

# Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons

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## SUMMARY

The neurotransmitter phenotype of sympathetic neurons is specified by interactions with the surrounding embryonic tissues. Adrenergic differentiation is elicited early during development in the vicinity of notochord and dorsal aorta and the importance of axial midline tissues for adrenergic differentiation has been well documented. We now provide evidence that bone morphogenetic proteins, BMP-4 and BMP-7 are signals produced by the dorsal aorta that direct sympathetic neuron differentiation. BMP-4 and BMP-7 are

expressed in the dorsal aorta at critical times during sympathetic neuron differentiation and have the ability to enhance the formation of adrenergic sympathetic neurons both in cultures of neural crest cells and when ectopically expressed in the developing embryo.

Key words: neural crest, bone morphogenetic protein-4, bone morphogenetic protein-7, sympathetic, adrenergic, noradrenergic, dorsal aorta

## INTRODUCTION

The processing of information in the nervous system is brought about by the highly coordinated action of neurons distinguished by their functional properties as well as their biochemical phenotypes, such as the neurotransmitter used by individual neurons. The problem of how such an intricate system is set up during embryonic development is certainly among the most challenging questions being asked by developmental biologists. Even though it is firmly established that tissue interactions mediated by growth factors play a decisive role during development of the nervous system, the molecular nature of the factors involved in the induction of a neurotransmitter phenotype *in vivo* has not been resolved. Factors that have been shown to induce cholinergic differentiation of sympathetic neurons in tissue culture (Fukada, 1985; Yamamori et al., 1989; Ernsberger et al., 1989; Saadat et al., 1989) turned out to be unlikely candidates for the induction of the cholinergic phenotype *in vivo* (Masu et al., 1993; Rao et al., 1993). However, detailed analysis of the development of sympathetic neurons and their precursor cells *in vitro* and *in vivo* have rendered this system one of the neuronal lineages most thoroughly analyzed with respect to development (see Anderson (1993), for review).

The precursors of postganglionic sympathetic neurons originate from the neural crest. After migrating ventrally, they

commence adrenergic differentiation in the vicinity of the dorsal aorta to form the primary sympathetic strands. In the chick embryo, tyrosine hydroxylase, the enzyme catalyzing the rate-limiting step in catecholamine biosynthesis, is first expressed (Ernsberger et al., 1995; Groves et al., 1995) shortly before the neurons display catecholamine storage (Enemar et al., 1965; Kirby and Gilmore, 1976; Allan and Newgreen, 1977; Rothman et al., 1978). This indicates that tyrosine hydroxylase, which has been used for demonstrating adrenergic differentiation in neural crest cells, is a useful marker for the development of the catecholaminergic phenotype both *in vivo* and *in vitro*.

Even though expression of adrenergic markers is first seen in the vicinity of the dorsal aorta, tissues lining the migration pathway of the neural crest cells to the sites of formation of primary sympathetic strands are considered to be important for the induction of the adrenergic character. The potency of notochord, ventral neural tube and somitic mesoderm to induce adrenergic differentiation in neural crest cells was shown after transplantation within the chick embryo as well as in tissue culture (Cohen, 1972; Norr, 1973; Teillet and Le Douarin, 1983; Howard and Bronner-Fraser, 1985, 1986). Removal of notochord and ventral neural tube from the developing chick embryo prevented the differentiation of adrenergic cells in the primary sympathetic ganglia (Teillet and Le Douarin, 1983; Stern et al., 1991; Groves et al., 1995). Even though neural

crest cells were found to migrate to the dorsal aorta following notochord removal and neural tube rotation, these cells did not acquire adrenergic properties (Groves et al., 1995), indicating that ventral neural tube and/or notochord are necessary for the development of the adrenergic transmitter phenotype. Since the expression of tyrosine hydroxylase does not start before the migrating neural crest cells have reached the dorsal aorta (Ernsberger et al., 1995; Groves et al., 1995; Stern et al., 1991), inductive events that occur at the aorta and are mediated by factors expressed at the aorta may be the trigger for adrenergic differentiation proper.

The importance of ventral neural tube and notochord for the development of adrenergic sympathetic neurons suggests that the secreted protein sonic hedgehog (shh) may be involved. This protein has an important signalling role in vertebrate spinal cord and motoneuron induction (Roelink et al., 1994), differentiation of midbrain dopaminergic neurons (Hynes et al., 1995), somite differentiation (Johnson et al., 1994; Fan and Tessier-Lavigne, 1994) and limb patterning (Laufer et al., 1994; Francis et al., 1994; Yang and Niswander, 1995). In *Drosophila*, hedgehog is involved in patterning of epidermis (reviewed in Perrimon, 1995) and acts by inducing expression of decapentaplegic, which is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily. This has led to the suggestion that the action of shh may be mediated by TGF- $\beta$  family members in vertebrates (O'Farrell, 1994). In the developing limb, shh controls anterior-posterior patterning and induces expression of bone morphogenetic protein-2 (BMP-2), which is a TGF- $\beta$  family member, although there is as yet no direct evidence that BMP-2 mediates the effects of shh on limb patterning. Interestingly, it has recently been observed that BMP and hedgehog genes are coexpressed at many sites of epithelial-mesenchymal interaction in the vertebrate embryo, suggesting the involvement of hedgehog and BMPs in the development of diverse embryonic structures (Bitgood and McMahon, 1995).

The requirement of ventral neural tube/notochord for adrenergic development *in vivo*, together with the recent report that osteogenic protein-1/BMP-7 induces adrenergic differentiation in avian neural crest cell cultures (Wehby et al., 1993) prompted us to analyze the relative roles of shh and different members of the TGF- $\beta$  family in the differentiation of adrenergic characters in the avian embryo. Here we demonstrate that BMP-2, BMP-4 and BMP-7 strongly induce the expression of the adrenergic marker enzyme tyrosine hydroxylase in neural crest cultures, whereas shh has only a small effect. Infection of chick embryos with a retrovirus that encodes BMP-4 leads to ectopic expression of tyrosine hydroxylase in cells found in the neural crest migration pathway of chick embryos. During normal development, mRNA for BMP-4 and BMP-7, but not for BMP-2, is found in the dorsal aorta before the expression of tyrosine hydroxylase. Explants of dorsal aorta were observed to induce adrenergic development in neural crest cultures. The expression of BMP-4 and BMP-7 in the dorsal aorta just before adrenergic differentiation of sympathetic neurons, as well as their effects on adrenergic differentiation *in vitro* and *in vivo*, suggests that these molecules may be responsible for induction of adrenergic differentiation *in vivo*.

## MATERIALS AND METHODS

### Secondary cultures of trunk neural crest cells

Primary culture of quail trunk neural crest (NC) was performed using a modification of the protocol described by Fauquet et al. (1981). Neural tubes from 2-day-old quail embryos were dissected from the trunk region, carefully cleaned from adhering connective tissue by pancreatin treatment, plated on a collagen substratum and grown in DMEM with 15% CEE (chicken embryo extract) and 15% HS (horse serum). After 24 hours at 37°C and 10% CO<sub>2</sub>, neural tubes were removed and the remaining migrating NC cells were detached with 0.1% trypsin in PBS. After stopping the reaction with trypsin inhibitor, pelleting by centrifugation and resuspending, 8000 cells were replated in wells (10 mm diameter) of collagen-coated dishes (Greiner). After 3 hours, when cells had attached, the protein factors to be tested were added. Cells were grown for 5 days after replating and were then analysed by immunocytochemistry or by *in situ* hybridization. Human recombinant BMP-2 and BMP-4 were kindly provided by Dr Wozney (Genetics Institute, Cambridge, USA); chicken TGF- $\beta$ 3 was from R&D Systems (Minneapolis, USA), and human GDNF from Pepro Tech (Rocky Hill, USA). Human recombinant BMP-7 was prepared as described previously (Sampath et al., 1992). The recombinant shh used was kindly provided by D. Brumcrot and A. McMahon (Harvard, USA) and is active in inducing ventral cell types in embryonic chick neural tissue (Marti et al., 1995).

### Co-cultures of neural crest and dorsal aorta

To enable the dissection of dorsal aorta, arteries of stage 17-18 chick embryos were perfused with india ink from the heart. Using sharpened tungsten needles, the neural tube was removed over a stretch of about 8-10 somites at wing level. For further dissection, the embryos were treated (10 minutes, RT) with pancreatin (Gibco BRL). This pretreatment allowed removal of notochord and somitic mesenchyme without rupturing of the dorsal aorta. Pieces of dorsal aorta were then cultured overnight in collagen-coated wells used for neural crest cultures. Neural crest cells were then plated onto the wells with dorsal aorta explants and cultured for 5 days under conditions described above for neural crest cells. As controls, similar sized pieces of the main extraembryonic artery (vitelline artery) were dissected and cultured similarly.

### Immunocytochemistry and quantification of the data

Staining for tyrosine hydroxylase (TH) was carried out using the biotin streptavidin method as previously described (Ernsberger et al., 1989). The distribution of TH-positive cells was uneven and thus the whole area of the culture dish was analysed. In most cases the total number of TH-positive cells was determined. In some experiments (Fig. 1B) where very high total numbers of TH-positive cells were expected a semiquantitative method was used. TH-induction was evaluated by determining the number of TH-positive cells per visual field. Visual fields that showed more than 20 TH-positive cells were counted. The results are expressed as percentage of total visual fields analysed (33 visual fields per well, corresponding to the total culture area). As shown in Fig. 1B, this evaluation method reliably reflects the massive changes in the number of TH-positive cells after BMP treatment. The number of melanocytes was determined by counting the total number of melanin-containing cells per culture dish. The total number of neural crest cells was determined by counting the total number of cell nuclei in 10 randomly selected visual fields after staining nuclei with Hoechst dye. Data from at least three independent experiments are given for both the BMP effects on TH-positive cells and on melanocyte development. Values are given as means  $\pm$  s.e.m.

### Immunohistochemistry

Staining for tyrosine hydroxylase and the neural crest marker HNK-

1 (Tucker et al., 1984) on frozen sections of chick embryos were carried out on alternate sections as described previously (Rohrer et al., 1986).

### Overexpression of BMP-4 by *in vivo* application of an avian retroviral vector encoding BMP-4

BMP-4/RCAS consists of the replication competent avian retroviral vector RCAS BP(A) (Hughes et al., 1987), containing the mouse BMP-4 coding region. Control virus contains the BMP-4 coding region in inverse orientation. Cultured 0 line chick embryo fibroblasts infected with BMP-4/RCAS produce functional BMP-4 (Duprez et al., 1996). Fertilized virus-free chicken eggs were obtained from Lohmann, Cuxhaven, Germany, and incubated for 2 days at 38°C in a humidified atmosphere. After opening the eggs and staging the embryos, aggregates of infected, BMP-4/RCAS-virus-producing (or control virus-producing) chick embryo fibroblasts (line 0) were implanted into the embryos at the brachial level, between the neural tube and the last somite generated. The eggs were sealed with tape and incubated for further 3 days. Then the embryos were staged according to Hamburger and Hamilton (1951), fixed in 4% paraformaldehyde in PBS for 2 hours and, after washing, stored in 100% methanol at -20°C until whole-mount staining. Untreated control embryos were fixed in the same manner.

### In situ hybridisation

For whole-mount *in situ* hybridization, embryos were processed using a modification of the protocol described by Wilkinson (1993). Detailed protocols are available upon request. Single-stranded RNA probes were prepared by the transcription of linearized plasmids with the DIG-RNA labelling kit (Boehringer) according to the manufacturer's instructions.

For non-radioactive *in situ* hybridization to cryostat sections or neural crest cultures, a modification of the protocol of Schaeren-Wiemers and Gerfin-Moser (1993) was used. Detailed protocols are available upon request.

Radioactive *in situ* hybridization to tissue sections was performed as described in Ernsberger et al. (1995). DIG- or <sup>35</sup>S-labelled antisense RNA probes for chicken BMP-2 or BMP-4 transcripts were prepared as described (Francis et al., 1994). Probes for chicken BMP-7 or tyrosine hydroxylase (TH) transcripts were prepared as described by Houston et al. (1994) and Ernsberger et al. (1995), respectively. BMP-4/RCAS virus transcripts were detected using a probe specific for mouse BMP-4 transcripts (Jones et al., 1991).

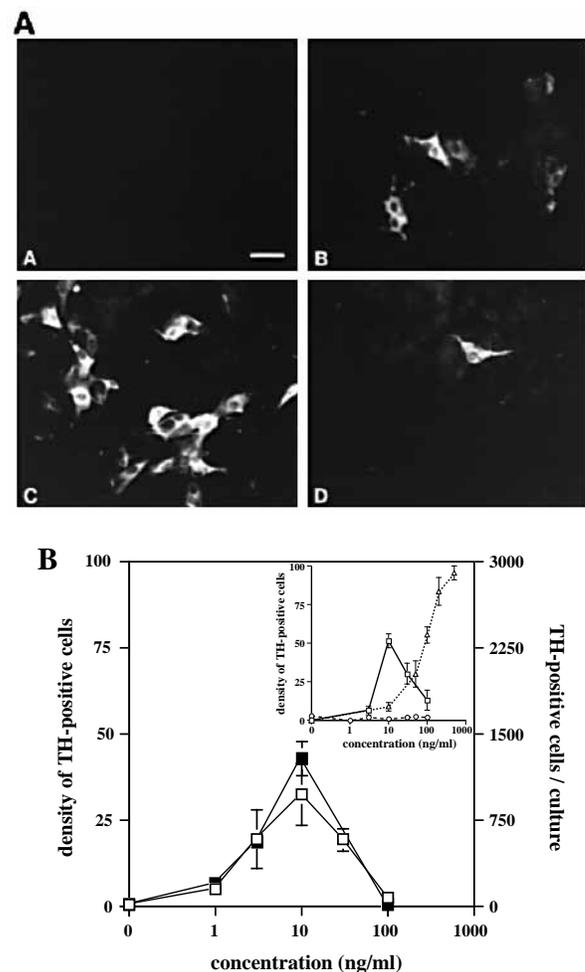
## RESULTS

### BMP-2, BMP-4 and BMP-7 induce the development of sympathoadrenergic cells in neural crest cultures

To identify and characterize factors involved in the development of adrenergic sympathetic neurons, neural crest cultures were established that contain sympathoadrenergic precursor cells. In the absence of added factors, only a low number of cells develop an adrenergic phenotype, characterized by the expression of the adrenergic marker enzyme tyrosine hydroxylase (TH). Upon addition of the biologically active NH<sub>2</sub>-terminal shh-peptide (Marti et al., 1995) the number of TH-positive cells was increased by a factor of  $2.1 \pm 0.8$  (mean  $\pm$  s.d.;  $n=3$ ) at a concentration of  $2 \times 10^{-9}$  M. The effect of 10-fold higher shh concentrations was the same. In contrast, BMP-2, BMP-4 and BMP-7 produced a large, up to 100-fold increase in the number of TH-positive cells in neural crest cultures (Fig. 1A,B). In view of this strong effect, the present study has

focused on the role of BMPs for adrenergic sympathetic development *in vitro* and *in vivo*.

The number of TH-positive adrenergic cells increased with the addition of BMP-4 up to a concentration of about 10 ng/ml. Interestingly, at this concentration, a maximum of adrenergic induction was reached. Higher concentrations resulted in only low numbers of adrenergic cells (Fig. 1B). Determination of the absolute number of TH-positive cells as well as a semi-quantitative analysis used in experiments involving large numbers of TH-positive cells (see Materials and Methods), provided similar results (Fig. 1B). It should be noted that, even under optimal conditions, only a small proportion (<10%) of



**Fig. 1.** Effect of BMP-4, BMP-2, BMP-7 and TGF- $\beta$ 3 on the development of TH<sup>+</sup> cells in neural crest cultures. Secondary neural crest cultures were maintained in the presence of different concentrations of the factors and analysed immunohistochemically for the density of TH<sup>+</sup> cells after 5 days. (A) Immunohistochemical staining for TH of cultures kept without BMP-4 (A) or with 3 ng/ml BMP-4 (B), 10 ng/ml BMP-4 (C) and 100 ng/ml BMP-4 (D). Scale bar 30  $\mu$ m. (B) Dose-response analysis of the effects of BMP-4, BMP-2, BMP-7 and TGF- $\beta$ 3 on the development of TH<sup>+</sup> cells. Main graph: Effect of BMP-4 analysed by determining the total number of TH<sup>+</sup> cells (■) or by a semi-quantitative determination of the density of TH<sup>+</sup> cells (□) (see Experimental Procedures). Insert: Effect of BMP-2 (□), BMP-7 (△) and TGF- $\beta$ 3 (○) on the density of TH<sup>+</sup> cells. Values are given as mean  $\pm$  s.e.m. of at least 3 independent experiments.

the total cell population expressed adrenergic properties, indicating that these factors may act only on a subpopulation of competent cells. This notion is further supported by the finding that the total number of cells is not affected by BMP treatment ( $33640 \pm 2050$  cells/culture in control as compared to  $34030 \pm 3700$  in cultures with 10 ng/ml BMP-4; mean  $\pm$  s.d.;  $n=3$ ).

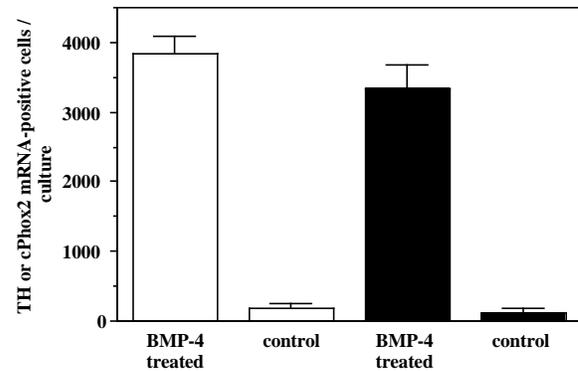
BMP-4 and BMP-2 are members of a subfamily of the large superfamily of TGF- $\beta$  related growth factors (Kingsley, 1994). To analyse the selectivity of the effects observed, BMP-2 and members of other subfamilies were assayed for their ability to induce adrenergic development. BMP-2 was as active as BMP-4 and displayed a similar dose-response relationship (Fig. 1B, insert). BMP-7, a member of the more distantly related 60A subfamily, also induced an increase in the number of adrenergic cells. However, the maximal number of adrenergic cells was higher than in BMP-4-treated cultures and no reduction of the biological effect at high concentrations was observed (Fig. 1B, insert). The concentration of BMP-7 required to elicit half-maximal effects was, however, at least 10-fold higher than BMP-2 and BMP-4 (Fig. 1B, insert). The results obtained with BMP-7 are in good agreement with effects recently observed by Varley et al. (1995). TGF- $\beta$ 3 (Fig. 1B, insert), TGF- $\beta$ 1 and GDNF (data not shown), which display a low sequence identity with BMP-2 and BMP-4, did not affect sympathoadrenergic differentiation. These results support the notion that only specific members of the TGF- $\beta$  superfamily affect the development of adrenergic sympathetic neurons. They demonstrate that BMP-4 and BMP-2 display effects only within a small concentration range.

In addition to the sympathoadrenergic lineage, the development of melanocytes is also affected by BMPs. Increasing concentrations of the factors led eventually to the complete suppression of melanocyte development (data not shown). This result demonstrates that BMP-2 and BMP-4 are active in these cultures at high concentrations and argues against the possibility that the lack of adrenergic differentiation at high BMP-2 and BMP-4 concentrations is due to artefactual inactivation of the factors (i.e. aggregation and precipitation).

The onset of adrenergic differentiation of sympathoadrenal precursor cells *in vivo* is characterized by the simultaneous expression of TH and the homeodomain transcription factor cPhox2 (Ernsberger et al., 1995). To investigate if the cells differentiating *in vitro* display similar properties, the expression of cPhox mRNA and TH mRNA was investigated by *in situ* hybridization. It was observed that BMP-4 addition resulted in a strong increase in the number of cells expressing Phox2 mRNA, which corresponded to the increase in the number of TH mRNA-expressing cells (Fig. 2). Thus, both molecular markers are regulated *in vitro* in response to BMPs in a similar way as *in vivo*.

#### BMPs must be present during the initial stage of neural crest cultures to stimulate adrenergic development

As a first step towards an analysis of the mechanism of action of BMPs in neural crest cultures, the timing of the BMP effects in neural crest cultures was investigated. It was observed that adrenergic cells are first detected during the third day of secondary culture and that their number then further increases during the next 2 days in culture (Fig. 3A). Interestingly, to



**Fig. 2.** Effect of BMP-4 on the development of cells expressing TH or cPhox2 mRNA. Secondary neural crest cultures maintained in the presence or absence of BMP-4 were analysed by *in situ* hybridization for the presence of cells positive for TH (■) or cPhox2 (□). The total number of positive cells/culture was determined after a culture period of 5 days. Values are given as mean  $\pm$  s.d. of at least 3 independent experiments.

affect adrenergic development, BMP-4 must be present during the first 2 days of secondary culture. Application during only the third day results in the lack of adrenergic cells (Fig. 3B). This can not be explained by a delayed expression of TH, since cultures that had been treated with factors during the third day in culture were devoid of TH-positive cells when assayed either 2 days or 6 days after BMP application.

These findings are in agreement with the notion that BMPs act on a step preceding the onset of adrenergic differentiation rather than affecting proliferation or survival of differentiated sympathoadrenergic cells.

#### Overexpression of BMP-4 in the developing chick embryo results in the generation of ectopic, TH-positive cells in the neural crest migration pathway

To investigate if BMPs are also sufficient to induce adrenergic differentiation *in vivo*, we used virus-mediated BMP-4 overexpression in the chick embryo. The overexpression of BMP-4 *in vivo* in the vicinity of migrating neural crest cells and developing sympathetic neurons may result in an increased number of sympathetic neurons, leading to increased ganglion size or adrenergic neurons in ectopic positions. To direct local overexpression of BMP-4, we used a replication-competent avian retrovirus (BMP-4/RCAS), which carries a cDNA insert encoding mouse BMP-4 (Duprez et al., 1996). To test the activity of the virus-produced BMP-4, neural crest cultures were infected with the BMP-4/RCAS virus, resulting in a virus-titer-dependent increase in the number of adrenergic cells (data not shown). Virus-producing chick embryo fibroblasts were then implanted into the neural crest migration pathway of E2 chick embryos (18-22 somites) at the level of the last somite. At E5, viral transcripts were found to be abundant in mesenchymal cells in the vicinity of the developing peripheral sensory and sympathetic ganglia, as judged by whole-mount *in situ* hybridization using a species-specific probe for mouse *Bmp-4* sequences (Fig. 4). Along the rostral-caudal axis, the virus-infected cells could be detected over a length of about two vertebrae (Fig. 4A). The infection was mainly restricted

to the side of the implant, with very little spread to the contralateral side (Fig. 4B).

Embryos with BMP-4-expressing viral implants were analysed for the expression of TH mRNA. We observed an ectopic, unilaterally enlarged domain of expression of TH mRNA in the region of implantation in 10 out of 20 embryos. Besides an increased size of sympathetic ganglia, TH mRNA-positive cells were also found in ectopic locations in these embryos, at positions where TH-positive cells are never found on the contralateral side (Fig. 5B) and in uninfected embryos (Ernsberger et al., 1995, and the present study). Ectopic TH mRNA-expressing cells were found lateral to the ganglia (Fig. 5B) but also at positions dorsolateral to the spinal nerve (not shown). When virus spread and ganglion size was analysed in serial sections by alternate *in situ* hybridizations for mBMP-4 and TH, respectively (Fig. 5), a close correlation between BMP-4 expression and increased ganglion size as well as ectopic TH-positive cells was observed along the rostral-caudal axis. We found that in embryos where virus-mediated mBMP-4 expression extended from the lateral body wall to the aorta (Fig. 5A), enlargement of sympathetic ganglia and ectopically located TH-positive cells were present (Fig. 5B). In contrast, in embryos where virus-mediated mBMP-4 expression was restricted to tissue lateral to the ventral migration pathway of neural crest cells, no enlargement of sympathetic ganglia was found. The increase in ganglion size and ectopic TH-positive cells were also not detected in embryos infected with control virus that carries the BMP-4 cDNA insert in antisense orientation (6 embryos analysed). To control for potential effects of BMP-4 on neural crest generation or migration, migrating neural crest cells and neural crest derivatives were identified on sections of embryos at different time points after infection using the HNK-1 antibody. We did not observe unilaterally increased volumes of DRG or other indications of increased migration of neural crest in the area of the implant when analysed 1, 2 or 3 days after infection (data not shown). These results demonstrate that overexpression of BMP-4 *in vivo* can result in a strong increase in the size of sympathetic ganglia and suggest that BMPs are involved in the specification of the adrenergic sympathetic phenotype.

### The timing and localization of BMP-7 and BMP-4 expression suggest a physiological role for sympathoadrenergic development

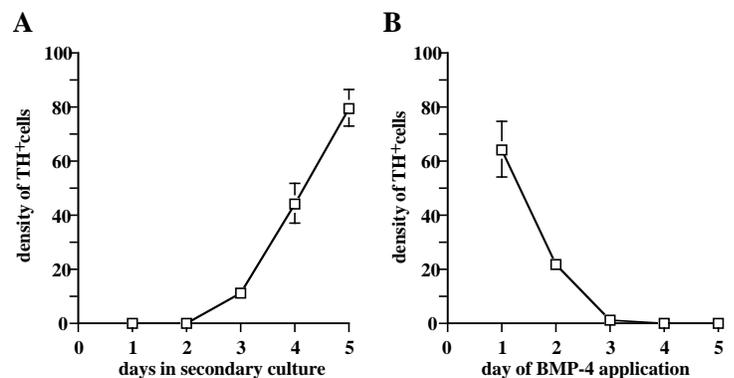
Induction of adrenergic characteristics in neural crest cells by BMPs both *in vitro* and *in vivo* indicates that these factors may have a physiological role for sympathetic neuron differentiation. The adrenergic phenotype, characterized by the expression of TH mRNA and the presence of catecholamines, is acquired at stage 18 during the third day of chick embryonic development (Ernsberger et al., 1995). The cells that express these markers are invariably located at the dorsolateral aspect of the dorsal aorta. Analysis of BMP-2, BMP-4 and BMP-7 expression by *in situ* hybridization revealed that both BMP-7 and BMP-4 mRNA were present in the dorsal aorta as early as at stage 16, that is before the onset of TH mRNA expression. *In situ* hybridisation of parallel sections with a probe for TH mRNA confirmed our previous findings (Ernsberger et

al., 1995) that TH mRNA is not expressed at stages 16 and 17 (Fig. 6), but from stage 18 onwards. BMP-4 and BMP-7 mRNA expression could be detected up to stage 25, the latest stage investigated. To define the identity of BMP-expressing cells in the dorsal aorta, non-radioactive *in situ* hybridisations were carried out (Fig. 6). Signals could be identified in the aorta on the innermost layer of cells as well as in the adjacent 2-3 cell layers. BMP-2 expression was not observed in the dorsal aorta at these stages, but could be demonstrated in nephric mesenchyme (not shown) and in the developing limb bud, as described previously (Francis et al., 1994). At stage 17, a weak signal was also observed in the notochord for BMP-7, but not for BMP-2 or BMP-4. Moreover, BMP-4 and BMP-7 signals were observed in the wing bud as well as in the dorsal neural tube. In addition, BMP-7 mRNA was detected in nephric mesenchyme.

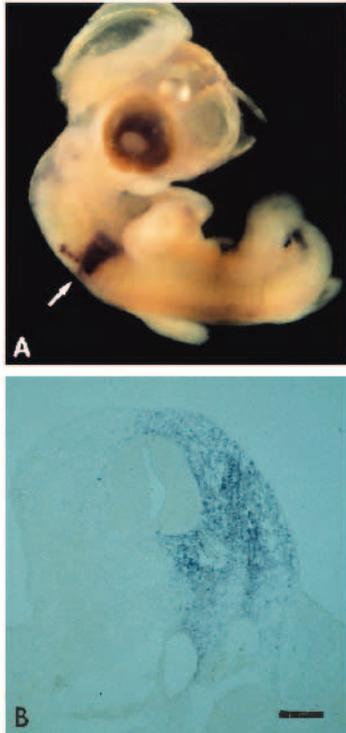
The expression patterns of BMP-7 and BMP-4 mRNA before the onset of TH expression indicates that BMP-7 and BMP-4 play a role in the induction of the adrenergic transmitter phenotype in sympathetic neurons at the dorsal aorta *in vivo*.

### Explants of dorsal aorta induce the development of sympathoadrenergic cells in neural crest cultures

To confirm the presence of factors in the dorsal aorta that are capable of stimulating adrenergic development, explants of dorsal aorta were co-cultured with neural crest. Dorsal aorta was dissected at stage 17-18 from chick embryos at the wing level and cultured either alone or in the presence of quail neural crest cells. As control, similar sized pieces of the vitelline artery from the extraembryonic region of the embryo were also co-cultured with neural crest cells. It was observed that dorsal aorta was able to strongly increase the number of TH-positive cells in neural crest cultures (Fig. 7). In cultures of dorsal aorta without the addition of neural crest cells only a few TH-positive cells were observed. Their presence is very likely due to a small number of sympathoadrenergic precursor cells present in the vicinity of the dorsal aorta that were not eliminated during the dissection procedure. Co-culturing pieces of the extraembryonic vitelline artery with neural crest cells did not result in



**Fig. 3.** Analysis of the onset of TH protein expression in neural crest cultures and of the timing requirements for BMP-4 addition. (A) Secondary neural crest cultures maintained in 10 ng/ml BMP-4 and analysed for the appearance of TH<sup>+</sup> cells at the times indicated. (B) BMP-4 (10 ng/ml) was added at the times indicated and the density of TH<sup>+</sup> cells was analysed after a total culture period of 5 days (see Materials and Methods for quantification).



**Fig. 4.** Infection of chick embryos with BMP-4/RCAS virus. Embryos were implanted at E2 with aggregates of BMP-4/RCAS virus-producing cells. The presence of viral RNA was investigated at E5 by whole-mount in situ hybridisation with a species-specific probe for mouse BMP-4. (A) Viral RNA extends rostral-caudally over a region of about two segments (arrow). (B) Sectioning reveals a unilateral domain of viral RNA that extends ventrally below the region of the dorsal aorta. Viral RNA is also evident in the somitic mesenchyme and in the base of the limb bud. Scale bar 250  $\mu$ m.

increased numbers of TH-positive cells (data not shown).

The finding that the dorsal aorta, dissected at the time when BMP-4 and BMP-7 mRNA is expressed in this tissue, secretes a biological activity that is able to induce adrenergic differentiation further supports the hypothesis that aorta-derived BMPs are involved in the specification of the adrenergic sympathetic phenotype.

## DISCUSSION

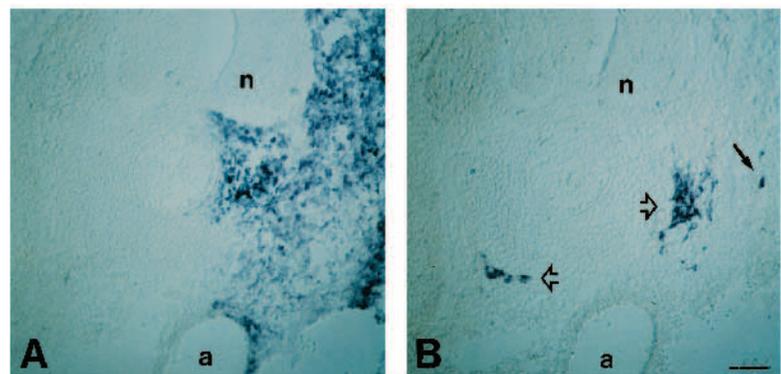
The importance of environmental cues, and in particular of the axial structures notochord and ventral neural tube, for sympathetic neuron development has been well established (Cohen, 1972; Norr, 1973; Teillet and Le Douarin, 1983; Howard and Bronner-Fraser, 1985, 1986). The requirement of axial structures for the acquisition of the adrenergic phenotype suggests that signals controlling this differentiation step are derived, either directly or indirectly, from these structures. On the contrary, adrenergic differentiation is not observed between notochord and

neural tube, but at a more ventral location, at the dorsolateral aspect of the dorsal aorta (Enemar et al., 1965; Kirby and Gilmore, 1976; Ernsberger et al., 1995; Groves et al., 1995). This suggests that signals derived from the circulation or from the vasculature itself contribute an essential signal to elicit the onset of catecholaminergic differentiation. Here we provide evidence to indicate that BMP-4 and BMP-7, produced in the dorsal aorta and the surrounding mesenchyme, represent this differentiation signal. In agreement with current literature, the inductive effects of tissues and factors leading to the appearance of cells with adrenergic phenotype *in vivo* and *in vitro* are referred to as differentiation effects, although the mechanism involved awaits further analysis.

### Catecholaminergic development is induced by dpp subfamily members

BMP-2, BMP-4 and BMP-7 were found to induce large increases in the number of catecholaminergic cells in neural crest cultures, identified by the expression of tyrosine hydroxylase, the rate limiting enzyme in catecholamine production. The biological effect was selective for BMP subfamily members, as revealed by the lack of response to TGF- $\beta$ 1, TGF- $\beta$ 3 and GDNF.

Members of the TGF- $\beta$  superfamily exert their effects through binding to specific cell surface receptors (Kingsley, 1994; Attisano et al., 1994). These receptors are heteromeric complexes between two types of transmembrane serine/threonine kinases known as type I and II receptors that are both needed for signalling. In the signal transduction of BMPs, three different type I receptors BMPR-IA, BMPR-IB and ActR-I (Koenig et al., 1994; Ten Dijke et al., 1994), and one type II receptor, BMPR-II (Liu et al., 1995; Rosenzweig et al., 1995), have been implicated. TGF- $\beta$  is not able to signal through BMPR-II, which is in agreement with the lack of TGF- $\beta$  effects on adrenergic development. The different dose-response relationship of BMP-7 and BMP-2/BMP-4 with



**Fig. 5.** Ectopic expression of TH mRNA upon BMP-4 overexpression. Embryos infected with BMP-4/RCAS virus were analysed for mBMP-4 mRNA expression (A) and the localisation of TH mRNA-expressing cells (B) at E5 by in situ hybridisation of alternate sections. (A) The representative section shown illustrates the unilateral expression of mBMP-4 on the right side of the embryo where virus-producing cells had been implanted. (B) TH mRNA detection in sympathetic ganglia (open arrows) shows ganglion enlargement on the infected side of the embryo. In addition, TH mRNA-expressing cells occur in ectopic lateral positions (filled arrow) in the area of BMP-4 overexpression. Scale bar 100  $\mu$ m.

respect to adrenergic differentiation may be explained by different receptor complexes assembled by BMP-2/BMP-4 and BMP-7 or by different signal transduction efficiencies through the same receptor complex (Liu et al., 1995).

The finding that adrenergic differentiation is observed only at concentrations of between 5 and 30 ng/ml of BMP-2 and BMP-4 is in line with other examples where a specific biological effect is elicited by TGF- $\beta$  family members only within a small concentration range. In particular, the determination of different dorsal fates in the early *Drosophila* embryo depends on the local concentration of dpp (Ferguson and Anderson, 1992), the *Drosophila* homologue of BMP-2 and BMP-4. Similarly, in *Xenopus*, it was shown that different mesodermal phenotypes are determined by different concentrations of activin (Green and Smith, 1990; Gurdon et al., 1994).

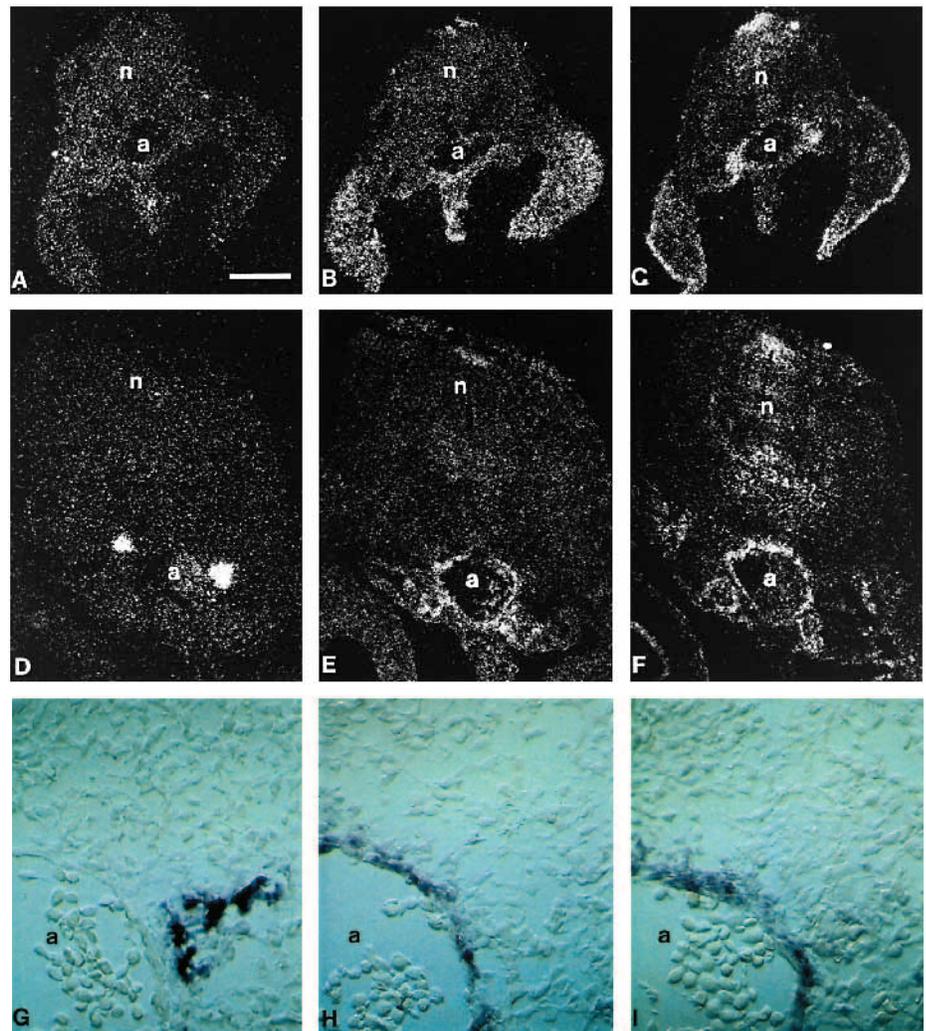
### BMPs are required during the initial stages of catecholaminergic development

Neural crest cells consist of a mixture of pluripotent cells and of cells with restricted developmental potential (Baroffio et al., 1988; Selleck et al., 1993). With time in culture, mature catecholaminergic sympathetic neurons are generated in a sequence of developmental steps that may be all controlled by extrinsic cues. Thus, BMPs could act on the determination of neuronal and/or adrenergic phenotype, on the differentiation to catecholaminergic cells, or on the proliferation and survival of both undifferentiated and differentiated cell types. To address this issue, we have analysed the timing of the onset of expression of adrenergic markers in neural crest cultures and defined the timing requirements of the action of BMPs. We found that tyrosine hydroxylase-positive cells were observed only after a delay of several days after BMP addition and that BMPs were only effective when added at the beginning of the culture, before the onset of adrenergic differentiation. Addition of BMPs at later stages to cultures that contained low numbers of differentiated sympathoadrenergic cells had no effect. It is thus unlikely that BMPs act on the survival or proliferation (Rohrer and Thoenen, 1987) of already differentiated cells. These results and conclusions are in agreement with a recent analysis of the effects of BMP-7 on neural crest cells (Varley et al., 1995). In this context, it should also be mentioned that BMPs were found to interfere with rather than to stimulate the proliferation of immature sympathetic neurons from

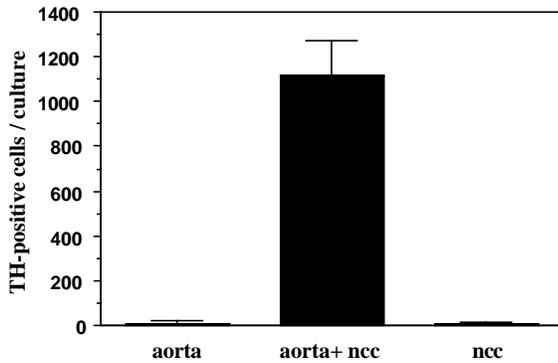
E7 chick embryos (H. R., unpublished observations). Taken together, these results suggest that the increased number of TH-positive sympathetic neurons induced by BMPs in neural crest cultures may be due to effects on pre-adrenergic precursor cells, for instance increasing survival or inducing neuronal and/or catecholaminergic differentiation.

### BMP-4 overexpression in vivo results in ectopic development of TH-positive cells

The dramatic effects of BMPs on adrenergic differentiation in vitro prompted the study of BMP overexpression in a normal physiological context, the chick embryo. As BMPs are involved in many developmental processes (Hogan et al., 1994), it is likely that a general overexpression of BMPs in the



**Fig. 6.** BMP-4 and BMP-7 mRNA are expressed in and around the dorsal aorta before the onset of TH mRNA expression. Adjacent thoracic sections of chick embryos at stage 17 (A-C) or stage 21 (D-I) were analysed by in situ hybridisation for the expression of TH-mRNA (A,D,G), BMP-4 mRNA (B,E,H) and BMP-7 mRNA (C,F,I). Please note the absence of signals for TH mRNA at stage 17 (A) and the localisation of TH<sup>+</sup> cells at a lateral position of the aorta (a) at stage 21 (D,G). In contrast, BMP-4 and BMP-7 signals are evident at the aorta at both stages. Additionally, BMP-4 and BMP-7 expression is evident in wing bud ectoderm and mesoderm at stage 17. Non-radioactive in situ hybridization demonstrates expression of BMP-4 and BMP-7 mRNA in the dorsal aorta adjacent to cells positive for TH mRNA. Scale bar 200  $\mu$ m (A-F), 50  $\mu$ m (G-I).



**Fig. 7.** Explants of dorsal aorta induce the development of TH<sup>+</sup> cells in co-cultures with neural crest. Parallel cultures of dorsal aorta alone, neural crest (ncc) alone or co-cultures of neural crest and dorsal aorta were kept for 5 days and analysed for the number of TH-immunoreactive cells. Values are given as mean  $\pm$  s.d. of at least three independent experiments.

developing embryo would be lethal. Thus, we used a strategy in which it was possible to overexpress BMPs in the vicinity of the developing sympathetic ganglia. When a replication-competent avian retrovirus encoding mouse BMP-4 was applied between neural tube and somites at the level of the notochord, at a time when neural crest migration just starts, it lead to a unilateral expression of viral RNA in the mesenchyme surrounding the developing sympathetic and sensory ganglia. Such embryos showed a unilateral increase in sympathetic ganglion size and, in addition, TH-expressing cells at ectopic locations where TH-positive cells are never seen in normal embryos. These results clearly demonstrate that BMPs are able to promote an adrenergic phenotype *in vivo*. However, these results also raise the question of why not all neural crest derivatives become adrenergic? There are several very interesting possibilities that have to be considered. (1) Our *in vitro* results suggest that BMPs may be important for one specific step in sympathoadrenergic development and thus their action may be restricted to this subpopulation of competent cells. The generation of these competent cells may require signals from ventral neural tube and notochord (Teillet and Le Douarin, 1983; Stern et al., 1991; Groves et al., 1995) such that only cells in the ventral part of the somite are able to respond to BMPs. (2) However, there is also evidence for dorsoventral patterning of the somite by the ectoderm and the neural tube (Fan and Tessier-Lavigne, 1994). A factor derived from these tissues may suppress the ability of neural crest cells in the dorsal part of the somite to respond to BMPs by adrenergic differentiation. Previous work demonstrated the presence of adrenergic precursor cells in sensory DRG and that their potential for adrenergic differentiation is repressed during normal development (Le Lievre et al., 1980; Xue et al., 1985; Rohrer et al., 1986). (3) Finally, as virus-expression and BMP-4 protein synthesis needs time, the majority of cells may already be specified and only a subpopulation of late migrating pluripotent cells may still be able to respond to BMPs. The observation that not all implanted embryos displayed increased numbers of adrenergic cells can thus be explained by the variations in the localisation of the implant relative to the developing ganglia and the extent of initial virus spread.

Recent evidence suggests that BMP-4 and BMP-7 are able to induce the generation of neural crest cells in lateral regions of neural plate (Liem et al., 1995). This raises the question whether the increased size of sympathetic ganglia could be due to the generation of additional neural crest cells from the neural tube. However, there are several findings that argue against this possibility. First, no evidence for increased generation of neural crest cells, i.e. enlargement of dorsal root ganglia or neural crest cells at ectopic places was observed in BMP-4-overexpressing embryos stained for TH and the neural crest marker HNK-1. Second, as the competence of neural plate cells to respond to inductive signals is lost rapidly (Yamada et al., 1993; Placzek et al., 1993), the neural tube cells may have lost the competence to respond to BMP-4 by the time it is expressed ectopically. Third, grafting of supernumerary neural crest cells does not result in increased size of sympathetic ganglia (Weston and Butler, 1966; Le Lievre et al., 1980).

### **BMP-4 and BMP-7 are expressed at the dorsal aorta at critical times for adrenergic differentiation**

*In situ* hybridization analysis revealed that BMP-4 and BMP-7 mRNA are expressed at the dorsal aorta, in the immediate vicinity of the site where the sympathoadrenergic precursor cells aggregate and start to differentiate. BMPs are also expressed in the dorsal aorta in other vertebrate embryos (Lyons et al., 1990, 1995). Interestingly, we found that both BMP-7 and BMP-4 mRNA are expressed at this location before the onset of adrenergic differentiation, i.e. before TH mRNA is expressed. The expression of BMP-4 and BMP-7 mRNA is evident in several cell layers of the aorta including the innermost cells. When pieces of dorsal aorta, dissected from stages when BMP-4 and BMP-7 mRNA is detectable in this tissue, are co-cultured with neural crest cells, a strong increase in the number of TH-positive cells is observed. As these cells are not restricted to the immediate vicinity of the explant, dorsal aorta must release a diffusible activity that stimulates adrenergic development. Similar results have recently been obtained in co-cultures of mammalian neural crest cells with dorsal aorta (A. Groves and D. Anderson, personal communication). The most parsimonious interpretation of the co-culture experiments is that dorsal aorta expresses BMP protein. The expression studies and co-culture experiments strongly suggest that BMP-7 and BMP-4 are produced at the required time and location and are of physiological importance for the development of the adrenergic sympathetic phenotype.

How can these findings, in particular the localisation of BMPs in the dorsal aorta, be reconciled with the previously demonstrated importance of the axial midline structures, notochord and ventral neural tube? It has been well documented that in the absence of axial midline structures, sympathetic ganglia form in the vicinity of dorsal aorta, but the cells are unable to express TH (Groves et al., 1995). There are several interesting possibilities to explain these findings. (1) Adrenergic differentiation requires the sequential action of signal(s) from axial structures and the dorsal aorta. If the axial signal is lacking, BMPs cannot act since the cells are not competent. (2) The signal(s) from the axial structures and from the dorsal aorta must act simultaneously. (3) The axial signals do not act directly on the sympathoadrenergic lineage but indirectly, inducing the expression of BMPs in the dorsal aorta. A sequential action of shh and BMPs on neural crest cells would explain that BMPs

are able to induce adrenergic differentiation *in vitro*, since cultured neural crest cells have been subject to shh signalling from the ventral neural tube. A requirement of simultaneous action of shh and BMPs seems unlikely since BMPs display strong effects on adrenergic development *in vitro* in the absence of shh and since the combination of shh and BMP-4 did not increase the number of adrenergic cells over that observed in the presence of BMP-4 alone (E. R., unpublished observation). An indirect action of axial signals by inducing BMPs in the dorsal aorta would be reminiscent of the induction of BMP-2 by sonic hedgehog in the developing limb bud (Laufer et al., 1994), although there is as yet no direct evidence that BMP-2 mediates the effects of sonic hedgehog in the limb. It would explain that adrenergic differentiation occurs exclusively in the vicinity of the dorsal aorta but requires signals from the axial structures (Stern et al., 1991). The exciting advantage of the chick embryo is that the different hypotheses can directly be tested. Thus, a full account of the factors involved in catecholaminergic differentiation in the peripheral and central nervous system seems to be within reach. This, together with the effect of shh on midbrain dopaminergic neuron development (Hynes et al., 1995), will provide the first description of transmitter phenotype differentiation in molecular terms. The understanding of factors involved in catecholaminergic differentiation may prove to be relevant for the development of new strategies for the treatment of certain neurodegenerative diseases, such as Parkinson's disease.

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