Retinoic acid signalling specifies intermediate character in the developing telencephalon

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Summary

The organisation of the telencephalon into its major structures depends on its early regionalisation along the dorsoventral axis. Previous studies have provided evidence that sonic hedgehog (SHH) is required for the generation of telencephalic cells of ventral character, and that sequential WNT and fibroblast growth factor (FGF) signalling specifies cells of dorsal telencephalic character. However, the signalling mechanisms that specify telencephalic cells of an intermediate character remain to be defined. We provide evidence here that retinoic acid has a crucial role in specifying telencephalic progenitor cells of intermediate character.

Supplemental data available online

Key words: Chick, Mouse, Retinoic acid

Introduction

The emergence of functionally distinct regions of the developing central nervous system (CNS) depends on the sequential actions of patterning signals that induce rostrocaudal (RC) and dorsoventral (DV) character in neural progenitor cells (Bronner-Fraser and Fraser, 1997; Jessell, 2000; Lumsden and Krumlauf, 1996; Stern, 2001). During neurulation, progenitor cells located in different RC domains of the neural tube become exposed to signals that impose DV regional identity (Appel and Eisen, 2003; Briscoe and Ericson, 2001; Gunhaga et al., 2003; Hebert et al., 2002; Lee and Jessell, 1999; Maden, 2002; Muroyama et al., 2002; Rallu et al., 2002a; Rubenstein, 2000). The mechanisms underlying the DV patterning of neural progenitor cells in caudal regions that give rise to spinal cord and hindbrain have been elucidated, whereas the events that control DV patterning in rostral regions of the CNS, notably telencephalon, are less well resolved (Appel and Eisen, 2003; Briscoe and Ericson, 2001; Briscoe et al., 2000; Jessell, 2000; Rallu et al., 2002a; Rubenstein, 2000).

The assignment of an initial regional identity to neural progenitor cells in the prospective telencephalon is an important step in the generation of the functional subdivisions within the telencephalon. The cerebral cortex derives from the dorsal region and the pallidum from the ventralmost region of the early developing telencephalon. In mammals, the intermediate region of the telencephalon contains neural progenitor cells of at least three different structures: the striatum, the olfactory bulb and parts of the amygdala (Campbell, 2003; Deacon et al., 1994; Olsson et al., 1998; Schuurmans and Guillemot, 2002). Studies in diverse vertebrate organisms have provided evidence that patterns of expression of transcription factors early in development delineate the future functional subdivisions of the telencephalon along its DV axis, and that these expression patterns are largely conserved among vertebrate embryos (Cobos et al., 2001a; Cobos et al., 2001b; Fernandez et al., 1998; Puelles et al., 2000; Puelles et al., 1999). Thus, examination of when and how these profiles of gene expression are established in the telencephalon may reveal how telencephalic cells acquire their early dorsal, intermediate and ventral characters.

The diverse and interdependent functions of patterning signals of the HH, WNT, BMP, FGF and retinoid classes during forebrain and craniofacial development have made it difficult to establish the precise role(s) of individual signals in the early development of the telencephalon, and to resolve whether these signals act directly on neural cells or indirectly on surrounding tissues. In chick embryos, prospective neural tissue can be separated from adjacent tissues at stages when forebrain cells normally acquire dorsoventral DV regional character (Cobos et al., 2001b; Couly and Le Douarin, 1987). Thus, the stage at which telencephalic cells acquire different DV characters can be defined, and the direct response of neural cells to putative inductive signals and their mode(s) of action can be elucidated. Studies in chick have provided evidence that anterior forebrain cells are specified as cells of ventral character in response to an early phase of SHH signalling that operates at gastrula stages (Gunhaga et al., 2000). More recent evidence suggests that at the neural fold stage, WNT signals block ventral character and induce early dorsal character in telencephalic cells (Gunhaga et al., 2003). Later, at the early neural tube stage, FGF signals derived from dorsal midline cells act

together with WNT signals to induce definitive dorsal/precortical character in early dorsal cells (Gunhaga et al., 2003). Collectively, these studies have provided information on the time at which telencephalic cells acquire ventral and dorsal character, and the molecular nature of some of the signalling molecules involved in these processes. Although, they have failed to account for the patterning events that specify telencephalic progenitor cells of an intermediate character. Emerging evidence in mouse indicates that cells characteristic of the intermediate region of the telencephalon are generated in *Shh* mutants (Rallu et al., 2002b) (H. Toresson, PhD thesis, Lund University, 2001), but WNTs, BMPs and FGFs have not been implicated directly in the specification of cells of intermediate character (Rallu et al., 2002a). Thus, additional signals that act in concert with these peptide growth factors are likely to participate in the induction of cells of an intermediate telencephalic character.

Over the past few years, several lines of evidence have indicated that retinoic acid (RA) signalling has an important role in patterning vertebrate neural progenitors, and in neuronal specification (Appel and Eisen, 2003; Diez del Corral et al., 2003; Halilagic et al., 2003; Maden, 2001; Maden et al., 1998; Muhr et al., 1997; Novitch et al., 2003; Pierani et al., 1999; Schneider et al., 2001). In the developing spinal cord, recent findings have lead to a model in which RA signalling establishes the intermediate region of the caudal neural tube, in part by opposing the influence of FGF signalling (Diez del Corral et al., 2003; Novitch et al., 2003). The fact that RA acts in a coordinated manner with WNT, FGF, BMP and SHH signals to impose DV cell pattern in the spinal cord raises the possibility that RA signalling also contributes to the developmental steps that specify intermediate cell character in the telencephalon. There are several potential sources of RA within or adjacent to the early developing telencephalon (Blentic et al., 2003; Mic et al., 2002), and there is evidence that RA signalling influences anterior forebrain development (Anchan et al., 1997; Halilagic et al., 2003; Schneider et al., 2001; Smith et al., 2001; Swindell et al., 1999; Toresson et al., 1999; Whitesides et al., 1998) (H. Toresson, PhD thesis, Lund University, 2001). The attenuation of RA signalling at early stages of development led to aberrant expression of BMP and SHH signals in ventral midline neural tube cells and in the prechordal plate mesoderm that underlies the anterior neural plate; it also led to impaired survival of head mesenchyme and ventral telencephalic cells (Halilagic et al., 2003). At early neural tube stages, the gene encoding the RA synthetic enzyme retinaldehyde dehydrogenase 3 (RALDH3) is expressed in the head ectoderm adjacent to the early developing telencephalon (Blentic et al., 2003), and inhibition of RA signalling at these stages results in a general perturbation of the growth and development of the forebrain and frontonasal processes (Anchan et al., 1997; Schneider et al., 2001; Whitesides et al., 1998). Thus, RA is required for the growth, patterning and survival of multiple tissues of the early developing rostral head. At later stages of telencephalic development, radial glial cells in the prospective striatum serve as a localised source of retinoids, and retinoid signalling appears to enhance neuronal differentiation in the striatum (H. Toresson, PhD thesis, Lund University, 2001) (Toresson et al., 1999). However, from these studies it has remained unclear whether RA signalling contributes to the initial specification of telencephalic progenitor cells of intermediate character, rather than solely to later aspects of cell differentiation, and if so whether RA acts directly on neural cells or indirectly on adjacent tissues.

Using assays of neural differentiation in chick neural tissue, in vitro and in intact chick embryos, we provide evidence that RA signalling has a crucial role in specifying telencephalic cells of intermediate character. Our data also support the idea that FGF signalling maintains ventral progenitor character, in part, by opposing the influence of RA signals. Thus, the opponent roles of RA and FGF signals in establishing an intermediate positional character of neural cells appears to be conserved along the anteroposterior axis of the neural tube.

Materials and methods

Isolation and culture of explants

Explants of the prospective ventral and dorsal telencephalon were dissected from HH stage 8 and 10 chick embryos as described (Gunhaga et al., 2003). Explants of the prospective intermediate region of the telencephalon were isolated from stage 10 embryos guided by fate maps (Fernandez et al., 1998). These explants were cultured for 50-56 hours as described (Gunhaga et al., 2003). Explants from the prospective intermediate telencephalon of HH stage 12 and 14 chick embryos were cultured for 40-48 hours.

Mouse recombinant soluble FGFR4 (R&D Systems) was used at 150 nM together with 0.5 μg/ml heparin (Sigma). FGF8 (R&D Systems) was used at 5 nM. Soluble WNT3A, mouse frizzled receptor 8 protein (mFrz8CRD-IgG) and control condition media were generated as described (Gunhaga et al., 2003). All-trans retinoic acid (Sigma) and BMS189453 (Chen et al., 1995; Schulze et al., 2001) were used at 100 nM. Cyclopamine (Incardona et al., 1998) was used at 1 μM. BMP4 (R&D Systems) was used at 3 nM.

Whole-embryo culture

New culture method was essentially carried out as previously described (Chapman et al., 2001). Heparin acrylic beads (Sigma) were soaked in PBS (control) or mouse recombinant FGFR4 (R&D Systems). AG 1-X2 resin converted to formate form were soaked in DMSO (control), all-trans retinoic acid (1 mM) or the synthetic retinoid BMS-189453 (Chen et al., 1995; Schulze et al., 2001) (4 mM). The beads were inserted into the prospective prosencephalon of HH stage 10-11 chick embryos and placed in contact with the neural ectoderm. The embryos were maintained in New culture for ~48 hours.

Cloning of chick *Meis2* and generation of MEIS2 and EMX1 antiserum

A cDNA fragment corresponding to nucleotides 246-748 of the chick *Meis2* sequence (GenBank Accession Number AF199011) was obtained by RT-PCR using total RNA isolated from E5 chick embryos as template and the following oligonucleotide primers 5′-AAG-GATGCGATCTACGG-3′ and 3′-CTAAACCATCCCCTTGCT-5′.

The synthetic peptide (NH_2) – MAQRYDELPHYGGMDGC – (COOH) was used to generate a rabbit anti-MEIS2 antibody. Rabbit anti-EMX1 was generated using a mix of (NH2-) CLATKQSSGEDIDVTSND (–COOH) and (Ac-) AGSEVS-QESLLLHGC (–COOH) (Agrisera AB).

In situ hybridisation and immunohistochemistry

In situ RNA hybridisation histochemistry using chick digoxigenin-labelled *Meis2* probe was performed essentially as described (Gunhaga et al., 2003). Whole-mount in situ RNA hybridisation using chick digoxigenin-labelled *Raldh3* probe was performed as described (Wilkinson and Nieto, 1993).

For staining with the anti-EMX1, anti-NKX2.1 (BIOPAT

immunotechnologies), anti-MEIS2 and anti-cleaved caspase 3 (Cell Signaling) rabbit antibodies and with the monoclonal anti-PAX6, anti-TTF1 antibodies (AbCam), embryos and explants were fixed as described (Gunhaga et al., 2003). To quantify the percentage of antigen-expressing cells, each explant was serially sectioned at 8 μm, stained and counted, the total numbers of cells were determined by counting the number of nuclei using DAPI (Boehringer Mannheim).

Results

Telencephalic cells acquire intermediate character sequentially

In the early developing telencephalon, distinct profiles of HD transcription factor expression define progenitor cells of an intermediate character (Skogh et al., 2003; Toresson et al., 2000). In the mouse embryo, MEIS2 expression marks striatal progenitor cells and neurons from early stages into adulthood (Toresson et al., 2000), and in Hamilton and Hamburger stage 22 (E3.5) chick embryos (Fig. 1A), MEIS2 is expressed at high levels in progenitor cells in the intermediate of the telencephalon (Fig. 1B,C). PAX6 is expressed in progenitor cells in both the dorsal region, and in a dorsal domain of the intermediate region (Fig. 1B,C). NKX2.1 and EMX1 are expressed in the ventral and dorsal regions, respectively, but are excluded from the intermediate region of the telencephalon (Fig. 1B,C). Thus, cells in the intermediate region of the telencephalon express MEIS2, and cells in most dorsal part of the intermediate region co-express MEIS2 and PAX6 (Fig. 1B,C).

To examine when telencephalic cells acquire intermediate character, we monitored the generation of MEIS2$^+$ and PAX6$^+$ cells in explants isolated from the prospective intermediate region of the telencephalon of stages 10, 12 and 14 embryos. At stage 10, cells in the ventral region of the telencephalon are specified as NKX2.1$^+$ progenitor cells characteristic of the future pallidum, whereas more dorsal cells are re-specified as early PAX6$^+$ cells, apparently in response to WNT signals (Gunhaga et al., 2003). Guided by fate maps (Fernandez et al., 1998), we isolated explants of the prospective intermediate region of stage 10 embryos, and grew them for 50-56 hours in vitro. Stage 10 intermediate (I) explants generated a large number of PAX6$^+$ cells, a small number of NKX2.1$^+$ cells, but no MEIS2$^+$ or EMX1$^+$ cells (data not shown). The generation of PAX6$^+$ cells is likely to reflect the exposure of cells in the prospective intermediate region of the telencephalon to WNT signals (Gunhaga et al., 2003). Stage 12 I explants generated EMX1$^+$, PAX6$^+$, MEIS2$^+$ and NKX2.1$^+$ cells in variable numbers (Fig. 2B), indicating that some of these cells have started to acquire an intermediate character. Stage 14 I explants, generated PAX6$^+$ (23±4%) and MEIS2$^+$ cells (22±6%), but no NKX2.1$^+$ or EMX1$^+$ cells (Fig. 2C) – a profile indicative of the intermediate region of the telencephalon. Thus, a domain of telencephalic cells of intermediate character emerges from stage 12, and is established by stage 14.

SHH, FGF, BMP and WNT signals do not induce MEIS2$^+$ cells of intermediate character

To examine whether SHH, FGF or BMP signalling contribute to the specification of cells of intermediate character, we exposed stage 14 I explants to SHH, WNT, FGF and BMP proteins. SHH (10 nM) blocked the generation of MEIS2$^+$ and PAX6$^+$ cells, and induced NKX2.1$^+$ cells (65±5% compared with 0% in untreated stage 14 I explants, P<0.01) **(**Fig. 2D) – a profile characteristic of the ventral telencephalon. FGF8 (1 nM) blocked the generation of PAX6$^+$ and MEIS2$^+$ cells, whereas in the presence of BMP4 (3 nM), MEIS2$^+$ (20±4%) and PAX6$^+$ (61±6%) cells were still generated (data not shown.). To test whether any of these signals were required for the generation of intermediate cells, we exposed stage 14 I explants to antagonists of these signals; cyclopamine, a steroidal alkaloid that inhibits SHH signal transduction, soluble FGF receptor 4, an inhibitor of

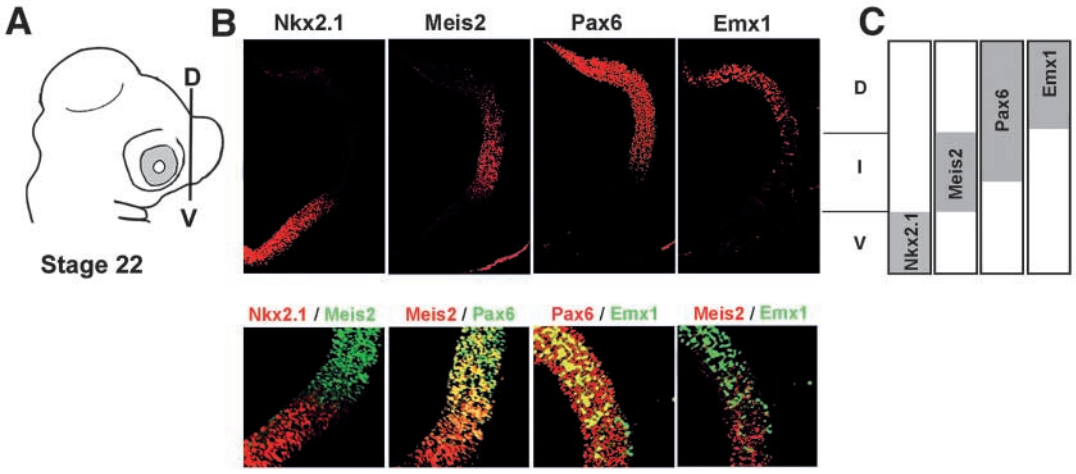

Fig. 1. Domains of expression of transcription factors define ventral (V), intermediate (I) and dorsal (D) subdivisions of the developing telencephalon. (A) A schematic drawing of a HH stage 22 (E3.5) chick embryo. The dorsal (D) to ventral (V) line indicates the level of the transverse sections shown in the corresponding panel. (B) In the telencephalon of a HH stage 22 chick embryo, MEIS2 was expressed at high levels in the intermediate region. PAX6 was expressed in the dorsal region and in the most dorsal domain of the intermediate region of the telencephalon. NKX2.1 was expressed exclusively in the ventral region and EMX1 exclusively in the dorsal region of the telencephalon (top panel). Below, enlargements depict overlapping (MEIS2/PAX6, PAX6/EMX1) and largely non-overlapping (MEIS2/NKX2.1, MEIS2/EMX1) regions of the telencephalon. Yellow staining represents double-labelled cells. (C) The expression domains of transcription factors in the developing telencephalon.

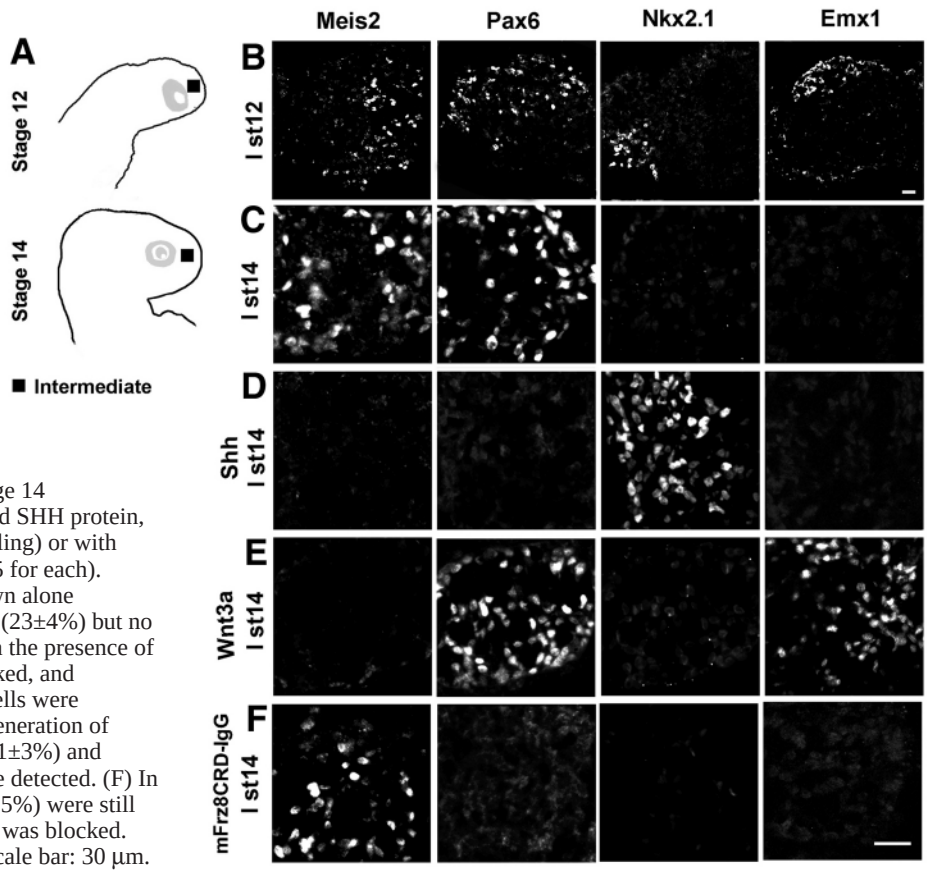

Fig. 2. Telencephalic progenitor cells gradually acquire their intermediate character. (A) A stage 12 and a stage 14 chick embryo (side view, rostral towards the right). Black squares indicate intermediate neuroectoderm explant regions of the embryo. Explants were analysed for expression of transcription factors after 40-48 hours in culture. (B) Stage 12 intermediate explants (n=15) generated MEIS2$^+$ (14±7%), PAX6$^+$ (22±7%), NKX2.1$^+$ (7±6%) and EMX1$^+$ (8±6%) cells in different domains. Scale bar: 30 μm. (C-F) Expression of transcription factors in stage 14 intermediate explants grown alone, with purified SHH protein, with mFrz8CRD (an antagonist of WNT signalling) or with conditioned medium containing WNT3A (n=15 for each). (C) Stage 14 intermediate explants (n=25) grown alone generated MEIS2$^+$ cells (22±6%), PAX6$^+$ cells (23±4%) but no NKX2.1$^+$ or EMX1$^+$ cells were detected. (D) In the presence of SHH, the generation of MEIS2$^+$ cells was blocked, and NKX2.1$^+$ (65±5%) but no PAX6$^+$ or EMX1$^+$ cells were detected. (E) In the presence of WNT3A, the generation of MEIS2$^+$ cells was blocked, and PAX6$^+$ cells (61±3%) and EMX1$^+$ (25±5%) but no NKX2.1$^+$ or cells were detected. (F) In the presence of mFrz8CRD, MEIS2$^+$ cells (20±5%) were still generated, while the generation of PAX6$^+$ cells was blocked. No NKX2.1$^+$ or EMX1$^+$ cells were detected. Scale bar: 30 μm.

FGF activity, and Noggin, an inhibitor of BMP signalling activity. In the presence of either of these antagonists, stage 14 I explants still generated MEIS2$^+$ (~20%) and PAX6$^+$ (~20%) cells, and no EMX1$^+$ or NKX2.1$^+$ cells appeared (data not shown). Collectively, these results suggest that neither SHH, FGF nor BMP signals are involved directly in the induction of telencephalic cells of an intermediate character.

The generation of PAX6$^+$ cells in stage 10 I explants indicates that prospective intermediate cells are exposed to early WNT signals. Consistent with this idea, in the presence of mouse frizzled receptor 8 protein (mFrz8CRD-IgG), an antagonist of WNT signals, the generation of PAX6$^+$ cells (4±2% compared to 23±4% in untreated 14 I explants, P<0.01) was suppressed in stage 14 I explants, but MEIS2$^+$ cells (20±5%) were still generated, and no EMX1$^+$ or NKX2.1$^+$ cells appeared (Fig. 2F). However, WNT3A exposure blocked the generation of MEIS2$^+$ cells, increased the number of PAX6$^+$ cells, and induced EMX1$^+$ (25±5% compared with 0% in untreated stage 14 I explants, P<0.01) cells – a profile characteristic of the dorsal telencephalon (Fig. 2E). Thus, early WNT signalling appears to contribute to the induction of PAX6$^+$ cells characteristic of the dorsal domain of the intermediate region, whereas high levels of WNT signals block intermediate and induce dorsal character in telencephalic cells.

RA is required and sufficient to induce cells of intermediate character

We next addressed the molecular nature of signal(s) that induce intermediate character in telencephalic cells. RA acts as an activator of MEIS1 and MEIS2 expression in the proximal region of the developing limb bud (Mercader et al., 2000) and MEIS2 was originally identified as a retinoid-inducible gene in P19 carcinoma cells (Oulad-Abdelghani et al., 1997), raising the possibility that RA signals contribute to the induction of MEIS2$^+$ cells of intermediate character in the developing telencephalon. Consistent with this possibility, the gene encoding the RA synthetic enzyme RALDH3 starts to be expressed in the head ectoderm adjacent to the rostral forebrain at ~stage 9/10 (Blentic et al., 2003), and by stage 14, the *Raldh3*-expressing domain is located adjacent to the ventral and intermediate regions of the telencephalon (Fig. 3A). Thus, telencephalic cells appear to be exposed to RA signals at the time they begin to acquire intermediate character. To examine whether RA signalling is required for the specification of cells of an intermediate character, we exposed stage 14 I explants to the RAR antagonist BMS-1895453 (BMS453) (100 nM) (Chen et al., 1995; Schulze et al., 2001). Under these conditions, the generation of MEIS2$^+$ cells was blocked (0% compared with 22±6% in untreated stage 14 I explants, P<0.01), the number of PAX6$^+$ cells (7±3%) was reduced, and a small number of NKX2.1$^+$ cells (5±3%) also appeared, but the growth of the explants was not affected (Fig. 3B). Thus, these results provide evidence that RA signalling is required for the specification of cells of intermediate character.

To examine whether RA signalling is sufficient to induce an intermediate character in telencephalic cells, we exposed dorsal (D) explants to RA at stage 8, when *Raldh3* expression has not

yet been initiated in the head ectoderm (Blentic et al., 2003). To exclude the possibility that, under these conditions, RA selectively induces the expression of MEIS2 but not other intermediate characteristics in early dorsal cells, we also monitored the generation of cells that express the LIM HD protein ISL1. ISL1 is expressed in postmitotic neurons in the ventral region of the telencephalon, and in the ventral domain of the intermediate region that generates MEIS2$^+$ cells, but not in the dorsal domain that generates MEIS2$^+$/PAX6$^+$ cells (Fig. 4C). Stage 8 D explants generated PAX6$^+$ cells (78±4%), whereas in the presence of RA (100 nM), MEIS2$^+$/PAX6$^-$ (22±4%) and MEIS2$^+$/PAX6$^+$ (60±5%) cells were generated in different regions of the explants (Fig. 4B). ISL1$^+$ neurons appeared (17±3%) in the region of the explants that generated

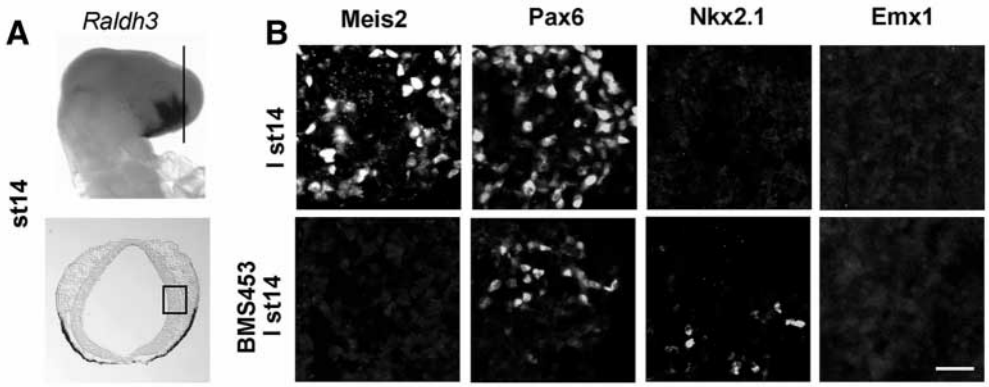

Fig. 3. RA signalling is required for the specification of intermediate cells. (A) Expression of *Raldh3* in the head ectoderm adjacent to the rostral forebrain in a stage 14 chick embryo (lateral view, rostral towards the right). The dorsal-to-ventral line indicates the level of the transversal section shown below. Boxed area indicates intermediate telencephalic region of the embryo. (B) Expression of transcription factors in stage 14 intermediate explants grown alone or with BMS189453 (an antagonist of retinoid signalling via receptors RARα/γ) (*n*=25). Stage 14 intermediate explants (*n*=25) grown alone generated MEIS2$^+$ cells (22±6%) and PAX6$^+$ cells (23±4%), but no NKX2.1$^+$ or EMX1$^+$ cells were detected. In the presence of BMS453, the generation of MEIS2$^+$ cells was blocked and the generation of PAX6$^+$ cells was markedly reduced (7±3%). A small number of NKX2.1$^+$ cells (5±3%) were generated, but no EMX1$^+$ cells were detected. Scale bar: 30 μm.

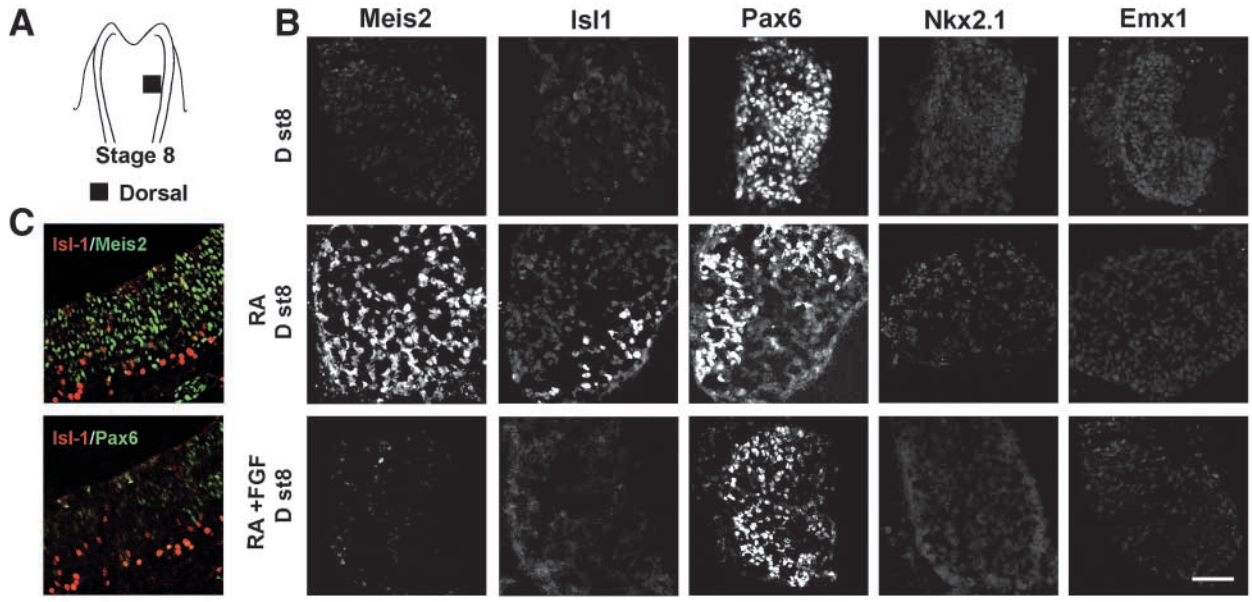

Fig. 4. RA induces intermediate character in early dorsal telencephalic cells. (A) A stage 8 chick embryo (dorsal view, rostral is upwards). Black square indicates prospective dorsal telencephalic neuroectoderm explant region. (B) Expression of transcription factors in stage 8 dorsal (D, st 8) explants cultured alone or with all-trans retinoic acid (RA) or a combination of all-trans retinoic acid and FGF8 for 48-52 hours. Stage 8 D explants cultured alone (*n*=20) generated PAX6$^+$ cells (78±4%) but no MEIS2$^+$, ISL1$^+$, NKX2.1$^+$or EMX1$^+$ cells were detected. Stage 8 D explants (*n*=20) exposed to all-trans retinoic acid generated MEIS2$^+$ cells (82±4%) distributed throughout the explants, while ISL1$^+$ cells (17±3%) and PAX6$^+$ cells (65±5%) were expressed in complementary regions in the explant. No NKX2.1$^+$ or EMX1$^+$ cells were detected. Stage 8 D explants (*n*=15) exposed to a combination of all-trans retinoic acid and FGF8 generated PAX6$^+$ cells (63±5%), but no MEIS2$^+$, NKX2.1 ISL1$^+$ or EMX1$^+$ cells were detected. Scale bar: 75 μm. (C) Expression of transcription factors in the ventral-most domain of the prospective intermediate telencephalon in a stage 22 chick embryo. ISL1$^+$ cells were located in the periphery of the proliferating neuroepithelium, while MEIS2$^+$ cells were situated throughout the neuroepithelium overlapping with the ISL1$^+$ domain. No PAX6$^+$ cells were detected in this ventral subdomain of the intermediate telencephalon.

MEIS2$^+$ but not MEIS2$^+$/PAX6$^+$ cells (Fig. 4B) – a profile indicative of the intermediate region of the telencephalon. There was a significant (P<0.01) difference between the number of MEIS2$^+$ and ISL$^+$ cells in stage 8 D explant compared with stage 8 D explants exposed to RA. Thus, RA appears to be able to induce independent markers of intermediate cell character in early dorsal telencephalic cells. We also examined whether RA produced cells of intermediate character in stage 8 ventral (V) explants (Fig. 5A). Stage 8 V explants grown alone generated NKX2.1$^+$ (>90%) and ISL1$^+$ (19±4%) neurons (Gunhaga et al., 2003), (Fig. 5B). RA (100 nM) blocked the generation of NKX2.1$^+$ cells and although ISL1$^+$ neurons (17±4%) were still generated, only a small number of MEIS2$^+$ (23±3% compared with 0% in untreated stage 8 V explants (P<0.01) and compared with 82±4% in stage 8 D explants exposed to RA, P<0.01) and PAX6$^+$ (15±4%) cells were induced (Fig. 5B). Thus, RA signalling is sufficient to induce intermediate character in both early dorsal and ventral telencephalic cells, albeit in a less efficient manner in ventral cells.

FGF maintains cells of ventral character by blocking their response to RA

The pattern of expression of *Raldh3* in the head ectoderm (Fig. 3A), suggests that at stages when telencephalic cells acquire an intermediate character (Fig. 2B,C) cells in ventral region of the telencephalon are also exposed to RA signals (Blentic et al., 2003; Li et al., 2000; Mic et al., 2000). Nevertheless, cells in the ventral region maintain their ventral character, which raises the possibility that ventral cells are exposed to a signal that suppresses their ability to respond to RA signalling. FGF8 opposes RA activity during DV patterning of neural progenitor cells in the spinal cord (Diez del Corral et al., 2003; Novitch et al., 2003), and cells in the ventral region of the prospective telencephalon are exposed to FGF8 derived from the adjacent anterior neural ridge (Crossley et al., 2001).

To examine whether FGF8 maintains ventral telencephalic character by suppressing RA signalling, we isolated V explants from stage 8 and 10 embryos, before and after the onset of *Raldh3* expression in the adjacent head ectoderm, and exposed them to soluble FGFR4 (6 μg/ml), an inhibitor of FGF8 signalling. Under these conditions, soluble FGFR4 did not block the generation of NKX2.1$^+$ cells, or induce MEIS2$^+$ cells in stage 8 V explants (data not shown). By contrast, in stage 10 V explants, soluble FGFR4 blocked the generation of NKX2.1$^+$ cells and induced a large number of PAX6$^+$ (56±5%) and MEIS2$^+$ (62±3%) cells (Fig. 6B) – a profile indicative of the intermediate region of the telencephalon, but did not suppress the growth of the explants. The changes in numbers of cells expressing NKX2.1, MEIS2 and PAX6 was significant (P<0.01) compared with the untreated stage 10 V explants. Thus, these results support the idea that that FGF8 blocks RA signalling in ventral telencephalic cells. We also tested whether FGF8 blocked the ability of RA to induce intermediate character in dorsal telencephalic cells by exposure of stage 8 D explants to RA (100 nM) and FGF8 (5 nM). Under these conditions, FGF8 blocked the ability of RA to induce MEIS2$^+$ (0% compared to 82±4% in stage 8 D explants only exposed to RA, P<0.01) and ISL1$^+$ neurons, but PAX6$^+$ (63±5%) cells were still generated (Fig. 4B). Collectively, these results provide evidence that FGF signals maintain cells of ventral character in the telencephalon by blocking the ability of RA to induce intermediate character in ventral telencephalic cells.

RA signalling is required and sufficient for the generation of intermediate cells in intact embryos

We next used New Culture methods (Chapman et al., 2001) to examine whether RA signalling is required and sufficient for the acquisition of intermediate telencephalic character in intact chick embryos. Control beads or beads soaked in BMS453 were placed adjacent to the neuroepithelium of the anterior prosencephalon of stage 9-10 embryos, and developed to stage 20 (E3). At this stage, *Meis2* mRNA is expressed in wide domain of the intermediate region of the telencephalon (Fig. 7B), but very few cells have started to express MEIS2 protein (Fig. 7D). All embryos grafted with control beads (n=10) showed normal morphology and dorsoventral patterning of the telencephalon (Fig. 7A). In 10 out of 12 embryos grafted with BMS453 beads, *Meis2* expression normal on one side and severely reduced or absent on the opposite side (Fig. 7B). There were no major differences in the patterns of expression of EMX1, PAX6 and NKX2.1 between the left and right sides of the telencephalon in these embryos (Fig. 7B). Cleaved caspase 3 is a marker of apoptotic cells (Fernandes-Alnemri et al., 1994). On the side of the telencephalon where *Meis2* was still expressed, 1±1% of the cells expressed cleaved caspase 3, when compared with 3±1% of the cells on the side where *Meis2* expression was suppressed (n=4) (see Fig. S1 at http://dev.biologists.org/supplemental), providing evidence that apoptosis did not contribute significantly to the loss or reduction of cells expressing *Meis2*. In addition, the number of mesenchyme cells that expressed cleaved caspase 3 was increased on the side where *Meis2* expression was blocked (see

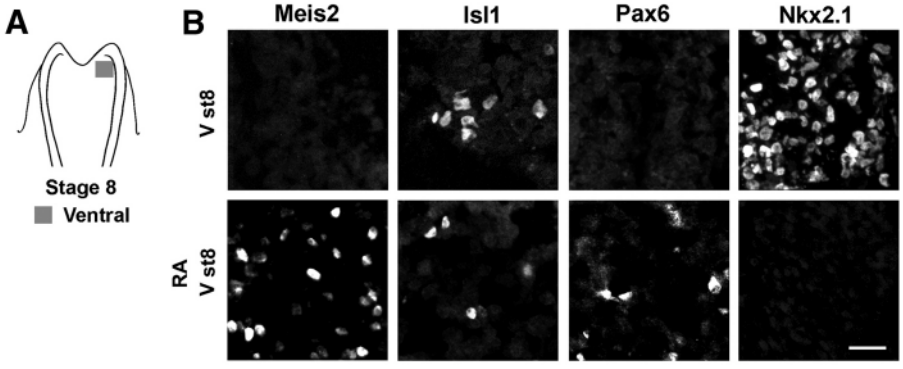

Fig. 5. Retinoic acid (RA) induces intermediate character in early ventral telencephalic cells. (A) A stage 8 chick embryo (dorsal view, rostral is upwards). Grey square indicates ventral telencephalic neuroectoderm explant region. (B) Expression of transcription factors in stage 8 ventral (V, st 8) explants cultured alone or with all-trans retinoic acid for 48-52 hours. Stage 8 V explants (n=15) generated NKX2.1$^+$ cells (>90%) and ISL1$^+$ cells (19±4%) but no MEIS2$^+$ or PAX6$^+$ cells. Stage 8 V explants (n=15) exposed to all-trans retinoic acid generated MEIS2$^+$ cells (23±3%), ISL1$^+$ cells (17±4%), PAX6$^+$ cells (15±4%) but no NKX2.1$^+$ or EMX1$^+$ cells. Scale bar: 30 μm.

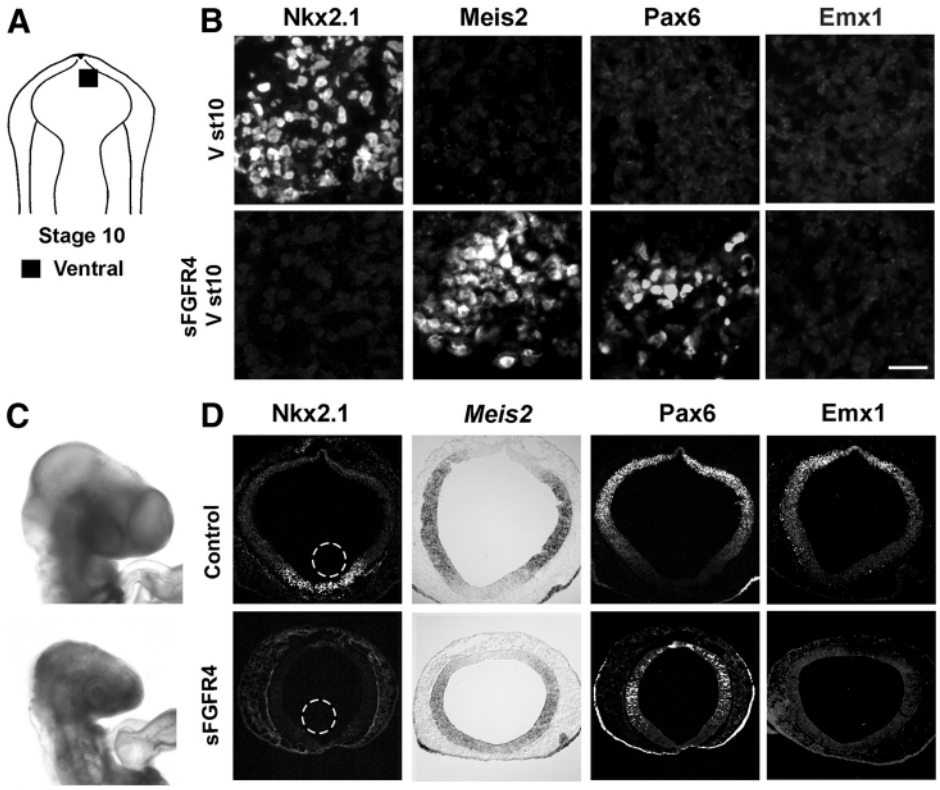

Fig. 6. FGF signals inhibit ventral telencephalic cells from responding to RA signalling. (A) A stage 10 chick embryo (dorsal view, rostral is upwards). Black square indicates ventral telencephalic neuroectoderm explant region. (B) Expression of transcription factors in stage 10 ventral (V) explants cultured alone or with soluble FGFR4 (sFGFR4) for 48-52 hours. Stage 10 V explants cultured alone (n=15 for each) generated NKX2.1$^+$ cells (>90%) but no MEIS2$^+$, PAX6$^+$ or EMX1$^+$ cells. In the presence of soluble FGFR4, the generation of NKX2.1$^+$ cells was blocked but MEIS2$^+$ cells (62±3%) and PAX6$^+$ cells (56±5%) were detected in stage 10 V explants. No EMX1$^+$ cells were detected. Scale bar: 30 μm. (C) Lateral views of stage 20-22 chick embryos generated in New Culture from stage 10 grafted with control or soluble FGFR4 beads. All control embryos showed normal morphology, while the embryos grafted with soluble FGFR4 had smaller telencephalic vesicles than did controls. (D) Consecutive transversal sections of New Culture embryos, showing the expression of transcription factors in the telencephalon. The broken circle indicates the position of the bead. Embryos (n=10) grafted with control beads in the prospective ventral telencephalon expressed NKX2.1$^+$ cells in the ventral region, *Meis2*$^+$ cells in the intermediate region and PAX6$^+$ cells in the dorsal domain of the intermediate region and in the dorsal region of the telencephalon. EMX1$^+$ cells were located exclusively in the dorsal region. Embryos (n=10) grafted with soluble FGFR4 beads in the prospective ventral telencephalon lacked or showed a severely reduced number of NKX2.1$^+$ cells, in four out of 10 embryos ventral cells ectopically expressed *Meis2*, PAX6 was still expressed in the intermediate and dorsal region of the telencephalon, and the generation of EMX1$^+$ cells was blocked.

Fig. S1 at http://dev.biologists.org/supplemental). These results provide evidence that BMS452 does not induce selective death of *Meis2*$^+$ cells, and that attenuation of RA signalling inhibits the expression of *Meis2* in the intermediate region of the telencephalon in intact chick embryos.

To examine whether RA is sufficient to induce MEIS2 cells in the telencephalon, we implanted beads soaked in RA adjacent to the dorsal neuroephithelium of stage 10 embryos that were maintained in culture to stage 20 (E3). In embryos grafted with control beads (n=10), the DV patterning of EMX1$^+$, PAX6$^+$ and NKX2.1$^+$ in the telencephalon was normal (Fig. 7D), but as mentioned previously, few if any cells in the telencephalon expressed MEIS2 protein. Embryos grafted with RA beads (n=4) had a morphologically normal telencephalon, but the developing eyes were enlarged (Fig. 7C). However, a large number of MEIS2$^+$ cells were induced throughout the dorsal and intermediate regions of the telencephalon, and the generation of EMX1$^+$ cells was almost completely blocked (Fig. 7D). Moreover, the expression of PAX6 was partially suppressed, but NKX2.1$^+$ cells were still generated in the ventral region of the telencephalon (Fig. 7D). Thus, RA induces intermediate character in prospective dorsal telencephalic cells in intact embryos.

FGF maintains telencephalic cells of ventral character in intact embryos

We also used New Culture methods (Chapman et al., 2001) to examine whether FGF activity maintains ventral and suppresses intermediate telencephalic character in intact chick embryos. Control beads or beads soaked in soluble FGFR4 were implanted into the ventral region of the anterior prosencephalon of stages 9-10 embryos, and permitted to develop to stages 20-22. All embryos grafted with control beads (n=10) showed normal morphology and dorsoventral patterning of the telencephalon (Fig. 6C). In embryos grafted with soluble FGFR4 beads (n=10), cells in the telencephalon failed to express NKX2.1 and (as expected) EMX1, and in four embryos, ventral cells ectopically expressed *Meis2* (Fig. 6D). All embryos also had smaller heads and telencephalic vesicles (Fig. 6C), with increased numbers of cells that expressed cleaved caspase 3 predominantly in the more dorsal regions of the telencephalon (see Fig. S1 at http://dev.biologists.org/supplemental). Thus, attenuation of FGF signalling does not lead to selective death or block of proliferation of ventral telencephalic cells. These results provide evidence that in intact chick embryos FGF activity maintains ventral and suppresses intermediate telencephalic character by inhibiting cells to respond to RA signalling.

Discussion

The establishment of different neural progenitor domains along the DV axis at early stages of development is an essential step

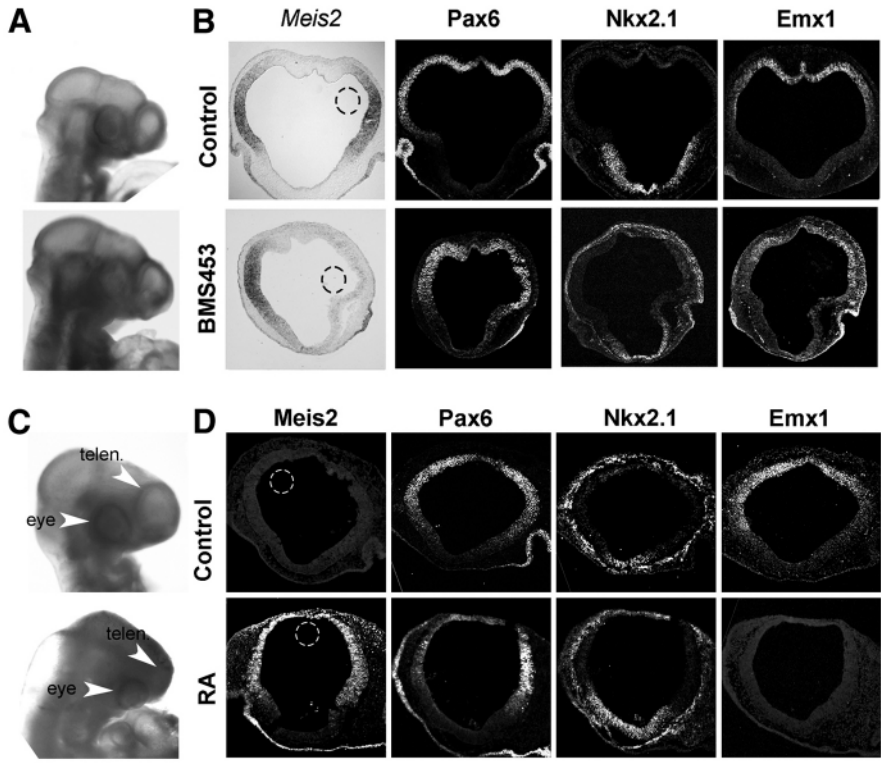

Fig. 7. RA signalling is required and is sufficient for the specification of intermediate cells. (A) Lateral views of stage 20-22 chick embryos generated in New Culture from stage 10 embryos grafted with either control beads (upper panel) or BMS453 beads (lower panel) into the prospective telencephalon. (B,D) Consecutive transversal sections of New Culture embryos, showing the expression of transcription factors in the telencephalon. The broken circle indicates the position of the bead. (B) Embryos (n=10) grafted with control beads in the prospective telencephalon (upper panel) expressed *Meis2*$^+$ cells in the intermediate region at mRNA level, PAX6$^+$ cells in the dorsal region and NKX2.1$^+$ in the ventral region of the telencephalon. Embryos (n=12) grafted with BMS453 beads into the prospective telencephalon (lower panel) had an asymmetric expression pattern of *Meis2*, being expressed only at one lateral side of the developing telencephalon. EMX1$^+$, PAX6$^+$ cells were located dorsally, while NKX2.1$^+$ cells were positioned in the ventral region of the telencephalon. (C) Lateral views of stage 20-22 chick embryos generated in New Culture from stage 10 embryos grafted with either control beads (upper panel) or RA beads (lower panel) into the prospective telencephalon. The heads of the RA-treated embryos were normal in size but the eyes were enlarged. (D) Embryos (n=10) grafted with control beads in the prospective telencephalon (upper panel) did not generate MEIS2$^+$ cells, but generated PAX6$^+$ and EMX1$^+$ cells in the dorsal region and NKX2.1$^+$ in the ventral region of the telencephalon. Embryos (n=4) grafted with RA beads into the prospective telencephalon (lower panel) generated a large number of MEIS2$^+$ cells in the dorsal and intermediate regions of the telencephalon, a reduced number of PAX6$^+$ cells, no EMX1$^+$ cells and a normal number of NKX.2.1$^+$ cells.

in the generation of different functional regions of the mature telencephalon. The growth, patterning and morphogenesis of the forebrain and the rostral head depend on intricate crossregulatory interactions between SHH, BMP, WNT, FGF and RA signal (Francis-West et al., 2003; Machon et al., 2003). The complexity of these interactions has made it difficult to establish the contribution of individual signals to the induction of DV regional neural identity in the telencephalon, and whether signals act directly on neural cells or indirectly on adjacent tissues.

To address these issues, we have established explant assays of neural differentiation in chick embryos, and previous studies have provided evidence that SHH signalling induces ventral character, and that WNT and FGF signals act sequentially to induce dorsal/precortical character in telencephalic cells (Gunhaga et al., 2000; Gunhaga et al., 2003). These studies have so far failed to identify the signal(s) that induces intermediate character in telencephalic cells. Our findings provide evidence that RA signalling in neural cells is required and sufficient to induce cells of intermediate character, and suggest that FGF signals maintain ventral character by opposing RA signalling in ventral telencephalic cells.

Taken together with previous findings (Campbell, 2003; Gunhaga et al., 2000; Gunhaga et al., 2003; Rallu et al., 2002a; Schuurmans and Guillemot, 2002), our results provide insights into the sequential steps involved in assigning an initial DV regional identity to telencephalic neural progenitor cells (Fig. 8). The following model emerges from these findings. At gastrula stages, most or all prospective telencephalic cells become specified as ventral (NKX2.1$^+$) cells in response to node-derived SHH signals (Gunhaga et al., 2000). At neural fold and early neural plate stages, cells in the prospective dorsal and intermediate regions of the telencephalon cells are exposed to WNT signals that induce PAX6$^+$ cells (Gunhaga et al., 2003). The head ectoderm adjacent to the telencephalon then starts to express *Raldh3* (Blentic et al., 2003), exposing telencephalic cells to RA signals that promotes the generation of intermediate (MEIS2$^+$) cells. From the neural plate stage, prospective ventral telencephalic cells are exposed to FGF8 derived from the anterior neural ridge (Crossley et al., 2001), and FGF8 maintains ventral telencephalic character by opposing the influence of RA signals in ventral cells. At early neural tube stages, the domain of *Fgf8* expression expands dorsally and FGF signals derived from the dorsal midline region induce definitive dorsal/precortical (EMX1$^+$) cells (Gunhaga et al., 2003), and cells that are exposed to RA and low levels of FGF8 acquire intermediate character.

Our results provide evidence that SHH signalling is not involved in generating cells of intermediate telencephalic character in the chick embryo. Consistent with these results, genetic evidence in mouse indicates that SHH signalling is required for the generation of ventral telencephalic cells characteristic of the medial ganglionic eminence, whereas cells in the lateral ganglionic eminence, which derive from the intermediate region of the telencephalon, are still generated in SHH-deficient embryos (Rallu et al., 2002b) (H. Toresson, PhD thesis, Lund University, 2001). A similar situation appears to exist in the developing spinal cord, where SHH signalling is not

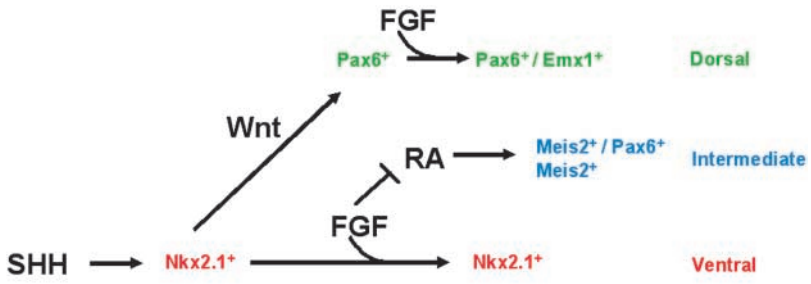

Fig. 8. Model of the initial dorsoventral patterning of the telencephalon. Proposed signalling events in neural cells during the initial specification of telencephalic cells of ventral, intermediate and dorsal character. At gastrula stages, most or all prospective telencephalic cells become specified as ventral NKX2.1$^+$ cells in response to node-derived SHH signals (Gunhaga et al., 2000). WNT signals derived from adjacent dorsal ectoderm induce early dorsal PAX6$^+$ cells (Gunhaga et al., 2003). RA promotes the generation of intermediate MEIS2$^+$ and MEIS2$^+$/PAX6$^+$ cells. FGF8 derived from the anterior neural ridge (Crossley et al., 2001) maintains ventral character by opposing RA signalling in ventral cells, and FGF signals derived from the dorsal midline region induce definitive dorsal/precortical PAX6$^+$/EMX1$^+$ cells (Gunhaga et al., 2003). The specification of the most dorsal midline cells of the telencephalon appears to also require BMP signalling (Hebert et al., 2002), but is not indicated in the model.

required to induce Dbx$^+$ intermediate progenitor cells, and RA signalling can establish many molecular aspects of this intermediate domain independent of SHH signalling (Pierani et al., 2001). RA signalling promotes PAX6 expression in the intermediate region of the developing spinal cord (Diez del Corral et al., 2003; Novitch et al., 2003), and we find that RA induces PAX6 in telencephalic cells. Thus, RA signalling appears to contribute to the generation of an intermediate progenitor cell domain in both the telencephalon and the spinal cord, in part by inducing the generation PAX6$^+$ progenitor cells.

FGF signalling appears to maintain ventral character in telencephalic cells (Shinya et al., 2001; Walshe and Mason, 2003), providing a potential explanation for the transient requirement for SHH in the generation of ventral cells in the telencephalon (Gunhaga et al., 2000). Our results extend these findings by showing that that FGF signalling also maintains ventral progenitor character by suppressing the RA-mediated induction of intermediate character in ventral cells. The opponent activities of RA and FGF signals also has a parallel in the developing spinal cord, where RA promotes and FGF suppress the expression of class I HD proteins (Diez del Corral et al., 2003; Gunhaga et al., 2000; Novitch et al., 2003). Thus, in addition to the conserved function of SHH in inducing ventral progenitor cells (Briscoe and Ericson, 2001; Ericson et al., 1995), RA and FGF signals appear to have similar opponent roles in establishing intermediate character at different levels of the developing neural tube.

Finally, our results, together with previous studies, indicate that RA signalling has important roles at several sequential stages in the generation of striatal neurons. At gastrula stages, RA signalling appears to refine the rostrocaudal identity of the anterior neural plate by modulating signalling involved in axial mesoderm specification (Halilagic et al., 2003). As shown in this study, during the initial DV patterning of neural progenitor cells in the telencephalon, RA signalling induces intermediate cells. At later stages of telencephalic development, glial cells in the developing striatum start to express *Raldh3* (Smith et al., 2001), and serve as a localized source of retinoids (H. Toresson, PhD thesis, Lund University, 2001) (Toresson et al., 1999), which appear to enhance neuronal differentiation in the striatum (H. Toresson, PhD thesis, Lund University, 2001) (Toresson et al., 1999).

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