Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube

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SUMMARY

For neural crest cells to engage in migration, it is necessary that epithelial premigratory crest cells convert into mesenchyme. The mechanisms that trigger cell delamination from the dorsal neural tube remain poorly understood. We find that, in 15- to 40-somite-stage avian embryos, BMP4 mRNA is homogeneously distributed along the longitudinal extent of the dorsal neural tube, whereas its specific inhibitor noggin exists in a gradient of expression that decreases caudorostrally. This rostralward reduction in signal intensity coincides with the onset of emigration of neural crest cells. Hence, we hypothesized that an interplay between Noggin and BMP4 in the dorsal tube generates graded concentrations of the latter that in turn triggers the delamination of neural crest progenitors. Consistent with this suggestion, disruption of the gradient by grafting Noggin-producing cells dorsal to the neural tube at levels opposite the segmental plate or newly formed somites, inhibited emigration of HNK-1-positive crest cells, which instead accumulated within the dorsal tube. Similar results were obtained with explanted neural tubes from the same somitic levels exposed to Noggin. Exposure to Follistatin, however, had no effect. The Noggin-dependent inhibition was overcome by concomitant treatment with BMP4, which when added alone, also accelerated cell emigration compared to untreated controls. Furthermore, the observed inhibition of neural crest emigration in vivo was preceded by a partial or total reduction in the expression of cadherin-6B and rhoB but not in the expression of slug mRNA or protein. Altogether, these results suggest that a coordinated activity of Noggin and BMP4 in the dorsal neural tube triggers delamination of specified, slug-expressing neural crest cells. Thus, BMPs play multiple and discernible roles at sequential stages of neural crest ontogeny, from specification through delamination and later differentiation of specific neural crest derivatives.

Key words: Avian embryo, Cadherin, Cell delamination, Epithelial-mesenchymal conversion, RhoB, Slug, Somite, TGFβ.

INTRODUCTION

The neural crest is a discrete structure that exists transiently in the vertebrate embryo. Its component cells yield an extraordinary variety of cell types, from peripheral neurons and satellite cells to bones, tendons, connective and adipose tissues, dermis, melanocytes and endocrine cells. Neural crest cells become specified in a rostrocaudal fashion along the body axis. Subsequently, they are released from the neuroepithelium as mesenchymal cells that follow definite migration routes, finally reaching target embryonic sites where they settle and differentiate (Le Douarin and Kalcheim, 1999).

In spite of significant progress achieved in the neural crest field over the past years, little is known about the mechanisms underlying the onset of crest cell migration, a process that involves dramatic changes in cell-cell and cell-matrix interactions, resulting in the loss of epithelial conformation and the consequent generation of cell motility. Intercellular adhesions involving calcium-dependent mechanisms were proposed to play a role in regulating the epithelial state of premigratory neural crest cells (Newgreen and Gooday, 1985; Takeuchi, 1988). Consequently, loss of N-cadherin and c-cad-6B, expressed in presumptive epithelial crest cells, was found to correlate with their emergence from the neural tube (Akitaya and Bronner-Fraser, 1992; Duband et al., 1988; Nakagawa and Takeichi, 1995; Newgreen and Gooday, 1985; Newgreen and Minichiello, 1995). Under normal conditions, however, cadherins might not be exclusively involved in maintaining intercellular cohesion as several members of this family, including c-cad-7 (Nakagawa and Takeichi, 1995, 1998), Xenopus cadherin-11 (Vallin et al., 1998) and U-cadherin (Winklbauer et al., 1992) were shown to be expressed on subsets of migrating mesenchymal progenitors.

In avians, the earliest available marker that enables to distinguish neural crest cells from the rest of the neuroepithelium is the zinc-finger transcription factor Slug (Liem et al., 1995; Nieto et al., 1994). Interference with the function of chick Slug by antisense oligonucleotide treatment in early embryos has been suggested to inhibit the emergence of cranial neural crest cells from the neural tube (Nieto et al.,
In mice, however, the slug gene is expressed only in migrating crest cells and its inactivation has no effect on crest development (Jiang et al., 1998) but when expressed in the rat bladder carcinoma cell line NBT-II, mouse slug caused dissociation of desmosomes, a necessary step for growth factor-induced epithelio-mesenchymal transition (Savagner et al., 1997). Recently, Sefton et al. (1998) have provided evidence that, in murine embryos, the related gene Snail is expressed in premigratory crest cells, as slug is in avians. The complex process of epithelio-mesenchymal transition of neural crest cells was also shown to be triggered by events involving protein phosphorylation (Newgreen and Minichiello, 1995; Minichiello et al., 1999). Furthermore, it has been reported that rhoB, a recently cloned member of the rho family of GTP-binding proteins, is expressed in the dorsal neural tube and inhibition of rho activity by C3 exotoxin prevents delamination of neural crest cells from explanted neural tubes (Liu and Jessell, 1998). Alternative means of detachment from the neuroepithelium were proposed that involve changes in the plane of cell division (Erickson, 1993). It was observed that 70% of all mitotic spindles in the dorsal neural tube are oriented such that one of the daughter cells would detach from the epithelium, whereas elsewhere along the neural tube the cleavage planes are oriented so that the progeny retains a connection with the luminal surface.

The data summarized above suggest that the onset of neural crest cell migration is a complex morphogenetic process requiring the coordinated action of several categories of molecules upon which environmental signals would act. The identity of possible triggers remains, however, unknown. Delannet and Duband (1992) have shown that exogenously added TGFβ1 and TGFβ2 were able to stimulate the emigration of neural crest cells in vitro, possibly by increasing the substratum adhesion of the cells. Their presence in the microenvironment of the dorsal neural tube was, however, not shown. Yet, specific members of the BMP family of TGFβ-related proteins were shown to be expressed first in the epidermal ectoderm that flanks the neural plate and later in the dorsal midline of the neural tube (Basler et al., 1993; Jones et al., 1991; Lee et al., 1998; Liem et al., 1995; Lyons et al., 1995; Arkell and Beddington, 1997; Dudley and Robertson, 1997; Watanabe and Le Douarin, 1996). Ectodermal BMPs were shown to induce at early stages the dorsalization of the neural tube by inducing de novo differentiation of neural crest cells expressing slug, rhoB and specific cadherins (Liem et al., 1995, 1997; Liu and Jessell, 1998). Somewhat later, dorsal tube-derived BMPs were implicated in patterning of dorsal spinal cord interneurons (Lee et al., 1998; Liem et al., 1997). Similarly, in Xenopus, a BMP gradient attained in the ectoderm has been shown to induce the neural crest fate (Marchant et al., 1998). Whether the same or different environmental signals control specification and delamination of crest cells remains unknown. It is also unclear whether these two processes are part of a single molecular cascade or are separable in time and space.

In the present study, we report that following initial specification of neural crest cells, a balance between the activities of BMP4 and its inhibitor Noggin, both localized in the dorsal neural tube, regulates emigration of neural crest progenitors from the neuroepithelium. This conclusion is supported by the following findings: (1) while the expression of BMP4 is homogeneous along the dorsal neural tube, that of noggin is progressively downregulated caudorostrally, coinciding in time and axial level with initial delamination of crest cells; (2) perturbation of this gradient by overexpression of Noggin along the tube in ovo and in vitro, inhibits neural crest emigration without affecting the expression of slug, but downregulates that of cadherin-6B and rhoB; (3) inhibition of neural crest delamination is sensitive to Noggin, which predominantly inhibits BMP4 activity but not to Follistatin, which inhibits BMP6, BMP7 and activin; (4) Added BMP4 both accelerates the normal timing of cell emigration and overcomes the Noggin-induced inhibition. Altogether, these results suggest that BMP4 has successive and distinguishable roles on neural crest development, first the induction of the neural crest fate and later, stimulation of neural crest delamination.

MATERIALS AND METHODS

Embryos

Chick (Gallus gallus) and quail (Coturnix coturnix japonica) eggs from commercial sources were used for this study.

In ovo grafting of Noggin-secreting cells

CHO cells producing Xenopus Noggin and the dhfr-CHO control line were a kind gift from Richard Harland and Dale Frank. Cell lines were grown as previously described (Lamb et al., 1993). Confluent cultures were harvested, cells were washed in phosphate-buffered saline (PBS), pelleted and resuspended in a minimal volume of PBS. The vitelline membrane of 16- to 20-somite-stage quail embryos was removed. A slit was performed along the dorsal edge of the neural tube at the level corresponding to the segmental plate and two last-formed epithelial somites with no removal of ectoderm. Concentrated cell suspensions were locally applied with a micropipette (30 μm tip opening, approximately) on top of the neural tube. Cell implants were performed under a Zeiss dissecting microscope with a 40× total magnification. Embryos were further incubated for 10 or 20-24 hours and then fixed either for immunocytochemistry or in situ hybridization.

Explants of neural primordia

The trunk region 16- to 20-somite-old quail embryos was separately sectioned at the level of the segmental plate or of the last five epithelial somite pairs. Neural primordia consisting of the neural tube and premigratory neural crest cells were isolated from adjacent tissues with 25% pancreatin in PBS, transferred to PBS supplemented with 5% newborn calf serum to stop enzymatic activity and washed in serum-free culture medium prior to explantation. Neural primordia were explanted onto multwell chamber slides (Nunc) that were precoated with fibronectin (50 μg/ml) for 1 hour. Culture media consisted of serum-free SFRI (Berganton, France) to which the following factors were added: Noggin (as medium conditioned over the CHO lines), Follistatin (National Hormone and Pituitary Program), BMP2, BMP4 and BMP7 (a gift from Avraham Fainsod and from Genetics Institute). Alternatively, substrata of control or Noggin-producing CHO cells were prepared, on top of which were placed the explants. Control and experimental neural primordia were grown for various times, fixed and processed for immunostaining. Each experiment was performed at least three times in quadruplicate cultures.

Fixation and immunocytochemistry

Embryos were fixed with Bouin’s fluid, embedded in paraffin wax, serially sectioned at 8 μm and immunostained with the monoclonal
HNK-1 antibody as previously described (Goldstein and Kalcheim, 1991) using secondary antibodies conjugated to horseradish peroxidase. Whole-mount immunostaining with HNK-1 was performed according to Loring and Erickson (1987) following fixation in 4% formaldehyde. Cultured neural primordia were fixed in 4% formaldehyde and immunostained with monoclonal antibodies HNK-1 (Debby-Brafman et al., 1999) or anti-Slug (mAb1E6, 1:100 dilution, according to Liem et al., 1997).

In situ hybridization
Whole-mount in situ hybridization was performed as described in Kahane et al. (1998) followed by paraffin embedding and serial sectioning at 8 μm. The following probes were employed: chick BMP4 (Francis-West et al., 1994), chick noggin (Reshef et al., 1998), chick rhoB (Liu and Jessell, 1998), slug (Nieto et al., 1994) and e-cad-6B (Nakagawa and Takeichi, 1995).

RESULTS

The relationship between the onset of neural crest migration, somitogenesis and expression of specific markers.

Previous studies have shown that the onset of migration of neural crest cells is related to the dynamic process of somitogenesis (Loring and Erickson, 1987; Teillet et al., 1987). In 15- to 30-somite-stage embryos, crest cells at the level of the segmental plate are still confined to the dorsal neural tube (Figs 1, 2F; Teillet et al., 1987). Emigration of the first neural crest cells becomes apparent at levels opposite the epithelial somites. These early cells express the HNK-1 marker in 25- to 30-somite, but not younger, embryos where they are only visible by morphology or with the quail marker (Fig. 2I, arrowheads; Teillet et al., 1987). Upon somite dissociation, visible by morphology or with the quail marker (Fig. 2I, 30-somite, but not younger, embryos where they are only shown for later stages). The expression of HNK-1 continues their emigration from the neuroepithelium and begin simultaneously invading the longitudinal pattern of BMP4 expression, as already detailed (Fig. 2C,L; Loring and Erickson, 1987; Teillet et al., 1987; Debby-Brafman et al., 1999).

As previously described, premigratory crest cells localized opposite the unsegmented mesoderm express cadherin-6B and rhoB (Figs 1E,F, 8; Liu and Jessell, 1998; Nakagawa and Takeichi, 1995). These two genes continue to be detected in premigratory crest cells at the dorsal tube in areas corresponding to dissociated somites where migration away from the neural primordium is well underway (Figs 1, 8; data not shown). The transcription factor Slug is also expressed by the premigratory crest cells along significant lengths of the axis in embryos having less than 22 somites (Fig. 1A,B). However, in contrast to cadherin-6B and rhoB, slug is progressively downregulated in a rostrocaudal direction as development proceeds (Fig. 1C,D), in spite of the observations that, in those areas lacking slug, neural crest cells are being continuously produced and progressively leave the neural tube up to late stages (see Discussion). These observations suggest that the expression of slug is transient and that it characterizes only the early specified crest progenitors at any given axial level.

The decreasing caudorostral distribution of noggin in the dorsal neural tube correlates with the onset of neural crest migration

We have analyzed the expression patterns of BMP4 and of its inhibitor noggin in relation to the emigration of crest progenitors. BMP4 transcripts were homogeneously expressed in the dorsal neural tube along the axis from regions adjacent to the rostral segmental plate to the most rostral somites. This pattern was consistently observed in 15- to 40-somite embryos (Fig. 2A,D,G,J; data not shown; Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998; Tonegawa and Takahashi, 1998). In striking contrast to the uniform longitudinal pattern of BMP4 expression, noggin mRNA was

![Fig. 1. Stage-dependent changes in the expression pattern of slug in the avian embryo. Comparison with cadherin-6B and rhoB. Embryos were hybridized with digoxigenin-labeled probes to detect slug, cadherin-6B and rhoB transcripts. Slug mRNA is present on both premigratory and migrating crest cells at the level of the mesencephalon (A) and rhombencephalon (B). In contrast, it characterizes only premigratory crest cells in trunk regions of the neuraxis (see also Fig. 7). Note that slug is expressed throughout the entire length of the axis in 16-somite-stage embryos but, at increasing developmental stages, its expression pattern becomes progressively restricted to caudal regions (arrowheads in C and D indicate the rostral boundary of expression, see also Fig. 7). For example, in 22-somite embryos, slug signal extends along the tube opposite the segmental plate and the 10 last-formed somites. In 34-somite embryos, it spans only the segmental plate level of the axis. The expression of cadherin-6B and rhoB, however, is apparent along the dorsal neural tube at equivalent stages (see E,F and data not shown for later stages).]
dynamically distributed along the rostrocaudal axis of the embryos (Fig. 2B,E,H,K). While in 10-somite-stage embryos, noggin expression in midline tissues was confined to Hensen’s node and notochord (data not shown; Capdevila and Johnson, 1998; Tonegawa and Takahashi, 1998), in older embryos (15-30 somites), expression of noggin transcripts was also detected in the dorsal neural tube. Expression in this site was strong opposite the segmental plate mesoderm, somewhat weaker adjacent to the newly formed epithelial somites (B,H) and totally absent opposite dissociated somites (B,K). The observed disappearance of noggin from the dorsal neural tube opposite dissociated somites corresponds approximately to the shift in mesodermal noggin expression from the lateral somite to the medial portion of the dermomyotome and to its disappearance from the notochord/floor plate (B,E,H, and see also Capdevila and Johnson, 1998; Hirsinger et al., 1997 for detailed mesodermal patterns). Note that the expression pattern of Noggin is reciprocal to the emergence of crest cells from the tube. (F) Crest cells are still an integral part of the epithelium. (I) The first cells begin migrating dorsoventrally (arrowheads) but do not express yet significantly the HNK-1 epitope. (L) HNK-1-positive crest cells migrate into the paraxial mesoderm. Abbreviations, DM, dermomyotome; LPM, lateral plate mesoderm; NT, neural tube; NO, notochord; S, somite. Bar, 60 μm for D-L.

Overexpression of Noggin along the axis prevents emigration of neural crest cells in ovo and in cocultures

The above observations led us to the hypothesis that the changing levels of Noggin create graded effective concentrations of BMP along the axis, which in turn affect the emigration of specified neural crest cells from the neuroepithelium. To begin testing this hypothesis, the normal caudorostral gradient displayed by noggin along the axis was disrupted by grafting Noggin-producing CHO cells adjacent to the dorsal aspect of the neural tube. Grafting was performed on embryos aged 16-20 somite pairs at the level corresponding to the segmental plate mesoderm and the two last-formed somites. At this stage and axial levels, the dorsal midline of the neural tube strongly expresses the crest-specific markers slug, rhoB and cadherin-6B (Figs 1, 8; data not shown), implying that neural crest cells are indeed already specified by the time Noggin concentrations were modified.

20 hours following grafting, the mesoderm at the grafted axial levels developed into fully dissociated somites (Fig. 2A,B). Analysis of serial transverse sections revealed that, in 90% of embryos that received implants of control CHO cells (n=16), neural crest cells expressing the HNK-1 epitope were present along characteristic migratory pathways. These included the rostral domain of the sclerotome, the subdermomyotomal pathway and the intersomitic spaces (Fig.

**Fig. 2.** The expression patterns of BMP4 and noggin along the dorsal neural tube in relation to the emigration of neural crest cells. (A,D,G,J) In situ hybridization of a 22-somite-stage embryo with a BMP4-specific probe. (B,E,H,K) In situ hybridization of a 24-somite-stage embryo with a Noggin-specific probe. (C,F,I,L) A 22-somite-stage embryo immunostained with the HNK-1 antibody. Sections were subsequently counterstained with Hematoxylin. Note the homogeneous expression of BMP4 signal along the axis, also revealed in the transverse sections from distinct areas along the axis (D,G,J). In contrast, noggin expression in the dorsal tube follows a caudocranial gradient, strong opposite the segmental plate (B,E), weaker opposite epithelial somites (B,H) and totally absent opposite dissociated somites (B,K). The observed disappearance of noggin from the dorsal neural tube opposite dissociated somites corresponds approximately to the shift in mesodermal noggin expression from the lateral somite to the medial portion of the dermomyotome and to its disappearance from the notochord/floor plate (B,E,H, and see also Capdevila and Johnson, 1998; Hirsinger et al., 1997 for detailed mesodermal patterns). Note that the expression pattern of Noggin is reciprocal to the emergence of crest cells from the tube. (F) Crest cells are still an integral part of the epithelium. (I) The first cells begin migrating dorsoventrally (arrowheads) but do not express yet significantly the HNK-1 epitope. (L) HNK-1-positive crest cells migrate into the paraxial mesoderm. Abbreviations, DM, dermomyotome; LPM, lateral plate mesoderm; NT, neural tube; NO, notochord; S, somite. Bar, 60 μm for D-L.
Moreover, the morphology of the dorsal neuroepithelium apposed to the grafted cells was normal resembling that of intact embryos (Fig. 3A). In striking contrast, in 80% of embryos (*n*=15) that received CHO-Noggin cell implants, no migration into the mesoderm was observed along the grafted area despite normal somitogenesis and subsequent somite dissociation into dermomyotomal and sclerotomal domains (Fig. 3B). Instead, crest cells remained confined to the dorsal tube, some protrude into its lumen and express HNK-1. In both cases, somite development is normal. Note that brown cells in the dermomyotome in B reflect non-specific staining of erythrocytes as separately assessed at high magnification.

To further characterize the effects of Noggin on emigration of neural crest cells, explants of neural primordia (neural tube comprising premigratory crest cells) were explanted on top of control CHO (C) or CHO-Noggin (D) monolayers. A day later, cocultures were stained with HNK-1. Similar results were obtained in at least 12 explants within each category. Abbreviations, DM, dermomyotome; NO, notochord; NT, neural tube; Scl, sclerotome. Bar, 30 μm.

**Fig. 3.** Overexpression of Noggin inhibits emigration of neural crest cells from the neural tube. (A,B) Transverse sections through the thoracic areas of embryos that received grafts of control CHO cells (A) or CHO-Noggin cells (B). Aggregates of CHO cells were grafted dorsal to the neural tube (asterisks) in 16- to 20-somite-stage embryos as described under Methods. Embryos were further incubated for 24 hours, fixed and processed for HNK-1 immunostaining. Note the normal pattern of migration of neural crest cells into the mesoderm of embryos that received control cells. In contrast, no crest cells are observed in the mesoderm of Noggin-treated embryos. Instead, cells remained confined to the dorsal tube, some protrude into its lumen and express HNK-1. In both cases, somite development is normal. Note that brown cells in the dermomyotome in B reflect non-specific staining of erythrocytes as separately assessed at high magnification. (C,D) Explants of isolated neural primordia (neural tube comprising premigratory crest cells) were explanted on top of control CHO (C) or CHO-Noggin (D) monolayers. A day later, cocultures were stained with HNK-1. Abbreviations, DM, dermomyotome; NO, notochord; NT, neural tube; Scl, sclerotome. Bar, 30 μm.

**Fig. 4.** Antagonistic effects of BMP4 and Noggin on emigration of neural crest cells from cultured neural tubes. Isolated neural tubes containing premigratory neural crest cells were seeded onto fibronectin-coated dishes and grown overnight in serum-free medium alone (A), in medium containing Noggin (B), BMP-4 (C), a combination of Noggin and BMP4 (D), and Follistatin (E). Whereas Noggin inhibited emigration of HNK-1-positive crest cells, BMP4 alone caused increased emigration when compared to controls and overcome the effect of Noggin when both factors were added simultaneously. Follistatin had no effect on the exit of crest cells from the tube. Similar results were obtained in at least 10 explants within each category. Bar, 180 μm.
emigrated when neural tubes were explanted on top of the Noggin-producing line (Fig. 3D). Thus, overexpression of Noggin inhibits emigration of neural crest cells both in vivo and in explants.

A balance between Noggin and BMP4 activities specifically inhibits emigration of neural crest cells from the neural tube

To further examine whether inactivation of BMP4 accounts for the Noggin-dependent inhibition of neural crest emigration, explants of neural tubes containing premigratory crest were seeded onto fibronectin-coated dishes in serum-free medium (A-C), in the presence of soluble Noggin (D-F) or BMP4 (G-I). Individual tubes were observed at 2 hour intervals and characteristic times are presented. Note in B, the epithelioid outgrowth of the first neural crest cells, which become mesenchymal after overnight growth. In contrast, in BMP4-treated dishes, the first fully mesenchymal cells are seen by 5-6 hours after explantation and, by 8 hours, a significant number is present on the substratum (H). In the presence of Noggin, cells fail to exit at all time points examined. Bar, 400 μm.

2), we examined the effects of BMP4 added in excess to the endogenous factor on neural crest emigration. As shown in Fig. 4C, addition of BMP4 caused a dramatic outgrowth of HNK-1-positive cells from all explants tested (see also Fig. 5G-I) when compared to controls (Fig. 4A). Moreover, addition of BMP4 to Noggin-treated premigratory tube fragments reversed the Noggin-induced inhibition (Fig. 4D). These results suggest that a balance between BMP4 and Noggin plays a role in the onset of neural crest migration.

In previous studies, both BMP4 and BMP7 of ectodermal origin were found to be required for initial specification of neural crest cells (Liem et al., 1997). BMP7 is also expressed in the dorsal neural tube following its closure, and its activity is blocked by Follistatin but not by Noggin (Liem et al., 1997). Emergence of neural crest cells upon treatment of the explanted tubes with Follistatin (up to 100 nM) was similar to control wells (Fig. 4E). We conclude that it is highly unlikely that BMP7, as well as other factors that are sensitive to Follistatin (i.e.; BMP6, activin) have physiological relevance in this process.

Thus, although both BMP4 and BMP7 are required for specifying the neural crest phenotype, BMP4 but not BMP7 stimulates at a later stage the emigration of specified precursors (see Discussion).

Noggin/BMP4 interactions affect initial delamination of neural crest cells from the neural primordium

To analyze the possible effects of modulating the levels of BMP4 on initial delamination of crest cells from the neural primordium, the behavior of neural tube explants was followed as a function of time in culture. Neural tubes from levels adjacent to either the segmental plate (Fig. 5) or epithelial somites (Fig. 6) were grown on fibronectin-coated dishes. In control explants derived from segmental plate levels and grown in a serum-free medium, no apparent migration of crest cells was observed prior to 8 hours after adhesion to the substratum. By this time, an epithelial-like sheath of cells could be seen around the tubes. These cells were previously identified as ‘epithelioid’ neural crest progenitors as they were of
intermediate morphology between the initial epithelial and the subsequent mesenchymal conformation (Newgreen and Minichiello, 1995). Only a few mesenchymal cells were apparent at this stage in the periphery of the epithelioid sheath (Fig. 5B). By 20 hours following explantation, a significant amount of neural crest cells with mesenchymal morphology was present around the entire neural tube, resulting both from increased delamination as well as proliferation of the crest progenitors that had migrated away from their source (Fig. 5C and data not shown). In contrast to this dynamic process of cell emigration, no significant exit of neural crest cells could be detected in explants treated with soluble Noggin at any of the examined time points, except for a general flattening of the tubes apparent 20 hours postexplantation (Fig. 5D-F). The opposite effect was obtained in explants treated with BMP4 (50 ng/ml). As early as 8 hours following seeding, many neural crest cells bearing a full mesenchymal phenotype were already present on the substratum. Interestingly, no epithelioid sheath of cells was apparent around the tube, suggesting that BMP4 causes a rapid de-epithelialization of progenitors that would have otherwise undergone a stepwise delamination (Fig. 5 compare H with B). Consistent with the picture seen at 8 hours, 12 hours later a dramatic outgrowth of mesenchymal crest cells could be seen in all explants treated with BMP4 when compared to untreated controls (Figs 4A,C, 5I). As most crest cells proliferate under the present culture conditions (Ziller et al., 1987 and data not shown), the increased number of cells at this time point most likely reflects continuous proliferation of an initially higher number of cells that had delaminated from the BMP4-treated tubes. In contrast to the effect of BMP4 on crest cell delamination, which was clearly detectable both at 10 and 50 ng/ml, similar concentrations of BMP7 or BMP2 were without effect on crest delamination when compared to untreated controls (not shown). However, at high BMP concentrations (300 ng/ml), all three factors were similarly active (data not shown). Since the emergence of crest cells from the neural tube is inhibited by Noggin, which preferentially inactivates both BMP4 and BMP2, but not by Follistatin, which inactivates BMP7, and since BMP2 is not expressed in the dorsal part of the avian neural tube, the delamination induced by high doses of either BMP2 or BMP7 is likely to reflect a pharmacological rather than a physiological effect of these factors.

Next we examined whether a similar, Noggin-dependent mechanism, accounts for regulated emigration of crest cells from the tube at more rostral levels of the axis. To this end, a time course of crest emigration was performed on neural tubes isolated from the five epithelial somite pairs of 16- to 20-somite-stage embryos. At this axial level, crest cells already begin delaminating from the tube and endogenous noggin signal is very low to undetectable whereas that of BMP4 is unchanged (Fig. 2) indicating that the effective levels of BMP4 protein are higher than more caudally along the axis. Consistent with this fact, control neural tube explants from this area revealed a very rapid exit of neural crest progenitors when compared with the premigratory segmental plate-level explants (compare Figs 5 and 6). Initial cell emigration was already clearly visible 1 hour after attachment of the tissue fragments to fibronectin and the number of migrating cells progressively increased by 4 and 8 hours (Fig. 6A-C). Unlike the younger explants, in the somewhat older tubes, emigrating cells were readily mesenchymal and no intermediate epithelioid conformation could be observed. This resembles the situation observed in young explants treated with BMP4 (Fig. 5H) (see also Newgreen and Gibbins, 1982, for differences in crest outgrowth between segmental plate and epithelial levels). Similar to the inhibition exerted by Noggin treatment on the premigratory explants and in striking contrast to the control situation, no emigration of neural crest cells from the epithelial-level tubes was observed upon Noggin addition during the 8 hour period examined (Fig. 6D-F), further confirming that Noggin inhibits the initial separation of crest
cells from the neural primordium. Taken together, these results suggest that an intrinsic gradient of BMP activity, created by varying levels of Noggin along the rostrocaudal axis of the dorsal tube, regulates delamination of specified neural crest cells at trunk regions of the neuraxis.

**Effects of Noggin-dependent BMP-4 inhibition on the expression of genes that characterize the premigratory phase of neural crest cells**

*slug* is not part of the BMP4-induced cascade related to neural crest delamination

As BMP4 was previously shown to induce de novo expression of *slug*, so far the earliest marker of neural crest specification (see Introduction), the expression of this gene was examined in control and Noggin-treated embryos. Because experimental embryos already expressed *slug* along the axis at the beginning of the treatments, we wished to assay for possible changes in maintenance of the *slug* signal. In situ hybridization performed after overnight incubation, when embryos attained between 28-30 somite pairs, revealed that similar to the situation in intact embryos (Fig. 1), *slug* mRNA was almost totally downregulated from relevant regions of the axis in both CHO-control and CHO-Noggin-treated embryos (data not shown). Thus, experimental embryos were fixed 10 hours following grafting when an average of 6 somite pairs were added to the axis. In both control and CHO-Noggin-grafted cases, *slug* expression became restricted to the premigratory crest up to the level of the 10-13 last-formed somites when compared to younger embryos, where the entire length of the dorsal tube expressed *slug*. In addition, no apparent differences were detected in signal intensity between the treatments in any of the experimental embryos (*n* = 11 for CHO control and *n* = 11 for Noggin treatments, Fig. 7A,B; data not shown).

Likewise, treatment of explanted neural tubes with Noggin, which resulted in the inhibition of neural crest emigration, had no effect on the expression of Slug immunoreactivity, which both under control and experimental conditions remained confined to the neural tube (Fig. 7C,D, see for comparison the...
pattern of HNK-1 immunoreactivity in E,F). Significantly, in vitro Slug protein could still be detected 20 hours following explantation of the neural tubes, whereas *slug* mRNA (Fig. 1) and protein (data not shown) in ovo are downregulated at an equivalent time. This apparent discrepancy might therefore reflect differences in the regulation of the expression of *Slug* in vivo compared to culture conditions.

Taken together, these results suggest that in spite of BMP4 being one of the factors responsible for the initial expression of *slug*, inactivation of BMP4 at a stage that follows its initial expression and thus specification of crest cells, does not affect maintenance of Slug production and therefore is unlikely to alter the state of neural crest commitment.

The expression of both *cadherin-6B* and *rhoB* is downregulated by Noggin-induced BMP4 inactivation. *Cadherin-6B* characterizes premigratory neural crest cells and is expressed along the neuraxis by the time the levels of BMP4 are experimentally modified (Fig. 1). Grafting of control-CHO cells dorsal to the neural tubes had no effect on the expression of *cadherin-6B* transcripts visualized 10 hours following the operation, as revealed both in whole mounts and in serially sectioned embryos (Fig. 8A,B, *n*=9 out of 10 embryos, 90%). In contrast, at an equivalent time, 82% of embryos that received grafts of CHO-Noggin cells showed greatly reduced up to undetectable levels of signal in the dorsal tube (Fig. 8C,D, *n*=11).

A similar situation was observed regarding the regulation of *rhoB* expression. Grafting of CHO-Noggin cells caused, 10 hours later, a dramatic decrease in *rhoB* signal in the dorsal neural tube of all experimental embryos (*n*=7, Fig. 8G,H), whereas no effect could be detected upon grafting of the respective control line (*n*=7, Fig. 8E,F). Moreover, the observed downregulation of *rhoB* mRNA was still apparent 24 hours following grafting in all embryos (*n*=6 and *n*=8 for control and CHO-Noggin treatments, respectively; data not shown). Thus, the Noggin-induced downregulation of both *cadherin-6B* and *rhoB* precedes the observed failure of neural crest cells to emigrate from the neural tube in ovo. These results show that dorsal neural tube-derived BMP4 is required for the continuous transcription of both *cadherin-6B* and *rhoB*, and therefore suggest that these genes are involved in the BMP4-dependent cascade leading to neural crest delamination.

**DISCUSSION**

In the present study, we report that BMP4, expressed in the
dorsal part of the neural tube, triggers the delamination of specified neural crest cells from the neural primordium. Increasing concentrations of BMP4 are likely to be reached by progressive downregulation of the expression of its specific inhibitor Noggin, which occurs in a caudorostral direction along the dorsal domain of the neural tube. Thus, BMP4 and Noggin act as a regulatory unit to affect the epitheliomesenchymal conversion of premigratory progenitors. Furthermore, we have begun to uncover the molecular cascade that acts downstream of BMP4 to drive crest cell delamination. We find that two genes, cadherin-6B and rhoB, are likely to be involved in this process, as their levels are affected by modifying the activity of BMP4 at stages that follow their initial expression along the axis. In contrast, the levels of slug remain unchanged, suggesting that this gene may not be involved in the BMP4-mediated delamination process at trunk levels of the axis.

Successive and separable effects of BMP4 on specification and emigration of neural crest cells

The experiments described in this study were all performed at levels of the trunk in which the neural folds had completed their closure into a tube-like structure. At these levels, slug, cadherin-6B and rhoB are already expressed in the dorsal tube, thereby distinguishing presumptive neural crest cells from other neuroepithelial cells. Precisely at these stages, single-cell analysis of dorsal neural tube progenitors revealed the earliest segregation of crest cells from other neural tube lineages (Bronner-Fraser and Fraser, 1988, 1989). Thus, neural crest cells represent already a specified population of dorsal neural tube cells by the time that we expose the system to experimental manipulations. Further support for the idea that Noggin antagonistically affects delamination of specified progenitors comes from results of our experiments revealing significant inhibition of crest delamination in the presence of Noggin even in neural tubes, opposite epithelial levels of the axis, that contain crest cells ready to leave the neural primordium (in fact, at this level a few cells had already left the tube, Fig. 2). Consistent with the present findings, Selleck et al. (1998) have reported that grafting Noggin-producing cells around the area of the closing neural tube also suppressed neural crest cell emigration. The lack of effect of the Noggin-producing cells on emigration of neural crest progenitors observed by the same authors at more rostral levels of the axis may be due to grafting of the cells into the lumen of the closed neural tube, a procedure that in our hands caused the precipitation of the grafted cells to the bottom of the lumen. This grafting method precluded direct contact of the cells with the dorsal tube and consequently an effect on crest cells (our unpublished observations).

Significantly, we observed no effect of Noggin treatment at this stage on the expression of slug transcripts following grafting of Noggin-producing cells in ovo, nor did we detect any apparent decrease in Slug immunoreactivity in Noggin-treated explants. Moreover, in the continuous presence of Noggin, crest cells expressing HNK-1 had accumulated within the dorsal neural tube where they normally do not express this epitope (Figs 3, 4B), suggesting altogether no adverse effect of the treatment on cell survival. These results show that following initial specification of neural crest cells, BMP4 signaling is required for the emigration of crest progenitors from the neural anlage but not for the maintenance of slug expression. This notion is further supported by observations of continuous expression of BMP4 in the dorsal tube and of crest emigration despite a progressive downregulation of slug in the same area (see Fig. 1). The present results are at apparent variance with those reported by Selleck et al. (1998), who found a decrease in slug expression upon grafting of Noggin-producing cells following closure of the neural tube. We find it difficult to address the reasons for this difference because the exact timing of slug examination following cell grafting was not mentioned in the above study. Since we find that slug mRNA expression follows a highly dynamic spatiotemporal pattern, knowledge of the time of embryo fixation postmanipulation is critical for understanding possible variations.

Altogether, our findings indicate that misexpression of BMP4/Noggin did not affect the specification state of neural crest cells. Consistent with this notion, Liem et al. (1997) have shown that the competence of neural epithelium to generate neural crest cells in response to BMP is transient. The switch in the ability of BMP4 to generate neural crest cells or to stimulate their delamination may coincide with the downregulation of BMP4 in the ectoderm and the beginning of its synthesis in the dorsal neural tube. Previous experiments were unable to discriminate between these two rather separable activities of BMP, as they challenged naïve explants of neural primordium with factors for several days, thus inducing the whole cascade of crest development that includes expression of slug followed by cadherin 6, rhoB and then HNK-1 immunoreactivity and crest migration (see for example Liem et al., 1995, 1997; Liu and Jessell, 1998).

Consistent with the present findings implying a role for BMP4 in epitheliomesenchymal conversion of premigratory neural crest cells, members of the TGF superfamily, including BMPs, were reported to be expressed in sites where these morphogenetic changes occur (Bitgood and McMahon, 1995) and to mediate the transition between epithelium and mesenchyme. For instance, TGFβ3 and BMP2 were implicated in the endothelial-mesenchymal transition that gives rise to the endocardial cushions of the heart (Nakajima et al., 1997, 1998; Potts et al., 1991; Yamagishi et al., 1998). In addition, BMPs were found to mediate interactions between epithelium and mesenchyme in various developmental systems such as the tooth (Aberg et al., 1987; Dassule et al., 1998; Keranen et al., 1998), the limb (Hofmann et al., 1996) and the face (Francis-West et al., 1994).

The specificity of BMP4-induced neural crest emigration

Previous studies have shown selectivity in the inhibitory effects of Noggin and Follistatin towards distinct BMP family members. For instance, Noggin preferentially binds and inactivates BMP4 and BMP2 (Liem et al., 1997; Zimmerman et al., 1996) and Follistatin blocks the activity of activin, BMP6 and BMP7 (Lee et al., 1998; Nakamura et al., 1990; Yamashita et al., 1995). In contrast, Dorsalin-1 is not inhibited by either of them (Liem et al., 1997). Our data show that emigration of neural crest cells is fully inhibited by Noggin both in vivo and in explants and that Follistatin has no effect. Moreover, BMP2 is not expressed in the dorsal tube of avian embryos (Liem et al., 1997). Consistent with the above, treatment of explants...
with exogenous BMP2 or BMP7 at concentrations in which BMP4 already revealed delamination activity, had no measurable effect (yet, high concentrations of all factors were similarly active). These results strongly suggest that BMP4, but not other BMPs among those present in the dorsal neural tube, is the endogenous factor responsible for crest emigration. Consistently, BMP4 is expressed in the dorsal tube at the appropriate stages, accelerates emigration of crest cells when added in excess to the endogenous factor and rescues the Noggin-induced inhibition.

GDF 6/7 is a new member of the BMP family that has been recently cloned in chick and shown to be present at stage 20 (43 somite pairs, approximately) of development in the dorsal neural tube (Lee et al., 1998). As GDF 6/7 is also sensitive to Noggin (Lee et al., 1998), the possibility remains open that this factor is involved as well in driving emigration of neural crest cells, provided it is also expressed at somewhat earlier stages when crest cells leave the neural primordium at various levels along the neuraxis (10-35 somite pairs). Additional members of this large family, TGFβ1 and TGFβ2, were shown to reduce in vitro the delay of neural crest cell emigration from premigratory-level explants (Delanmet and Duband, 1992), but it remains unclear whether they are expressed at the appropriate time in the dorsal neural tube. So, although several members of the family could affect crest delamination when added pharmacologically (see also Basler et al., 1993; Liem et al., 1995, 1997), only selective inactivation of the endogenous factor/s, like the Noggin-dependent inhibition of BMP4 activity shown here, provides direct evidence for a physiological role.

It is interesting to point out that induction of crest cells in the neuroepithelium requires both BMP4 and BMP7 of ectodermal origin, as only combined treatment with Noggin and Follistatin entirely abolish cell specification (Liem et al., 1997). In contrast to the above, BMP4 appears to be sufficient to control emigration of already specified neural crest cells (this study). Likewise, only GDF7 is required for specification of D1A neurons and other BMPs for generation of the D1B neuronal subtype (Lee et al., 1998). Thus, while ectodermal BMPs appear to act in a redundant fashion, dorsal neural tube-derived BMPs have distinct functions towards various dorsal cell types.

It is unknown at present whether the interplay between Noggin and BMP4 here reported to regulate neural crest cell emigration in avian embryos, is also operative in other species. In both Xenopus and mouse, noggin is expressed in the dorsal part of the neural tube (Smith and Harland, 1992; McMahon et al., 1998); however, it is unclear whether its pattern is homogeneous or alternatively, whether it decreases gradually along the axis in correlation with emigration of crest progenitors. Additional inhibitors of BMP activity are being continuously discovered (Gilka et al., 1998; Hsu et al., 1998; Joseph and Melton, 1997; Mariani and Harland, 1998; Pearce et al., 1999; Stanley et al., 1998) and their possible expression in the dorsal tube and physiological relevance to crest delamination remains an open question. Along the same line, the mouse dorsal neural tube expresses GDF7, BMP6 and BMP7 (Lee et al., 1998). Thus, the possibility exists that different members of the TGFβ superfamily as well as different BMP inhibitors might account for the regulation of crest cell delamination in different species.

**Molecules involved in neural crest delamination**

The local downregulation in the expression of cadherin-6B and rhoB upon noggin overexpression, suggests that these genes are part of a molecular cascade triggered by BMP4, that leads to the separation of neural crest cells from the neural tube. A role for rhoB in this process has been already suggested based on localization and inhibition experiments (Liu and Jessell, 1998). The present results are consistent with and further extend the findings by Liu and Jessell (1998), who reported that inactivation of rhoB inhibits crest cell delamination without altering expression of slug. Altogether, the present and previous results (Liu and Jessell, 1998) demonstrate that BMP4 is required both for de novo induction as well as for continuous maintenance of rhoB, and they indicate that BMP4-dependent maintenance of rhoB expression is required for delamination of crest progenitors.

In contrast to RhoB, the function of Cad6B remains unknown. On one hand, we observe a downregulation in expression of cad6B mRNA upon inactivation of BMP4, suggesting that BMP4 is required for continuous transcription of the gene. These results are consistent with previous findings showing that BMP4 stimulates de novo expression of cad6B (Liu and Jessell, 1998). On the other hand, BMP4 triggers neural crest delamination (this study), a process that eventually results in the disappearance of Cad6B from the emigrating cells. In light of these findings, the role/s of Cad6B in delamination of premigratory crest cells remain to be clarified. Based on its identity as an adhesion molecule, continuous expression of this protein would presumably be required for crest cells to maintain the strong intercellular interactions that characterize the epithelial state. At the same time, maintenance of cadherin-6B expression by BMP4 signaling could be important to modulate events leading to subsequent cell delamination, such as the status of protein phosphorylation (see Brennan et al., 1999 and refs therein).

In contrast to the effects on cadherin-6B and rhoB, inactivation of BMP4 following initial expression of slug, had no effect on the maintenance of Slug expression either at the mRNA or protein levels. This result would indicate that Slug activity is not sufficient for emergence of neural crest cells from the neuroepithelium, at least not as part of a BMP4-induced cascade. In further support of this notion, it was reported that neural crest cells still leave the neural primordium by stages 18-20 (35-45 somite pairs) of development at trunk levels of the axis (Erickson et al., 1992; Reed et al., 1998). At these stages and axial levels, cadherin-6B and rhoB are still expressed (unpublished data and see Liu and Jessell, 1998) but slug is not transcribed any longer (see Fig. 1 and unpublished results). By contrast, previous results have implicated this transcription factor in crest delamination (Nieto et al., 1994). However, several differences exist between both experimental systems. First, inhibition of slug was performed by Nieto et al. (1994) at cranial levels of the axis in 8- to 10-somite-stage embryos. At these levels, slug is expressed both in premigratory as well as in migrating progenitors (Nieto et al., 1994; Fig. 1A), whereas in the trunk it is exclusively expressed in the premigratory population (Fig. 1; data not shown). This difference may be significant, as at these two axial levels, Slug may play distinct functions by regulating transcription of different genes. Second, Nieto et al. (1994) have noticed that
following application of the antisense oligonucleotides, there was a transient downregulation in slug expression that recovered at later stages. Thus, the possibility cannot be excluded that under those experimental conditions, Slug inhibition had an adverse effect on the actual specification of neural crest cells, which was the actual reason for the phenotype obtained.

The significance of an intrinsic BMP/Noggin balance in various developing systems

Recent evidence reveals that the secreted proteins Noggin, Chordin and Follistatin, directly bind BMP in dorsal regions of early embryos, thus preventing it from binding its receptor (Fainsod et al., 1997; Piccolo et al., 1996; Zimmerman et al., 1996). For instance, different concentrations of BMP4 or Noggin were shown to shift the specification of progenitors to more ventral or dorsal fates, respectively, suggesting that a modulation of BMP levels specifies different cell types along the dorsoventral axis (reviewed in Graff, 1997; see also Dosch et al., 1997; Knecht and Harland, 1997; Neave et al., 1997; Wilson et al., 1997). Somite patterning and formation of different somite derivatives was also shown to involve opposing activities of BMP and Noggin (Capdevila and Johnson, 1998; Hirsinger et al., 1997; Marcelle et al., 1997; McMahon et al., 1998; Reshef et al., 1998; Tonegawa and Takahashi, 1998). Furthermore, a Noggin-mediated antagonism of BMP signaling was found to be essential for neural tube ventralization (McMahon et al., 1998) and an interaction between BMP7 and Chordin was reported to be important for differentiation of ventral midline cells at dienecephalic levels of the neuraxis (Dale et al., 1999).

As for neural crest induction, studies in Xenopus revealed that overexpression of Noggin in ventral regions can induce ectopic neural crest (Mancilla and Mayor, 1996; Mayor et al., 1997) and that low levels of BMP within the context of a gradient created by interactions with Noggin may be required, along with additional factors, for specification of this cell type (Marchant et al., 1998; Mayor et al., 1997). Similarly, in zebrafish, a pathway involving BMP2b/Swirl was recently suggested to underly development of neural crest cells (Nguyen et al., 1998). In avians, both BMP 4 and BMP7 from the ecdectom were suggested to specify the neural crest phenotype (Liem et al., 1995), yet Noggin alone was not sufficient to abolish the acquisition of crest-specific markers (Selleck et al., 1998), most likely because of residual BMP7 activity. Here we show that, following specification of crest progenitors, both BMP4 and noggin are expressed in the dorsal midline of the neural tube, the region where delamination of crest cells is about to take place. Whereas BMP4 is expressed in the dorsal portion of the neural tube in a homogeneous and rather stable manner over a wide developmental period, noggin reveals a highly dynamic pattern. The latter gene is strongly expressed in caudal areas of the axis and is downregulated at a time that coincides with the onset of crest cell emigration. These expression patterns, together with experimental data showing that disruption of the Noggin gradient by overexpression of either noggin or BMP4, alters crest delamination, provide additional evidence for the pivotal role and the general significance of the antagonistic interactions between BMPs and their inhibitors in patterning crucial events in morphogenesis.

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