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Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord

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SUMMARY

In the vertebrate spinal cord, oligodendrocytes originate from a restricted region of the ventral neuroepithelium. This ventral localisation of oligodendrocyte precursors (OLPs) depends on the inductive influence of sonic hedgehog (Shh) secreted by ventral midline cells. We have investigated whether the ventral restriction of OLP specification might also depend on inhibiting signals mediated by bone morphogenetic proteins (BMPs). BMPs invariably and markedly inhibited oligodendrocyte development in ventral neural tissue both in vitro and in vivo. Conversely, in vivo ablation of the dorsal most part of the chick spinal cord or inactivation of BMP signalling using grafts of noggin-producing cells promoted the appearance of neuroepithelial OLPs dorsal to their normal domain of emergence, showing that endogenous BMPs contribute to the inhibition of oligodendrocyte development in the spinal cord. BMPs were able to oppose the Shh-mediated induction of OLPs in spinal cord neuroepithelial explants dissected before oligodendrocyte induction, suggesting that BMPs may repress OLP specification by interfering with Shh signalling in vivo. Strikingly, among the transcription factors involved in OLP specification, BMP treatment strongly inhibited the expression of Olig2 but not of Nkx2.2, suggesting that BMP-mediated inhibition of oligodendrogenesis is controlled through the repression of the former transcription factor. Altogether, our data show that oligodendrogenesis is not only regulated by ventral inductive signals such as Shh, but also by dorsal inhibiting signals including BMP factors. They suggest that the dorsoventral position of OLPs depends on a tightly regulated balance between Shh and BMP activities.

Key words: Oligodendrocytes, BMPs, Shh, Spinal cord, Chick embryo

INTRODUCTION

Oligodendrocytes, the myelinating cells of the central nervous system (CNS), are ubiquitously distributed in the mature nervous system. However, their precursor cells are not produced in the whole neuroepithelium, but originate from restricted domains. In the spinal cord, in particular, culture experiments have shown that the ability to generate oligodendrocytes is limited to the ventral region (Warf et al., 1991; Ono et al., 1995; Timsit et al., 1995; Trousse et al., 1995). Furthermore, early markers for the lineage initially define a small population of oligodendrocyte precursors (OLPs) located in the ventral neuroepithelium, close to the floor plate (Pringle and Richardson, 1993; Yu et al., 1994; Ono et al., 1995; Timsit et al., 1995). From their location of origin, OLPs migrate as committed but still proliferative cells throughout the entire developing spinal cord and differentiate in their final microenvironment (Noll and Miller, 1993; Ono et al., 1995; Timsit et al., 1995). A similar pattern of oligodendrogenesis has also been described in the human neural tube (Hajihosseini et al., 1996), suggesting a conserved origin of the lineage between vertebrate species.

We and others have reported that OLP induction depends on the local influence of the morphogen protein sonic hedgehog (Shh), which is secreted by the notochord and the floor plate (Trousse et al., 1995; Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996) and is also responsible for the genesis of ventral neuronal cell types (Yamada et al., 1991; Yamada et al., 1993). Shh induces oligodendrocytes in vitro and in vivo, in territories that normally do not generate these cells, such as the dorsal part of the spinal cord (Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996) and the dorsal brain (Nery et al., 2001). In fact, Shh is both necessary and sufficient to promote the oligodendrocyte fate in neuroepithelial cells of the ventral spinal cord (Orentas et al., 1999; Soula et al., 2001). In the neural tube, Shh controls the expression and repression of a number of homeodomain transcription factors (including the Nkx, Pax and Dbx families) that subdivide dorsoventrally the spinal cord neuroepithelium (Briscoe and Ericson, 1999). Neuronal subtype specification at each dorsoventral level of the neural tube has been shown to largely depend upon the combination of factors expressed in each domain (Ericson et al., 1997a; Briscoe et al., 2000). Detailed analysis of oligodendrocyte origin has established that in the chick spinal cord, OLPs always lie within the most ventral Nkx2.2-expressing domain of the neuroepithelium, and not in the Pax6-

expressing domain located just dorsally (Soula et al., 2001). In addition, a fraction of OLPs, and later on most oligodendrocytes, express Olig2, a bHLH transcription factor also expressed at earlier stages in motor neurone precursors (Lu et al., 2000; Zhou et al., 2000; Zhou et al., 2001). Loss of function of either factor leads to impairment of oligodendrocyte development (Qi et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002). Furthermore, combined misexpression of Olig2 and Nkx2.2, but not that of either gene alone, promotes oligodendrocyte specification in the spinal cord (Zhou et al., 2001).

The fact that oligodendrocyte development can be induced in the dorsal part of the spinal cord (Trousse et al., 1995; Orentas and Miller, 1996; Poncet et al., 1996; Chandran et al., 1998), indicates that dorsal neural tube cells are competent to adopt an oligodendrocytic fate, even though they normally never differentiate along this lineage. This raises the interesting hypothesis that oligodendrogenesis could be negatively controlled by signals from the dorsal neural tube. The existence of such signals, at least in rodents, has been suggested by Wada and collaborators (Wada et al., 2000) who showed that cultivating dorsal neural tube tissue in close contact with ventral neural tube tissue reduced the number of oligodendrocytes in the ventral region of the neural tube. Bone morphogenetic proteins (BMPs) are among the signals likely to repress oligodendrogenesis. These members of the TGFβ superfamily are expressed at the dorsal aspect of the spinal cord, where they regulate the production of dorsal neural cell types (for a review, see Lee and Jessell, 1999). In addition, BMPs alter the response of ventral neuroepithelial cells to Shh signalling (Liem et al., 2000), indicating that the specification of ventral neural cell types in the early developing spinal cord is under the control of both dorsal and ventral signals. Moreover, BMPs are able to orientate phenotypic choices of neural precursors in vitro (Gross et al., 1996; Shah et al., 1996; Mabie et al., 1997; Mabie et al., 1999). In particular, in rodents, they promote the elaboration of astrocytes while suppressing oligodendrocyte differentiation from brain neural precursors in vitro (Gross et al., 1996; Mabie et al., 1997; Mabie et al., 1999; Zhu et al., 1999a; Zhu et al., 1999b). Finally, BMPs and Shh act in opposite fashion on the production of oligodendrocytes from rat embryonic forebrain derived neurospheres (Zhu et al., 1999b). These data taken as a whole could suggest that the BMPs expressed in the chick dorsal neural tube contributes to the ventral restriction of oligodendrogenesis by repressing the emergence of the lineage in the dorsal neural tube.

We have addressed this issue. First, in vitro and in vivo experiments indicate that BMPs can exert a suppressive effect on oligodendrocyte development and act early in the specification of this lineage. Next, we show that ablation of the chick dorsal neural tube and in vivo inactivation of BMP signalling results in the dorsal expansion of the original foci of OLPs. Furthermore, BMPs were able to oppose the Shh-mediated induction of oligodendrocytes in ventral neuroepithelial explants. Interestingly, BMP4-mediated inhibition of oligodendrogenesis in neural explants was associated with a strong repression of Olig2 expression but not with a simultaneous repression of Nkx2.2 expression. Our data indicate that dorsal factors, including BMP4 and BMP7, regulate oligodendrocyte development in the chick spinal cord by repressing the lineage specification in its intermediate and dorsal regions in vivo.

MATERIALS AND METHODS

Fertilised White Leghorn chicken eggs, obtained from commercial source, were incubated at 38°C until they reached the appropriate stage (Hamburger and Hamilton, 1992).

Cultures of neural tissues and cell lines

Regions of the medial neural plate were isolated from E1.5 chick embryos (stage 10) as previously described (Yamada et al., 1993). Ventral spinal cord explants were dissected from E4-E4.5 embryos (stages 23 to 25), as in Trousse and collaborators (Trousse et al., 1995). Ventral neuroepithelial explants were obtained from E4.5-E5 and E6 spinal cords (stages 25-26 and 29, respectively), as described by Soula et al. (Soula et al., 2001).

In all culture experiments, neural explants were grown on collagen gel-coated 12 mm plastic coverslips placed in 14 mm wells of four-well dishes (Nunc), in DMEM (Gibco, BRL) for ventral spinal cord tissues or in SFRI 4 (SFRI Laboratory) for medial neural plate and ventral neuroepithelial explants, supplemented with 10% or 1% FCS (DAP), respectively. Recombinant human BMP4 protein (R&D Systems) was used at a final concentration of 0.6 nM, except otherwise mentioned. The recombinant N-terminal fragment of the human-Shh protein (Biogen) was used at concentrations of 4 or 12 nM.

For long-term cultures of the embryonic chick spinal cord, we used a flat whole-mount preparation modified from that already described for the mouse embryonic spinal cord (Wada et al., 2000). Briefly, the cervicobrachial spinal cord from E4 chick embryos was dissected free of surrounding tissue, opened along the dorsal midline, and flattened on a nitrocellulose membrane (Sartorius) with the neuroepithelial layer upwards. The explants were then grown as organotypic cultures, e.g. at air-medium interface, for 2 or 8 days in DMEM with 10% FCS (Sigma), with or without 0.6 nM human BMP4 protein. Flat-mount explants were fixed in 4% PFA for 4 hours at 4°C, sectioned on a vibratome and processed for immunohistochemistry.

CHO cells producing *Xenopus* noggin were a gift from Drs Richard Harland and Dale Frank. Q2bn quail fibroblasts expressing human BMP2 and their control counterpart were kindly provided by Dr Delphine Duprez (Lamb et al., 1993; Duprez et al., 1996). Prior to some in vivo grafts, the aggregates were labelled using the fluorescent tracer di-I (Molecular Probes). The biological activity of noggin-expressing aggregates was verified on somite specification (Tonegawa and Takahashi, 1998).

In ovo microsurgery

Removal of the dorsal part of the spinal cord

On E3 embryos, the dorsal most region of the trunk spinal cord was removed along three to six somites using the Gastromaster microsurgery instrument (Xenotek Engineering). At the most, one quarter of the total height of the neural tube and overlying ectoderm were removed. The manipulated spinal cord region (exhibiting spina bifida) was dissected at E7-E7.5, fixed in 3.7% formaldehyde in PBS overnight at 4°C. Control embryos were obtained by making a long anteroposterior slit in the roof plate, leading to a spina bifida.

Grafts of BMP or Noggin-producing cells

BMP2- and Noggin-producing cell aggregates were implanted at E3 at trunk level into a slit made between neural tube and somites in ovo, on a length of one to four somites. In the case of BMP2-producing cells, aggregates were pushed as ventrally as possible, whereas they were kept more dorsally for noggin-expressing aggregates. At E7-E7.5, a large embryonic region surrounding the manipulated area was dissected and fixed in 3.7% formaldehyde in PBS either 4 hours or overnight at 4°C.

Immunohistochemistry

Antibodies

Cells of the oligodendrocyte lineage were identified using O4, OL-1

or AA3 antibodies. The mouse monoclonal O4 antibody recognises sulfatides at the surface of immature and differentiated oligodendrocytes (Sommer and Schachner, 1981; Bansal et al., 1989; Ono et al., 1995), and the so called 'prooligodendroblast antigen', which is expressed before sulfatides by oligodendrocyte progenitors and early differentiating oligodendrocytes (Bansal et al., 1992). Culture supernatant obtained from O4 hybridoma cells, a gift from Dr R. Bansal, was used undiluted. OL-1 is a rat monoclonal antibody also directed against sulfatides at the surface of immature and mature oligodendrocytes (Ghandour and Nussbaum, 1990). It recognises more differentiated oligodendrocytes than does O4 antibody (Giess et al., 1992). AA3, a rat monoclonal antibody directed against the DM20 isoform of the proteolipid protein (PLP/DM20) that is expressed in mature oligodendrocytes (Yamaura et al., 1991), was kindly given by Dr B. Zalc. The anti-Olig2 antiserum (Takebayashi et al., 2000) was a generous gift of Dr H. Takebayashi and was used at 1:2000 dilution. The anti-GLAST antiserum (Shibata et al., 1997) directed against the glutamate-aspartate transporter (GLAST) was a gift from Dr M. Watanabe and was used at 1:500. Astrocytes were evidenced by an antiserum directed against glial fibrillary acidic protein (GFAp; DAKO A/S, Denmark) used at 1:500. All the following monoclonal antibodies (culture supernatants) were obtained from the Developmental Studies Hybridoma Bank. They were used undiluted, except where indicated. Quail Q2bn fibroblasts were recognised using QCPN antibody (from Drs B. M. Carlson and J. A. Carlson) (Selleck and Bronner-Fraser, 1995) unless di-I labelled prior to the grafting procedure. Floor plate cells were visualized by their expression of HNF3β (4C7) (Ericson et al., 1996). Motoneurones were evidenced by their expression of Islet1 (39-4D5, used at 1:5) (Ericson et al., 1992). The ventral-most neuroepithelial cells were identified by their expression of Nkx2.2 (74.5A5) (Ericson et al., 1997b). Shh protein secreted by floor-plate cells was detected using 5E1 at a 1:5 dilution.

Tissue sections

Transverse sections (60-80 μm) from fixed manipulated and control spinal cords or from flat-mount experiments were performed using a vibratome (Leica). O4, anti-Olig2, anti-Nkx2.2, anti-GLAST, anti-Shh, anti-GFAp and AA3 primary antibodies were applied overnight at 4°C. Sections were rinsed and incubated for 30 minutes with biotinylated secondary antibody directed against mouse or rabbit Ig (Amersham, diluted 1:50). Biotinylated antibodies were revealed by 15 minutes incubation with fluorescein isothiocyanate (FITC)-coupled streptavidin (Amersham, diluted 1:50). In order to detect both oligodendrocytic cells and grafted quail fibroblasts, double detection immunohistochemistry was performed sequentially, using first O4 antibody, as above, then, after permeabilisation with Triton X-100 (Sigma, 0.5% in PBS); QCPN antibody which was revealed using the Biotin secondary antibody/rhodamine (TRITC)-conjugated/streptavidin combination. To detect simultaneously Olig2 and Nkx2.2, immunohistochemistry was performed sequentially, using first anti-Olig2 antiserum then biotinylated secondary antibody and fluorescein isothiocyanate (FITC)-coupled streptavidin as described above; this was followed by anti-Nkx2.2 antibody, revealed with goat anti-mouse Alexa-546 antibody (Molecular Probes).

Tissue cultures

Explants were fixed in 3.7% formaldehyde in PBS for 30 minutes and stained with O4, OL-1 or GFAP antibodies, as previously described (Giess et al., 1992). For the detection of intracellular antigens, fixed cultures were permeabilised using Triton-X 100 (0.5% in PBS) and adequate primary antibody was further applied at the appropriate dilution in 0.1% Triton-X 100/PBS and incubated overnight at 4°C. Primary antibodies were revealed with the appropriate FITC-conjugated antibodies as described for tissue sections. Double labelling experiments were performed sequentially as described above.

Sections and cultures were analysed with either a Zeiss LSM-410 or a Leica SP2 confocal microscope, equipped with argon and helium-neon lasers and appropriate filters combinations for fluorescein and rhodamine, respectively. In all cultures, labelled cells were counted and results are expressed as the mean ± s.e.m. of labelled cells per culture or per explant. Astrocytes were counted in at least five separate fields in each culture. For quantification of astrocytes in spinal cord flat mounts, the fibrillar nature of GFAp filaments precluded direct counting of the number of astrocytes. Therefore, astrocyte development was measured on series of confocal slices by quantifying the surface area covered by GFAp immunoreactive material, using Image Tool software, after threshold elimination of background pixels and image binarisation. At least ten sections from three different spinal cord flat mounts were used in control and BMP4-treated explants. Results are expressed as the mean percentage of labelled pixels. Significance of the results was analysed using the Student's *t*-test.

RESULTS

In this study, cells of the oligodendrocyte lineage were identified using, among other markers, the O4 monoclonal antibody, a highly specific marker for this cell type in most vertebrate species (Bansal et al., 1989; Bansal et al., 1992; Ono et al., 1995; Wada et al., 2000). In the embryonic chick spinal cord, initial expression of O4 antigen can be used as a reliable index of OLP determination as it occurs at the time of their specification in the ventral neuroepithelium (Soula et al., 2001). In addition, in the ventral neuroepithelium, O4 antibody and probes for PDGF α receptor, another well established marker of the lineage (Pringle and Richardson, 1993; Yu et al., 1994), define neural precursors located within the exact same domain (Soula et al., 2001).

BMP4 inhibits oligodendrocyte development in ventral explants

We first tested the effect of BMP4 on the medial region of the caudal neural plate isolated from E1.5 embryos (stage 10), which produces oligodendrocytes after a week in culture (Poncet et al., 1996; Pringle et al., 1996). At 0.6 nM, a dose sufficient to dorsalise the medial neural plate tissue as shown by enhancement of Pax3 mRNA expression (data not shown) (Liem et al., 1995), BMP4 led after 7 days in vitro to a drastic decrease in the number of $O4^+$ cells present in the explants (Fig. 1A-C). At 1.4 nM, the highest concentration tested, oligodendrocyte development was almost completely abolished. A similar reduction in the number of oligodendrocytes was also observed in explants cultivated for 11 days and stained with OL-1 monoclonal antibody (Fig. 1D-F) (Ghandour and Nussbaum, 1990), a marker for more mature cells of the oligodendrocyte lineage (Giess et al., 1992). Similarly, BMP4 also led after 2 days in vitro to a sharp decrease in the number of motoneurones, indicated by their expression of Islet 1 (Fig. 1G-I), without affecting the development of floor-plate cells, expressing HNF3β (Fig. 1H). Together, these results indicate that BMP4 treatment prevents ventral neural development, including oligodendrocytes, presumably by dorsalising early tissue.

Oligodendrocyte determination in the chick neural tube does not occur before E5.5 (stage 28) in the cervicobrachial region (Soula et al., 2001), a stage at which BMPs are still expressed in the dorsal spinal cord (Takahashi et al., 1996; Nifuji et

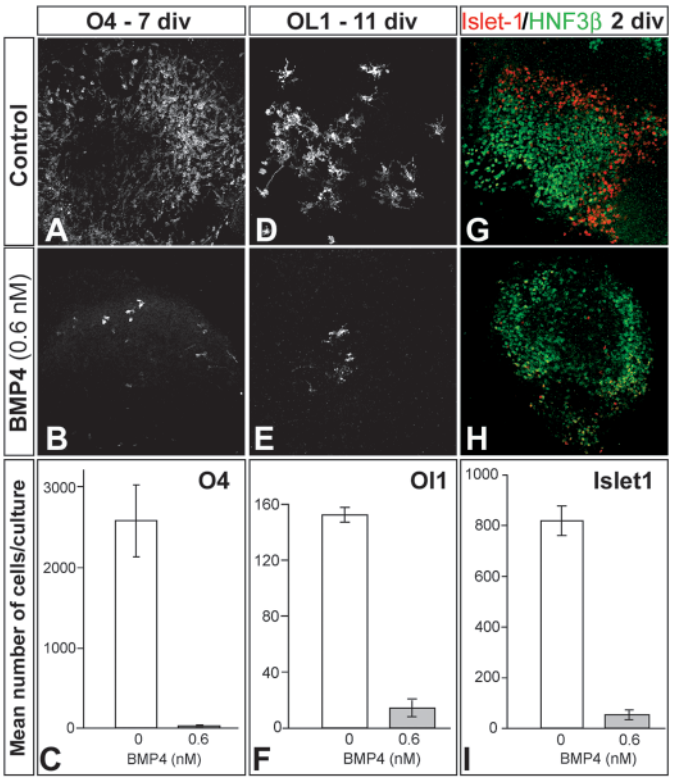

Fig. 1. BMP4 inhibits the development of oligodendrocytes and motor neurones in medial neural plate cultures. Medial neural plate explants were dissected from E1.5 embryos (stage 10), and grown in vitro in the absence (A,D,G) or in the presence (B,E,H) of 0.6 nM BMP4. Oligodendrocytes were stained after 7 days in vitro (div) by O4 antibody (A,B) and after 11 div by OL-1 antibody (D,E), which labels more mature oligodendrocytes. Motoneurones and floor-plate cells were stained after 2 div (G,H) with anti-Islet 1 antibody (red) and by anti-HNF3β antibody (green). Note that BMP treatment strongly inhibited the development of oligodendrocytes and motoneurones but had no effect on the development of floor plate cells. (C,F,I) The mean number of cells of the oligodendrocyte lineage (C-F) and of motoneurones (I) was determined in control and treated conditions. Scale bars represent the mean±s.e.m. of three to five independent experiments.

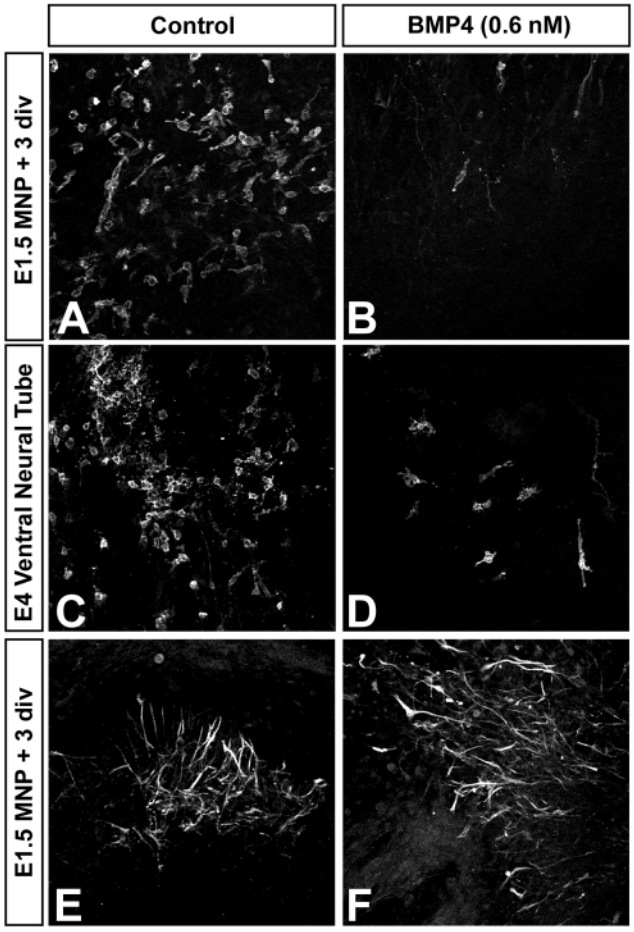

Fig. 2. BMP4 inhibits the development of oligodendrocytes in cultures of ventral neural tube explants, with no obvious stimulation of astrocyte differentiation. E1.5 medial neural plate explants (MNPs; A,B,E,F), initially grown for 3 days (final age 4.5 days), and ventral neural tube explants (VNT; C,D) dissected at E4-4.5 were left untreated (A,C,E) or treated (B,D,F) with 0.6 nM BMP4. After 4 and 7 days of treatment of MNP and VNT explants, respectively, cultures were stained with O4 antibody (A-D) or GFAp antibody (E,F). Note the drastic reduction in the number of O4+ cells (quantified in Table 1) in BMP treated explants (B,D). By contrast, the density of GFAp-positive cells is not increased upon BMP treatment (E,F); see text for quantification.

al., 1997). Therefore, we investigated BMP effect on neuroepithelial precursors just before their commitment towards the oligodendrocyte lineage. BMP4 treatment (0.6 nM) was applied to two different types of 'late' ventral explants: medial neural plate explants dissected at E1.5 and allowed to develop without the protein for 3 days (final age 4.5 days) or ventral neural tube explants directly dissected at E4-4.5. Medial neural plate explants were cultivated in the presence of BMP4 for 4 days, to reach 7 days in vitro, as in the previous experiments, and ventral neural tube explants were treated for 7 days.

In both types of explants, BMP4 treatment greatly reduced the number of oligodendrocytes. The most important decrease was observed for medial neural plate tissue matured in vitro, in which the mean number of $O4^+$ cells per treated culture was lowered to about 5% of controls (n=3) (Fig. 2A-C; Table 1). In ventral neural tube explants, the mean number of remaining oligodendroglial cells represented about 30% of controls (n=4) (Fig. 2D-F; Table 1). In those experiments, motoneurone development was not affected (data not shown), as expected, because most somatic motoneurones have already been produced by the time of treatment of the ventral spinal cord (Hollyday and Hamburger, 1977).

It has been shown that in certain culture conditions, BMPs can promote the differentiation of OLPs into type 2 astrocytes (Mabie et al., 1997; Gregori et al., 2002). Therefore, BMP treatment may not directly affect oligodendrocyte precursor specification but may convert already specified OLPs to an astrocyte fate. To address this possibility, we first monitored expression of the specific astroglial marker GFAp in matured medial neural plate explants treated with 0.6 nM BMP4 for 10 days in vitro (Fig. 2E,F). No difference in the number of GFAp stained cells was noticed between control cultures (27±3

Table 1. BMPs inhibit oligodendrocyte development in ventral neural tube explants

	Number of O4$^+$ cells per culture*			
	Treatment		Co-cultures with Q2bn cells	
Developmental stage	No BMP4	With BMP4	Control cells	BMP2-producing cells
E1.5 MNP plus 3 days in vitro†	120±40 (*n*=3)	6±4 (*n*=3)	777±313 (*n*=5)	7±5 (*n*=5)
E4.5 VNT§	656±128 (*n*=4)	199±66 (*n*=4)	910±319 (*n*=4)	181±87 (*n*=4)

*Mean±s.e.m.
†MNP, medial neural plate cultures (four to eight explants per culture).
§VNT: ventral neural tube cultures (40 explants per culture).
n, number of independent culture experiments.
E1.5 medial neural plate explants aged for 3 days in vitro or E4.5 ventral neural tube explants were cultivated in the absence or presence of human recombinant BMP4 protein (0.6 nM), or co-cultured with Q2bn control cells or Q2bn-BMP2-producing cells. Medial neural plate explants were grown for 4 days and ventral neural tube explants were grown for 7 days. Fixed cultures were then stained with O4 antibody and developing oligodendrocytes were counted.

cells per field) and BMP4-treated cultures (26±7 cells per field).

BMP2 inhibits oligodendrocyte development in the spinal cord in vivo

To gain further insights into the mode of action of BMPs, we next asked whether BMP proteins could also affect oligodendrogenesis in the spinal cord in vivo. For these experiments, we used the Q2bn quail fibroblast cell line, engineered to overexpress human BMP2 (Duprez et al., 1996), a protein very closely related to BMP4 (Zimmerman et al., 1996). Q2bn-BMP2 cells also drastically inhibited oligodendrocyte development in co-culture experiments with 'late' ventral explants, while control Q2bn cells, containing the human BMP2 coding sequence in antisense orientation, produced no effect (Table 1).

Aggregates of BMP2-producing cells or their control counterpart, were grafted in ovo along the trunk neural tube of E3 embryos, the latest stage at which this microsurgery can be performed with acceptable survival rates. They were introduced in a slit between the neural tube and the somites and pushed ventrally. Grafted embryos were fixed at E7-7.5 and sections were stained with O4 antibody. Grafted quail cells were localised either using QCPN antibody or by DiI fluorescence used to label Q2bn cells prior to the grafts.

Sections rostral and/or caudal to the grafted region displayed the well-established distribution pattern of O4-positive OLPs (Ono et al., 1995; Poncet et al., 1996; Soula et al., 2001): bilateral foci of O4-positive precursor cells were present in the ventral part of the neuroepithelium, close to the floor plate (Fig. 3A). At this stage, numerous O4-positive differentiating oligodendrocytes were located in the ventral and lateral marginal zones of the neural tube. Similarly, in embryos grafted with control Q2bn cells, no modification in this pattern of oligodendrocyte development was observed (Fig. 3C), despite the presence of a large mass of Q2bn cells flanking the lateral part of the spinal cord (Fig. 3B).

Strikingly, at the level of grafted Q2bn-BMP2 cells, the development of O4-positive cells was strongly inhibited (Fig. 3D,F). In six grafted embryos (*n*=10), O4-positive cell bodies were entirely absent from the sections. They were seen neither in the mantle and marginal layers, nor in the neuroepithelium, on both sides of the spinal cord (Fig. 3D). In the remaining four embryos, O4-positive cells were absent from the neuroepithelial, mantle and marginal layers of the spinal cord in the grafted side but were present in the contralateral side in all three layers, indicating a more limited effect, probably related to the size of the graft (Fig. 3F).

One of the possible mechanisms to explain the lack of oligodendrocyte development is that BMPs could repress Shh expression, as shown in early hindbrain development (Arkell and Beddington, 1997). To address this point, Shh protein expression was examined in grafted spinal cords using 5E1 antibody. As shown in Fig. 3H,I, Shh immunoreactivity was not modified in sections at the level of the graft, compared with that found in control sections (Fig. 3G). Therefore a mechanism in which BMPs repress Shh production is unlikely to occur in the spinal cord at these stages.

BMPs do not apparently modify development of the astroglial lineage

We next studied the consequences of BMP misexpression in vivo on the development of the astroglial lineage, by analysing the expression of two markers of this lineage: the glutamate-aspartate transporter (GLAST) and GFAp. GLAST is expressed early during spinal cord development, specifically labelling radial glial cells and, later on, astrocytes (Shibata et al., 1997; Hartfuss et al., 2001). In E7 control spinal cords, GLAST was expressed at the surface of neuroepithelial cell bodies and of their processes extending radially within the mantle layer (Fig. 3J). Although expression extended all along the dorsoventral axis of the neuroepithelium, it was particularly prominent in the medial region, and less pronounced in basal and alar regions, as previously described (Shibata et al., 1997; Hartfuss et al., 2001). In sections made at the level of the graft, which showed total disappearance of O4 expression in neuroepithelial cells and almost complete lack of oligodendrocyte development in the marginal layer (Fig. 3K), GLAST expression remain unchanged (Fig. 3L). In particular, there was no reinforcement of GLAST immunoreactivity in the region located just above the floor plate, and from which OLPs normally emerge.

To strengthen these data, we then used GFAp, the marker of mature astrocytes. For technical reasons (low survival rate of operated embryos at the late stage of GFAp expression, E12-E13 in the chick spinal cord), we turned to an ex vivo procedure and used the flat whole-mount preparation protocol already described for the mouse embryonic spinal cord (Wada et al., 2000). In this paradigm, normal development of spinal structures can be observed, and glial cells develop on schedule compared with in vivo development (E. A. and P. C.,

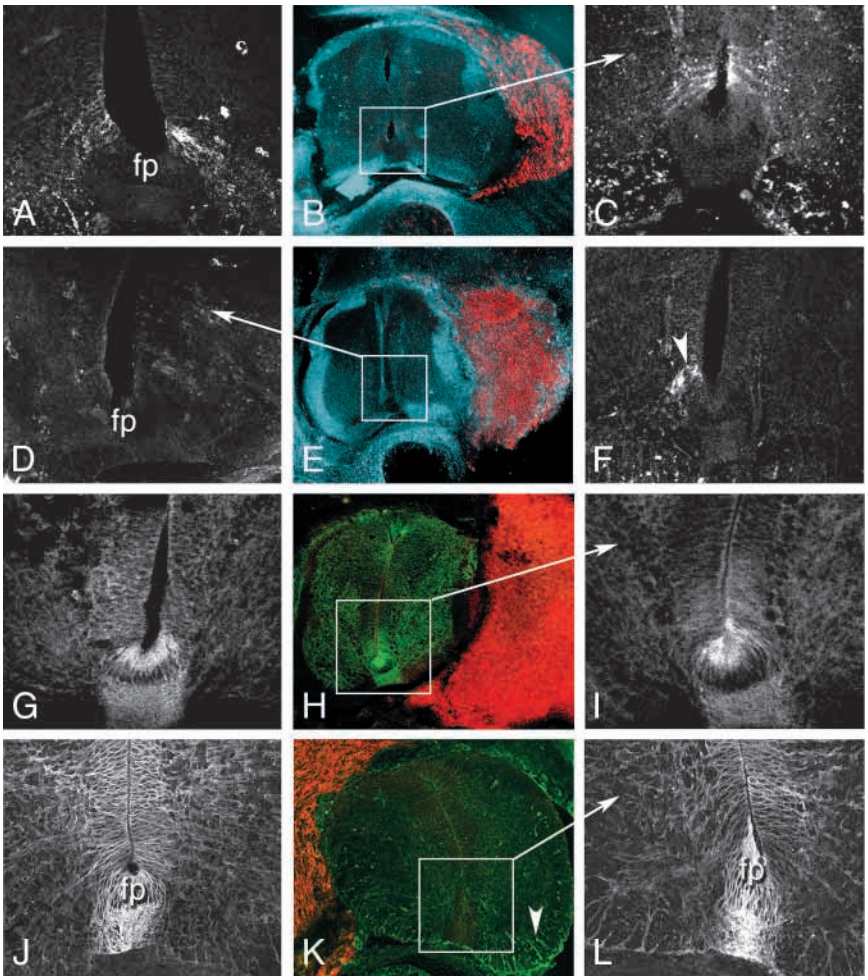

Fig. 3. Grafts of BMP2-producing cells obliterate the development of oligodendrocytes in the spinal cord in vivo. Such grafts, however, neither block SHH production nor affect the development of cells of the astrocyte lineage. Pellets of BMP2-overexpressing Q2bn cells or control cells were implanted at E3-E3.5 between neural tube and somites and embryos were fixed at E7-7.5. (A) Section outside the manipulated region in an embryo grafted with BMP2-producing cells. Ventricular O4-positive cells are found in normal position within the ventral ventricular zone, close to the floor plate (fp). (B,C) Embryo grafted with control-Q2bn cells. (B) Dark field/fluorescence view showing the position of DiI-labelled grafted cells (red). (C) Confocal image of the field framed in B, showing O4 immunostaining. Note the normal development of O4-positive cells in the ventral ventricular zone. (D-F) Sections in embryos grafted with BMP2-producing cells. (D) At the level of the graft, shown in red in E, O4-positive cells are totally absent. In particular, they are not found in the ventricular region above the floor plate (fp). (F) Section at the level of the graft in another embryo. There are no O4-positive neuroepithelial cells on the grafted side (right hand side of the micrograph), but some are discernible on the contralateral side (arrowhead). (G-I) Sections stained with anti-Shh antibody. (G) Control section. Floor-plate cells display Shh immunoreactivity. (H,I) Section in an embryo grafted with BMP2-producing cells. (H) Low-power view showing the large mass of grafted cells (red) flanking the lateral wall of the neural tube. (I) Enlargement of the area boxed in H. Distribution and intensity of Shh immunoreactivity in the floor plate appear comparable with those seen in control sections. (J) Control section stained with anti-GLAST antibody. GLAST is expressed at the plasma membrane of neuroepithelial cells and floor plate cells (fp). Dispersed staining is also found in radial fibres and dispersed cells within the mantle layer. (K) Section in an embryo grafted with BMP2-producing cells, stained with O4 antibody. Grafted cells appear in red on the left-hand side of the micrograph. Note that the ventricular region lacks O4-positive cells. A few are discernible in the marginal layer on the contralateral side. (L) Enlargement of the area boxed in K. The section has been counterstained with anti-GLAST antibody. Staining is comparable with that seen in control sections, both in the neuroepithelium and in the floor plate (fp).

unpublished). These preparations were treated with 0.6 nM BMP4 and examined after the appropriate time in culture for the expression of various markers.

After 2 days in vitro, normal domains of O4 progenitors were observed in the neuroepithelium flanking the floor plate (Fig. 4A). BMP4 treatment resulted in total disappearance of this marker (Fig. 4B), in agreement with the above in vitro and in vivo results. After 7 days in vitro, GFAp-stained astrocytes developed in both control (Fig. 4C) and treated (Fig. 4D) conditions. To quantify astrocyte development, confocal sections were subjected to area measurements (see Materials and Methods). GFAp quantification showed no significant changes in the surface area covered by astrocytes and their processes between control and treated spinal cords (Fig. 4E), although successful inhibition of oligodendrocyte development was confirmed at this stage using antibody directed against the specific myelin protein PLP/DM20 on parallel sections (compare Fig. 4F,G).

Taken together, these data indicate that BMP4 can suppress oligodendrocyte formation from precursors at the time they get committed towards the oligodendrocyte lineage, without apparently modifying radial glial and astroglial differentiation. The absence of O4-expressing cells within the neuroepithelium itself strongly suggests that BMPs impede oligodendrocyte development by preventing the specification of OLPs.

Dorsal signals inhibits development of OLPs in the intermediate and dorsal regions of the neuroepithelium in vivo

From the above results, it could be inferred that endogenous dorsal signals, including BMPs, might repress dorsally OLP specification, thus contributing to the ventral restriction of oligodendrogenesis. To address this possibility, we first sought to document the consequences of the removal of dorsal signals through the ablation of dorsal spinal cord tissue. The presence in the dorsal spinal cord of signals inhibiting oligodendrocyte development has already been demonstrated (Wada et al., 2000). However, these experiments were performed on mouse spinal cords explanted in vitro and did not establish whether

such signals could affect the number and dorsoventral localisation of early precursors still located in the neuroepithelium. We performed the surgical ablation in ovo of the dorsal part of the E3 (stage 19-20) trunk neural tube and overlying ectoderm. At the most, we removed one quarter of the total height of the neural tube. As above, operated embryos were analysed at E7-7.5 (stages 31-32).

Transverse sections located rostral and caudal to the operated region displayed the normal pattern of O4-positive cell distribution. By contrast, in all operated embryos (n=5), the foci of OLPs were expanded dorsally within the ventral half of the neuroepithelium compared with control spinal cord sections (Fig. 5B,C). More strikingly, dispersed O4-positive cells appeared at distance from these foci along the neuroepithelium (Fig. 5C,D,F). In some cases, they were found in the remaining dorsal region of the neuroepithelium (Fig. 5E), i.e. dorsal to the sulcus limitans. Some of those precursor cells were accompanied by O4-positive cells migrating in the mantle region of the spinal cord, suggesting local production of oligodendrocytes from ectopic precursors. In most cases, we also noted an increase in the density of O4-positive cells in the marginal zone compared with control sections, suggesting stimulated production of oligodendrocytes, as already described (Wada et al., 2000).

As excision of dorsal tissue invariably led to the formation of a spina bifida, as a further control, O4 expression was analysed in spinal cords with surgically induced spina bifida. In such spinal cords (Fig. 5A), the pattern of oligodendrogenesis was identical to that found in unoperated spinal cords, indicating that formation of a spina bifida without excision of dorsal tissue was without effect on oligodendrogenesis.

These data confirm that dorsally secreted factors inhibit OLP development (Wada et al., 2000) and further indicate that the lack of such factors not only leads to an enlargement of the domain of origin of OLPs, but also to the recruitment of OLPs at distance from this domain.

BMP inactivation in the spinal cord promotes the development of OLPs in the neuroepithelium in vivo

To assess whether BMPs are among the dorsal signals inhibiting OLP development, BMP signalling was specifically blocked by grafts of noggin-producing cells. The only known function of noggin is to sequester several members of the BMP family, thus preventing their binding to the corresponding receptors (Zimmerman et al., 1996). Aggregates of CHO cells that were stably transfected to express noggin (Lamb et al., 1993) were DiI-labelled and grafted in ovo, alongside the E3 trunk neural tube, as performed with BMP2-producing cells, except that cell aggregates were kept as dorsal as possible.

In seven out of 11 embryos examined, grafts of noggin-producing cells resulted in dorsal expansion of the domain of oligodendrocyte emergence on the ipsilateral side of the graft (brackets in Fig. 6B,D). In most cases, the dorsoventral extent of the domain of origin of OLPs roughly doubled that of the original domain. When the dorsal expansion of O4-positive foci was particularly important (four out of seven), the effect was associated with the appearance of individual O4-positive neuroepithelial precursors located several cell diameters dorsal to the enlarged site of OLPs (star in Fig. 6D). In all cases, however, these ectopic precursors were found ventral to the sulcus limitans. The four other embryos displayed a normal pattern of oligodendrogenesis. This could likely be due to the fact that CHO cells rarely persisted as a compact clump of cells, as in the case of quail fibroblasts aggregates, but dispersed in the mesenchyme surrounding the spinal cord (Fig. 6C), probably limiting the concentration of noggin protein available at the level of the dorsal spinal cord.

These results show that noggin expression along the spinal cord is sufficient to expand the domain of OLPs, indicating that endogenous BMPs inhibit the development of OLPs dorsal to their normal domain of origin.

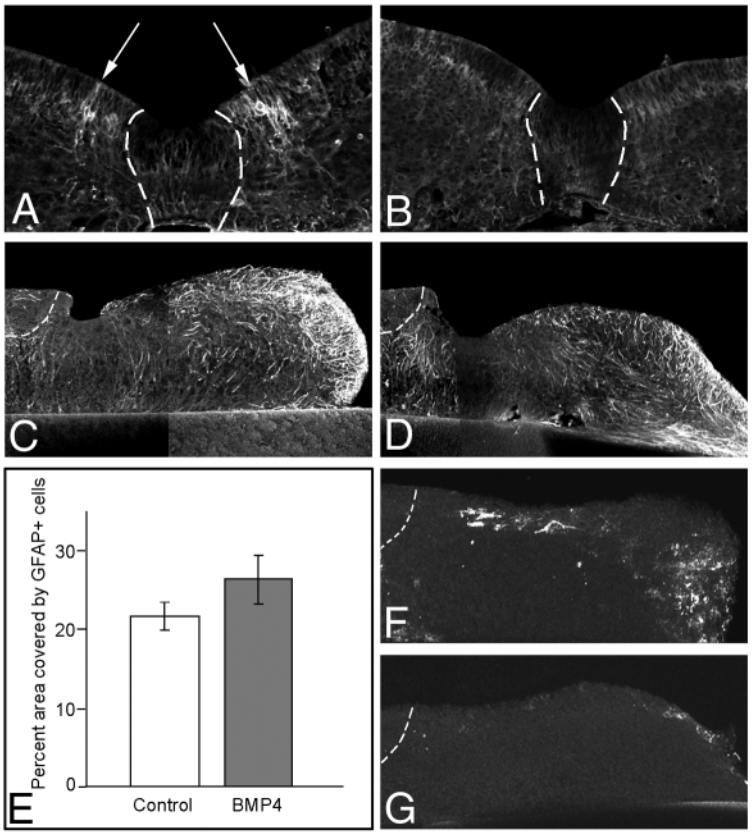

Fig. 4. BMP4 inhibits oligodendrocyte development without significantly affecting astrocyte development. E4 spinal cord flat mounts were organ cultured in control conditions (A,C,F) or treated (B,D,G) with 0.6 nM BMP4. In all micrographs, broken lines delineate the floor plate area.
(A,B) Transverse sections from spinal cords grown for 2 days, stained with O4 antibody. In control spinal cords, oligodendrocyte precursors are found in the ventral neuroepithelium, close to the floor plate (arrows in A). BMP4 treatment alone totally abolishes the emergence of O4-positive cells (B).
(C-G) Transverse sections from spinal cords grown for 8 days and stained with antibodies directed against GFAp (C,D) or PLP/DM20 (F,G). In control sections, numerous astrocytes (C) and oligodendrocytes (F) are observed, with higher density at the lateral edge of the flat mount. BMP4 treatment alone abolishes oligodendrocyte development (G) but does not appear to modify the pattern of expression of GFAp (D). (E) Quantification of the area covered by GFAp immunoreactive material in control or BMP4 treated spinal cord flat mounts, expressed in percent of total section surface.

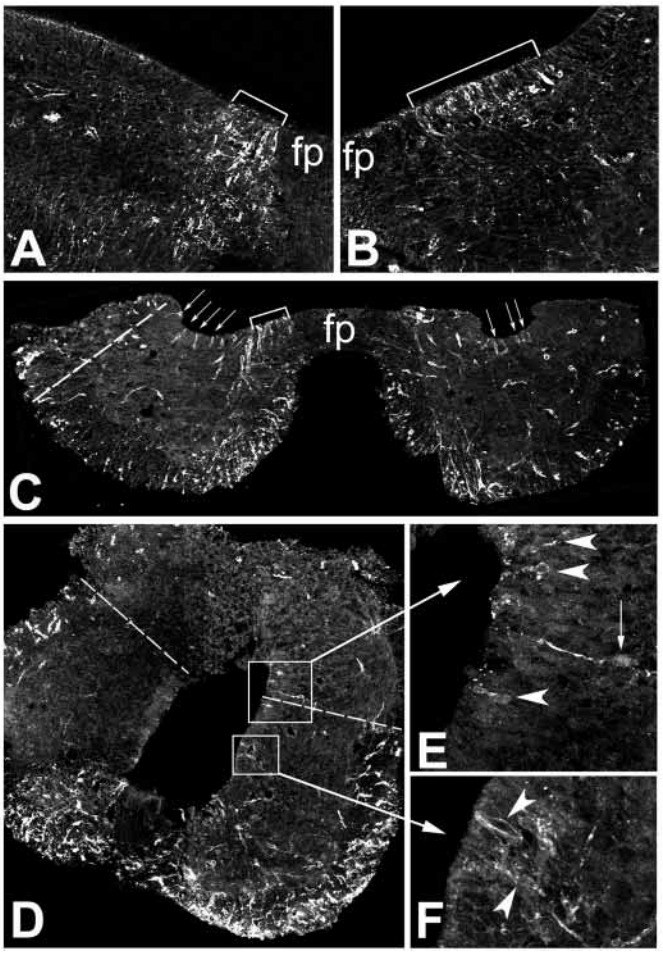

Fig. 5. OLPs arise from the intermediate and dorsal regions of the neuroepithelium after dorsal spinal cord ablation. The dorsalmost region of the spinal cord in E3 embryos was excised in ovo or, in control experiments, was simply opened without removing dorsal tissue. In both cases, this led to the formation of a spina bifida. Operated embryos were fixed at E7-7.5. Transverse sections were stained with O4 antibody. (A) Left hemisection from a control spinal bifida embryo (dorsal is towards the top). The spinal cord is widely opened and the ventricular surface is exposed dorsally. O4 antibody delineates a ventricular focus of OLPs of normal extension (bracket), close to the floor plate (fp). (B) Right hemisection from a dorsal spinal cord excision. The ventricular focus of O4-positive cells (bracket) is enlarged compared with that found in the control embryo. (C) Section in another embryo with dorsal spinal cord excision. The position of the sulcus limitans is denoted by the broken line on the left side. Ectopic O4-positive cells (arrows) are found away from the original focus of oligodendrogenesis (bracket), evenly spaced along the neuroepithelium up to the sulcus limitans. (D-F) Section in an embryo with dorsal spinal cord excision, at a level where the spinal cord remained closed. (D) The broken lines indicate the level of the sulcus limitans. Note the important development of O4-positive cells in the marginal zone. (E,F) Enlargements of the areas boxed in D. Ectopic O4-positive cells (arrowheads) develop dorsally to the sulcus limitans (E) and in the intermediate neuroepithelium (F). Note one O4-positive cell that seems to have migrated away from the dorsal neuroepithelium (arrow in E).

BMP4 antagonises the Shh-mediated induction of oligodendrocytes in vitro

Shh signalling is required in oligodendrocyte specification (Orentas et al., 1999; Soula et al., 2001). BMP-mediated inhibition of oligodendrogenesis could reflect an alteration of the response of ventral neuroepithelial cells to Shh at the time of OLP specification. To test this possibility, we analysed the effect of BMP4 on ventral neuroepithelial cells stimulated by Shh to produce oligodendrocytes. Explants of E5 ventral neuroepithelium, a stage which just precedes oligodendrocyte determination (Soula et al., 2001), were grown for 48 hours in 4 and 12 nM of Shh-N, with or without added BMP4 (0.6 nM). Oligodendrocyte differentiation was monitored after 2 days by expression of O4 antigen and after 7 days by expression of PLP/DM20.

As previously described (Soula et al., 2001), E5 ventral neuroepithelial explants grown alone did not produce significant numbers of O4-positive (Fig. 7A,D) or PLP/DM20-positive (Fig. 7E) cells. By contrast, upon Shh treatment, they reliably produced oligodendrocytes expressing these markers (Fig. 7B,F), in a dose-dependent manner (Fig. 7D) (Soula et al., 2001), 12 nM being the optimal concentration in our system (Fig. 7B,D). Strikingly, when cultures were simultaneously exposed to Shh and to BMP4, oligodendrocytes totally failed to develop, irrespective of the concentration of Shh used (4 or 12 nM) (Fig. 7C,D,G). This establishes that BMP4 can antagonise the Shh-mediated induction of OLPs in the chick E5 ventral neuroepithelium.

We then sought to determine BMP effect on similar neuroepithelial explants isolated at E6, i.e. soon after initial OLP induction. As previously described (Soula et al., 2001), after 48 hours, most E6 ventral neuroepithelial explants cultivated alone (88%, n=17), produced numerous O4-positive cells (Fig. 8A,C). BMP4 treatment (0.6 nM) reduced both the number of positive explants (58%, n=19) and the mean number of O4-positive cells per explant (Fig. 8B,C), but this treatment failed to totally block oligodendrocyte production.

Taken together, our data indicate that BMP4 completely blocks the specification of ventral neuroepithelial cells by Shh, but does not change the fate of already committed precursors, confirming that this protein mainly acts at the early steps of the specification of the lineage.

BMP4 represses Olig2 but does not affect NKX2.2 expression

To obtain insights into the mechanism of action of BMPs, we next analysed whether BMP-mediated inhibition of oligodendrocyte development could be related to modifications of Nkx2.2 or Olig2 expression, transcription factors that have been shown essential for oligodendrocyte development (Qi et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002; Takebayashi et al., 2002). Ventral neuroepithelial explants removed at E5 were grown as above in the combined presence of Shh and BMP4 and stained for Nkx2.2 and Olig2. In parallel cultures, O4 expression was also monitored to control oligodendrocyte development (not shown).

In control conditions, explants contained separate populations of Olig2- and Nkx2.2-labelled nuclei with a small number of double-labelled cells (Fig. 9A), perhaps corresponding to the few O4-positive cells that sometimes also develop in control cultures. Treatment with Shh-N (12 nM),

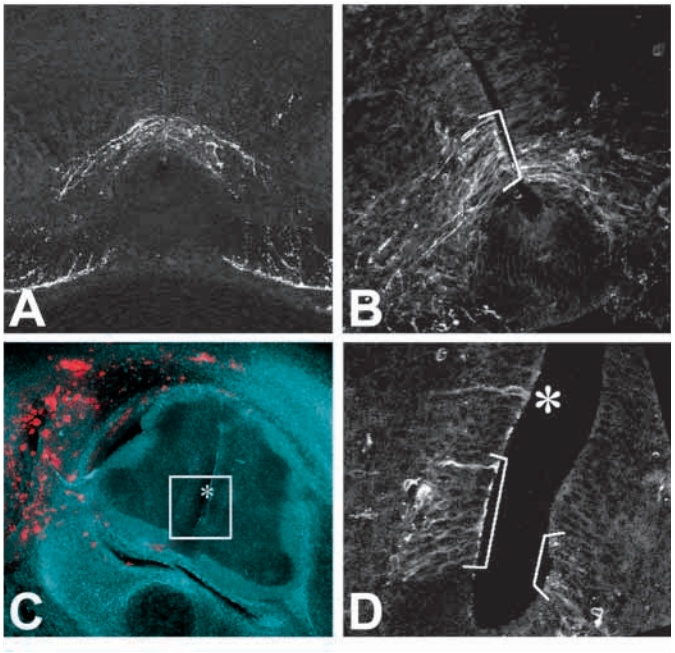

Fig. 6. Grafts of noggin-producing cells cause ectopic OLPs development in the neuroepithelium. Pellets of noggin-overexpressing CHO cells were implanted at E3 between neural tube and somites. Embryos were fixed at E7-7.5 and transverse sections were stained with O4 antibody. (A) Control section showing the normal appearance of O4-positive OLPs in the ventral ventricular zone. (B) Section at the level of the graft in an operated embryo. Note the dorsal enlargement of the O4-positive ventral ventricular zone on the grafted side (bracket), while the contralateral side displays normal development of O4-positive cells. (C,D) Section at the level of graft in another operated embryo.
(C) Darkfield/fluorescence view. CHO cells (red) are found dispersed all along the neural tube. (D) Confocal image at higher magnification of the area boxed in C. The $O4^+$ ventricular zone is enlarged on the operated side compared with the contralateral side (brackets). Note also the presence of an ectopic neuroepithelial O4-positive cell located dorsal to the normal level of origin of OLPs (asterisks in C and D).

which readily promoted oligodendrocyte development, also strongly induced both transcription factors, most positive nuclei co-expressing Olig2 and Nkx2.2 (Fig. 9C), with a 2.2- to fourfold increase in the average number of Olig2-positive and Nkx2.2-positive nuclei per explant, respectively, compared with control conditions (Fig. 9E). BMP4 treatment alone caused a 12-fold reduction in the number of Olig2-positive cells, without significantly modifying the number of Nkx2.2-positive cells (Fig. 9B,E). Strikingly, co-treatment of ventral neuroepithelial explants with 12 nM of Shh and 0.6 nM of BMP4, which in parallel cultures resulted in complete disappearance of oligodendrocytes, caused almost complete disappearance of Olig2-labelled cells (Fig. 9D), representing a 26-fold reduction compared with Shh treatment alone (Fig. 9E). Noteworthy, Shh-BMP co-treatment still resulted in strong induction of the expression of Nkx2.2 compared with untreated explants (Fig. 9D,E).

To extend these data, we also analysed the expression of Olig2 and Nkx2.2 in embryos grafted with BMP2-overexpressing cells. At E6.5, in control sections, the domain

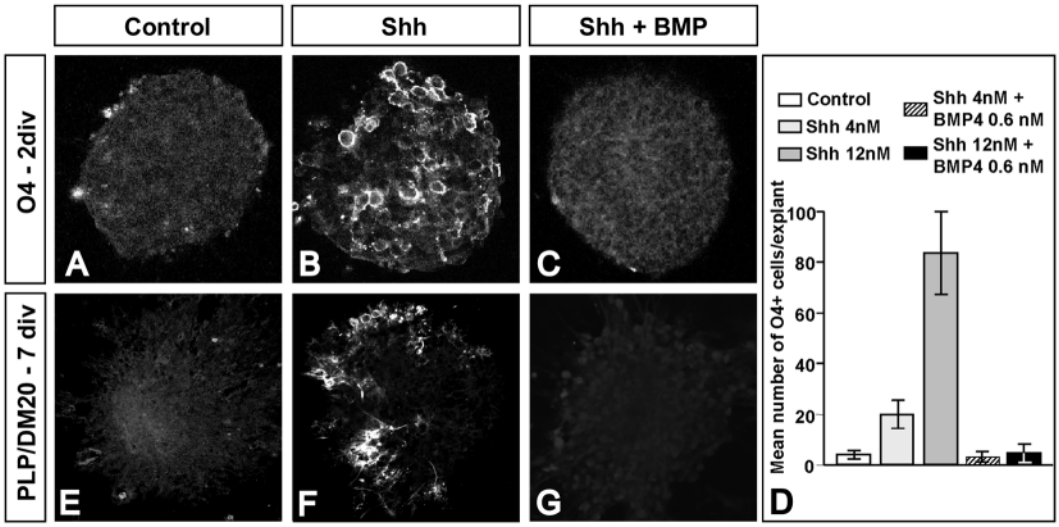

Fig. 7. BMP4 abolishes Shh-mediated induction of oligodendrocytes. Ventral neuroepithelial explants were dissected from E5 embryos, before oligodendrocyte induction has occurred. They were grown for 2 days or 7 days in the absence or presence of Shh, with or without BMP4, and stained with O4 antibody (A-C) or with anti-PLP/DM20 antibody (E-G). (A-C) When isolated at E5, the ventral neuroepithelium is unable to generate oligodendrocytes, as shown by absence of expression of O4 antigen (A). In the presence of 12 nM Shh, such explants generate numerous O4-positive cells within 2 days (B). However, explants treated simultaneously with 12 nM Shh and 0.6 nM BMP4 failed to generate O4-positive cells (C). (D) Quantification of the number of O4-positive cells developing in ventral neuroepithelial explants. Bars represent the mean±s.e.m. of at least 12 separate explants. Oligodendrocyte production is maximal with 12 nM Shh. Note that BMP4 totally blocks the Shh-mediated induction of oligodendrocyte formation, irrespective of Shh concentration. (E-G) Confirming the above results, E5 ventral neuroepithelial explants do not generate PLP/DM20-positive oligodendrocytes after a week in culture (E). Shh treatment induces the development of PLP/DM20-positive cells, which display typical oligodendrocyte morphology (F). Again, SHH/BMP double treatment results in the absence of oligodendrocyte development (G).

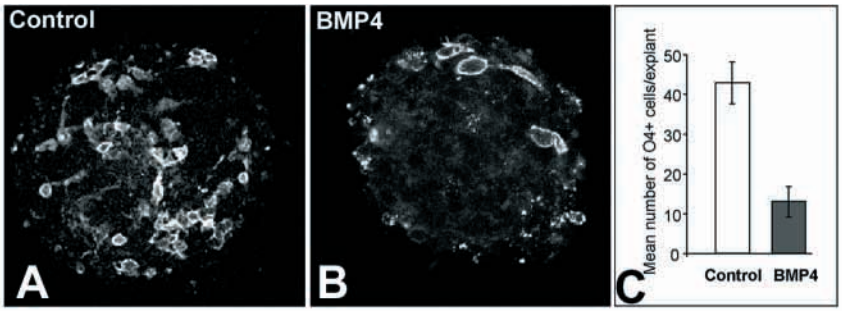

Fig. 8. BMP4 effect on E6 neuroepithelial explants. Ventral neuroepithelial explants were dissected from E6 embryos, i.e. just after oligodendrocyte induction has occurred. They were grown for 2 days in the absence (A) or presence (B) of 0.6 nM BMP4. (A) Control explant. E6 ventral neuroepithelial explants generate numerous O4-positive cells. (B) Upon BMP4 treatment, explants generate reduced numbers of O4-positive cells. (C) Quantification of the number of O4-positive cells developing in ventral neuroepithelial explants. Bars represent the mean±s.e.m. of at least 17 separate explants. At this stage, BMP4 treatment does not totally block O4-positive cell production.

of Nkx2.2 expression was restricted to the most ventral neuroepithelial region, while the domain of Olig2 was located more dorsally in the ventral neuroepithelium (Fig. 9F-H). At this stage, which is posterior to the time of induction of the oligodendrocyte lineage, the domains of expression of these two transcription factors overlap, leading to co-expression in several neuroepithelial nuclei (Fig. 9H). In all grafted embryos examined (n=5), BMP2 cells caused a drastic downregulation in Olig2 mRNA (not shown) and protein (Fig. 9I), while leaving Nkx2.2 expression unaffected (Fig. 9J). In three embryos, Olig2 expression was abolished on both sides of the neuroepithelium. In the two other embryos, Olig2 expression was extinguished on the grafted side but remained observable on the contralateral side (Fig. 9I-K).

Altogether, these results indicate that Olig2 expression is inhibited by BMP4 even in conditions of strong induction mediated by Shh. Thus, BMP4-mediated inhibition of oligodendrocyte development is accompanied by a drastic Olig2 downregulation without concomitant inhibition of Nkx2.2.

DISCUSSION

In the developing spinal cord, OLPs are generated in restricted domains of the ventral neuroepithelium. This ventral origin of OLPs appears related to local inductive signals produced by the notochord and floor plate, including Shh. We show that part of the spatial control of oligodendrogenesis is additionally controlled by dorsal signals of the BMP family.

Inhibition of oligodendrogenesis is mediated by members of the BMP family

Previous work in explant cultures of the mouse spinal cord has indicated that the dorsal spinal cord negatively regulates oligodendrocyte development, by limiting the proliferation of OLPs and inhibiting their maturation (Wada et al., 2000). Our own in vivo experiments confirm and extend this view, as shown by increased density of developing oligodendrocytes in dorsally truncated spinal cords. Increased proliferation of OLPs can also explain our observation of the expansion of the original foci of OLPs but is not likely to account for the appearance of dispersed, isolated OLPs in more dorsal regions of the neuroepithelium, seen in both dorsal ablations and grafts of noggin-producing cells. As migration within the neuroepithelium itself is very limited (Cameron-Curry and Le Douarin, 1995), these precursors probably do not arise from more ventrally located migrating OLPs. Whether they arise through increased survival of local progenitors that otherwise would have died or through the specification of new OLPs remains to be studied in future experiments. In any case, our experiments further indicate that dorsal signals affect not only proliferation and differentiation of OLPs, but also their number and position along the neuroepithelium at the time at which they are specified.

Among factors identified in the dorsal spinal cord (Roelink and Nusse, 1991; Basler et al., 1993; Liem et al., 1997), members of the BMP family were good candidates to influence oligodendrocyte development as they consistently inhibited the generation of oligodendrocytes from various types of neural cell lines (Gross et al., 1996; Mabie et al., 1997; Mabie et al., 1999). Our in vitro and in vivo results indicate for the first time that BMPs are able to suppress the normal process of oligodendrogenesis in the chick embryo. BMP4/BMP2 treatment of ‘late’ ventral neural tissue, just before the determination of oligodendrocyte precursors, led to a drastic reduction in the number of oligodendrocytes showing that neuroepithelial cells are sensitive to BMP effect long after initial ventralisation of the tissue and until determination of the oligodendrocyte lineage.

In principle, BMPs could suppress oligodendrocyte development by shifting the differentiation of newly specified OLPs towards the formation of astrocytes, as shown in various cell lines (Mabie et al., 1997; Gregori et al., 2002). However, several lines of evidence suggest that this is not the case.

(1) The development of early GLAST-positive astroglial precursors and number of GFAp-positive astrocytes did not appear modified in conditions that suppress oligodendrocyte development.

(2) If BMPs were acting after OLP specification, O4-positive cells should still be seen emerging within the ventral neuroepithelium region. In both in vivo BMP grafts and ex vivo BMP4 treatments, neuroepithelial emergence of O4-positive precursors was totally abolished.

(3) BMP treatment specifically inhibited Olig2 expression, a gene required for oligodendrocyte specification, at both mRNA and protein levels

(4) In neuroepithelial explants isolated at E6, after the initial step of oligodendrocyte specification has occurred, BMP treatment did not totally suppress oligodendrocyte development, suggesting that BMPs do not seem able to modify the fate of already committed oligodendrocyte

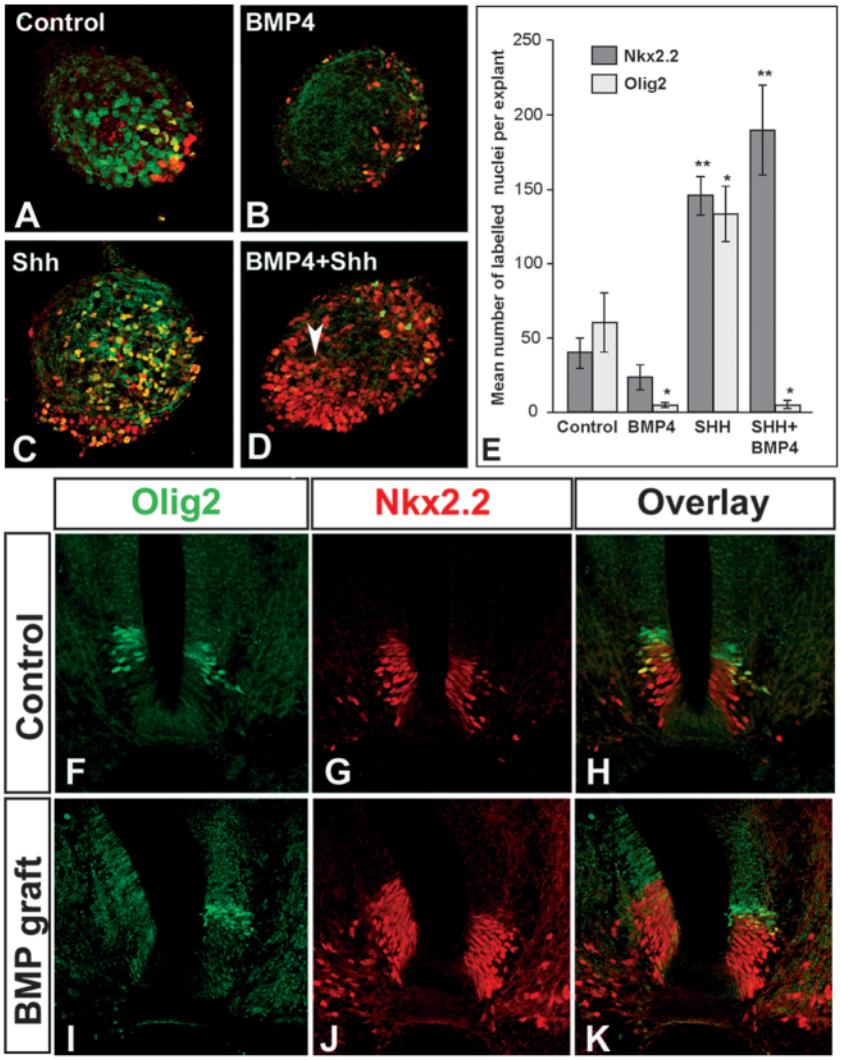

Fig. 9. BMP4-mediated inhibition of oligodendrocyte development is accompanied, in vitro as in vivo, by strong repression of Olig2 but does not depend on inhibition of Nkx2.2 expression. (A-D) Ventral neuroepithelial explants were dissected from E5 embryos, before oligodendrocyte induction has occurred. They were grown for 2 days in the absence or presence of 12 nM Shh, with or without 0.6 nM BMP4, and double-stained with Olig2 antiserum (green) and anti-Nkx2.2 monoclonal antibody (red). (A) Control explant displaying small numbers of Olig2 and Nkx2.2-positive nuclei. Only few nuclei are double-labelled. (B) BMP4 treatment alone inhibits Olig2 expression but does not significantly affect Nkx2.2 expression. (C) Shh treatment, which induces oligodendrocytes, also enhances the expression of Olig2 and Nkx2.2. Note that most nuclei are double labelled. (D) Co-treatment with Shh and BMP4, which abolishes Shh-mediated oligodendrocyte induction, also strongly inhibits Olig2 expression but does not affect Nkx2.2 expression. Note, in particular, Nkx2.2-positive nuclei in neuroepithelial cells forming the side of the explant (arrowhead). (E) Quantification of the number of Nkx2.2-positive and Olig2-positive nuclei per explant (20-33 explants per experiment). Bars represent the mean±s.e.m. of at least three independent experiments. Significance of the differences between treated and control conditions, assessed by Student's *t*-test: *, $P<0.01$; **, $P<0.001$. (F-K) Grafts of BMP2-overexpressing Q2bn cells were implanted at E3-E3.5 between neural tube and somites and embryos were fixed at E7-7.5. Transverse spinal cord sections were double stained with Olig2 antiserum (green) and Nkx2.2 antibody (red). (F-H) Control section. (F) Olig2-positive neuroepithelial cell nuclei are found several cell diameters above the floor plate. (G) Nkx2.2-positive nuclei are located more ventral in the neuroepithelium. (H) The overlay shows co-expression of the transcription factors in some nuclei (yellow), indicating that their respective domains of expression overlap at this stage. (I-K) Section at the level of the graft from an embryo implanted with BMP2-Q2bn cells. (I) Expression of Olig2 is totally abolished on the grafted side. (J) By contrast, Nkx2.2 expression appears not to be affected by the implanted BMP2-producing cells. (H) The overlay shows co-expression of the transcription factors in some nuclei on the contralateral side.

progenitors. Thus, our results strongly argue for a role of BMPs at the step of precursor specification.

Both ectopic expression of BMPs and BMP-inactivation experiments using noggin-expressing cell grafts indicate that endogenous BMPs are responsible, at least in part, for correct spatial distribution of OLPs within the neuroepithelium, in particular by inhibiting oligodendrocyte specification dorsal to their normal domain of emergence. Interestingly, the pattern of ectopic oligodendrocyte emergence in such grafted embryos resembled that of embryos in which the dorsal region of the spinal cord was removed. However, the effect of these grafts was less pronounced than the consequences of the removal of the dorsal part of the neural tube. Several possible explanations could account for this discrepancy. The amount of secreted Noggin reaching dorsal tissue might not be sufficient to block the activity of BMP4 and BMP7 proteins completely. Alternatively, other members of the TGF-β superfamily insensitive to Noggin inhibition could add to the effect of BMP4 and BMP7 on oligodendrogenesis regulation. Cells in the chick dorsal neural tube express additional members of the TGF-β family, including BMP5, DSL1, GDF6/7 and activin B, in nested domains (Liem et al., 1997; Lee et al., 1998), some of which share common effects with BMP4 and BMP7. For example, DSL1, which is not sensitive to noggin inhibition (Liem et al., 1997), mimics the ability of BMP4 to enhance Pax3 expression (Liem et al., 1995), to induce D1 interneurones (Liem et al., 1997), and to suppress the differentiation of motoneurones in intermediate neural plate explants exposed to ventralising signals from the notochord and floor plate (Basler et al., 1993). Thus, it is possible that additional BMPs or BMP-like factors act together with BMP4 and BMP7 in our system. Furthermore, factors of the Wnt family, which are positively controlled by the non neural ectoderm (Dickinson et al., 1995), may also play a role in this process.

Surprisingly, Wada et al. (Wada et al., 2000) failed to observe any effect of BMP4 treatment on oligodendrocyte development from mouse spinal cord cultures, despite the fact

that BMP4 affected the development of astrocytes in the same cultures. This discrepancy may be related to differences in the experimental system used or to the timing of the experiment. In their paradigm, BMP4 was applied at E12, just after O4-positive cells have appeared in the ventral neuroepithelium, which would correspond to E6 in the chick. If BMP4 mainly acts at the determination step, as discussed above, E12 may already be a too late stage for a significant inhibition. Alternatively, although rodent precursors isolated from the brain at later stages of development are sensitive to BMP4 (Gross et al., 1996; Mabie et al., 1999), early mouse spinal cord precursors may not be sensitive to BMP4 but respond to the other BMP-related factors listed above. In any case, at least in the chick embryo, our results allow us to propose that BMPs negatively control oligodendrocyte specification in the developing spinal cord.

BMPs antagonise the Shh oligodendrocyte-inductive effect

Hedgehog signalling appears to be absolutely required for oligodendrocyte specification (Orentas et al., 1999; Soula et al., 2001). This is the case not only for oligodendrocytes from ventral origin, but also for those that develop, in certain conditions, from the dorsal spinal cord (Sussman et al., 2000). Although cultivated CNS tissue from *Shh*$^{-/-}$ embryos can give rise to oligodendrocytes (Nery et al., 2001), other Shh-related molecules such as Indian and Desert Hedgehog might functionally replace Shh in these cultures. Such a possibility is strongly suggested by the results of Tekki-Kessaris et al. (Tekki-Kessaris et al., 2001), showing the role of these various hedgehog-related factors in oligodendrocyte specification in the neocortex.

Our in vivo grafting experiments showing that BMPs were able to prevent OLP emergence from the ventral neuroepithelium, although surrounded by endogenous inductive signals such as Shh produced by the floor plate, suggested that BMP signalling could oppose Shh effects. Our findings that E5 ventral neuroepithelial explants simultaneously exposed to Shh and BMP4 totally failed to generate oligodendrocytes confirm this view and establish that BMP signalling is indeed able to antagonise the Shh induction of OLPs. In a similar way, BMP2 was also able to negate the Shh-mediated stimulation of oligodendrocyte production by mouse forebrain-derived neurospheres (Zhu et al., 1999b). An antagonism between BMP and Shh has recently been proposed in the early neural tube, where BMP signalling has been shown to alter the response of neural cells to Shh, eliciting a ventral-to-dorsal switch in progenitor cell identity and neuronal fate (Liem et al., 2000; Patten and Placzek, 2002). Conversely, blocking BMP signalling led to a dorsal-to-ventral shift in progenitor cell domains and postmitotic neuronal identity (Liem et al., 2000). The present work indicates that BMPs and Shh have opposing activities in glial fate decisions as well and provides evidence that the antagonistic relationship between the two signalling systems persists during extended developmental period, at least up to oligodendrocyte determination (E5.5). Thus, BMP/Shh antagonism appears as a general feature governing neural development in the spinal cord and our data allow to propose that the position at which OLPs will be specified along the dorsoventral axis of the spinal cord depends on a tightly regulated balance between Shh and BMP activities.

The mechanism of BMP/Shh antagonism remains unknown. In the absence of evidence showing direct interaction between Shh and BMPs proteins, two non-mutually exclusive possibilities may be invoked. First, BMPs could repress Shh expression. For example, implanting BMP7-producing cells against the ventral hindbrain greatly reduced Shh expression in the floor plate (Arkell and Beddington, 1997). Such a mechanism has been invoked to explain the surprising finding that oligodendrocytes developed by a Shh-dependent mechanism in long-term cultures of the dorsal spinal cord (Sussman et al., 2000). As dorsal spinal cord tissue does not normally express Shh, local dorsalising signals present in the embryo (such as BMPs) may be lost in culture and dorsal explants may then assume some characteristics of ventral spinal cord such as expression of Shh. Thus, in our experiments of noggin-mediated inactivation of BMP activity, Shh expression may extend further dorsally to promote oligodendrocyte development at more dorsal levels, as recently suggested in the early neural tube (Patten and Placzek, 2002). However, in the converse experiments of grafts of BMP-producing cells, BMP did not seem to reduce the levels of Shh protein. Therefore, it is unlikely that BMPs would act through the regulation of Shh expression.

Alternatively, BMPs could antagonise Shh activity at the cellular level, as shown by the fact that BMP signalling altered neural cell responses to Shh at a step downstream of the availability and presentation of extracellular Shh protein (Liem et al., 2000). This second mechanism appears more likely as, in our own experiments, strong suppression of oligodendrocyte development was observed in ventral neuroepithelial explants despite the presence of high concentrations of Shh.

BMP affects Olig2 but not Nkx2.2 expression

In the chick embryo, O4-positive or PDGF-Rα-positive neuroepithelial OLPs are all included in the Nkx2.2 neuroepithelial domain (Soula et al., 2001) and are partly included in the Olig2 domain (Zhou et al., 2001) (E. A., P. K. and P. C., unpublished). Both of these transcription factors are involved in the development of this lineage. First, inactivation of *Nkx2.2* in mice results in drastic reduction and long delay in oligodendrocyte development (Qi et al., 2001). Second, loss of function of Olig genes leads to a complete loss of oligodendrocytes (Zhou and Anderson, 2002; Lu et al., 2002; Takebayashi et al., 2002). Finally, combined misexpression of these factors, but not of either factor alone, is sufficient to promote the oligodendrocyte fate in the chick spinal cord (Zhou et al., 2001).

In our in vivo experiments, BMP treatment inhibited Olig2 expression in progenitor cells without affecting Nkx2.2 expression, suggesting differential sensitivity to this factor along the neuroepithelium. In E5 ventral neuroepithelial explants, Shh treatment alone promoted strong expression of both transcription factors, in agreement with previous findings classifying them among class II factors (Briscoe and Ericson, 1999; Lu et al., 2000). In these conditions, despite the presence of inducing concentrations of Shh in the medium, BMP4 repressed both Olig2 and oligodendrogenesis, but not Nkx2.2, further substantiating the primordial role of Olig genes in oligodendrocyte formation, demonstrated by results of the loss of function of Olig genes (Zhou and Anderson, 2002; Lu et al., 2002). Such an uncoupling between BMP effect on

oligodendrocyte development and lack of inhibition of the expression of Nkx2.2 shows that BMPs do not antagonise all Shh responses.

These results bring further support to the idea that Olig genes are directly involved in initial specification of oligodendrocyte progenitors (Alberta et al., 2001; Lu et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002), whereas Nkx2.2 is more likely to regulate later stages of oligodendrocyte differentiation and/or maturation (Qi et al., 2001).

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