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Sonic hedgehog signaling at gastrula stages specifies ventral telencephalic cells in the chick embryo

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SUMMARY

A secreted signaling factor, Sonic hedgehog (Shh), has a crucial role in the generation of ventral cell types along the entire rostrocaudal axis of the neural tube. At caudal levels of the neuraxis, Shh is secreted by the notochord and floor plate during the period that ventral cell fates are specified. At anterior prosencephalic levels that give rise to the telencephalon, however, neither the prechordal mesoderm nor the ventral neural tube expresses *Shh* at the time that the overt ventral character of the telencephalon becomes evident. Thus, the precise role and timing of Shh signaling relevant to the specification of ventral telencephalic identity remains unclear. By analysing neural cell differentiation in chick neural plate explants we provide evidence that neural cells acquire molecular properties characteristic of the ventral telencephalon in response to Shh signals derived from the anterior primitive streak/Hensen's node region at gastrula stages. Exposure of prospective anterior prosencephalic cells to Shh at this early stage is sufficient to initiate a temporal program of differentiation that parallels that of neurons generated normally in the medial ganglionic eminence subdivision of the ventral telencephalon.

Key words: Sonic hedgehog, Prosencephalon, Telencephalon, Neural cell differentiation, Neural plate, Chick

INTRODUCTION

The early subdivision of the neural tube into molecularly distinct domains provides a structural foundation for later aspects of neuronal differentiation (Lumsden and Krumlauf, 1996). At caudal levels of the neural tube that give rise to the spinal cord and hindbrain, patterning mechanisms involving Sonic hedgehog (Shh) and Bone Morphogenetic Protein (BMP) signaling appear to operate along the dorsoventral axis of the neural tube to subdivide the neural epithelium into discrete progenitor domains, from which distinct classes of post-mitotic neurons emerge (Tanabe and Jessell, 1996; Lee and Jessell, 1999). There is increasing evidence that a similar regional subdivision of neural tissue operates at anterior prosencephalic levels that later give rise to the rostral diencephalon and telencephalon (Fishell 1997; Puelles et al., 1999). The ventral half of the embryonic telencephalon has been shown to have two major cellular subdivisions (Smart and Sturrock, 1979). Cells in the more ventral region of the ventral telencephalon form the medial ganglionic eminence (MGE), a structure which subsequently generates the globus pallidum (Kohtz et al., 1998; Puelles et al., 1999). More dorsally located cells make up the lateral ganglionic eminence (LGE), a region which later supplies projection neurons within the corpus striatum (Anderson et al., 1997; Kohtz et al., 1998; Puelles et al., 1999; Toresson et al., 1999). The central role of these two forebrain regions in extrapyramidal motor control and other neural functions (Gerfen 1992), has prompted increasing interest in the mechanisms that generate the regional character of cells located in the ventral telencephalon.

The analysis of early telencephalic patterning has been facilitated by the identification of molecular markers, notably transcription factors, that delineate regional domains within the telencephalon. In particular, members of the Nkx, Dlx, LIM/Lhx and Pax classes of homebox genes are expressed in different regions of the developing forebrain, including domains within the ventral telencephalon (for review see Puelles et al., 1999). The homeobox gene *Nkx2.1* is expressed in the ventral region of the embryonic telencephalon that later gives rise to the MGE (Lazzaro et al., 1991; Kimura et al., 1996). The related Nkx class gene, *Nkx2.2*, is also expressed by ventral forebrain cells, but with a rostral boundary of expression in the diencephalon (Price et al., 1992). The *Dlx* class homeobox genes *Dlx1, Dlx2, Dlx5* and *Dlx6* are expressed in overlapping regions in the ventral telencephalon and diencephalon (Anderson et al., 1997; Fernandez et al., 1998; Puelles et al., 1999). The LIM homeodomain protein Isl1 is expressed by neurons arrayed along most of the ventral region of the neural tube including both cholinergic and GABAergic neurons of the adult basal forebrain and septum

(Thor et al., 1991; K. Campbell, personal communication). Several other LIM homeobox genes are expressed in the developing telencephalon but in a more diffuse pattern (Porter et al., 1997; Bertuzzi et al., 1999; Grigoriou et al., 1998; Zhao et al., 1999a,b).

Evidence that these regulatory genes control aspects of telencephalic differentiation has been provided by analyses of mutant mice. In *Nkx2.1* mutant mice, cells that normally generate the globus pallidum instead appear to acquire a striatal character (Sussel et al., 1999), and genetic inactivation of other members of these classes of homeobox genes also affects the development of the telencephalon (Anderson et al., 1997; Dahl et al., 1997; Porter et al., 1997; Zhao et al., 1999b). The functional roles played by these regionally restricted transcription factors in the specification of telencephalic cell fates, in turn, raises the issue of the nature of the extrinsic signaling factors that initially establish their differential patterns of expression.

A combination of genetic and cellular studies have provided evidence that the specification of regional identity in the ventral half of the prospective telencephalon requires Shh signaling (Ericson et al., 1995; Fishell, 1997; Shimamura and Rubenstein, 1997; Chiang et al., 1996). However, at the time that the overt ventral character of telencephalic cells becomes evident, neither adjacent mesodermal cells nor neural cells themselves express *Shh* (Ericson et al., 1995; Fishell, 1997; Kohtz et al., 1998). Thus, the onset of cell specification in the ventral telencephalon occurs before the telencephalic *Shh* expression (Ericson et al., 1995; Fishell, 1997; Kohtz et al., 1998), apparently excluding Shh derived from the telencephalon itself in the initial induction of the ventral identity of telencephalic cells. Chick fate maps have revealed that telencephalic cells are initially derived from the lateral and dorsal regions of the rostral neural plate (Couly and Le Douarin, 1988; Fishell, 1997; Fernandez et al., 1998). The ventral telencephalon forms as cells in this region move ventrally during the morphogenetic movements associated with the closure of the anterior neuropore (Couly and Le Douarin, 1988; Fishell, 1997). Thus the specification of ventral telencephalic fates could, in principle, occur soon after neural tube closure. Nevertheless, even during this early phase of neural development, prospective telencephalic cells do not pass close to cell groups that express *Shh* (Ericson et al., 1995; Fishell, 1997).

The initial phase of *Shh* expression occurs at gastrula stages within the anterior primitive streak and Hensen's node. *Shh* is subsequently expressed in axial mesodermal cells of the notochord and transiently in prechordal plate cells that migrate anteriorly, under regions of the prospective forebrain (Ericson et al., 1995; Chiang et al., 1996; Kohtz et al., 1998; Pagan-Westphal and Tabin, 1998; Dale et al., 1999). These observations raise the possibility that Shh signaling at gastrula stages initiates the patterning of the ventral telencephalon. In this study we have tested this possibility by examining the cellular origin of Shh signals involved in specifying the early ventral identity of telencephalic cells. We provide evidence that prospective forebrain cells become specified as cells with characteristics of the ventral telencephalon in response to Shh signaling derived from the anterior primitive streak and Hensen's node, at the gastrula stage. Our findings also provide evidence that the exposure of prospective forebrain cells to Shh at this early stage is sufficient to initiate a sequential program of progenitor cell and post-mitotic neuronal differentiation that culminates in the differentiation of neurons with the molecular features of neurons that populate the medial ganglionic eminence.

MATERIALS AND METHODS

Embryos

Fertilized white Leghorn chicken eggs were obtained from Agrisera AB, Umeå, Sweden. Chick embryos were staged according to the protocol of Hamburger and Hamilton (1951).

Isolation and culture of explants

Explants of the prospective telencephalon, diencephalon and rhombencephalon were dissected from HH stages 4 and 6 chick embryos (Muhr et al., 1999; Ericson et al., 1995) and neural-mesendoderm explants were dissected from HH stage 4 embryos. Explants of the ventral telencephalon were dissected from HH stages 9, 12 and 15 embryos (Fernandez et al., 1998). Explants were cultured for 20-55 hours as described by Roelink et al. (1994); Tanabe et al. (1995) in collagen in OPTI-MEM medium with N-2 supplement (GIBCO BRL) and fibronectin (Sigma). Human recombinant FGF2 was used at 50-200 μg/ml, recombinant FGF8 was used at 500-1000 μg/ml (GIBCO BRL), baculovirus-SHH-N was used at 10^{-8} M and monoclonal anti-Shh antibody 5E1 was used at 2.5-7.5 μg/ml (Ericson et al., 1996). BMP4-protein was obtained from COS cells transfected with *BMP4* in pRK5 (provided by R. Derynck) as described by Alder et al. (1999). BMP4-conditioned medium, prepared in this manner, was used at a 25-fold dilution which consistently generated cell fate changes equivalent to those elicited by 12.5 nM recombinant BMP7/OP1.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization histochemistry using a chick digoxigenin-labelled *Shh* probe (Ericson et al., 1995) was performed essentially as described by Schaeren-Wiemers and Gerfin-Moser (1993). Immunohistochemical localization of proteins was performed as described by Tsuchida et al. (1994). Nkx2.1 was detected with an anti-Nkx2.1 rabbit antibody (Lazzaro et al., 1991), Isl1 with an anti-Isl1/2 rabbit antibody (Thor et al., 1991), Dlx with a pan anti-Dlx rabbit antibody (Panganiban et al., 1995), Nkx2.2 with mAb Nkx2.2 (Ericson et al., 1997), Pax7 with mAb Pax7 (Ericson et al., 1996), Msx1 with mAb 4G1 (Liem et al., 1995) and Otx2 with an anti-Otx2 rabbit antibody (Mallamaci et al., 1996). The MPM2 monoclonal antibody was used to detect cells in M-phase of the cell cycle (Westendorf et al., 1994; Upstate Biotechnology Inc). The total number of cells in rostral neural plate explants was determined by counting the number of $Otx2^{+}$ cells and the number of nuclei, using DAPI (Boehringer Mannheim), in serial sections. In stage 4 neural-mesendoderm explants, neural cells were identified using anti-Sox2 (Kamachi et al., 1998), anti-Sox3 (Wilson et al., 2000) and anti-Otx2 (Mallamaci et al., 1996) antibodies. Otx2 is downregulated in mesoendodermal cells during culture.

BrdU incorporation studies

Determination of BrdU incorporation into cells was performed by labelling the explants with 5 μM BrdU between 47 hours and 48 hours of culture or with 170 nM BrdU for the last 18-22 hours of the total 48-hour culture period. Double labelling experiments were performed essentially as described by Ericson et al. (1996) using an anti-Isl1/2 or anti-Nkx2.1 or anti-Dlx rabbit antibody and biotinylated anti-rabbit antibody and Cy3-streptavidin (Jackson Labs) together with an anti-BrdU antibody (Becton Dickinson) and FITC-conjugated goat anti-mouse IgG antibody.

RESULTS

To examine when cells fated to give rise to the ventral region of the telencephalon first acquire their ventral character we analyzed the differentiation of neuroepithelial cells grown in explant culture. To define neural character, the expression of several transcription factors that normally define cells generated in anterior and ventral regions of the neural tube was monitored (see below and Table 1).

The Nkx class homeobox gene *Nkx2.1* is initially expressed in regions of the neural tube fated to generate regions of the prosencephalon, midbrain and hindbrain, and expression of *Nkx2.1* is detected as early as HH stage 8 (Shimamura and Rubenstein, 1997; Pera and Kessel, 1998; Qui et al., 1998). Expression of *Nkx2.1* is subsequently downregulated in the midbrain and hindbrain regions. In the telencephalon, expression of Nkx2.1 is first detected by HH stages 13-14 and from this stage onwards Nkx2.1 is expressed selectively in the ventral regions of the telencephalon and rostral diencephalon (Lazzaro et al., 1991; Ericson et al., 1995; Pera and Kessel, 1998; Qui et al., 1998; see also Fig. 4A). The related Nkx class gene, *Nkx2.2*, is also expressed by ventral neural cells, but has a rostral boundary of expression in the diencephalon (Price et al., 1992). The *Dlx* class homeobox genes *Dlx1, Dlx2, Dlx5* and *Dlx6* are expressed in overlapping regions in the ventral telencephalon and diencephalon (Anderson et al., 1997; Fernandez et al., 1998; Puelles et al., 1999). At early developmental stages, the expression domains of these *Dlx* genes share a common border in the ventromedial region of the telencephalon (Fernandez et al., 1998). We analyzed the expression of Dlx proteins using an anti-Dlx antibody that appears to recognize many or all vertebrate Dlx proteins (Panganiban et al., 1995). Dlx$^+$ cells can first be detected in the ventral telencephalon at HH stage 18 (data not shown and Fig. 4C). The homeodomain gene *Otx2* is expressed by many or all neural tube cells located rostral to the hindbrain (Mallamaci et al., 1996). The LIM homeodomain protein Isl1 is expressed by neurons positioned along most of the ventral region of the neural tube, and in the ventral telencephalon, expression is first detected at HH stage 18 (Thor et al., 1991; Ericson et al., 1995; see also Fig. 4B). The Pax class homeodomain protein Pax7 is expressed at neural plate stages in the dorsal region of the neural tube, with a rostral boundary in the diencephalon. (Ericson et al., 1996). Thus, ventral telencephalic cells can be defined by the expression of Otx2, Nkx2.1, Dlx and Isl1, and by the absence of expression of Nkx2.2 and Pax7.

Table 1. Transcription factor markers expressed in different regions of the chick neural tube

Transcription factor	Telencephalon	Diencephalon	Hindbrain/ spinal cord
Nkx2.1	+	+	−
Nkx2.2	−	+	+
Otx2	+	+	−
Dlx	+	+	−
Isl1	+	+	+

The homeodomain transcription factors used as markers are expressed at different anteroposterior regions of HH stage 20 neural tube. Nkx2.1, Nkx2.2 and Otx2 are expressed in progenitor cells. Dlx is expressed in both progenitor cells and in post-mitotic neurons, and Isl1 is selectively expressed in post-mitotic neurons.

Prospective forebrain cells isolated from gastrula stage embryos acquire markers of ventral telencephalic identity

Previous studies (Muhr et al., 1999), have shown that rostral (R) epiblast cells isolated from HH stage 4 (definitive primitive streak stage) chick embryos, generate neural cells of forebrain

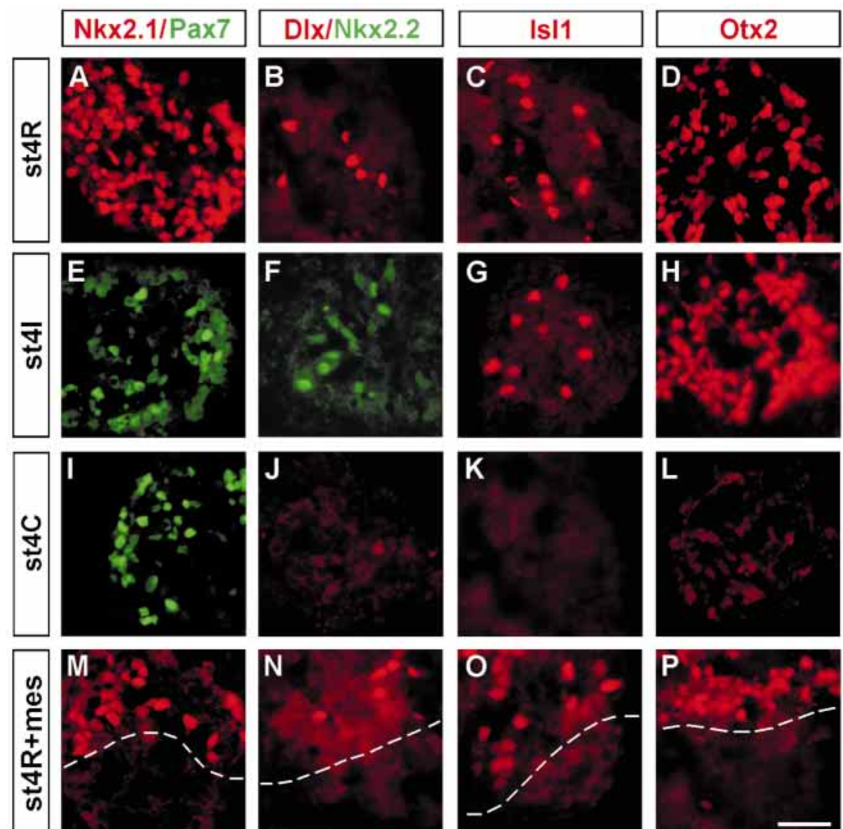

Fig. 1. Prospective rostral forebrain cells isolated from gastrula stage embryos acquire markers of ventral telencephalic identity. (A-P) Expression of neural markers in HH stage 4 rostral (R), intermediate (I) and caudal (C) explants after 52 hours in culture. (A-D) R explants (n=15) generate Nkx2.1$^+$ cells (85±3%; A), Dlx$^+$ cells (9±2%; B), Isl1$^+$ cells (23±3%; C) and Otx2$^+$ cells (90±5%; D) but no Pax7$^+$ cells (A) or Nkx2.2$^+$ cells (B). (E-H) I explants (n=10) generate Pax7$^+$ cells (30±3%; E), Nkx2.2$^+$ cells (10±3%; F), Isl1$^+$ cells (18±3%; G) and Otx2$^+$ cells (90±5%) but no Nkx2.1$^+$ cells (E) or Dlx$^+$ cells (F). (I-L) C explants (n=10) generate Pax7$^+$ cells (35±4%; I) but no Nkx2.1$^+$ cells (I), Dlx$^+$ cells (J), Nkx2.2$^+$ (J), Isl1$^+$ cells (K) or Otx2$^+$ cells (L). (M-P) R neural-mesendodermal explants (n=15) generate Nkx2.1$^+$ cells (85±3%; M), Dlx$^+$ cells (10±2%; N), Isl1$^+$ cells (22±3%; O) and Otx2$^+$ cells (90±5%; P) but no Pax7$^+$ cells (M) or Nkx2.2$^+$ cells (N). Broken line indicates the border between neuroectoderm and mesendoderm. Scale bar, 30 μm (A-P).

character when grown alone, whereas epiblast cells from intermediate (I) and caudal (C) regions generate cells of a more caudal neural character. These studies did not, however, define when prospective forebrain cells first acquire the capacity for ventral differentiation.

To address this issue we examined whether cells in HH stage 4 R explants acquire ventral character when grown in isolation for 52h. HH stage 4 R explants (Fig. 2B) generated Otx2$^+$, Nkx2.1$^+$, Dlx$^+$ and Isl1$^+$ cells but not Nkx2.2$^+$ nor Pax7$^+$ cells (Fig. 1A-D; Muhr et al., 1999). Thus, cells in these explants express a combination of transcription factors characteristic of cells in the ventral telencephalon. In contrast, HH stage 4 I explants (Fig. 2B) generated Otx2$^+$, Nkx2.2$^+$, Isl1$^+$ and Pax7$^+$ cells but no Nkx2.1$^+$ or Dlx$^+$ cells (Fig. 1E-H); a combination of markers characteristic of neural cells located caudal to the telencephalon. HH stage 4 C explants (Fig. 2B) generated Pax7$^+$ cells but no Otx2$^+$, Nkx2.1$^+$, Dlx$^+$, Isl1$^+$ or Nkx2.2$^+$ cells (Fig. 1I-L; Muhr et al., 1999), indicating that these cells exhibit a caudal character.

We were concerned that the acquisition of ventral character by HH stage 4 prospective forebrain cells might be affected by the isolation of neural tissue from signals potentially provided by surrounding mesendodermal tissue. To assess this possibility, HH stage 4 rostral explants were isolated without removing the underlying mesendoderm and placed in culture for 52 hours. In these neural-mesendodermal explants Otx2$^+$, Nkx2.1$^+$, Dlx$^+$ and Isl1$^+$ neural cells were still generated and no Nkx2.2$^+$ or Pax7$^+$ cells were detected (Fig. 1M-P). This finding indicates that the presence of mesendoderm does not markedly influence the generation of neural cells characteristic of the ventral telencephalon.

Shh signaling at gastrula stages is required for the specification of ventral forebrain cells

Shh signaling has been implicated in the generation of ventral cell types along the entire rostrocaudal extent of the neuraxis (Lumsden and Krumlauf, 1996). To determine whether Shh signaling might be involved in the acquisition of ventral forebrain character by cells in HH stage 4 R explants, we first examined the expression of *Shh* in gastrula stage embryos. At HH stage 3, *Shh* expression was detected in cells in the primitive streak (Fig. 2A), and at HH stage 4 *Shh* expression was detected in cells in Hensen's node (Fig. 2B), a pattern of expression consistent with the findings of Pagan-Westphal and Tabin (1998).

We next examined whether the generation of Nkx2.1$^+$, Dlx$^+$ and Isl1$^+$ cells in HH stage 4 R epiblast explants depends on Shh signaling at these early stages. To test this possibility we exposed explants to a function-blocking anti-Shh monoclonal antibody (mAb 5E1; Ericson et al., 1996). Addition of anti-Shh IgG completely blocked the generation of Nkx2.1$^+$, Isl1$^+$ and Dlx$^+$ cells in HH stage 4 R explants grown in vitro for 52 hours. In contrast, the number of Otx2$^+$ cells was not changed by the addition of anti-Shh IgG (Fig. 2C-E; data not shown). Thus, ongoing Shh signaling appears to be required for the induction of expression of ventral markers in prospective forebrain cells isolated from HH stage 4 epiblast.

To examine when HH stage 4 prospective rostral neural plate

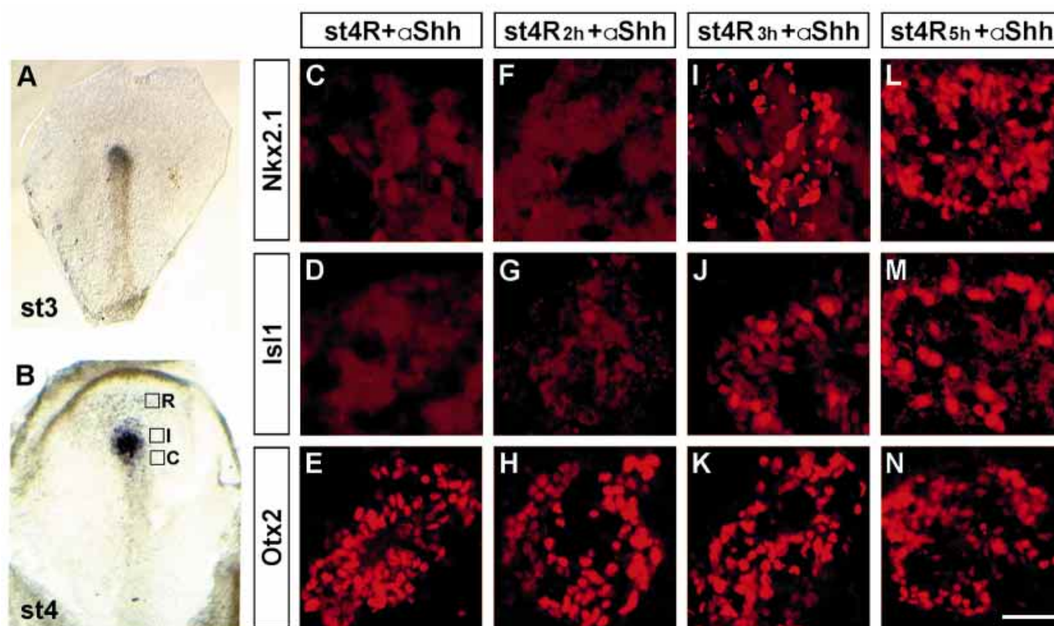

Fig. 2. Shh signaling at gastrula stages is required for the specification of ventral forebrain cells. (A,B) Expression of *Shh* in HH stage 3 (A) and HH stage 4 (B) chick embryos shown by whole-mount in situ hybridization. The boxes in B indicate the regions from which rostral (R), intermediate (I) and caudal (C) explants were isolated for in vitro assays. (C-N) Expression of ventral neural markers in HH stage 4 rostral (R) explants after 52 hours inculture with addition of an anti-Shh function blocking antibody at different times after start of culture. (C-H) When added at the start or after 2 hours in culture the anti-Shh antibody blocked the generation of Nkx2.1$^+$ cells (C,F) and Isl1$^+$ cells (D,G) in R explants (n=15) whereas Otx2$^+$ cells (>95%) (E,H) were generated. (I-K) The anti-Shh antibody partially blocked the generation of Nkx2.1$^+$ cells (45±4%; I) and Isl1$^+$ cells (12±2%; J) in R explants (n=10) when added at 3 hours after the start of culture, whereas Otx2$^+$ cells (>95%; K) were generated. (L-N) The anti-Shh antibody did not block the generation of Nkx2.1$^+$ cells (80±4%; L), Isl1$^+$ cells (20±3%; M) or Otx2$^+$ cells (>95%; N) when added 5 hours after the start of culture. Scale bar, 30 µm (C-N).

cells acquire independence from ongoing Shh signaling during the generation of Nkx2.1⁺ and Isl1⁺ cells, anti-Shh IgG was added to HH stage 4 R explants at various times during the culture period. Addition of anti-Shh IgG after 2 hours still resulted in a complete block in the generation of Nkx2.1⁺ and Isl1⁺ cells (Fig. 2F and G). In contrast, when anti-Shh IgG was added after 3 hours, approx. 50% of the normal number of Nkx2.1⁺ and Isl1⁺ cells was generated (Fig. 2I and J). Addition of anti-Shh IgG approx. 5 hours after onset of culture failed to inhibit in the number of Nkx2.1⁺ and Isl1⁺ cells generated, when compared to control cultures grown in the absence of blocking antibody (Fig. 2L and M). Thus, prospective forebrain cells grown in vitro rapidly lose their dependence on Shh signaling for the acquisition of ventral neural character.

Sequential acquisition of ventral character in prospective telencephalic cells in vivo

The location of prospective telencephalic and diencephalic cells in the chick epiblast at HH stage 4 has not been clearly defined. By HH stages 6-8, however, existing fate maps (Couly and Le Douarin, 1988; Dale et al., 1999; Ericson et al., 1995; Fernandez et al., 1998) permit prospective telencephalic and diencephalic regions of the early neural plate cells to be isolated separately. At HH stage 6, prospective telencephalic cells are located in a lateral and rostral region of the neural plate, approx. 150 μm from axial mesodermal cells in the node and prechordal mesoderm that have been shown to express *Shh* (Couly and Le Douarin, 1988; Dale et al., 1999; Ericson et al., 1995; Fishell, 1997). To examine the state of specification of prospective telencephalic cells at these stages, we monitored the dorsoventral character of cells isolated from HH stage 6 prospective telencephalic (T) and diencephalic (D) regions of the neural plate (Fig. 3V; Ericson et al., 1995) after culture in vitro for 48 hours.

HH stage 6 T explants generated Nkx2.1⁺, Isl1⁺, Dlx⁺ cells but no Nkx2.2⁺ cells (Fig. 3A-D), a marker combination characteristic of the ventral telencephalon. HH stage 6 D explants generated Nkx2.1⁺, Isl1⁺, Dlx⁺ cells and in addition Nkx2.2⁺ cells (Fig. 3E-H), a combination of markers characteristic of cells that populate the ventral region of the rostral diencephalon. Explants of HH stage 6 medial hindbrain (H) (Fig. 3V; Ericson et al., 1995) generated Nkx2.2⁺ cells and Isl1⁺ neurons but no Nkx2.1⁺, Dlx⁺ or Pax7⁺ cells were

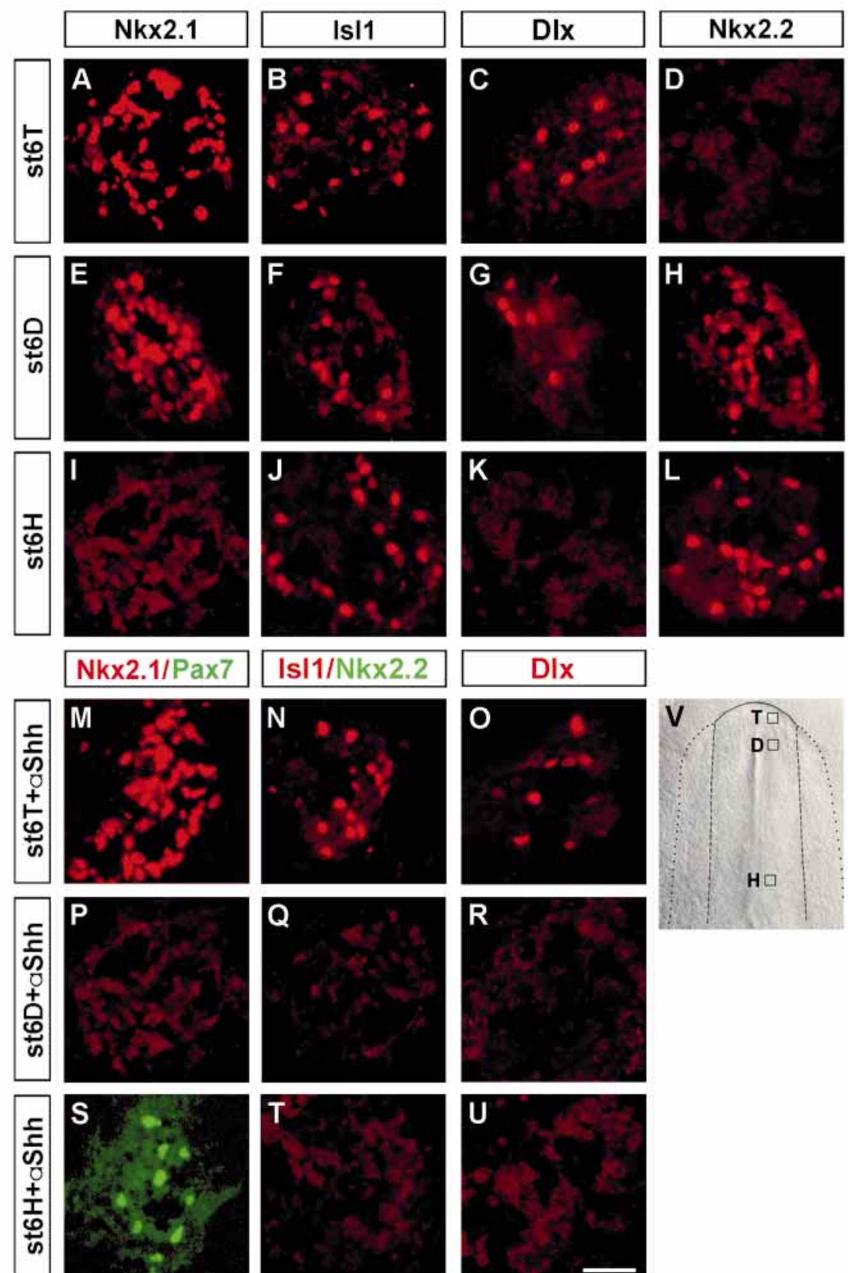

Fig. 3. Ventral telencephalic neurons are specified by stage 6. (A-U) Expression of ventral neural markers in HH stage 6 telencephalic (T), diencephalic (D) and medial hindbrain (H) explants after 48 hours in culture with or without the anti-Shh antibody. (A-D) T explants (*n*=30) cultured alone generate Nkx2.1⁺ cells (85±4%; A), Isl1⁺ cells (22±3%; B) and Dlx⁺ cells (11±3%; C) but no Nkx2.2⁺ cells (D). (E-H) D explants (*n*=15) cultured alone generate Nkx2.1⁺ cells (80±4%; E), Isl1⁺ cells (20±3%; F), Dlx⁺ cells (6±3%; G) and Nkx2.2⁺ cells (45±3%; H). (I-L) H explants (*n*=20) cultured alone generate Isl1⁺ cells (22±4%; J) and Nkx2.2⁺ cells (24±4%; L) but no Nkx2.1⁺ cells (I) or Dlx⁺ cells (K). (M-O) The anti-Shh antibody did not block the generation of Nkx2.1⁺ cells (75±3%) (M), Isl1⁺ cells (20±4%) (N) or Dlx⁺ cells (10±2%) (O) in T explants (*n*=30). No Nkx2.2⁺ cells (N) or Pax7⁺ cells (M) were detected. (P-R) In the presence of the anti-Shh antibody the generation of Nkx2.1⁺ cells (P), Isl1⁺ cells (Q), Dlx⁺ cells (R) and Nkx2.2⁺ cells (Q) were blocked in D explants (*n*=15). No Pax7⁺ cells (P) were detected. (S-U) In the presence of the anti-Shh antibody the generation of both Nkx2.2⁺ cells and Isl1⁺ cells (T) was blocked but Pax7⁺ cells (15±5%) (S) appeared in H explants (*n*=20). No Nkx2.1⁺ cells (S) or Dlx⁺ cells (U) were detected. (V) HH stage 6 chick embryo. Boxes indicate the regions from which prospective telencephalic (T), diencephalic (D) and medial hindbrain (H) explants of the neural plate were isolated for *in vitro* assays. Scale bar, 30 μm (A-U).

detected (Fig. 3I-L; data not shown). Thus by HH stage 6, cells isolated from a region fated to generate telencephalic cells express markers of ventral character when cultured in vitro.

HH stage 4 prospective forebrain cells grown in vitro acquire independence from Shh signaling approx. 5 hours after onset of culture: the developmental equivalent of approx. HH stage 6 in ovo (Fig. 2L,M). We therefore examined whether anti-Shh IgG was able to block the generation of cells of ventral neural character in prospective T, D and H neural tissue isolated from the epiblast of HH stage 6 embryos. In HH stage 6 T explants, Nkx2.1$^+$, Dlx$^+$ and Isl1$^+$ cells were generated, despite the continued presence of anti-Shh IgG (Fig. 3M-O). In contrast, anti-Shh IgG blocked both the generation of Nkx2.1$^+$, Dlx$^+$, Isl1$^+$ and Nkx2.2$^+$ cells in D explants (Fig. 3P-R) and the appearance of Nkx2.2$^+$ cells and Isl1$^+$ neurons in H explants (Fig. 3T). Under these conditions, Pax7$^+$ cells characteristic of the dorsal caudal neural plate appeared in HH stage 6 H explants (Fig. 3S). Thus at HH stage 6, prospective telencephalic cells acquire a ventral progenitor character independent of further exposure to Shh. In contrast, cells in the prospective diencephalon and hindbrain require ongoing Shh signaling for the generation of ventral neuronal cell types. These results indicate that a period of exposure to Shh prior to HH stage 6 appears to be sufficient to specify the ventral character of telencephalic cells.

Sequential acquisition of ventral telencephalic neural markers in vitro

To assess further the differentiated character of ventral cells generated in HH stage 6 T explants, we monitored the temporal pattern of generation of Nkx2.1$^+$, Isl1$^+$ and Dlx$^+$ cells and the proliferative state of the cells that express each of these markers. Nkx2.1$^+$ cells can first be detected at HH stages 13-14 in the telencephalon, and at this stage Nkx2.1 expression is confined to proliferative progenitor cells in the ventral telencephalon (Fig. 4A). In contrast, Dlx$^+$ and Isl1$^+$ cells start to appear in the ventral telencephalon only approx. 15 hours later, at HH stage 18 (Fig. 4B and C). At this stage Dlx is expressed by both proliferative progenitor cells and by post-mitotic neurons (Anderson et al., 1997; Puelles et al., 1999). In contrast, Isl1 expression is restricted to post-mitotic neurons in the ventral telencephalon from the onset of its expression, at HH stage 18 (Fig. 4D) and by HH stage 20, approx. 20% of the Isl1$^+$ neurons coexpressed Dlx proteins and approx. 20% of the Dlx$^+$ cells coexpressed Isl1 (Fig. 4E). Thus, the profile of expression of Nkx2.1, Dlx and Isl1 in vitro appears to define sequential stages in the differentiation of ventral telencephalic neurons in vivo.

To examine whether the sequence of marker expression by progenitor cells and postmitotic neurons is maintained in vitro, we monitored the time course of generation of Nkx2.1$^+$, Isl1$^+$ and Dlx$^+$ cells in HH stage 6 T explants and also analyzed the proliferative state of these cells. Nkx2.1$^+$ cells were first detected in these explants after 25 hours in culture and by 30 hours, approx. 85% of the cells expressed Nkx2.1 (Fig. 4F). In contrast, Dlx$^+$ cells and Isl1$^+$ neurons were first detected only after 38-40 hours in culture (Fig. 4F). At 48 hours, approx. 10% of the cells expressed Dlx and approx. 20% of cells expressed Isl1 (Fig. 4F).

Addition of BrdU 26 hours after the start of the culture

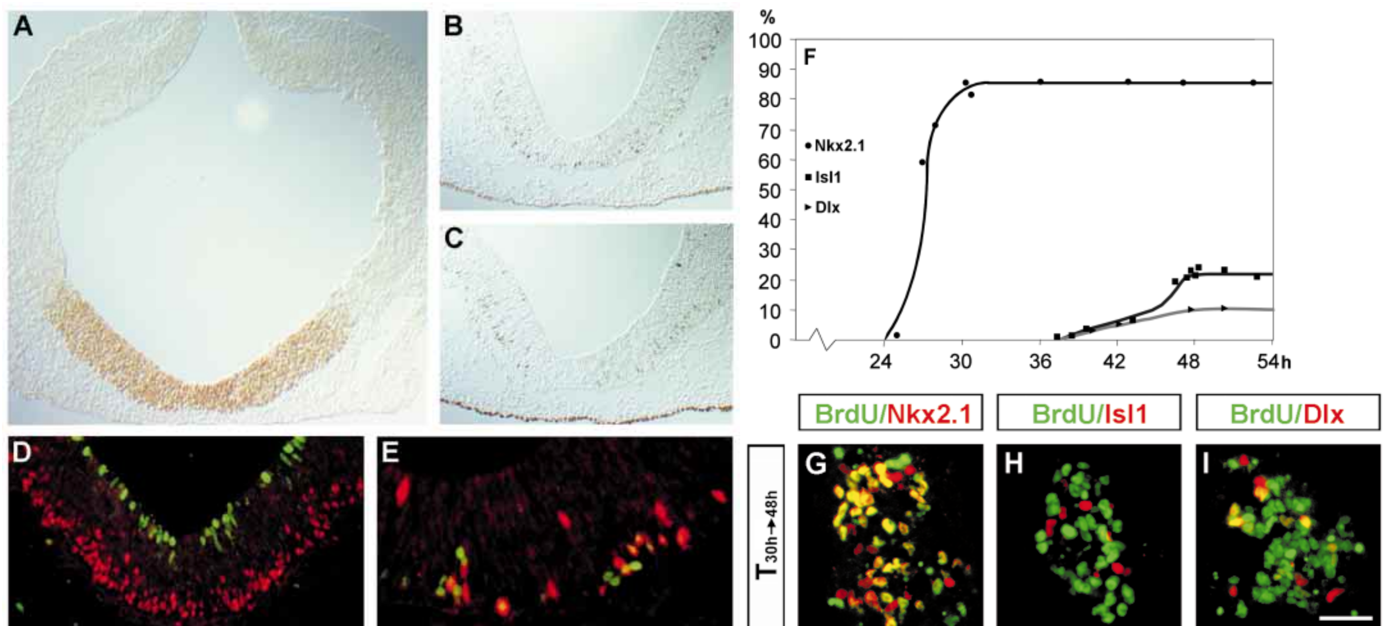

Fig. 4. Sequential acquisition of ventral telencephalic neural markers in vitro. (A-E) Patterns of expression of Nkx2.1 (A), Isl1 (B) and Dlx (C) in the ventral telencephalon of a HH stage 20 chick embryo. (D) No Isl1$^+$ cells (red) were double-labelled with mpm2 (green), which detects cells in M-phase of the cell cycle (Kuang et al., 1989). (E) Double-labelling with anti-Isl1 (green) and anti-Dlx antibodies (red) shows that approx. 20% of the Isl1$^+$ cells express Dlx (yellow). (F) Temporal patterns of generation of Nkx2.1$^+$ cells, Isl1$^+$ neurons and Dlx$^+$ cells in HH stage 6 T explants (n=30). After 48 hours in culture, 85±4% of the cells were Nkx2.1$^+$ cells, 22±3% were Isl1$^+$ neurons and 11±3% were Dlx$^+$ cells. (G-I) HH stage 6 T explants (n=10) were cultured for 30 hours and then BrdU was added and its incorporation determined at 48 hours to detect cells in the S and G_2 phase of the cell cycle. After 30 hours in culture >90% of the Nkx2.1$^+$ cells (G) but no Isl1$^+$ neurons (H) incorporated BrdU and ~50% of the Dlx$^+$ cells incorporated BrdU (I). Scale bar, 30 μm (G-I).

period and analysis after 48 hours in vitro revealed labelling of >90% of Nkx2.1$^+$ cells and approx. 50% of Isl1$^+$ neurons (data not shown). Addition of BrdU at 30 hours, 36 hours or 44 hours and analyses at 48 hours still resulted in labelling of >90% of Nkx2.1$^+$ cells, but under these conditions no BrdU-labelled Isl1$^+$ neurons were detected (Fig. 4G and H; data not shown). Thus prospective Isl1$^+$ neurons appear to exit the cell cycle between 26 and 30 hours in vitro, that is, approx. 8 hours before Isl1 expression is first detected. Under these labelling conditions, approx. 50% of Dlx$^+$ cells incorporated BrdU (Fig. 4I).

From these studies we infer that the Nkx2.1$^+$ cells generated in HH stage 6 T explants represent proliferative progenitors, whereas Isl1$^+$ cells are solely postmitotic neurons. The Dlx$^+$ cell population appears to contain a mixture of proliferative cells and postmitotic neurons. Thus, it appears that the time of differentiation, the proliferative state and the relative number of Nkx2.1$^+$ cells, Dlx$^+$ cells and Isl1$^+$ neurons in HH stage 6 T explants closely parallels the generation of the equivalent cell populations in vivo.

Time of consolidation of ventral telencephalic identity

We next attempted to determine the stage at which prospective ventral telencephalic cells commit to a ventral progenitor fate, and whether this step is rate limiting in the progression to a postmitotic ventral telencephalic neuronal phenotype.

Bone Morphogenetic Proteins (BMPs) represent a class of factors that can induce dorsal character in neural cells and can antagonise Shh signaling in neural plate explants (Liem et al., 1995; Furuta et al., 1997; Muhr et al., 1997; Golden et al., 1999; Lee and Jessell, 1999). In particular, BMPs can induce Msx1/2$^+$ cells characteristic of the dorsal neural tube in HH stage 6 T explants (Muhr et al., 1997). We therefore used the sensitivity of neural cells to BMPs as an operational measure of the state of commitment of ventral progenitor cells: commitment being defined here as the time at which cells in explants of the ventral telencephalon retain their ventral character upon exposure to BMP signaling. Since Nkx2.1$^+$ cells are first detected in the ventral telencephalon at HH stages 13/14, we isolated explants of the ventral telencephalon (VT) at HH stages 9, 12 and 15. VT explants were cultured in the absence or presence of BMP4 for 24-40 hours and the generation of Nkx2.1$^+$ cells, Isl1$^+$ neurons and Msx1/2$^+$ cells was assayed.

In HH stage 9 and 12 VT explants (Fig. 5M and N), the generation of Nkx2.1$^+$ cells and Isl1$^+$ neurons was completely blocked by exposure to BMP4, and conversely, many Msx1/2$^+$ cells (approx. 55% of cells in each explant) were induced (Fig. 5A-D). In contrast in HH stage 15 VT explants (Fig. 5O), the number of Nkx2.1$^+$ cells and Isl1$^+$ neurons was comparable in the presence and absence of BMP4 (Fig. 5E and F). Thus, telencephalic cells appear to commit to a ventral progenitor fate over a 5 hours period, between HH stages 12 and 15.

We next examined whether Nkx2.1$^+$ ventral progenitor cells are also programmed to progress to a post-mitotic neuronal state at HH stages 12 and 15. Several *Fgfs* are expressed by

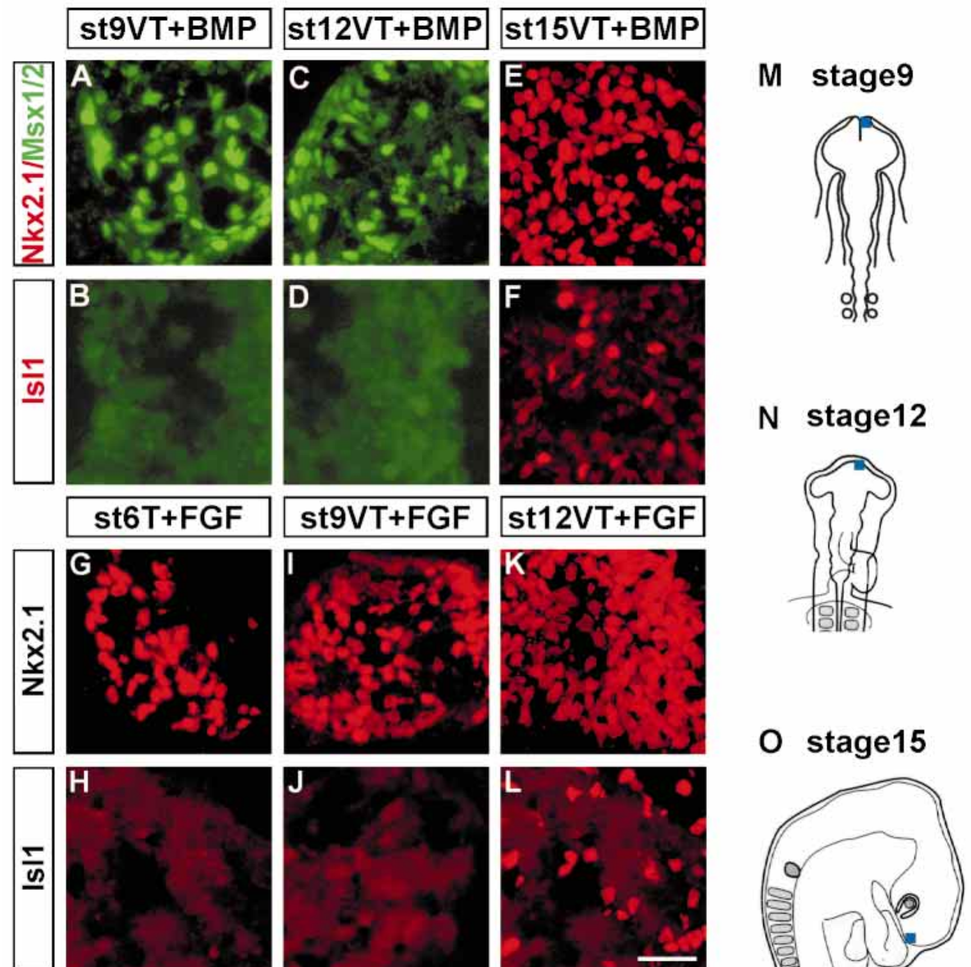

Fig. 5. Ventral telencephalic identity is consolidated by HH stage 12 (A-D) HH stage 9 VT explants (n=10) (A,B) and HH stage 12 VT explants (n=10) (C,D) cultured for 40 hours in vitro in the presence of BMP4 generate Msx1/2$^+$ cells (55±5%) (A,C) but no Nkx2.1$^+$ cells (A,C) or Isl1$^+$ cells (B,D). (E,F) HH stage 15 VT explants (n=10) cultured in the presence of BMP4 for 24-40 hours generate Nkx2.1$^+$ cells (85±4%) (E) and Isl1$^+$ cells (20±3%) (F) but no Msx1/2$^+$ cells (E). (G-J) HH stage 6 T (G,H) and 9 VT (I,J) explants cultured *in vitro* for 48 hours in the presence of FGF2 generate Nkx2.1$^+$ cells (85±4%) (G,I) but no Isl1$^+$ cells (H,J). (K,L) HH stage 12 VT explants cultured *in vitro* for 40 hours in the presence of FGF2 generate Nkx2.1$^+$ cells (85±4%) (K) and Isl1$^+$ cells (25±3%) (L). (M-O) Drawings of HH stage 9 (M) and HH stage 12 (N) embryos, dorsal view, and of HH stage 15 (O) embryo, side view. Blue boxes indicate the regions from which ventral telencephalic (VT) explants were isolated. Scale bar, 30 μm (A-L).

cells in the anterior neural ridge and ventral telencephalon over the period that Isl1$^+$ neurons first appear (McWhirter et al., 1997; Shimamura and Rubenstein 1997; Meyers et al., 1998; Xu et al., 1999). Moreover, FGFs are known to maintain forebrain neuroepithelial cells in a proliferative state (Bartlett et al., 1998). We therefore analyzed whether Nkx2.1$^+$ progenitor cells in telencephalic explants are able to generate postmitotic Isl1$^+$ neurons in the presence of FGFs.

In HH stage 6 T explants grown for 48 hours in the presence or absence of FGF2 or FGF8, Nkx2.1$^+$ cells were generated in near equal numbers. In contrast, no Isl1$^+$ neurons were generated in the presence of FGF2 or FGF8 (Fig. 5G and H; data not shown). In addition, the number of cells that incorporated BrdU after a 1 hour pulse at approx. 48 hours of culture increased from approx. 15% to approx. 45% in the presence of FGFs (n=10; data not shown). Similarly, HH stage 9 prospective ventral telencephalic explants grown in the presence of FGF2 generated Nkx2.1$^+$ cells in normal numbers, but the generation of Isl1$^+$ neurons was blocked (Fig. 5I,J). In contrast, explants derived from HH stage 12 ventral telencephalon generated both Nkx2.1$^+$ cells and Isl1$^+$ neurons, even in the presence of FGF2 (Fig. 5K and L). These results suggest that by the time ventral telencephalic progenitors have committed to a Nkx2.1$^+$ progenitor identity they are no longer sensitive to the proliferative actions of FGFs, and thus progress to a postmitotic Isl1$^+$ neuronal state.

DISCUSSION

In this study we have examined the timing of specification of cells destined to populate the ventral telencephalon, and the role of Shh signaling in this specification process. Our findings in the chick embryo provide evidence that prospective ventral telencephalic cells are first exposed to Shh signals derived from the anterior primitive streak and Hensen's node region at gastrula stages. They suggest that exposure of prospective forebrain cells to Shh signaling at this stage is sufficient to generate both proliferative progenitor cells and post-mitotic neurons of ventral telencephalic character. We discuss these findings in the general context of the mechanisms involved in specifying the regional pattern of the vertebrate forebrain.

Involvement of Shh signaling at gastrula stages in the specification of ventral telencephalic character

Shh has been implicated in the specification of ventral cell fates at spinal cord, hindbrain, midbrain and forebrain levels of the neuraxis (Ericson et al., 1995; Roelink et al., 1994; Chiang et al., 1996; Hynes et al., 1995; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997; Kohtz et al., 1998). Genetic studies in mice have further established the essential role of Shh signaling in the specification of ventral cell types in the telencephalon (Chiang et al., 1996). Nevertheless, the precise role of Shh in ventral telencephalic patterning remains unclear.

One major reason for this uncertainty has been the difficulty in pinpointing a source of *Shh* expression relevant to the patterning of the ventral telencephalon. At spinal cord, hindbrain, midbrain and caudal diencephalic levels of the neuraxis, the notochord and floor plate provide an appropriately positioned ventral source of *Shh* over the period that ventral cell fates are established (Ericson et al., 1995; Dale et al., 1997). In contrast, at the time that the overt identity of cells in the ventral telencephalon becomes evident, midline mesodermal and neural cells at telencephalic levels are devoid of *Shh* expression, and the nearest source of *Shh* is located in the floor plate of the caudal diencephalon (Ericson et al., 1995; Fishell, 1997). Although *Shh* is eventually expressed by cells in the ventral telencephalon itself (Ericson et al., 1995; Fishell, 1997), the onset of intrinsic telencephalic expression of *Shh* occurs only after the identity of cells in the ventral telencephalon has been specified (Ericson et al., 1995; Fishell 1997; Kohtz et al., 1998).

The present in vitro analyses reveal that the expression of several molecular markers characteristic of ventral telencephalic cells in vivo is specified prior to HH stage 6, in response to Shh signals derived from the anterior primitive streak/Hensen's node region, and possibly also from the prechordal plate region. These studies have also indicated that the early involvement of Shh signaling is transient: prospective telencephalic level neural plate cells become independent of a requirement for further Shh signaling at around HH stage 6. Yet the results with BMP addition suggest the commitment of prospective telencephalic cells to their ventral fate in vitro appears to occur only approx. 25 hours later, at HH stages 12-15. Thus, an early phase of Shh signaling appears to initiate a program of differentiation that proceeds in the absence of further Shh exposure, but only at a later stage do cells progress to a state of ventral commitment.

The progression of ventral telencephalic differentiation

How do telencephalic cells acquire their early independence from Shh signaling? In vivo, the profile of expression of Nkx2.1, Dlx, and Isl1 defines temporally distinct phases in the differentiation of Isl1$^+$ neurons. Nkx2.1 expression appears first and is confined to ventral progenitor cells; Dlx$^+$ cells and Isl1$^+$ cells are first detected approx. 15 hours later. At this stage, Dlx proteins are expressed both by proliferative progenitor cells and by post-mitotic neurons (Anderson et al., 1997; Puelles et al., 1999), whereas Isl1 is restricted to post-mitotic neurons, some of which also express Dlx proteins. The temporal patterns of generation of Nkx2.1$^+$, Dlx$^+$ and Isl1$^+$ cells in HH stage 6 T explants mimic closely that observed in vivo. The late onset of Dlx protein expression raises the possibility that Dlx expression defines a late step in the differentiation of ventral telencephalic progenitor cells, perhaps analogous to the onset of expression of MNR2 and Lim3 during the final division cycle of motor neuron progenitors at more caudal levels of the neuraxis (Ericson et al., 1997; Tanabe et al., 1998; Briscoe et al., 1999).

The sufficiency of a brief period of Shh exposure for the generation of ventral telencephalic character is in apparent contrast with results obtained at more caudal levels of the neuraxis, where a prolonged period of Shh exposure appears to be required for the specification of motor neuron differentiation (Ericsson et al., 1996). This difference may relate in part to the identity of the ventral cell type under study. Even at caudal levels of the neural tube, the inhibition of Shh signaling at late stages does not block the differentiation of V0 and V1 interneurons; neurons generated in the dorsalmost region of the ventral neural tube (Ericson et al., 1997; Pierani et al., 1999). Thus, at caudal levels of the neuraxis prolonged

Shh exposure may be necessary for the differentiation of only certain ventral neuronal subtypes.

Prospective telencephalic cells are initially specified as cells of MGE character

The ventral half of the prospective telencephalon contains two major regional subdivisions at early stages in its differentiation, the medial ganglionic eminence (MGE) and the lateral ganglionic eminence (LGE), regions which later give rise to the globus pallidum and the corpus striatum, respectively (Smart and Sturrock, 1979). At a molecular level, cells that contribute to the MGE can be distinguished from LGE cells by their expression of the Nkx class homeobox gene *Nkx2.1*. Moreover, *Nkx2.1* function is required for the specification of cells of MGE character. In *Nkx2.1* mutant mouse embryos the MGE fails to differentiate and cells of LGE character are now found at a more ventral position of the neural tube (Kimura et al., 1996; Takuma et al., 1998; Sussell et al., 1999). This early distinction between the MGE and LGE appears to depend, at least in part, on a limitation in the range of Shh signaling. In support of this idea, Nkx2.1 is expressed along most of the dorsoventral axis of the telencephalon in mice lacking *Ptc* function, a situation in which neural cells receive high level activation from the Shh signal transduction pathway (Goodrich et al., 1997). This finding implies that cells arrayed along the entire dorsoventral axis of the telencephalon are initially competent to generate an MGE-like character if exposed at an early stage to Shh signaling.

The spatial limitation in generation of cells of MGE character could reflect exposure of more dorsal telencephalic cells to a Shh concentration insufficient to induce a MGE fate. Alternatively, dorsal cells may be exposed to signals that antagonize Shh signaling. BMPs have been shown to antagonize Shh signaling at caudal levels of the neural tube (Liem et al., 1995; Lee and Jessell, 1999) and are normally required to induce dorsal interneurons (Lee and Jessell, 1999; Lee et al., 2000). We find that BMP4 can block the expression of Nkx2.1 by cells in explants of HH stage 6 and HH stage 9 ventral telencephalon and that telencephalic progenitor cells become resistant to this action of BMP4 during a approx. 5 hours period, between HH stages 12 and 15. BMPs are expressed in the dorsal region of the forebrain and in the surrounding epidermal ectoderm at these stages (Golden et al., 1999; Streit and Stern, 1999; data not shown). BMPs have also been shown to promote the expression of Msx1/Msx2 and thus may act to specify dorsal cell fates in the telencephalon as in the spinal cord and hindbrain (Liem et al., 1995; Furuta et al., 1997; Muhr et al., 1997; Golden et al., 1999; Lee and Jessell, 1999; Lee et al., 2000). During development of the telencephalon, the ability of BMP signaling to antagonize Shh signaling could therefore contribute to the generation of cell types of more dorsal character.

In addition, our findings show that early exposure of prospective ventral telencephalic cells to FGF prevents the generation of $Isl1^+$ neurons. FGF signaling enhances the incorporation of BrdU and prevents the generation of $Isl1^+$ neurons, but does not affect the generation of $Nkx2.1^+$ progenitor cells in explants of the ventral telencephalon. The FGF family members, *Fgf8, Fgf15, Fgf17* and *Fgf8,* are expressed in the anterior neural ridge and later in the ventral telencephalon (McWhirter et al., 1997; Shimamura and Rubenstein, 1997; Meyers et al., 1998; Xu et al., 1999). Reduced levels of expression of *Fgf8* in mouse leads to an impairment in the growth of the telencephalon (Meyers et al., 1998). Moreover, in chick, *Fgf8* starts to be expressed by cells in the anterior neural ridge by HH stage 9, approx. 30 hours before $Isl1^+$ neurons start to accumulate in the ventral telencephalon. One interpretation of these observations is that FGFs normally have a role in promoting the proliferation and in delaying the differentiation of forebrain progenitor cells. In this respect the actions of FGFs in the ventral telencephalon parallel the role of FGF signaling in the developing pituitary gland (Ericson et al., 1998; for review see Sheng et al., 1999). Here *Fgf 8* is expressed in the infundibular region of the ventral diencephalon, which later develops into the posterior pituitary (Ericson et al., 1998). Isl1 is expressed by cells in the adjacent anterior pituitary and FGF signaling can block the expression of Isl1 in cells in the dorsal region of the anterior pituitary. Thus FGFs may act to inhibit the expression of Isl1 in both neuroepithelial and neuroendocrine cells.

When and how cells in more dorsal regions of the telencephalon are intially specified remains to be determined. The present neural plate explant system may provide an experimental approach to address these issues.

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