Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin

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SUMMARY

Ventral midline cells in the neural tube have distinct properties at different rostrocaudal levels, apparently in response to differential signalling by axial mesoderm. Floor plate cells are induced by sonic hedgehog (SHH) secreted from the notochord whereas ventral midline cells of the rostral diencephalon (RDVM cells) appear to be induced by the dual actions of SHH and bone morphogenetic protein 7 (BMP7) from prechordal mesoderm. We have examined the cellular and molecular events that govern the program of differentiation of RDVM cells under the influence of the axial mesoderm. By fate mapping, we show that prospective RDVM cells migrate rostrally within the neural plate, passing over rostral notochord before establishing register with prechordal mesoderm at stage 7. Despite the co-expression of SHH and BMP7 by rostral notochord, prospective RDVM cells appear to be specified initially as caudal ventral midline neuroectodermal cells and to acquire RDVM properties only at stage 7. We provide evidence that the signalling properties of axial mesoderm over this period are regulated by the BMP antagonist, chordin. Chordin is expressed throughout the axial mesoderm as it extends, but is downregulated in prechordal mesoderm coincident with the onset of RDVM cell differentiation. Addition of chordin to conjugate explant cultures of prechordal mesoderm and neural tissue prevents the rostralization of ventral midline cells by prechordal mesoderm. Chordin may thus act to refine the patterning of the ventral midline along the rostrocaudal axis.

Key words: Prechordal mesoderm, Notochord, Rostral diencephalic ventral midline, Floor plate, BMP7, SHH, chordin, Chick

INTRODUCTION

Ventral midline cells in the neural tube provide signals that control the differentiation of adjacent neural cells and that guide developing axons (Placzek et al., 1993; Ericson et al., 1995, 1996; Hynes et al., 1995; Serafini et al., 1996; Tanabe and Jessell, 1996). At different rostrocaudal levels of the neuraxis, ventral midline cells exhibit distinct molecular properties and signalling activities (Daikoku et al., 1982; Placzek et al., 1990; Dale et al., 1997). Floor plate cells are confined to caudal levels of the neural tube and are induced by sonic hedgehog (SHH)-mediated signals from the notochord (Chiang et al., 1996; Ericson et al., 1996; Tanabe and Jessell, 1996; Dale et al., 1997). At forebrain levels, the specialized properties of rostral diencephalic ventral midline cells appear to be induced by prechordal mesoderm through a process that can be recapitulated in vitro by the coordinate actions of SHH and bone morphogenetic protein 7 (BMP7) (Dale et al., 1997). The distinct properties of ventral midline cells at different levels of the rostrocaudal axis may therefore be induced by distinct signals from adjacent axial mesoderm. How these inducing and responding cell groups interact in the embryo to generate the spatial precision of ventral midline cell specification remains unclear.

Fate mapping studies in the chick have shown that ventral midline cells positioned along the entire rostrocaudal axis derive from a small region of epiblast, termed area a (Schoenwolf et al., 1989a,b; Garcia-Martinez et al., 1993), which at stage 4 lies directly above the prechordal mesoderm (Nicolet, 1970, 1971). As gastrulation and neurulation proceed, cells from area a migrate in the midline of the neural plate so that the ventral midline becomes extended into distinct regions of the neuraxis. Axial mesoderm shows a similar pattern of migration: both notochord and prechordal mesoderm cells originate in Hensen’s node but then migrate under the midline of the neural plate (Spratt, 1955; Rosenquist, 1966; Sellenk and Stern, 1991). By the end of neurulation (stage 12), prechordal mesoderm lies adjacent to floor plate cells of the
mesencephalon, rather than to the forebrain (Seifert et al., 1993).

To provide insight into when and where the induction of RDVM cells is initiated, we have examined the relative movements of prechordal mesoderm and prospective RDVM cells in vivo and have used in vitro assays to determine when RDVM cell properties are specified. We find that prechordal mesoderm and prospective RDVM cells migrate out of register. Prospective RDVM cells extend adjacent to rostral notochord and re-establish contact with prechordal mesoderm only at stage 7, at the level of the prospective rostral diencephalon. Even at this stage, these two cell groups remain in contact only transiently, but this brief period of apposition appears sufficient for the specification of RDVM cell properties in the neur ectoderm.

These results are consistent with the idea that ventral midline cells are patterned by spatially restricted signals from the underlying axial mesoderm. However, the co-expression of BMP7 and SHH by axial mesoderm is not restricted to regions or times at which RDVM induction occurs. In particular, BMP7 and SHH are expressed in rostral notochord both when this tissue underlies prospective RDVM cells prior to the specification of their rostral properties and later when it lies below rostral floor plate. This raises the issue of why floor plate cells rather than RDVM are induced by rostral notochord. The identification of endogenous secreted antagonists of BMP activity (Piccolo et al., 1996; Zimmerman et al., 1996; Hsu et al., 1998) led us to examine whether such inhibitors might regulate the signalling properties of the axial mesoderm and thus provide a mechanism for limiting the induction of RDVM cells. We show that from stages 4 to 7, all axial mesoderm cells express the BMP inhibitor, chordin. The regulation of chordin expression in axial mesoderm and the ability of chordin to block the rostralizing activity of prechordal mesoderm suggest that chordin acts to refine patterning of the ventral midline along the rostrocaudal axis, preventing premature and ectopic differentiation of RDVM cells and permitting the development of floor plate cells in caudal regions.

MATERIALS AND METHODS

Fate mapping

Chick embryos were incubated to HH stages 5-8 (Hamburger and Hamilton, 1951). A small region of egg shell and the underlying vitelline membrane were removed to expose the embryo. India ink was injected under the embryo to enhance visual contrast. The lipophilic carbocyanine dye, 1,1'-dioctadecyl-3,3,3'-tetramethyl indocarbocyanine perchlorate (DiI) (Molecular probes Inc.) was injected into small groups of cells at the anterior midline of the neural plate/ neural groove (Fig. 2E,F). 2-6 cells were injected in each case, judged qualitatively (Fig. 2F, inset) and quantitatively (mean spot diameter of initial fluorescent patch = 16 μm; n=21). The dye was driven from a capillary needle, attached to a micromanipulator, by making a circuit with a 9 V battery. The distance of the injection site from the anterior neuropore was recorded in microns using a graticule eye-piece. The window in each shell was sealed and the embryos were incubated for another 15-18 hours until they reached stage 11. At the end of the experiment, progeny of injected cells came to lie as cohorts occupying either the rostral floor plate, the RDVM, rostral diencephalic lateral regions or the telencephalon. To assess the position of labelled progeny more precisely, the embryos were dissected in L15 and fixed in 4% paraformaldehyde in 0.12 M PB (pH 7.4) overnight at 4°C. After incubation in Dispase (Boehringer Mannheim: 1 mg/ml in L15 at room temperature for 15 minutes), the neur ectoderm layer was isolated using tungsten needles, flat-mounted in Glycergel (DAKO) on Superfrost Plus slides and examined on a Zeiss Axioptih microscope under phase and epifluorescence optics (Fig. 2G-I). The positions of labelled progeny relative to the anterior neuropore were recorded.

Prechordal mesoderm identification

The position of the prechordal mesoderm at stages 5-11 was identified morphologically, on the basis of its shape (Fig. 2) and position, relative to the prechordal plate at the rostral tip of the foregut (Seifert et al., 1993) and to the thinner, rod-like notochord (Fig. 2A-D). Scanning EM studies confirmed the position of the prechordal mesoderm at different stages (Fig. 2A). Our studies indicate that, in addition to morphological differences between prechordal mesoderm and prechordal plate mesenchyme (Adelman, 1922; Meier, 1981; Wachtler et al., 1984; Seifert et al., 1993), these tissue express distinct combinations of markers over stages 4-7. Midline cells in the prechordal plate mesenchyme express BMP7 from stage 5 (not shown) and express SHH relatively late (st6). They do not express chordin (except for a very weak and transient expression at stage 4) or netrin-1. Prechordal mesoderm and notochord cells express SHH throughout stages 4-7. However, whereas from stage 5 prechordal mesoderm cells express BMP7, notochord cells begin to express BMP7 only at stage 6 (Fig. 2F). Finally, prechordal mesoderm cells transiently express vanishingly low levels of netrin-1, between stages 5 and 7, whereas notochord cells express netrin-1 strongly from stages 4 to 7 (Fig. 2C).

Heterogeneity and definition of ventral midline neurectodermal cells

The properties of telencephalic midline, RDVM, floor plate of the mesencephalon/caudal diencephalon and floor plate cells of the spinal cord/ hindbrain were defined on the basis of the array of markers expressed in the stage 11 embryo (Fig. 1). At this stage, telencephalic midline expresses none of the markers examined (Fig. 1A-C). RDVM cells express SHH, BMP7 and Nkx2.1 but not HNF3β or netrin-1 (Fig. 1A-C, I-M), floor plate cells of the mesencephalon/caudal diencephalon express SHH, netrin-1, BMP7 (Fig. 1A-C) and HNF3β but not Nkx2.1 (not shown). Floor plate cells of the spinal cord and hindbrain express SHH, netrin1, HNF3β but not Nkx2.1 (Fig. 1A-H). Weak expression of BMP7 is detected in the rostral hindbrain, but not in the caudal hindbrain/spinal cord (Fig. 1C).

Tissue dissection and explant culture

The position of prospective RDVM cells was assessed using the fate map (stages 5-8). Area a was defined according to Schoenwolf et al. (1989a). Prospective RDVM cells, with or without underlying axial mesoderm, were isolated using Dispase and cultured in collagen gels for 24 hours and 40 hours (Dale et al., 1997; Placzek and Dale, 1998). 5-10 explants were examined for each marker, in each experiment. Individual explants were examined at either 24 or 40 hours, i.e. times that are predicted to encompass the time in vivo at which committed RDVM cells would be observed. Both time points were examined in every experiment and, in all cases, the same marker profile was observed at the 24 and 40 hour time points.
In situ hybridization
Embryos and explants were examined using standard techniques (Schaeren-Wiemers and Gerfin-Moser, 1993; Hume and Dodd, 1993; Dale et al., 1997). Digoxigenin-labelled antisense RNA probes were prepared by in vitro transcription from linearised template DNAs. The pcm21 plasmid encoding netrin-1 (Serafini et al., 1994) was linearised with EcoRI and transcribed with T7 polymerase. The pcp7 plasmid encoding HNF3b was linearised with HindIII and transcribed with SP6 polymerase (Ruiz i Altaba et al., 1995a). The pcvhh1 plasmid encoding SHH was linearised with SalI and transcribed with SP6 polymerase (Ericson et al., 1995). The plasmid encoding chordin was linearised and transcribed as described (Streit et al., 1998). Following antisense analysis, embryos and explants were either serially sectioned or examined as whole mounts of isolated neuroectoderm.

Immunocytochemistry
Embryos (n=3 minimum for each marker and each age) and explants were examined using standard techniques (Placzek et al., 1993). Antibodies were used at dilutions: 4C7, anti-HNF3b mAb (1:40); Ttf-1, anti-Nkx2.1(1:5000) (Lazzaro et al., 1991). Second antibodies (TAGO and Boehringer-Mannheim) were conjugated to fluorescein isothiocyanate (FITC), Cy-2 or Cy-3.

Analysis of chordin activity
Supernatant from S2 cells infected with baculovirus carrying the VL1E construct encoding recombinant chordin protein (Streit et al., 1998), was diluted 1:40 in culture medium and then added at the onset of culture. As a control, supernatant of insect cells infected with a baculovirus carrying a control construct VL1C, was used in similar conditions.

RESULTS

RDVM cells and prechordal mesoderm extend out of register
To assess the pattern of rostral migration of prospective RDVM cells and prechordal mesoderm, we constructed a fate map of the neurectodermal midline of chick. At stage 4, cells destined to populate the ventral midline of the neural tube along its entire rostrocaudal axis, including prospective RDVM cells, are located in register with underlying prospective prechordal mesoderm (Nicolet, 1970, 1971; Schoenwolf et al., 1989a; Schoenwolf and Sheard, 1990; Fig. 2J). At stages 5 and 6, both prospective RDVM cells and prechordal mesoderm move rostrally but do not remain in register. Prechordal mesoderm migrates in advance of prospective RDVM cells and reaches its rostral-most location at stage 6. In contrast, midline neural cells, including prospective RDVM cells, continue to migrate rostrally until at least stage 8. Thus, between stages 5 and 8, neur ectodermal cells pass over the prechordal mesoderm. Cells

**Fig. 1.** Heterogeneity in ventral midline neur ectodermal cells in the HH stage 11 chick. In situ hybridization (A-G,I-L) and immunocytochemical (H,M) analyses in flat-mounted neur ectoderm (A-C; ventral view) or transverse sections (D-M) of stage 11 chick embryos. (I-M) Sections at the level of the rostral diencephalon, indicated as RD in C. (D,E,G,H) Sections at the level of spinal cord; section shown in F is at the level of the midbrain, indicated as M in C. Floor plate cells of the spinal cord and caudal hindbrain express SHH (A,E), HNF3b (D) and netrin-1 (B) but do not express BMP7 (C,G) or Nkx2.1 (H); floor plate cells of the mesencephalon and caudal diencephalon express SHH (A), netrin-1 (B, F) and also BMP7 (C). Ventral midline cells of the rostral diencephalon (RDVM cells) do not express HNF3b (I) or netrin-1 (B, K) but do express SHH (A,J), BMP7 (C and arrow in L) and Nkx2.1 (M). In addition, SHH but not BMP7 is expressed in cells immediately lateral to RDVM cells. Cells of the incipient telencephalon express none of the markers examined (A-C). The notochord expresses HNF3b (D), SHH (E) and netrin-1 (F). Abbreviations: T, prospective telencephalon; RD, rostral diencephalon; CD, caudal diencephalon; M, mesencephalon; HB, hindbrain. Scale bar: A-C, 162 μm; D-M, 100 μm.
Fig. 2. Fate-mapping the chick neurectodermal midline. (A-C) Positioning prechordal mesoderm. (A) Scanning electron micrograph of stage 6+ chick mesendoderm (i.e. neurectodermal layer is removed). At its most rostral limit, the notochord (nc) forms a knob-like structure that abuts the caudal end of the fan-shaped prechordal mesoderm (pm). The prechordal mesoderm is well delineated laterally from the adjacent prechordal plate (pp) and, although the rostral end of the prechordal mesoderm appears to merge with prechordal plate medially, the rostrolateral edges of the prechordal mesoderm can be distinguished. Brackets in A, B, D show length and position of prechordal mesoderm. Inset: the characteristic shapes of notochord and prechordal mesoderm remain apparent after enzymatic dissection (stage 8 embryo). (B,C) In situ hybridization analyses of stage 6+ chick embryos, showing (B) expression of BMP7 in flat-mounted rostral mesendoderm (ie neurectodermal layer removed), and (C) expression of netrin-1 in isolated axial mesoderm. Notochord cells are netrin-1^-/BMP7^-; prechordal mesoderm cells are netrin-1^+/BMP7+. (D) The fan-shaped prechordal mesoderm can be distinguished in a whole-mounted stage 6+ embryo. (E) Whole-mount view of the rostral region of a stage 6+ embryo, with red dots depicting injection points. (F) An example of a focal injection into the neurectodermal midline. Inset: High power magnification view showing two cells weakly labelling and a medial cluster of approximately 6 cells strongly labelling. (G) Ventral view of flat-mounted isolated stage 11 neurectoderm, analysed for expression of BMP7. Prospective telencephalon (T), rostral diencephalic ventral midline (RDVM), rostrolateral diencephalon (RDL) and rostral floor plate (FP) are shown. (H,I) Flat-mount ventral views of neurectoderm showing examples of stage 11 embryos that had received focal midline injections at stage 6. The embryo shown in H received an injection 370 µm caudal to the anterior neuropore (starred red arrowhead in J). Labelled progeny migrated forwards to populate the RDVM. The embryo shown in I received an injection 130 µm posterior to the anterior neuropore (starred blue arrowhead in J). Labelled progeny migrated forwards, some moving laterally upon reaching the anterior neuropore, populating the prospective telencephalon, including the telencephalic midline (arrow in I; labelled cells are out of focus). In H and I, dotted white line depicts the outline of the neurectoderm. (J) Diagram of the relative positions of neurectodermal midline cells and axial mesoderm at stage 4-11. Rostral is to the right. All positions are shown in µm (see scale bar at bottom). Position 0 µm marks the site of the anterior neuropore (AN). At stage 4 and 5, the entire embryo back to Hensen’s node (HN) is shown. At stage 6-11, the embryos are cut off at 700 µm. Injection sites made within the midline neurectoderm over stages 5-8. Each injection site is represented by an arrowhead coloured to correspond to the final position of the marked cells in the midline. The data shown for stage 4 are taken from the fate map of stage 3-5 chick of Schoenwolf and Sheard (1990) and are shown as open arrowheads. The data obtained here for stage 5 correspond with the observations of Schoenwolf’s group for that stage. The final position occupied by labelled cells at stage 11 was recorded and allocated to one of four domains of the neurectoderm (see cartoon). Thus, injections depicted by blue, red, pink and brown arrowheads fate-mapped to, respectively, the telencephalon (T), the RDVM, the rostrolateral diencephalon (RDL) and the rostral floor plate (FP). The stars above triangles at stage 6 show the examples documented in H and I. The position of the neurectodermal mesoderm at each stage is indicated by the solid black bar, with hatched ends to show the extent the prechordal mesoderm moves at a particular stage. The position of the notochord at each stage is shown as a grey bar. Prospective RDVM cells in area a at stage 4 migrated into a domain spanning approximately 130 µm, at stage 7. Subsequently, RDVM cells coalesced into a region spanning approximately 90 µm at stage 8-11 that co-expressed BMP7, SHH and Nkx2.1. Scale bar: A, 80 µm; B-E, 70 µm; F, 50 µm; G-I, 60 µm.

Rostrolateral diencephalon
migrating above the prechordal mesoderm at stages 5 and 6 disperse rostrally and laterally, giving rise at stages 7 to 11 to telencephalic midline and to some lateral diencephalic cells (Fig. 2I,J). Prospective RDVM cells migrate rostrally over the prechordal mesoderm only between stages 7 and 8 (Fig. 2H,J). Subsequently, the expansion of the neurectodermal and mesendodermal cell layers results in further changes in the relative positions of the two cell layers (Seifert et al., 1993) and, as a consequence, the prechordal mesoderm becomes located at a more caudal position with respect to RDVM cells. By stage 11, the prechordal mesoderm lies adjacent to floor plate cells of the caudal diencephalon. RDVM cells now overlie the prechordal plate mesenchyme (Seifert et al., 1993; Fig. 2J).

This analysis reveals that prospective RDVM cells and the prechordal mesoderm do not migrate in register but exhibit a dynamic spatial relationship between stages 4 and 11. Prospective RDVM cells lie adjacent to prechordal mesoderm only at two stages: stage 4 and stage 7-8.

**RDVM cells first express rostral character at stage 7**

We next examined when the onset of RDVM character occurs in relation to the time of apposition of RDVM cells and prechordal mesoderm.
mesoderm. At stage 4, cells in area a express SHH, but not other midline markers examined (not shown). At stages 5 and 6, when prospective RDVM cells lie adjacent to rostral notochord, they express SHH and HNF3β but not netrin-1 or BMP7 (Figs 3E-L, 8 and not shown) and cannot be distinguished from prospective floor plate cells at these stages (not shown; summarized in Fig. 8). RDVM properties were first observed in ventral midline cells at stage 7. At this stage, HNF3β was downregulated (Fig. 3M,U) and netrin-1 was not expressed (Fig. 3O,W), whereas BMP7 expression was initiated (Figs 3P,X, 8) and SHH was maintained at a high level (Figs 3N,V, 8). By stage 11, Nkx2.1 expression was also detected in RDVM cells (Fig. 1M). The defining features of RDVM cells (the downregulation of HNF3β and the expression of BMP7; Dale et al., 1997) are thus first acquired at stage 7, the time at which prospective RDVM cells re-establish register with prechordal mesoderm.

A discrepancy between the onset of RDVM cell differentiation and the expression of BMP7 and SHH by axial mesoderm

The ability of chick prechordal mesoderm to induce RDVM cells in neural plate explants can be mimicked by the coordinate actions of BMP7 and SHH (Dale et al., 1997). To examine whether the acquisition of RDVM properties simply reflects the onset of BMP7 and SHH co-expression by stage 7 prechordal mesoderm, we analysed the pattern of expression of BMP7 and SHH by axial mesoderm between stages 4 and 11.

SHH was expressed by all axial mesoderm cells throughout stages 4-11 (Figs 3B,F,J,N,R, 8 and not shown), as previously described (Ericson et al., 1995; Levin et al., 1995; Marti et al., 1995; Dale et al., 1997). However, BMP7 was also widely expressed by axial mesodermal cells over an extended period. At stage 4, BMP7 was not expressed by prechordal mesoderm cells that underlie the midline cells in area a that represent the precursors of both the floor plate and the RDVM (not shown). By stage 5+, expression of BMP7 was observed in prechordal mesoderm (Fig. 3D) and expression was maintained until stage 10 (Figs 3P, 8 and not shown). Thus, BMP7 is expressed in prechordal mesoderm cells at the time that they re-establish register with prospective RDVM cells. However, BMP7 is also expressed by the prechordal mesoderm before and after the stages at which it encounters prospective RDVM cells. Furthermore, BMP7 expression was also detected in the rostral notochord from stage 6, a time at which the notochord underlies prospective RDVM cells (Fig. 3L), and was maintained after stage 10 (not shown). These results are inconsistent with a simple model in which the onset of RDVM markers by neural cells is governed by the coincidence of expression of BMP7 and SHH.

RDVM cell differentiation occurs only after exposure to prechordal mesoderm

We have found previously that notochord does not induce

Fig. 4. Prospective RDVM cells are initially specified with caudal fate. Explants of prospective RDVM cells, isolated, cultured for 40 h ours and examined for expression of ventral midline markers. Prospective RDVM explants isolated at stage 5, 6 or 7 express HNF3β, SHH and netrin-1, but do not express BMP7 or Nkx2.1 (A-O). RDVM explants isolated at stage 8 express SHH, BMP7 and Nkx2.1 but do not express HNF3β or netrin-1 (P-T). HNF3β and Nkx2.1 expression were detected by immunohistochemical analysis of sectioned explants. SHH, netrin-1 and BMP7 expression were detected in whole-mount explants using in situ hybridization. Scale bar: A-T, 100 μm.
RDVM cell properties in rat lateral neural plate explants (Dale et al., 1997). Thus, the co-expression of SHH and BMP7 by rostral notochord at the time that it contacts prospective RDVM was unexpected. We therefore re-examined the possibility that rostral notochord does indeed specify RDVM cells within the chick ventral midline but that the expression of RDVM properties is delayed, becoming evident only after the neural cells have migrated further rostrally.

RDVM cells are not specified until stage 8

Using the fate map described above, prospective RDVM cells from the neurectoderm of stage 4-8 embryos were cultured as isolated explants for 24 and 40 hours and then examined for the expression of ventral midline markers. Explants of area a, isolated at stage 4 (containing cells that contribute to all levels of the neuraxis, including RDVM cells), did not express midline markers after culture (100% n=6 explants, not shown). Explants of prospective RDVM cells isolated and cultured from stage 5 embryos expressed the ventral midline markers HNF3β and SHH (100%, n=9) and also the floor plate marker, netrin-1 (66%, n=9), but not the rostral markers BMP7 or Nkx2.1 (100%, n=5; Fig. 4A-E). At stage 5, therefore, midline cells that are destined to populate the rostral diencephalon appear to be specified to give rise to the floor plate. Prospective RDVM cells in explants cultured from stage 6 and stage 7 embryos also expressed HNF3β, SHH and netrin-1, but not BMP7 or Nkx2.1 (100%, n=7, 12 explants at stages 6 and 7 respectively; Fig. 4F-O), again suggesting their specification as floor plate. Explants isolated from stage 8 embryos, however, expressed SHH, BMP7 and Nkx2.1 in the absence of HNF3β and netrin-1 (100% n=8; Fig. 4P-T), indicating that at the time they were isolated they were specified as RDVM cells. RDVM cells thus appear to be specified only after stage 7, the time at which they come into register with prechordal mesoderm.

The expression of RDVM properties in vitro was observed only in explants isolated from stage 8 embryos. Thus, the rostral character of stage 7 prospective RDVM cells evident at the time of isolation (see Fig. 3M-P) appears to be lost during the culture period. This suggests that prolonged exposure to inductive signals is required for specification of RDVM cells and that the relevant signals operate before stage 8. To test whether prechordal mesoderm provides signals that promote the stable expression of RDVM properties, explants of stage 7 prechordal mesoderm were cultured with stage 7 prospective RDVM. In contrast to the generation of floor plate-like character in stage 7 explants grown alone (Fig. 4K-O), explants cultured with prechordal mesoderm acquired RDVM properties (100%, n=5; Fig. 5A-C). Thus, at stage 7 prospective RDVM cells can respond to a prechordal mesoderm signal that promotes expression of RDVM properties.

These results show (i) that at the time that prospective RDVM cells come to lie adjacent to prechordal mesoderm (stage 7), a rostralizing signal is still required for their specification, (ii) that the prechordal mesoderm can provide such a signal and (iii) that sustained exposure to prechordal mesoderm is required for the specification of RDVM cells.

Prechordal mesoderm, but not rostral notochord, induces RDVM fate in midline neural cells

The finding that a sustained exposure to axial mesoderm signals is required for the stable expression of RDVM properties leaves open the possibility that RDVM cell differentiation is initiated at stage 6, when prospective RDVM cells first encounter SHH and BMP7 from rostral notochord (Figs 2J, 3J,L). To test this, we compared the differentiation of stage 6 prospective RDVM cells grown together with stage 6 rostral notochord or stage 7 prechordal mesoderm. Stage 6 prospective RDVM cells grown with rostral notochord expressed floor plate markers (Fig. 5D-F), whereas they expressed RDVM properties in the presence of stage 7 prechordal mesoderm (Fig. 5G-I). Thus, prospective RDVM cells isolated from a stage 6 embryo are competent to respond to a rostralizing signal but this signal is not provided by the rostral notochord, despite its co-expression of BMP7 and SHH.

Chordin is expressed in axial mesoderm and midline neurectoderm

The acquisition of RDVM character by midline neural cells in
response to prechordal mesoderm in vitro appears to depend on BMP7. In the presence of anti-BMP7 IgG, prechordal mesoderm induces floor plate, rather than RDVM cells (Dale et al., 1997). This observation suggests a constraint on the potential inductive activity of rostral notochord that is predicted on the basis of BMP7 and SHH expression. One possibility is that endogenous factors that block BMP signalling act to regulate the inductive properties of the axial mesoderm. The BMP-binding proteins follistatin and chordin can block the activity of BMP7 (Liem et al., 1997; Lee et al., 1998) and thus represent candidates for restricting the activity of BMP7 in the axial mesoderm. The late onset of expression of follistatin in rostral axial mesoderm of developing chicks (Patel et al., 1996) suggests that follistatin does not provide the limit to BMP7 activity implicated in RDVM development. We therefore examined the distribution of chordin in the axial mesoderm and neural tube over the period of RDVM cell specification.

From stages 4 to 6, chordin was expressed by all axial mesoderm cells (Figs 6A–L, 8) and by midline neurectodermal cells (Figs 6F–HJL, 8). From stage 6+, the rostral domain of expression of chordin decreased, so that by stage 7 neither prechordal mesoderm nor prospective RDVM cells expressed chordin (Fig. 6M). However, expression was maintained caudally, both in the notochord and overlying neurectoderm (Figs 6N,O, 8). Between stage 8 and stage 9, however, once RDVM cell fate has been specified, expression of chordin in caudal diencephalon and underlying mesoderm was also lost (Figs 6R,V, 8). The pattern of chordin expression is therefore consistent with the possibility that its activity restricts BMP7 signalling by axial mesoderm.

### Chordin limits the spatial and temporal pattern of differentiation of RDVM cells

To test the idea that chordin might interfere with mesodermal induction of RDVM properties, stage 7 prospective RDVM cells, together with underlying prechordal mesoderm, were cultured in the presence of recombinant chick chordin. When cultured with prechordal mesoderm alone, stage 7 prospective RDVM cells expressed RDVM properties (Figs 5A–C, 7A–F; Table 1). In contrast, in the presence of chordin, floor plate cells expressing

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<th>SHH</th>
<th>Netrin-1</th>
<th>BMP7</th>
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<tr>
<td>RDVM-pm alone</td>
<td>100% (n=3)</td>
<td>16%*(n=6)</td>
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<tr>
<td>RDVM-pm + con</td>
<td>100% (n=3)</td>
<td>11%‡(n=9)</td>
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<tr>
<td>RDVM-pm +chordin</td>
<td>100% (n=3)</td>
<td>100% (n=9)</td>
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*1 explant, very weak netrin-1 expression.
‡1 explant, very weak netrin-1 expression.
§1 explant BMP7+.
netrin-1, but not BMP7, were generated instead of cells with RDVM character (Fig. 7G-I; Table 1). Thus, chordin can inhibit the rostralizing action of BMP7 on ventral midline cells. This finding supports a model in which chordin provided by the axial mesoderm limits the rostralizing capacity of axial mesoderm to the prechordal mesoderm at stage 7, thus constraining the timing and position of differentiation of RDVM cells.

**DISCUSSION**

Previous studies have implicated the prechordal mesoderm in forebrain patterning (Adelman, 1922; Mangold, 1933; Rubenstein and Beachy, 1998), yet its precise role remains uncertain. In particular, it has been unclear whether the prechordal mesoderm provides solely ventralizing signals, patterning neural tissue that has already been regionalized along its rostrocaudal axis (Ericson et al., 1995; Shimamura and Rubenstein, 1997; Qiu et al., 1998; Rubenstein and Beachy, 1998), or independent ventralizing and rostralizing signals (Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997). Our experiments suggest that prior to stage 7 the prechordal mesoderm provides only a ventralizing signal, mediated by SHH. Initially, this may be because BMP7 is not expressed and subsequently it may reflect the occlusion of BMP7 activity by chordin. From stage 7, prechordal mesoderm imparts both ventralizing and rostralizing cues to adjacent neurectoderm, possibly as a consequence of the downregulation of chordin.

We discuss these findings with respect to the potential roles of mesodermal sources of BMP7 and chordin in the generation and refinement of midline neural cell identity and pattern along the rostrocaudal axis of the neural tube.

**Ventral midline neural cell differentiation**

Analysis of floor plate function and marker expression have

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**Fig. 7.** Chordin prevents the rostralizing action of prechordal mesoderm. Explants of stage 7 RDVM cells and underlying prechordal mesoderm were cultured for 40 hours alone (A-C), in the presence of control viral supernatant (D-F) or in the presence of chordin-containing viral supernatant (G-I). Explants cultured alone or with control supernatant express SHH and BMP7 but not netrin-1 (A-F). Explants cultured with chordin viral supernatant express SHH and netrin-1 but not BMP7 (G-I). Scale bar: A-I, 80 μm.

**Fig. 8.** Summary of expression of signalling molecules and transcription factors in axial mesoderm and neurectodermal midline cells in stages 4–11 chick embryos in comparison to positions of prospective RDVM and prechordal mesoderm. Within the centre of each panel, a scale bar marks the position, in microns, from the anterior neuropore (AN). Expression of each marker mRNA is depicted in a separate colour (see key). The neurectodermal and mesodermal expression profiles of each marker are shown above and below the scale bar, respectively. Rostral is to the right. The position of the prechordal mesoderm is shown as a black bar with hatched ends to show the extent of prechordal mesoderm movement during a particular stage. The position of prospective and definitive rostral diencephalic ventral midline (RDVM) cells is judged from the fate-mapping analysis (see Fig. 2J) and shown as a red bar.
previously suggested that the differentiation of ventral midline neural cells occurs over a protracted period (Yamada et al., 1991; Placzek et al., 1993; Dale et al., 1997). The work described here supports and extends this idea, identifying the times at which, first, ventral midline character and, subsequently, distinct rostral and caudal properties of RDVM cells and floor plate are specified. In addition, our results suggest that prolonged mesodermal signalling is required for the acquisition of ventral midline neural properties.

The expression of SHH by cells in area a raises the question of when the induction of ventral midline cells is initiated. Many studies indicate that SHH plays an essential role in inducing ventral midline neural expression of HNF3β and SHH itself (Echelard et al., 1993; Roelink et al., 1994; Sasaki and Hogan, 1994; Hynes et al., 1995, 1997; Ruiz i Altaba et al., 1995b; Epstein et al., 1996). Expression of SHH is detected in Hensen’s node from stage 4 (Levin et al., 1995), and in prechordal mesoderm underlying area a cells at stage 4. Hensen’s node or the prechordal mesoderm might therefore initiate SHH expression in area a. However, although ventral midline precursors in area a express SHH, it is downregulated when this region is cultured in isolation and other ventral midline markers are not expressed in area a or in isolated explants. These results suggest that SHH expression in area a cells is unstable and, moreover, that the level of SHH to which area a cells have been exposed is insufficient to specify or consolidate ventral midline cell fate. Both prospective floor plate and prospective RDVM cells are committed to a ventral midline fate only over stage 5 to stage 6, as they migrate in apposition with SHH-expressing notochord. These observations, together with in vitro studies showing that high levels of SHH are required to specify cells to a floor plate fate (Roelink et al., 1995; Ericson et al., 1996), suggest that a prolonged period of SHH signalling operates in vivo to generate ventral midline specification. Between stages 4 and 6, ventral midline cells are continuously exposed to SHH, first from Hensen’s node and prechordal mesoderm and then from notochord. SHH signalling from the prechordal mesoderm at stage 4 may therefore play a part in the gradual specification of cells to a ventral midline fate.

Our results with isolated explants suggest that, prior to stage 7, midline cells that are destined to populate the floor plate and the RDVM are indistinguishable. Both express HNF3β and SHH and both give rise to caudal floor plate (assessed by netrin-1 expression) in the absence of further signalling. However, these midline neural cells can be triggered to an RDVM fate by their exposure to prechordal mesoderm, suggesting that the rostralization of midline cells can occur several hours after they first acquire a ventral midline fate.

**Prechordal mesoderm and RDVM cells in forebrain patterning**

That the acquisition of rostral properties by ventral midline neural cells is critical to the normal differentiation of the forebrain is evident in mice that are mutant for Nkx2.1 expression and lack a hypothalamus (Kimura et al., 1996). The induction of RDVM cell properties may also have other consequences for neural patterning. The axon guidance molecule, netrin-1, which is expressed in the floor plate and, at later stages, in the telencephalic ventral midline (Kennedy et al., 1994), is not expressed by RDVM cells. RDVM cells do not exhibit chemoattractive activity for commissural growth cones (Placzek et al., 1990), a finding that may underlie the absence of a rostral commissure in the ventral CNS. The lack of axons in this region may, in turn, enable the RDVM to continue to signal to adjacent structures. Rathke’s pouch, the precursor of the anterior pituitary, comes to lie adjacent to the developing hypothalamus, and may depend upon signalling from prospective hypothalamus for pituitary patterning (Ericson et al., 1998; Treier et al., 1998; Watkins-Chow and Camper, 1998).

In the embryonic mouse, the establishment of rostral character in the neural plate appears to begin prior to gastrulation, under the influence of signals from the anterior visceral endoderm (Thomas and Beddington, 1996; Beddington and Robertson, 1998; Ruiz i Altaba, 1998). An outstanding question is why midline neural cells might require a distinct rostralizing signal. Our fate mapping experiments address this issue, in part, by demonstrating a relatively late migration of ventral midline cells to their final positions (see also Woo and Fraser, 1995; Heisenberg and Nusslein-Volhard, 1997). Prospective RDVM cells migrating into the diencephalon may not encounter the early endodermal signals that appear to impart rostral character to more lateral regions of the neural plate. It is unclear at present whether a subset of ventrolateral forebrain cells also acquires rostral identity only after neural plate formation. Cells that in chick and rat migrate into lateral diencephalon from area a or from more caudal regions at neural tube stages (Schoenwolf et al., 1990; Morriss-Kay and Tuckett, 1987) may also require rostralization by signals from prechordal mesoderm or RDVM cells.

**BMP7 and chordin delineate the rostrocaudal pattern of ventral midline neural cell differentiation**

The acquisition of rostral diencephalic fate by ventral midline neural cells correlates with the exposure of the neural midline to prechordal mesoderm co-expressing BMP7 and SHH. This supports a model in which the two molecules can act in concert to trigger RDVM induction. In the axial mesoderm, however, the sites of co-expression of BMP7 and SHH are more widespread than would be predicted from the timing and position of differentiation of RDVM cells. The ability to specify RDVM differentiation appears to correlate with the downregulation of chordin in prechordal mesoderm. The results of our in vitro assays suggest that chordin may act to block the capacity of rostral notochord to induce RDVM fate in vivo, thereby limiting the action of BMP7 to stage 7 prechordal mesoderm and, consequently, the induction of RDVM properties to overlying neural cells. Although it is likely that chordin is responsible for regulating prechordal mesoderm activity, we cannot exclude the possibility that chordin also acts directly on neural cells to inhibit the development of RDVM properties in neural explants. The expression of BMP7 itself by RDVM cells may be an essential component of the subsequence development of RDVM properties. The addition of chordin to conjugate explants of stage 7 prechordal mesoderm and RDVM may thus interfere directly with the ability of the neurectodermal cells to differentiate into RDVM.

Our results suggest a model in which BMP7 and chordin act as regulators of rostralizing activity in prechordal mesoderm. The absence of expression of other known BMP family
members in rat and chick prechordal mesoderm (S. Shah and J.
D., unpublished observations; K. Liem and T. Jessell, personal
communication) and the blockade of prechordal mesoderm activity by anti-BMP7 IgG (Dale et al., 1997) suggest that
BMP7 mediates the rostralizing activity of the prechordal
mesoderm. We cannot exclude that, in some vertebrate species,
distinct TGFβ family members exist in prechordal mesoderm
and act in parallel or in conjunction with BMP7 (Lyons et al.,
1995). It is also possible that additional BMP antagonists
contribute to the regulation of BMP activity at the midline. The
expression of noggin, follistatin and follistatin-like gene (flik)
have been observed in the axial mesoderm of the chick (Patel
et al., 1996; Connolly et al., 1997) and the DAN family member,
cerberus is expressed in mouse prechordal mesoderm (Biben,
1998; Shawlot et al., 1998). However, unlike the distribution of
chordin, the patterning of these genes does not mirror the changes in the RDVM-signalling capacity of axial mesoderm.
Nonetheless, follistatin is expressed in rostral notochord (Patel
et al., 1996) and thus could have a role in occluding BMP
signalling from the mesoderm.

Taken together, these results support the idea that, in the
embryo, the ability of axial mesoderm to induce RDVM cells
requires both the co-expression of SHH and BMPs by axial
mesoderm and the absence of chordin signalling in mesoderm or
neurectoderm. These studies suggest that the primary role
of chordin in the midline is to prevent the premature acquisition
of RDVM characteristics by prospective RDVM cells as they
extend and, later, to delineate the boundary between RDVM
and floor plate cells.

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