Gerster, 1996; Li et al., 1997; Shimamura and Rubenstein, 1997) has shown that Shh signaling can induce the expression of ventral forebrain genes, such as *Nk2.2, zp-50* (zebrafish) and *Nkx2.1* (chicken and mouse, also called TTF-1 or T/ebp). While both Shh and Nkx2.1 are expressed in the MGE, neither are expressed at detectable levels within the LGE (Lazzaro et al., 1991; Price et al., 1992; Shimamura et al., 1995, Marti et al., 1995). Therefore, while these experiments suggest a role for Shh in inducing MGE, they do not address the question of whether Shh is involved in LGE induction. Direct evidence for an involvement of Shh in LGE induction has come from investigation of mice where the *Shh* gene has been ablated by targeted mutagenesis. In these animals, the striatal expression of *Dlx5* is lost (C. Chiang, personal communication), whereas the expression of the neocortical marker *Emx1* is expanded (Chiang et al., 1996). In addition, in these animals the LGE, as a morphological structure, fails to appear.

Previous work has shown that between 0-4 somites, telencephalic tissue responds to Shh by expressing the MGE-specific marker *Nkx2.1* (Ericson et al., 1995). As noted above, both morphological development (Smart and Sturrock, 1979) and gene expression analyses (Shimamura et al., 1995) suggest that the LGE is only generated later in development. Hence, we have devised an in vitro assay at this stage to evaluate the role Shh plays in this process. We find that during a narrow window of competence, between E10.5-E11.5 of rat development (10 to 23 somites), either ventral telencephalic midline tissue or Shh protein can induce telencephalic tissue to express genes characteristic of the LGE. Notably, even at the highest Shh concentration examined (960 nM), the MGE/pallidal marker Nkx2.1 was not induced at this stage of development. Furthermore, if Shh activity is blocked between the time the MGE and LGE are induced, Dlx expression is greatly reduced. Together, this suggests that the progressive development of the various ventral telencephalic structures is critically dependent on changes in the competence of the telencephalon to respond to Shh. We hypothesize that early in development, Shh signaling within the telencephalon results in MGE/pallidal induction, whereas later, Shh signaling induces telencephalon to adopt a LGE/striatal fate.

MATERIALS AND METHODS

Explant dissection and culture

Rat telencephalic explants were isolated by mechanical dissection from stage E9.5-E15.5. E10.5-E15.5 explants were cultured on 0.02 μm Nucleopore filters for 3 days in serum-free defined medium (DMEM:F12 medium supplemented with 2 mM glutamine, N2 and B27 serum-free supplements [Gibco], mito C [1×; Collaborative Research]). While the mesenchyme can be mechanically dissected from the explants, it is not possible to remove the ectodermal layers without enzymatic treatments. Under these conditions, dorsal-intermediate explants maintained Emx1 expression but with a non-uniform pattern of expression (results not shown), consistent with only the dorsal-most tissue being specified as cortex. All explants were negative for all ventral markers tested. Regional marker expression was assayed by immunocytochemistry and PCR analysis. A range of Shh concentrations between 6 and 960 nM was used in these experiments. The number of explants used in all the experiments ranged from 6-50. For the Shh titration experiments, highly concentrated N-terminally myristolyated Shh was used.

Co-cultures of explants and culturing of the younger explants (E9.5) was performed in collagen as described by Guthrie and Lumsden (1994), and fixing and staining as described below except that the concentration of Triton X-100 was increased to 0.5% in all incubations. The Shh blocking experiments were performed using the monoclonal antibody 5E1 (obtained from the Developmental Studies Hybridoma Bank [DSHB]), which has been shown previously to block Shh activity (Dale et al., 1997). This monoclonal was used at a concentration of 5 μg/ml in all blocking experiments.

Immunohistochemistry and in situ hybridization

Detection of Dlx was performed with an affinity-purified anti-Dlx polyclonal antibody (kindly provided by G. Panganiban; Panganiban et al., 1995) at a concentration of 1 μg/ml on 4% paraformaldehyde-fixed, frozen sections (15 μm) from E11.5 or E13.0 rat brains, and visualized with a Vector Labs ABC kit and DAB. Notably, multiple Dlx proteins are expressed within the basal telencephalon, including Dlx1, 2, 5, and 6. Given that this antibody was raised against the conserved homeodomain of *Drosophila* Dll it is reasonable to assume that this antibody recognizes all of these proteins. Nonetheless, all of these proteins have very similar expression patterns within the basal telencephalon, with the caveat that Dlx1 and 2 are expressed within the ventricular zone, whereas Dlx5 and 6 are only expressed in subventricular cells. For detection of Dlx in explants, explants were fixed in 4% paraformaldehyde for 20 minutes, blocked in PBS containing 20% goat serum and 0.1% Triton X-100 (PBT-GS), incubated at 4°C with anti-Dlx antibody in PBT-GS overnight, and visualized with FITC-conjugated goat anti-rabbit FAB fragment antibody at 1:30 (Boehringer Mannheim). Islet-1/2 was detected using mAb 40.2D6 (ascites) at 1:5000 (obtained from the DSHB). For Nkx2.1 (also called TTF-1) detection, staining, and fixation procedures were similar to those stated above, except that Triton X-100 was excluded from the primary antibody incubation. Nkx2.1 staining was visualized using rabbit polyclonal antibodies (Lazzaro et al., 1991; kindly provided by Dr DiLauro). For double staining of Dlx and Nkx2.1, a 1:1000 solution of a monoclonal antibody against Nkx2.1 was used (Holzinger et al., 1996; kindly provided by J. Whitsett) and Nkx2.1 immunoreactivity was visualized using Cy3-conjugated secondary antibodies. To visualize nestin expression, anti-nestin mAb (rat 401, culture supernatant, obtained from the DSHB) was used at 1:5 and visualized using goat anti-mouse secondary antibody conjugated to Cy3 (Jackson). The same procedure was used for detection with anti-Emx1 antibodies except that explants were fixed in 2% paraformaldehyde for 10 minutes (Briata et al., 1996). Both the blocking of Shh activity and Shh immunostaining were done using mAb 5E1 (obtained from the DSHB).

In situ hybridization was performed with digoxigenin-labeled antisense RNA probes according to Schaeren-Wiemers and Gerfin-Moser (1993). The *Evf-1* transcript was made from a *Bam*HI linearized 2.7 kb cDNA template, and the mouse Shh transcript was made from a *Hin*dIII linearized 1 kb cDNA template (Echelard et al., 1993). Both were transcribed with T7 polymerase.

RT-PCR analysis of explants

RT-PCR on explants was performed after isolation of RNA (Chomczynski and Sacchi, 1987) from 2 explants grown in culture. Reverse transcription was performed using MoMuLV(Gibco-BRL) and random hexanucleotides as primers. For a typical 20 μl reaction, half of the yield from 2 explants was used for reverse transcription, the other half served as a reverse transcriptase negative control; 1/50th of the resultant cDNA was used for PCR. Both the amount of template cDNA for amplification, and the number of cycles (28-32) were determined to be in the linear range. Each primer set generated the expected band size only from samples generated in the presence of reverse transcriptase, and not in its absence. Therefore, Dnase

treatment of RNA prior to reverse transcription was not found to be necessary for this particular set of primers. S17 was used as control for RNA input.

1. *Dlx-2*: (5′-ACACCGCCGCGTACACCTCCTA-3′)
   (5′-CTCGCCGCTTTTCCACATCTTCTT-3′)
2. *Emx1*: (5′-CGAGAAGAACCACTACGTGG-3′)
   (5′-AGGTGACATCGATGTCCTCC-3′)
3. *Evf-1*: (5′-CCAGACTCACTTAGGTCCAAGC-3′)
   (5′-CCAGATGAGAGCATCACTGG-3′)

Note that the *Evf-1* primers were selected so they recognized the rat, but not the mouse, form of the ventral telencephalic marker *Evf-1* (Fig. 2Bi). As shown in Fig. 2Bi, using RT-PCR, our primer sets for *Evf-1* only amplify the rat, and not the mouse, form of this gene. Using this approach we could distinguish *Evf-1* derived from the target and the inducing tissues.

4. *T-brain*: (5′-AACTCTCTCCTGTCTTGGACG-3′)
   (5′-TGTTGCACAGGTATACTTGCG-3′)
5. *GAD-67 ES* (glutamate decarboxylase): (5′- TTGTGAAGGAG-AAAGGCCC-3′)
   (5′-TAGAGTTGTTTGGCAGTGCG-3′)
6. *Ikaros 4* (Molnar et al., 1994): (5′-GCCTGTCCCTGAG-GACCTGTC-3′)
   (5′-TCTGAGGCATGAGCTCTTAC-3′)
7. *Shh*: (5′-CTCCGATGTGTTCCGTTACC-3′)
   (5′-TGCACCTCTGAGTCATCAGC-3′)
8. *S17*: (5′-AAGCTCCGCAACAAGATAGC-3′)
   (5′-TGAAGGTTGGACAGACTGCC-3′)

RESULTS

Regional telencephalic markers and Shh expression

To address the nature of ventral patterning within the E11.5 telencephalon, we first defined markers that indicate the regional patterns of differentiation during this period of development. Notably, regional markers within the different territories of the telencephalon appear prior to morphological differentiation. Two cortical markers have been used previously, Emx1, which is expressed during early corticogenesis (Simeone et al., 1992), and the early cortical differentiation marker, *Tbr-1* (Bulfone et al., 1995). Emx1 is expressed within both the proliferative and postmitotic regions of the cortex. In contrast, *Tbr-1* is expressed only in postmitotic cortical cells (Bulfone et al., 1995).

We used the following as markers of ventral telencephalon: Shh, Nkx2.1 (Lazzaro et al., 1991), Dlx (Porteus et al., 1991), Islet-1/2 (Ericson et al., 1995, 1996), *Evf-1* (Kohtz and Fishell, unpublished data), *GAD-67-embryonic stop (ES)* (Behar et al., 1994), and *Ikaros* (Georgopoulos et al., 1992, 1994). The ventral telencephalic genes/proteins *GAD-67 ES, Evf-1*, Islet-1/2, and *Ikaros* collectively provide sequential markers for cells as they pass through the various stages of maturation. Cells within the proliferative ventricular zone express *GAD-67 ES*, more mature neuroblasts within the subventricular zone express *Evf-1*, early postmitotic cells are positive for Islet-1/2, and, finally, mature neurons express *Ikaros*. With the exception of *Ikaros*, all markers used are expressed within the MGE. While both Shh and Nkx2.1 are expressed strongly within the MGE, neither is present within the LGE (Lazzaro et al., 1991; Shimamura and Rubenstein, 1995). In *Nkx2.1* homozygous mutant animals, while the ventral forebrain is grossly deformed, the striatum is relatively unaffected (Kimura et al., 1996). Dlx, Islet-1/2, *Evf-1* (Fig. 1), and *GAD-67 ES* (results not shown) are present within both the MGE and the LGE. Although *Ikaros* is an LGE-specific marker (B. Morgan, personal communication), its expression only becomes detectable by in situ hybridization near birth (E21). Therefore, we use the absence or presence of Shh or Nkx2.1 in combination with the more widespread ventral telencephalic markers described above (Dlx, *Evf-1*, Islet-1/2, *GAD-67 ES*) to distinguish LGE- and MGE-derived ventral neurons.

The LGE and MGE become morphologically distinct between E11.5 and E13.0. In contrast, regional patterns of gene expression are already apparent within the ventral telencephalon at E11.5 (Fig. 1). At this time, the cells in the midline of the ventral telencephalon express both Shh and Nkx2.1 in overlapping domains (Fig. 1A,B,F,G). Also, at E11.5, small numbers of ventral telencephalic neurons express Dlx and *Evf-1* (Fig. 1C,D,H,I) as they exit the ventricular zone. Dlx/*Evf-1*-positive cells can be detected near the midline region, where they co-express Shh and Nkx2.1, as well as in a small region further from the midline which is negative for both Nkx2.1 and Shh expression (note the arrowheads in Fig. 1A-D and F-I, which indicate the MGE/LGE division in E11.5 telencephalon). This latter region containing cells positive for Dlx and *Evf-1*, and negative for Nkx2.1 and *Shh*, presumably constitutes part of the region that will give rise to the LGE. Islet-1/2 is expressed at very low levels within the telencephalon at E11.5 and then only within the MGE by whole-mount staining (data not shown). Islet-1/2 is not detectable in E11.5 telencephalic section staining. Interestingly, the order in which these markers appear is consistent with the morphological evidence suggesting that the MGE is induced prior to the generation of the LGE.

By E13.0, the LGE can be distinguished from the MGE morphologically (Fig. 1J-R) and it becomes apparent that Nkx2.1 and Shh are restricted to the MGE; note the absence of expression in the LGE (Fig. 1J,K,N,O). Hence the restriction of these expression domains at the earlier age (i.e. E11.5) is maintained. Notably, the numbers of cells expressing Dlx and *Evf-1* (Fig. 1L,M,P,Q) increases significantly by E13.0 compared to that seen a day earlier. Similarly, Islet-1/2 expression appears within postmitotic cells of the LGE (Fig. 1R). Together with the observation that the largest portion of the nascent Dlx and *Evf-1* expressing cells are within the LGE, a region that is Nkx2.1 and Shh negative, these results are consistent with this being the period during which LGE/striatum is induced.

Ventral telencephalic midline can induce ventral markers in telencephalic explants

The known involvement of Shh in generating basal telencephalic structures (Chiang et al., 1996) led us to ask whether ventral telencephalic midline cells expressing Shh can act as inducers of ventral telencephalic differentiation. To test this, we established in vitro conditions in which rat or mouse telencephalic explants can be maintained in culture for up to 3 days. We then used mouse ventral midline tissue and apposed it to rat telencephalon and incubated these co-cultures in vitro for 3 days. In order to distinguish ventral genes of the inducing and responding tissues, we generated rat-specific primers for the ventral marker *Evf-1*.

When E10.5 mouse ventral telencephalic midline is apposed to E11.5 rat dorsal/intermediate telencephalic explants (see below for definition of this piece), the latter is induced to

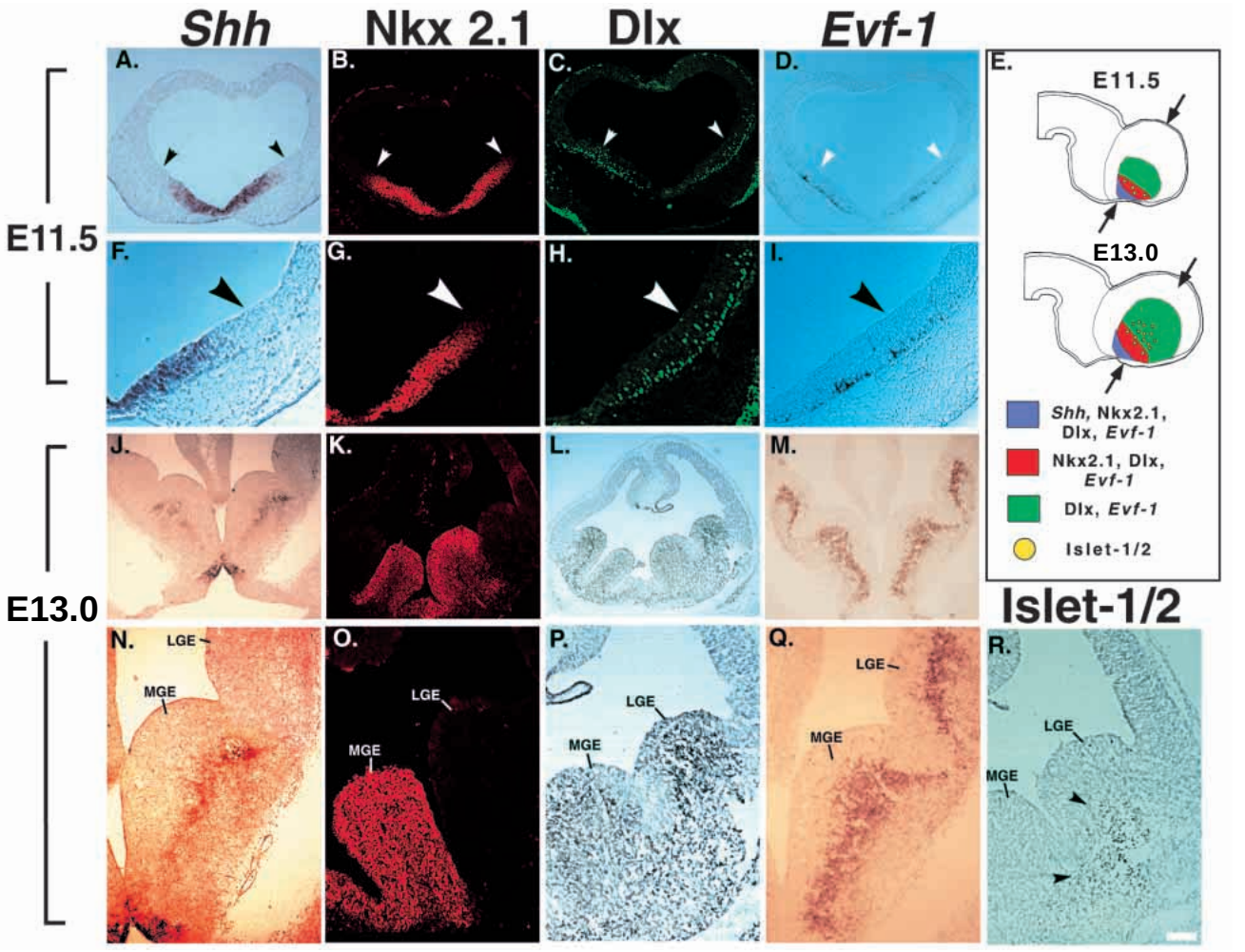

Fig. 1. In situ and antibody localization of *Shh*, Nkx2.1, Dlx, *Evf-1*, and Islet-1/2 in E11.5 and E13.0 telencephalon. Coronal sections of rat E11.5 and E13.0 telencephalon (the plane of section is indicated by the arrows in the schematics in E) probed with *Shh* anti-sense RNA (A,F,J,N); rabbit anti-Nkx2.1 (B,G,K,O); rabbit anti-Dlx (C,H,L,P); *Evf-1* anti-sense RNA (D,I,M,Q); mouse anti-Islet-1/2 (R). The in situ hybridizations shown in A,D,F,I,J,M,N and Q were performed as described by Schaeren-Wiemers and Gerfin-Moser (1993). Visualization of primary antibodies was achieved using fluorescently conjugated secondary antibodies (fluorescein, green; Cy3, red) in B,C,G,H,K and O. Visualization of primary antibodies was performed using biotinylated secondary antibodies and a Vector ABC peroxidase kit followed by DAB (L,P,R). A-D and F-I show E11.5 telencephalic sections, whereas J-R show E13.0 telencephalic sections. E shows the distribution of Dlx, Shh, Nkx2.1, and Islet-1/2 proteins in a schematic of E11.5 and E13.0 rat telencephalon, as viewed in a flat-mounted explant, visualized from the ventricular surface. All sections are coronal taken through the middle of the telencephalon, as indicated by the arrows in E. (A,F) E11.5 and (J,N) E13.0 *Shh* expression within the telencephalon. Note that *Shh* is expressed within the MGE but not within the LGE at E13.0. (B,G) At E11.5, Nkx2.1 expression extends more laterally than the *Shh* expression domain. By E13.0 (K,O), Nkx2.1 expression extends throughout the MGE but is absent from the LGE. (C,H) Dlx expression at E11.5 becomes apparent in cells as they exit the ventricular zone. At E13.0 (L,P), Dlx is expressed widely throughout the LGE and the MGE. (D,I) At E11.5, *Evf-1* expression shows a very similar distribution to Dlx, extending laterally beyond the Nkx2.1 expression domain. By E13.0 (M,Q), *Evf-1* expression is restricted to the subventricular zone, whereas Dlx expression is also found within the ventricular zone. (R) By E13.0, Islet-1/2 expression is found in a small population of cells in the postmitotic region of both the LGE and MGE (indicated with arrowheads). The arrowheads in A-D and F-I indicate the lateral extent of Nkx2.1 expression. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bar in R, 100 μm (A-D); 40 μm (F-I); 150 μm (J-M) and 60 μm (N-R).

express *Evf-1* (Fig. 2Bii, lanes 5-8). To examine if Shh signaling is necessary for the ventral telencephalic midline-mediated induction of *Evf-1* in this assay, we examined whether a monoclonal antibody that blocks Shh signaling (5E1; Ericson et al., 1996) could block *Evf-1* induction in our assay. Whereas, 6/13 co-cultured telencephalic explants were induced to express *Evf-1*, 0/9 co-cultured telencephalic explants incubated with the anti-Shh antibody showed detectable *Evf-1* induction (Fig. 2Bii, lanes 3 and 4). Given that blocking Shh signaling prevents the induction of the ventral telencephalic marker *Evf-1*, we set forth to determine the character of the ventralized tissue. Specifically, we asked whether the ventralized telencephalic tissue was adopting an MGE or LGE phenotype.

Telencephalic tissues used in the in vitro assays

To determine which specific regions of the E11.5 telencephalon are competent to be ventralized, we examined specific portions of the dorsal or intermediate telencephalon for their ability to

express Dlx and/or Islet-1/2 in response to Shh treatment. Subdissection of the telencephalon was performed as indicated in Fig. 3. Specifically, three telencephalic pieces were assayed: (1) dorsal + intermediate, (2) dorsal alone, and (3) intermediate alone. Each of these pieces was incubated with Shh to determine which were responsive. Only pieces containing the intermediate region (i.e. 1 and 3) were able to express Dlx and Islet-1/2 positive cells in response to Shh. Notably, the intermediate region does not spontaneously express either of these markers in the absence of Shh. Therefore, the dorsal/ventral character of portions of this region at E11.5 is presumably unspecified.

Shh can induce the expression of LGE/striatal markers in telencephalon

In these experiments, we cultured pieces of E10.5 and E11.5 telencephalon (i.e. piece 1 in Fig. 3) in the presence or absence of Shh protein. In the absence of Shh, this piece non-uniformly expressed dorsal markers (Fig. 4II, lane 2) but not ventral markers (Fig. 4I, lane 2). In contrast, we found that E11.5 telencephalic tissue cultured in the presence of Shh expressed Dlx, *GAD-67 (ES)*, and more differentiated ventral markers such as *Evf-1*, Islet-1/2, and *Ikaros* (Fig. 4I, lane 1 and 4B). That telencephalon cultured in the presence of Shh adopted an LGE/striatal rather than an MGE/pallidal identity was suggested by the absence of *Shh* expression (Fig. 4I) and Nkx2.1 staining in preparations treated with recombinant Shh protein (Fig. 4D). Whereas, 6 nM Shh could induce Dlx expression, levels as high as 960 nM of Shh did not induce Nkx2.1 in E11.5 telencephalic explants (Fig. 4D). This higher concentration represents a concentration of Shh 200× greater than was necessary to induce either *HNF3β* in spinal cord (Ruiz i Altaba et al., 1995) or Nkx2.1 expression in younger telencephalic tissue (Ericson et al., 1995; Shimamura and Rubenstein, 1997). In contrast, E9.5 presumptive telencephalic explants (0-8 somites) cultured in a concentration of Shh that can induce Dlx in E11.5 telencephalic explants, results in widespread expression of Nkx2.1 (Fig. 4C). Quantitative analysis of the numbers of Dlx-positive cells in E11.5 telencephalic explants at different concentrations of Shh is shown in Fig. 5.

Shh protein can inhibit the expression of genes normally expressed in the dorsal telencephalon

Within the spinal cord, Shh has been shown to inhibit the expression of dorsal markers (Liem et al., 1995). To test if a similar repression of dorsal telencephalic markers is observed in telencephalic tissue treated with Shh, we used the early cortical marker, Emx1, and the early cortical differentiation marker, *Tbr-1* (Simeone et al., 1992; Bulfone et al., 1995). Consistent with what is seen in more posterior regions of the CNS, dorsal markers of E10.5-11.5 dorsal telencephalon, such as Emx1 and *Tbr-1*, are repressed at E10.5 (10-15 somite stage) and, to a lesser degree, at E11.5 (17-23 somite stage) in the presence of Shh (Fig. 4II,G-J).

Shh induces the expression of different ventral forebrain genes at different times during development

The observation that Shh does not induce the expression of Nkx2.1 within the E11.5 telencephalic explants supports the idea that the telencephalon undergoes progressive changes in its response to Shh. To test this question directly, we cultured head-fold stage explants, containing presumptive telencephalon, in the presence or absence of the Shh-blocking antibody 5E1 (5 μg/ml). If Shh induces the telencephalon to take on an MGE identity early during telencephalic development and later induces an LGE fate, blocking Shh

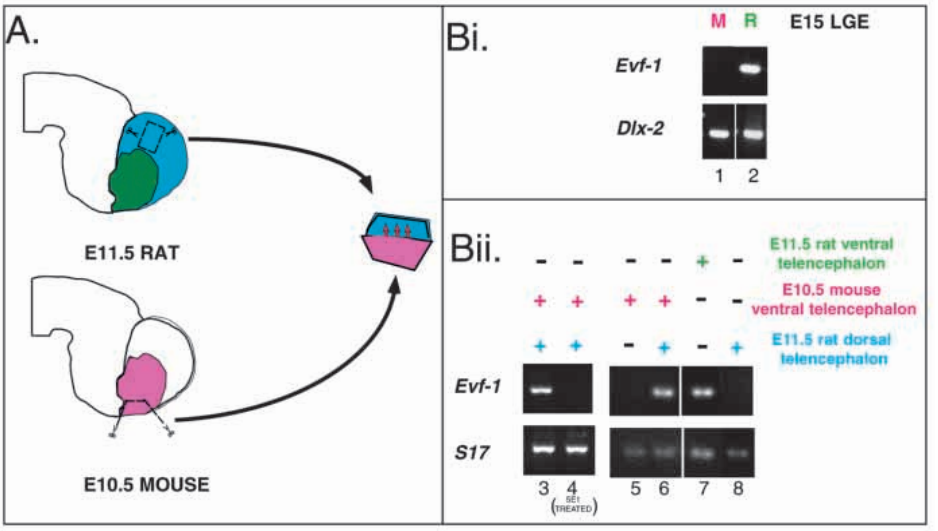

Fig. 2. Ventral midline explants can induce *Evf-1* in telencephalic explants in vitro. (A) Schematic of how the apposition experiments of E10.5 mouse ventral telencephalon (pink) and E11.5 rat dorsal telencephalon (blue, same as tissue used in Fig. 3A) were performed. (Bi) In order to distinguish *Evf-1* already present in the inducing ventral tissue, and *Evf-1* derived from the responding tissue, we utilized rat PCR primers. Differences in the sequence of the 3′ region of the *Evf-1* gene in rats and mice were exploited to produce a primer set that only recognizes the rat form. RNA from E15 lateral ganglionic eminence (LGE), which constitutes a large region of the ventral telencephalon at this age, was isolated from both mouse (M, pink, lane 1) and rat (R, green, lane 2) embryonic brain. *Evf-1* primers are specific for rat, and do not amplify products from mouse RNA. *Dlx-2* primers recognize both species. (Bii) RT-PCR analysis of RNA levels of the ventral-specific marker *Evf-1* (embryonic ventral forebrain 1, cloned in our laboratory) shows that mouse ventral telencephalon can induce rat telencephalon to express this ventral gene, *in vitro*. Rat E11.5 ventral telencephalon (rE11.5vt, green, lane 7) expresses *Evf-1*, as would be expected. Co-culturing of explants from mouse E10.5 ventral telencephalon (mE10.5vt, pink) and rat E11.5 dorsal telencephalon (rE11.5dt, blue) shows that *Evf-1* is induced in rat explants (lanes 3 and 6, 6 of 13 appositions). However, when the apposed explants are cultured in the presence of the Shh-specific antibody 5E1, *Evf-1* induction is blocked (lane 4, 0 of 9 appositions). mE10.5vt (lane 5) or rE11.5dt (lane 8) alone do not express *Evf-1*.

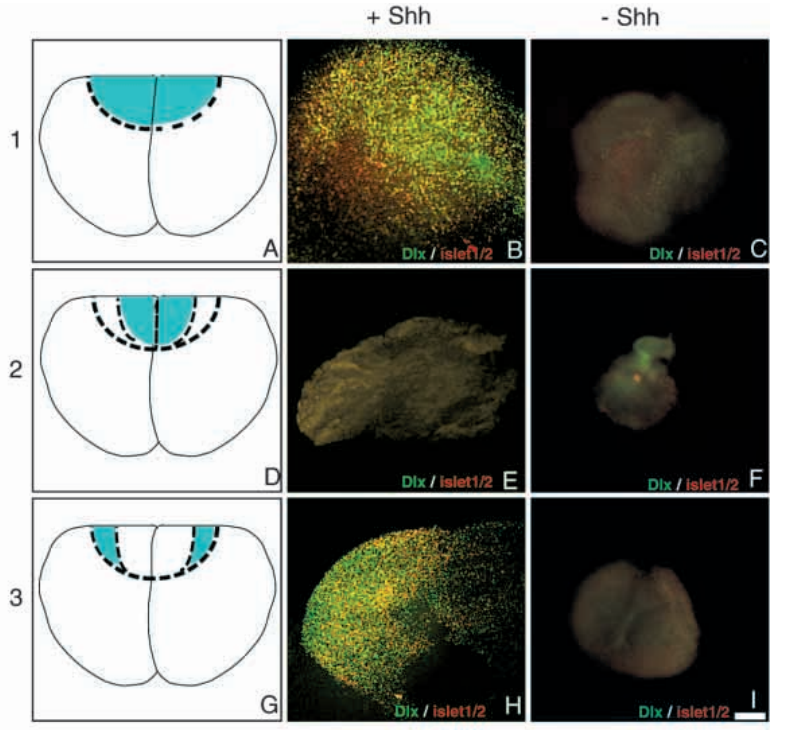

Fig. 3. Intermediate E11.5 telencephalic explants are induced to express ventral markers in response to Shh. Three different types of E11.5 telencephalic explants were tested for their responsiveness to Shh. 1: (A-C) dorsal + intermediate; 2: (D-F) dorsal alone; and 3: (G-I) intermediate alone (presumptive LGE). B,E and H were cultured in the presence of Shh protein, whereas C,F, and I were cultured in its absence. In the schematics the region of the telencephalon used in the in vitro assay is in blue (A,D,G). Explants were stained with anti-Dlx antibody (green) and anti-Islet-1/2 antibody (red) in B,C,E,F,H and I. (B) E11.5 dorsal + intermediate explants incubated with Shh express both Dlx (in green) and Islet-1/2 (in red) in response to Shh. While in a few cases, small numbers of induced ventral cells were observed (results not shown), typically E11.5 dorsal explants alone did not express Dlx or Islet-1/2 in response to Shh protein (E). E11.5 intermediate explants express both Dlx and Islet-1/2 in response to Shh protein (H). Scale bar in I 100 μm (B,C,E,F,I); 200 μm (H).

activity between the time when the MGE and LGE are induced should not effect Nkx2.1 expression but should greatly reduce Dlx expression. This is precisely what we observed (Fig. 6). In headfold explants taken at E9.5 (time=0), Nkx2.1 was detectable whereas Dlx was not (Fig. 6A,E). After culturing for 40 hours, Nkx2.1 is expressed in headfold explants in a region flanking the ventral midline (Fig. 6B), whereas Dlx expression shows a similar expression pattern that extends more laterally (Fig. 6F). In identical explants cultured in the presence of the anti-Shh antibody 5E1, the expression pattern of Nkx2.1 is unaffected (Fig. 6C) but the expression of Dlx is reduced greatly (Fig. 6G).

Shh-mediated ventralization of telencephalon is limited by a competence period

If Shh signaling can induce telencephalon to adopt a striatal identity, what prevents the entire telencephalon from becoming striatum? Three possible mechanisms are plausible. First, regions of telencephalon could be sufficiently distant from a source of Shh, so as to prevent this tissue from being exposed to a high enough level of Shh to induce ventral phenotypes. Second, a putative extrinsic dorsalizing signal (such as BMP's, (Liem et al., 1995; Dickinson et al., 1995; Golden et al., 1997; Muhr et al., 1997) could antagonize the striatal inducing effects of Shh (see below). Finally, this inductive event could be temporally limited by a competence period.

In order to investigate whether a striatal competence period can be defined, we assayed progressively older telencephalic explants for their ability to express Dlx in response to Shh. We found that Dlx can only be induced by Shh in the telencephalon during a limited time in development (Fig. 4B vs. F). While E11.5 rat telencephalon (17-23 somites) could still respond to Shh protein, E12.5 or older tissue (>30 somites) could not (Fig. 4).

Telencephalic tissue cultured in isolation loses it competence to be ventralized by Shh

In order to characterize the cellular mechanism by which loss of competence to assume a ventral fate occurs, we asked whether the E12.5 loss of competence to express Dlx in response to Shh is dependent on tissue extrinsic or intrinsic cues. If during development of the telencephalon, the transition from a plastic (E11.5) to a restricted state (E12.5 or older) requires external tissues or signals, E11.5 explants should remain competent in the absence of such signaling. However, as seen in Fig. 7, E11.5 explants in isolation were found to undergo a progressive loss of competence to express Dlx in response to Shh after one (Fig. 7A) or two (Fig. 7B) days in culture. These results suggest that the signals responsible for this loss of competence are inherent to E11.5 tissue.

The loss of competence of telencephalon to be ventralized by Shh is not caused by neural maturation

Is the loss of competence of telencephalic tissue to become striatum simply the result of these progenitors withdrawing from the cell cycle? It seems unlikely, as E12.5 represents a time very early during telencephalic neurogenesis (Bayer, 1989). Indeed, it has been shown that progenitors can be induced to change their regional fates through heterotopic transplantation until perinatal periods (approx. E18+) (Brustle et al., 1995; Campbell et al., 1995; Fishell, 1995). However, to examine this possibility directly we compared the numbers of progenitors in 'competent' versus 'non-competent' tissue using nestin, a marker for proliferating neural cells (Lendahl et al., 1990). The number of nestin-positive cells in untreated E11.5 versus E12.5 explants was comparable. In addition, the numbers of nestin positive cells in Shh-treated telencephalic tissue was roughly 50% greater than untreated tissue,

regardless of whether E11.5 or E12.5 explants were examined (Fig. 8A-D). Given the persistence of Shh expression within the telencephalon at later stages of embryonic and postnatal development, this suggests that Shh could act as a mitogenic or trophic factor in older animals. Indeed, a study by Miao et al (1997) shows that Shh is trophic for striatal cells at later times in development.

DISCUSSION

Our experiments suggest that the sequential induction of different ventral telencephalic structures is a consequence of a change in how the telencephalon responds to Shh signaling. The present data supports the hypothesis that Shh first induces E9.5 telencephalon to become MGE/globus pallidus (Nkx2.1/Shh positive) and later (E10.5-E11.5) LGE/striatum (Dlx/Islet-1/2 positive, Nkx2.1/Shh negative). Even though Shh persists within the forebrain at later times in development, telencephalic tissue is unable to be ventralized beyond E12.5.

Experiments in this study suggest that the loss of competence to adopt a ventral fate is not due to a general reduction in neural progenitor populations. It also does not appear to be mediated by inhibitors of Shh-signaling produced by tissues extrinsic to the telencephalon. Rather, it appears that loss of competence to be ventralized is an inherent time-dependent property of the telencephalon. These results suggest that ventral patterning within the telencephalon is dependent upon a series of progressive changes in the competence of this tissue to respond to Shh signaling.

MGE versus LGE induction within the telencephalon

Examination of *patched (Ptc)* null mice and in vitro explant studies suggest Shh acts to induce the MGE at early periods during telencephalic development. Numerous studies have shown that Shh's primary function is to repress the inhibition of *Smoothened* by *Ptc* (Alcedo et al., 1996; Marigo et al., 1996; Stone et al., 1996). Therefore, the ablation of the *Ptc* gene is functionally equivalent to constitutive over-expression of Shh. In accordance with the notion that the entire telencephalon is competent to become MGE during early stages of development, the majority of the telencephalon becomes Nkx2.1 positive in *Ptc* homozygous mutants (Goodrich et al., 1997). Furthermore, a number of groups have used neural plate explants, isolated prior to neural tube closure, to examine the role of Shh in ventralizing the forebrain between 0 to 8 somites of development (chick stage 6 presumptive telencephalon (Ericson et al., 1995); E8.5 mouse telencephalon (Shimamura and Rubenstein, 1997); or diencephalic intermediate neural plate (Dale et al., 1997)). In these studies as well as our own Shh (either alone or in combination with BMP-7) was shown to induce the expression of the homeobox-containing gene *Nkx 2.1* in the forebrain.

In this paper, we show that the MGE-specific marker Nkx2.1 is not induced in E11.5 telencephalic explants at concentrations as high as 960 nM. Furthermore, we show that blocking Shh

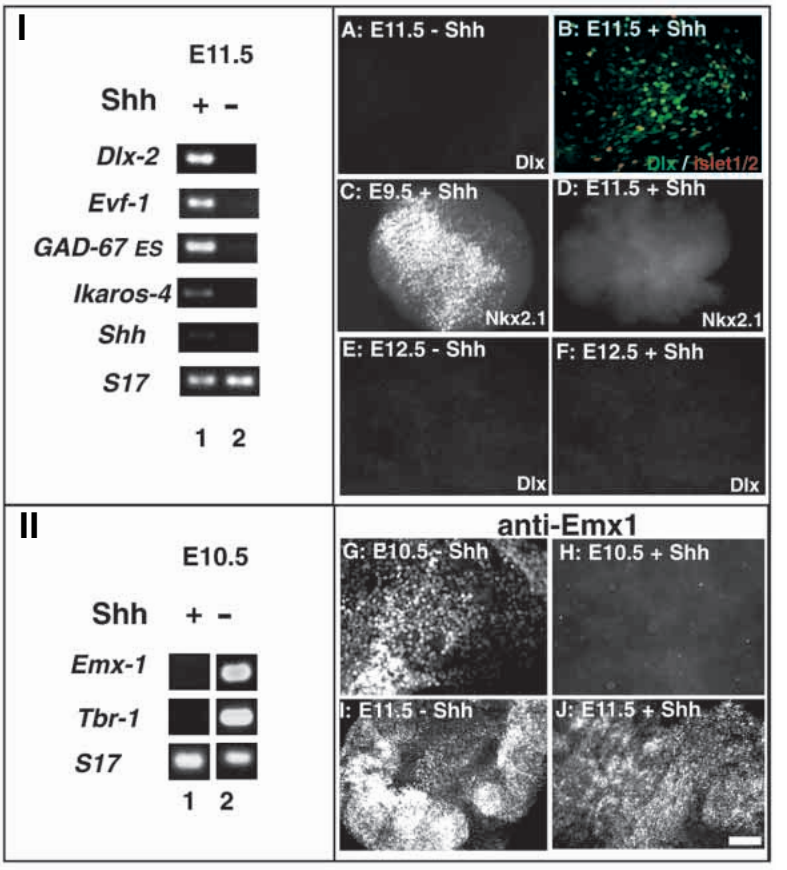

Fig. 4. Telencephalic explants can be ventralized by Shh during a competence period. (I) Shh induces ventral markers at E11.5. Explants were dissected from stage E11.5 (lanes 1 and 2) and cultured in the presence (+, lane 1), or absence (–, lane 2) of Shh. RT-PCR revealed that Shh can induce the ventral-specific genes, *Dlx-2*, *Evf-1*, *GAD-67 ES* and *Ikaros*, while induction of *Shh* is negligible. *S17* (a ribosomal binding protein) RNA was used as a control for input RNA. (A-J). Telencephalic explants from different stages during rat embryogenesis (E9.5-E12.5) were cultured for 3 days in the absence of Shh (–) or in its presence (+), then fixed and stained with antibodies to Dlx, Islet-1/2, Nkx2.1 or Emx1 as indicated. (A) E11.5 explant cultured in medium alone and stained with anti-Dlx antibodies. (B) E11.5 explant cultured in the presence of Shh and double-stained for Dlx (green) and Islet-1/2 (red) immunoreactivity. (C) E9.5 explant cultured in the presence of Shh and stained with anti-Nkx2.1 antibodies. (D) E11.5 explant cultured in the presence of Shh and stained with anti-Nkx2.1 antibodies. (E) E12.5 explant cultured in medium alone and stained with anti-Dlx antibodies. (F) E12.5 explant cultured in the presence of Shh and stained with anti-Dlx antibodies. (Y,G-J) Shh suppresses dorsal genes in rat telencephalic explants. (II) RT-PCR analysis of RNAs derived from E10.5 rat telencephalic explants were treated with Shh (+, lane 1) or cultured in medium alone (–, lane 2) and assayed for levels of the dorsal markers *Emx1* (mouse homeobox empty-spiracles homologue) or *Tbr-1* (mouse brachyury homologue). At E10.5, both dorsal genes are suppressed by Shh; *S17* is shown as a control for the level of input RNA. (G-J) Telencephalic explants from stages E10.5 and E11.5 incubated in the absence (–, G,I) or presence (+, H,J) of Shh for 3 days. The explants were fixed, stained with an anti-Emx1 antibody, and visualized with a fluorescein-conjugated secondary antibody. Shh suppresses Emx1 expression entirely at E10.5 (compare G and H) but not at E11.5 (compare I and J). Scale bar in J, 100 μm (A,B,D-H); 150 μm (C); 200 μm (I,J).

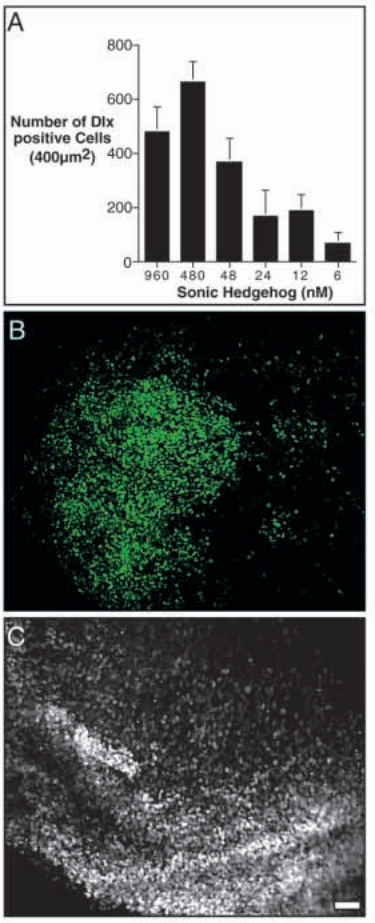

Fig. 5. Shh induces Dlx but not Nkx2.1 in E11.5 telencephalic explants over a wide range of concentrations. E11.5 telencephalic explants were isolated as shown in Fig. 3A and cultured in the presence of Shh protein in a range of concentrations between 6 and 960 nM. Explants were analyzed by double immunocytochemistry, using a FITC-conjugated secondary antibody to visualize Dlx staining, and a Cy3-conjugated secondary antibody to visualize Nkx2.1 staining. As seen in A, in all cases significant numbers of Dlx-positive cells were observed. In contrast, even when this tissue is exposed to extremely high concentrations of Shh, Nkx2.1 expression is not induced. B shows a representative explant which was cultured in the presence of 480 nM Shh. This explant was double-stained for Dlx (green) and Nkx2.1 (red). The absence of any red cells (in this and all other explants examined in this experiment) demonstrates that Shh cannot induce Nkx2.1-positive cells in E11.5 telencephalic explants. C shows control ventral tissue stained for Nkx2.1-positive cells. The numbers of Dlx-positive cells shown in A were obtained from five to eight 400 μm^2 regions, for each Shh concentration. All control explants, cultured in the absence of Shh protein, were negative for both Dlx and Nkx2.1 staining. Scale bar for C and D, 100 μm.

activity (using 5E1) within headfold explants during the time between MGE and LGE induction, greatly reduces the expression of Dlx within these explants but has no effect on Nkx2.1 expression (Fig. 6). These results suggest that later during telencephalic development Shh induces telencephalon to adopt an LGE/striatal rather than a MGE/pallidal fate. Therefore, the timing of telencephalic exposure to Shh is critical in determining which ventral phenotypes are induced.

Does Shh respecify neurons or act only on unspecified progenitors?

Based on nestin staining it appears that Shh increases the numbers of progenitors in treated explants. In addition, Shh-treated explants are consistently larger, indicating either increased proliferation or decreased cell death. This supports the idea that Shh acts by expanding and ventralizing unspecified progenitors within the telencephalon. However, the observation that Shh suppresses the dorsal genes *Emx1* and *Tbr-1* suggests that it can also act to respecify dorsal telencephalic cells as well. Similarly, it has recently been shown that an early function of Shh is the suppression of genes such as *Pax6*, a gene ultimately restricted to the dorsal-most regions of the ventral spinal cord (Ericson et al., 1996).

The demonstration that respecification occurs is particularly relevant in understanding the transition of the telencephalon from a competent to a non-competent state (discussed below). Without respecification, it might be argued that the loss of competence to be ventralized by Shh is only a reflection of a reduction in the number of progenitors. The persistence of a large population of nestin-positive cells in both competent and incompetent explants rules out this possibility (see Fig. 8).

The loss of competence of telencephalon to be ventralized by Shh

In this study, the extent to which competence and position determine dorsal/ventral patterning of the telencephalon is investigated. Hence the response to Shh signaling within the telencephalon is strongly dependent on when the tissue is exposed to this signaling molecule. Notably, *Shh* expression persists within the telencephalon throughout embryonic and postnatal development (Miao et al., 1997). Hence, the telencephalic transition from a competent (E11.5) to a non-competent (E12.5) state may be important in preventing the entire telencephalon from being ventralized. Investigation of the molecular basis of this loss of competence should contribute to our understanding of the factors involved in the establishment of dorsal/ventral determination within the telencephalon.

It remains possible, and indeed likely, that changes in expression and/or activity of other elements of the Shh-signaling pathway explain the loss of competence during the E11.5-E12.5 transition. In order to investigate the molecular basis of this loss of competence, we examined the expression of the Shh receptor molecules Ptc and Smoothened (Smo) in telencephalon that is no longer responsive to be ventralized by Shh. Changes in the expression of these molecules does not appear to explain the observed loss of competence (results not shown). Other possible candidates which could mediate this loss of competence include the various Gli proteins (Platt et al., 1997; Lee et al., 1997; Ruiz i Altaba, 1997), CBP, PKA, Cos2 and Fused (Robbins et al., 1997; Sisson et al., 1997). Important to our understanding of how telencephalic patterning is established will be the further characterization of this loss of competence.

Dlx is an evolutionarily conserved target of Shh signaling

The present study demonstrates that Shh induces members of the *Dlx* gene family. It has been shown previously that *hh* (the *Drosophila* homolog of Sonic hedgehog), through a mechanism

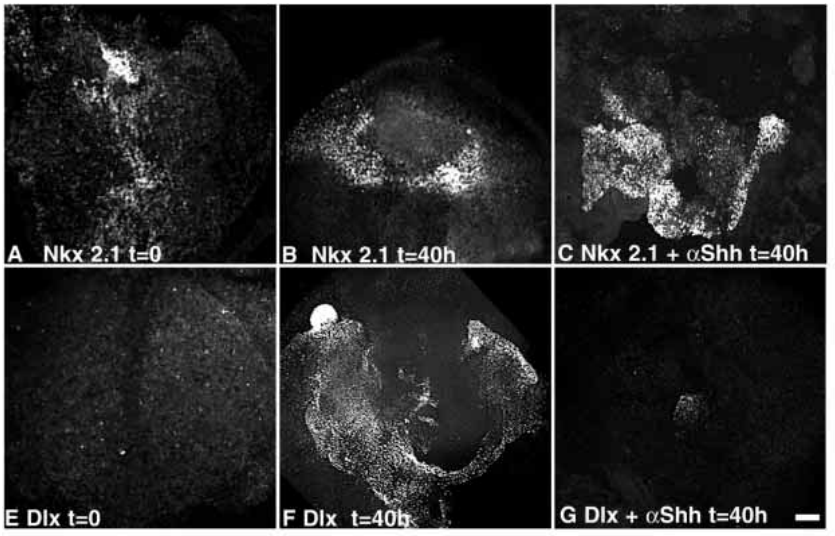

Fig. 6. Blocking Shh activity during the period of LGE induction greatly reduces the normal expression of Dlx in the forebrain. Headfold explants were isolated from E9.5 embryos (0-8 somites). These explants were stained at the time of dissection (t=0; A,E) or after being cultured for 40 hours in the absence (B,F) or the presence (C,G) of the anti-Shh antibody 5E1. (A-C) Explants stained with anti-Nkx2.1 and visualized with a Cy3-conjugated secondary antibody. (E-G) Explants stained with anti-Dlx antibody and visualized with a Cy3-conjugated secondary antibody. In the absence of the anti-Shh antibodies, explants express Nkx2.1 and Dlx. In contrast, explants in which Shh activity is blocked show similar Nkx2.1 expression to control explants but have greatly reduced levels of Dlx expression. This result suggests that Shh induces different ventral forebrain genes at different times during development. All explants are flat-mounted such that anterior is at the top and posterior is at the bottom. For each condition *n*=9 and the outcome of each replication gave an identical result. Scale bar in G, 50 μm.

involving *dpp* (decapentaplegic) and *wg* (wingless), regulates *dll* (distal-less) expression in *Drosophila* (Diaz-Benjumea et al., 1994). Specifically, the combined expression of *dpp* and *wg* results in the activation of *dll* (Diaz-Benjumea et al., 1994). It remains to be determined whether there is a similar requirement for BMPs (Gelbart, 1989; i.e. vertebrate homologs of *dpp*) and Wnts (Rijsewijk et al., 1987; i.e. vertebrate homologs of *wg*) for *Dlx* expression within the telencephalon. To date, the majority of BMP and Wnt expression has been found to be restricted to dorsal telencephalon, in areas distant from where the *Dlx*s are present (Parr et al., 1993; Furuta et al., 1997).

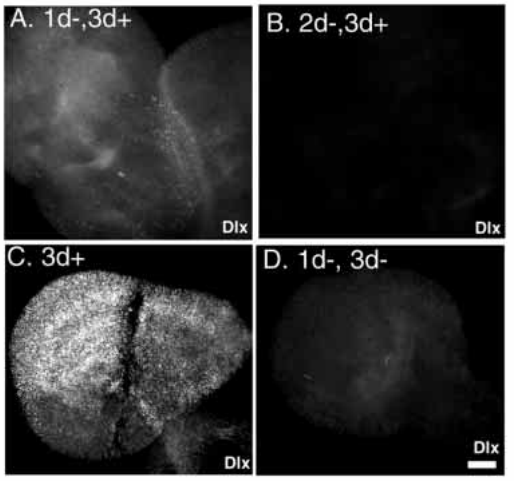

Fig. 7. Competence to be ventralized by Shh is determined by a tissue intrinsic mechanism. Competence of E11.5 telencephalic explants to express Dlx in response to Shh is lost after 1 or 2 days in vitro. E11.5 telencephalic explants were cultured in vitro for the number of days indicated, treated with Shh (+) or medium without Shh (–) and stained with anti-Dlx antibodies. (A) 1d–, 3d+: explant was cultured for 1 day in medium without Shh before adding Shh for 3 days. (B) 2d–, 3d+: explant was cultured for 2 days in medium without Shh before adding Shh for 3 days. (C) 3d+; explant was cultured in medium containing Shh for 3 days without any prior incubation. (D) 1d–, 3d–: explant was cultured for 1 day without Shh, fresh medium without Shh was added and the explant was cultured for an additional 3 days. This result is unaffected by an additional 1 or 2 days of culturing (results not shown). The loss of competence of telencephalic tissue to express Dlx in response to Shh follows a similar course in vitro to that observed in vivo. Explants incubated for 1 or 2 days before addition of Shh for 3 days fail to express Dlx, suggesting that a time-dependent intrinsic mechanism is responsible for the observed change in Shh responsiveness. Scale bar (in D), 150 μm.

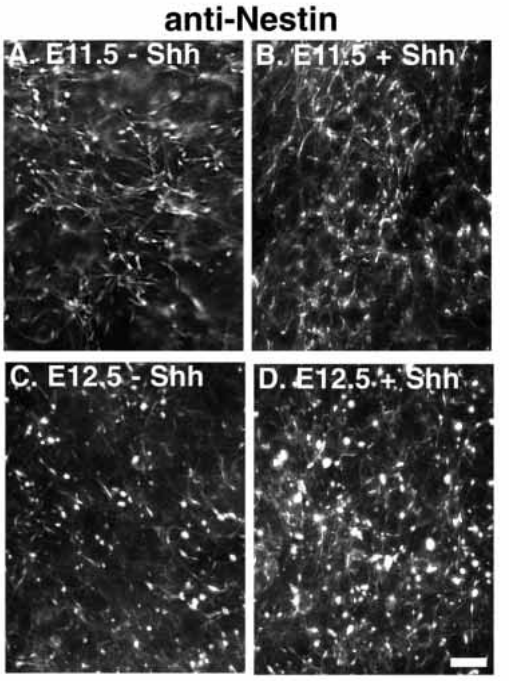

Fig. 8. The loss of competence of telencephalon to be ventralized by Shh is not caused by neural maturation. All photomicrographs show explants stained for the presence of nestin, an intermediate filament found in proliferating neural cells, using anti-nestin antibodies, and visualized with a Cy3-conjugated secondary antibody. (A,B) E11.5 telencephalic explants cultured for 3 days in the absence (A) or the presence (B) of Shh protein. C and D show E12.5 telencephalic explants cultured for 3 days in the absence (C) or the presence (D) of Shh protein. Note that in all cases abundant nestin is observed, consistent with the culture conditions maintaining progenitors in an undifferentiated state, as would be expected of aged-matched *in vivo* tissue. Scale bar in D applies to all panels and is 50 μm.

Nonetheless, it has recently been demonstrated that BMP-7 is expressed in the prechordal mesoderm, adjacent to ventral midline diencephalic cells, and that this protein cooperates with Shh to induce the production of forebrain ventral neurons (Dale et al., 1997). It will be important to determine whether there are other phylogenetically conserved elements controlling *Dlx/dll* regulation.

Temporally regulated changes in competence is vital to Shh signaling within the telencephalon

Changes in the way tissues respond to Shh signaling is central to its role in patterning. Shh induces differentiation events in a variety of tissues outside the nervous system, including the limb (Riddle et al., 1993), the eye (Levine et al., 1997), and the lungs (Bellusci et al., 1997). Within the nervous system the cell types induced by Shh depend both on its concentration and the specific anterior-posterior position being examined (Echelard et al., 1993; Roelink et al., 1994; Ericson et al., 1995; Hynes et al., 1995). The response to Shh can also be modified through cooperation with other molecules, such as BMP-7 (Dale et al., 1997). In addition, as has been shown in the spinal cord, where two critical periods of Shh signaling appear to exist (Ericson et al., 1996), timing plays an important role in the interpretation of the Shh signal.

At present, the mechanism by which Shh mediates different induction events within the telencephalon at different times in development is unclear. It will be important to determine whether temporally regulated autocrine or paracrine factors within the telencephalon act to modify Shh signaling in a manner distinct from that found in more caudal regions of the neuraxis. Whether changes in responsiveness to Shh in the telencephalon are mediated autonomously or through the action of as yet unidentified cooperative factors will be of interest to investigate in future studies.

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