Neural crest induction in *Xenopus*: evidence for a two-signal model

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SUMMARY

We have investigated the molecular interactions underlying neural crest formation in *Xenopus*. Using chordin overexpression to antagonize endogenous BMP signaling in whole embryos and explants, we demonstrate that such inhibition alone is insufficient to account for neural crest induction in vivo. We find, however, that chordin-induced neural plate tissue can be induced to adopt neural crest fates by members of the FGF and Wnt families, growth factors that have previously been shown to posteriorize induced neural tissue. Overexpression of a dominant negative XWnt-8 inhibits the expression of neural crest markers, demonstrating the necessity for a Wnt signal during neural crest induction in vivo. The requirement for

Wnt signaling during neural crest induction is shown to be direct, whereas FGF-mediated neural crest induction may be mediated by Wnt signals. Overexpression of the zinc finger transcription factor Slug, one of the earliest markers of neural crest formation, is insufficient for neural crest induction. Slug-expressing ectoderm will generate neural crest in the presence of Wnt or FGF-like signals, however, bypassing the need for BMP inhibition in this process. A two-step model for neural crest induction is proposed.

Key words: Neural crest, *Xenopus*, Slug, BMP, Wnt, FGF, Chordin, Molecular interaction

INTRODUCTION

During the process of neural induction in vertebrates, the ectoderm becomes divided into several distinct tissue types including neural plate, epidermis and neural crest. Much recent work, mainly in *Xenopus*, has focused upon signals that can direct prospective epidermis to adopt neural fates (reviewed in Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1997). In the models of neural induction that have emerged, a relatively high level of signaling by bone morphogenetic proteins (BMPs), which are initially expressed throughout the ectoderm, is sufficient to repress neural and induce epidermal fates (Wilson and Hemmati-Brivanlou, 1995). Neural-inducing molecules such as noggin (Lamb et al., 1993), chordin (Sasai et al., 1994) and follistatin (Hemmati-Brivanlou et al., 1994), which can bind to BMPs and, presumably, prevent them from activating their receptors (Zimmerman et al., 1996; Piccolo et al., 1996; Fainsod et al., 1997), inhibit BMP signaling causing cells to adopt neural fates. This process is accompanied by a loss of BMP4 transcripts from the induced neural plate. While this model of neural induction is clearly simplistic, and many complexities are sure to emerge, it nonetheless reflects the significant progress that has been made in understanding how two of the three major ectodermally derived tissues are specified. By comparison, much less is understood about the molecular basis of neural crest induction.

The neural crest is a population of cells that is induced at the border between neural plate and epidermis. These cells eventually become migratory and populate diverse regions throughout the embryo where they give rise to most of the peripheral nervous system (PNS), epidermal pigment cells and craniofacial cartilage (Le Douarin, 1982; Hall and Hörstadius, 1988). In *Xenopus*, prospective neural crest cells can be identified at late gastrula stages (stage 12) by their expression of the zinc finger transcription factor *Slug* (Mayor et al., 1995); this expression is initially restricted to the crest-forming boundary of the neural plate, although at later stages *Slug* is also expressed in lateral plate mesoderm. *Slug* expression is also the first molecular indicator of neural crest induction in avian embryos (Nieto et al., 1994), but its expression commences later, in the closing neural folds. This may indicate a later specification for this cell type in avians.

Early experiments on neural crest induction suggested that graded amounts of the same signal could be responsible for inducing both neural and neural crest cell types. When medial archenteron roof (prospective axial mesoderm) was grafted into the blastocoel of amphibian embryos, competent ectoderm could be induced to form both neural tissue and neural crest, while lateral archenteron roof (prospective paraxial and lateral plate mesoderm) induced only neural crest (Raven and Kloos, 1945). The authors proposed that a neural/neural crest 'evocator' produced by the mesoderm would induce neural crest at low levels, while at high levels it would also induce neural tissue. Interestingly, noggin and chordin are expressed in the axial mesoderm and could represent such an 'evocator.'

More recent experiments in amphibian and avian embryos have suggested that interactions between neural plate and epidermis are sufficient to induce neural crest derivatives (Moury and Jacobson, 1990; Selleck and Bronner-Fraser,

1995), as well as *Slug* expression (Dickinson et al., 1995; Mancilla and Mayor, 1996), when these tissues are juxtaposed in vitro or in vivo. In avian embryos, the ability of epidermis to induce neural crest markers in prospective neural plate can be mimicked by culturing neural plate explants in the presence of BMP4 or BMP7 (Liem et al., 1995). These experiments, together with recent results implicating graded levels of BMP signaling in neural versus epidermal cell fate decisions (Wilson and Hemmati-Brivanlou, 1995), suggest that BMPs may also specify neural crest cell fates in a concentration-dependent manner. Alternatively, the ability of epidermis to induce prospective neural plate to form neural crest may reflect a requirement for a second, non-BMP signal in this process. In addition to epidermal/neural plate interactions, there is also evidence that a signal from the mesoderm may be important for neural crest induction (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996).

We have investigated the molecular mechanisms underlying neural crest induction, using chordin overexpression to inhibit BMP signaling in explants of prospective ectoderm from *Xenopus* blastulae. We find that chordin can induce low level expression of early neural crest markers in a dose-dependent manner. These levels of expression are far lower than those found endogenously, however, and such explants fail to form melanocytes, a neural crest derivative. In contrast, melanocytes and robust expression of neural crest markers, are induced when chordin-expressing explants are cultured with prospective mesoderm. This induction can be mimicked by coculturing chordin-expressing explants with those expressing eFGF (Isaacs et al., 1992) or XWnt-8 (Christian et al., 1991). Similarly, overexpression of the early neural crest marker Slug is not sufficient to induce neural crest in naive ectoderm. Slug-expressing ectoderm can give rise to neural crest, however, when cocultured with explants expressing eFGF or XWnt-8. These results suggest a model in which reduced levels of BMP signaling at the lateral edges of the neural plate provide an initial, weak specification of neural crest fate. Additional signals from the adjacent non-neural ectoderm, the underlying mesoderm or both are then required to enhance and maintain this induction. Evidence is presented that a Wnt signal is required for this process.

MATERIAL AND METHODS

Preparation of embryos and explants

Pigmented and albino embryos were obtained and fertilised in vitro by established methods, dejellied and cultured in 0.1× MMR. Staging of embryos was done according to the normal table of Nieuwkoop and Faber (1967). Embryos were injected in 1× MMR + 3% Ficoll then transferred to 0.2× MMR until further manipulation or harvesting. In vitro transcribed capped messenger RNA was dissolved in water and injected at a volume of 10nl per blastomere. Concentrations of injected mRNA are as noted in the text. In some whole embryo experiments, nuclear GFP (kindly provided by Robert Davis) or nuclear β-galactosidase mRNA was co-injected as a lineage tracer to assist in identifying the injected side of the embryo. Animal pole explants were harvested from late stage 9 embryos unless otherwise noted and cultured in 1× MMR containing 50 μg/ml gentamycin until sibling embryos reached the noted stage. Conjugates were generated by physically juxtaposing explants with watchmaker forceps and allowing them to heal on agar-coated dishes.

Constructs

Xenopus chordin in the vector pCS2+ (Sasai et al., 1994) was a kind gift of Eddy DeRobertis. pSP64T XWnt-8 (Christian et al., 1991) and dominant negative XWnt-8 in pCS2 (Hoppler et al., 1996) were kindly provided by Randy Moon. BMP4 in the vector pCS2+ (Hemmati-Brivanlou and Thomsen, 1995) was a kind gift of Gerry Thomsen. The entire open reading frame of *Xslug* was amplified by low-cycle number PCR using PWO polymerase (Boehringer Mannheim) from stage 17 cDNA and cloned into the vector pCS2+. Amplified products were sequenced on both strands using an automated DNA sequencer. One sequenced clone was identical to the previously published sequence. A second clone differed from the published sequence at five positions, but translated to an identical protein. Both clones performed similarly in overexpression experiments.

Fixation and whole-mount in situ hybridization

Embryos and explants were fixed for 1 hour in MEMFA, and stored dehydrated in 100% methanol. For β-galactosidase staining, embryos were washed 2× in PBS and 2× in staining solution (10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS). Color reaction was carried out overnight at room temperature in staining solution containing 1.5 mg/ml X-gal. In situ hybridization was carried out with digoxigenin-labelled antisense RNA probes as described in Knecht et al. (1995). Alkaline phosphatase detection reactions were carried out using NBT/BCIP (Gibco BRL) as a substrate. *Xslug* (Mayor et al., 1995), *Xtwi* (Hopwood et al., 1989) and *Xsox2* (Mayor et al., 1995) probes were kindly provided by Roberto Mayor, Peter Vize and Rob Grainger, respectively. Embryos and explants were photographed in 100% methanol. For histology, embryos and explants were incubated in Histisol for 30 minutes, embedded in paraplast and sectioned at 10-12 μm.

Reverse transcription-PCR analysis

RT-PCR analysis was carried out as previously described (LaBonne and Whitman, 1994). Serial dilutions of whole embryo cDNA were used to ensure the quantitative nature of the PCR reactions with each primer pair. PCR cycles were for 30 seconds at 94°C, 60 seconds at 55-60°C and 30 seconds at 72°C. Primer pairs utilised were as follows:

Xslug (Mayor et al., 1995)
   U: CAATGCAAGAACTGTTCC
   D: TCTAGGCAAGAATTGCTC
Xsna (Essex et al., 1993)
   U: AAGCACAATGGACTCCTT
   D: CCAATAGTGATACACACC
Xtwi (Hopwood et al., 1989)
   U: AGTCCGATCTCAGTGAAGGGCA
   D: TGTGTGTGGCCTGAGCTGTAG
Epidermal Keratin (Jonas et al., 1985)
   U: CACCAGAACACAGAGTAC
   D: CAACCTTCCCATCAACCA
NCAM (Kintner and Melton, 1987)
   U: CACAGTTCCACCAAATGCCG
   D: GGAATCAAGCGGTACAGA
EF1α (Krieg et al., 1989)
   U: CAGATTGGTGCTGGATATGC
   D: ACTGCCTTGATGACTCCTAG

RESULTS

Manipulation of BMP signaling in whole embryos

To address whether graded amounts of BMP signaling play an instructive role in the specification of neural crest fates, we manipulated the levels of BMP signaling in whole embryos and assayed the effects on expression of an early neural crest

marker, *Xslug*. In order to upregulate BMP signaling in the ectoderm, increasing amounts of BMP4 mRNA were microinjected into one dorsoanimal blastomere at the 8-cell stage. Embryos were cultured until stage 17 when they were harvested for in situ hybridization using a *Xslug* antisense probe. While a range of phenotypes was obtained with each injected dose, the average phenotype increased in severity with dose and a representative phenotype for each dose is presented in Fig. 1.

At the lowest dose (50 pg, *n*=60), most embryos were mildly ventralized and *Xslug* expression was not markedly reduced. The two characteristic domains of *Xslug* expression in the neural folds of the hindbrain were deflected toward the midline (Fig. 1B), reflecting a loss of dorsoanterior character in the transverse neural folds. At higher doses (200 pg, *n*=68) increasing numbers of embryos were severely ventralized, exhibiting considerable microcephaly and *Xslug* expression domains were fused at the ventral aspect of markedly reduced head folds (Fig. 1C). This is likely due to an absence of forebrain and cement gland progenitors normally found in this region. *Xslug* expression was only lost at doses of BMP4 that eliminated the neural folds completely (1 ng, *n*=40) (Fig. 1D). Thus, *Xslug* expression does not appear to be eliminated in response to increases in BMP4 levels, but rather continues to reflect the boundary between the diminishing neural plate and the surrounding non-neural ectoderm. Significantly, no dose of BMP4 was observed to increase neural crest formation at the expense of the neural plate, as might be expected if

intermediate BMP doses were primarily responsible for directing the prospective ectoderm toward neural crest fates.

To examine the effects of decreased BMP activity on *Xslug* expression, 200 pg of chordin mRNA was injected into one cell of 2-cell-stage embryos. At stage 17, these embryos displayed increased levels of *Xslug* expression on the injected side. Ectopic *Xslug* expression was contiguous with endogenous expression domains and expansion was always into more lateral regions, at the expense of non-neural ectoderm, rather than into more medial neural plate regions (Fig. 1E). Ectopic *Xslug* expression was sometimes found in a band extending toward the ventral aspect of the embryo, as depicted in Fig. 1F. At doses of chordin that hyperdorsalized the embryo, *Xslug* expression was found in a band around the circumference of the radially symmetric embryo as has previously been described for noggin (Mayor et al., 1995; and not shown).

Chordin overexpression in ectodermal explants

It has recently been demonstrated that BMPs can specify distinct ectodermal cell fates, including epidermis, cement gland and neural plate, in a concentration-dependent manner (Wilson and Hemmati-Brivanlou, 1995; Wilson et al., 1997). Our observation that reductions in BMP activity following chordin overexpression lead to increases in *Xslug* expression suggested that specific levels of BMP signaling might play a role in the specification of neural crest cell fate. We therefore asked if chordin overexpression was sufficient to induce expression of neural crest markers in ectodermal explants. Chordin mRNA was injected in the animal poles of both cells of 2-cell embryos at doses ranging from 10 pg to 2 ng. Animal
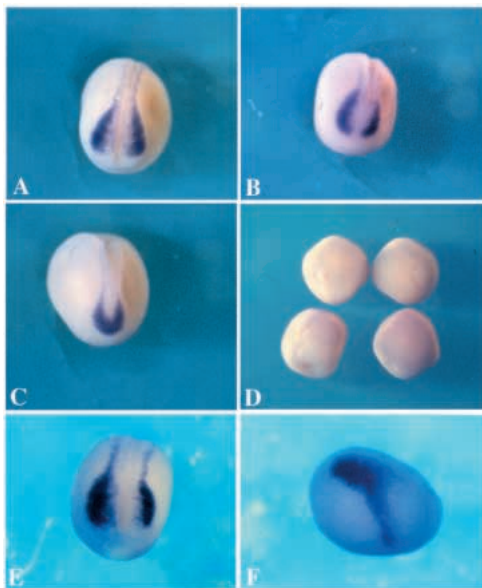
Fig. 1. *Xslug* expression is altered in response to changes in BMP activity. Whole-mount in situ hybridization showing *Xslug* expression in stage 17 control embryos (A) and in embryos injected in one cell at the 2-cell stage with 50 pg (B), 200 pg (C) or 1 ng (D) BMP4 mRNA. With increasing BMP concentration *Xslug* expression domains become deflected toward the midline (B), fuse (C) and are lost in ventralized embryos lacking a discernible neural plate (D). (E,F) *Xslug* expression in embryos injected in one cell at the 2-cell stage with 200 pg of chordin mRNA. *Xslug* expression is expanded laterally (E) and/or ventrally (F) on the injected side.
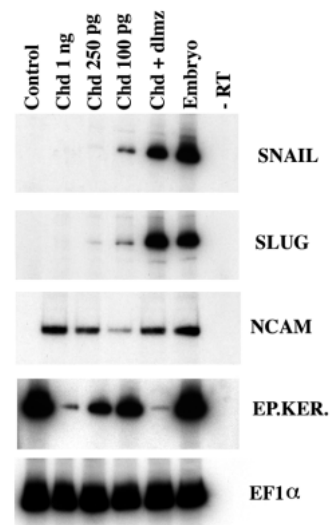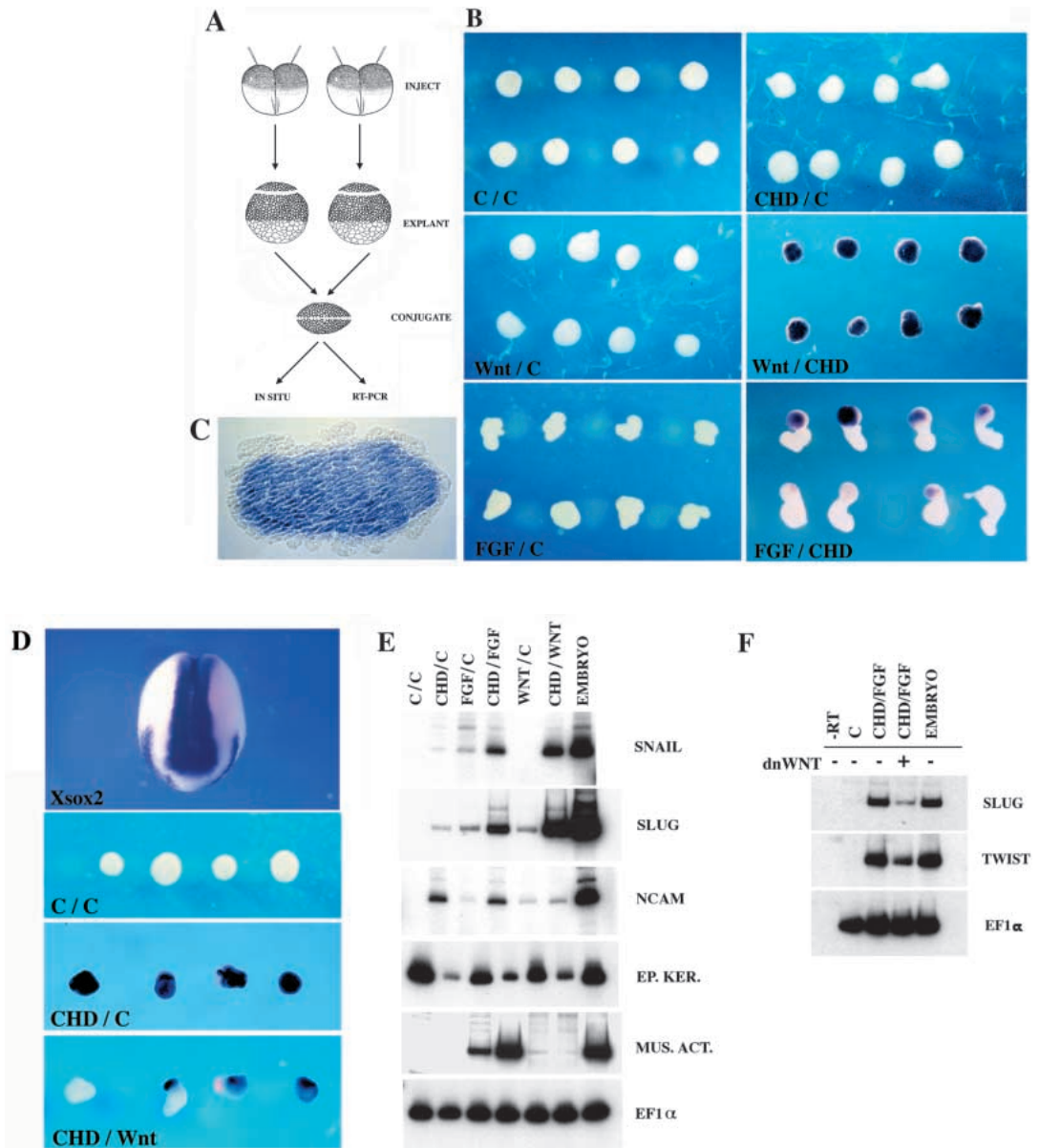

Fig. 2. Chordin induces low levels of neural crest gene expression in ectodermal explants. RT-PCR analysis of ectodermal explants from embryos injected with a range of concentrations (10 pg-2 ng) of chordin mRNA. Representative doses are shown. Explants were isolated at late stage 9 and harvested at stage 17. Control explants are from uninjected embryos. Whole embryos and chordin-expressing explants conjugated with dlmz serve as positive controls. All samples were analyzed with primers specific for the neural crest markers *Xsna* and *Xslug*, the pan-neural marker *NCAM*, the mesodermal marker *muscle actin* and *epidermal keratin*. *EF1α* serves as a loading control.

caps were explanted at late stage 9 and cultured to stage 17 when they were harvested for RT-PCR analysis.

We found that high doses of chordin, which significantly inhibited the expression of *epidermal keratin* (Jonas et al., 1985) and induced expression of *NCAM* (Kintner and Melton, 1987), did not induce expression of early neural crest markers such as *Xsna* (Essex et al., 1993) and *Xslug* (500 pg-2 ng, Fig. 2 and not shown). At lower doses of chordin, however, low level expression of these markers could be induced (100-250 pg, Fig. 2 and not shown). Induction of neural crest markers in these samples could be the result of secondary interactions between chordin-induced neural and epidermal cell types, as these samples also expressed significant amounts of *epidermal keratin*. The levels of neural crest markers induced in these experiments were below that which could be detected by in situ hybridization. (Fig. 3B and not shown) No dose of chordin was found that induced neural crest markers in the absence of neural and epidermal markers, or which induced neural crest markers to levels consistent with their endogenous expression.
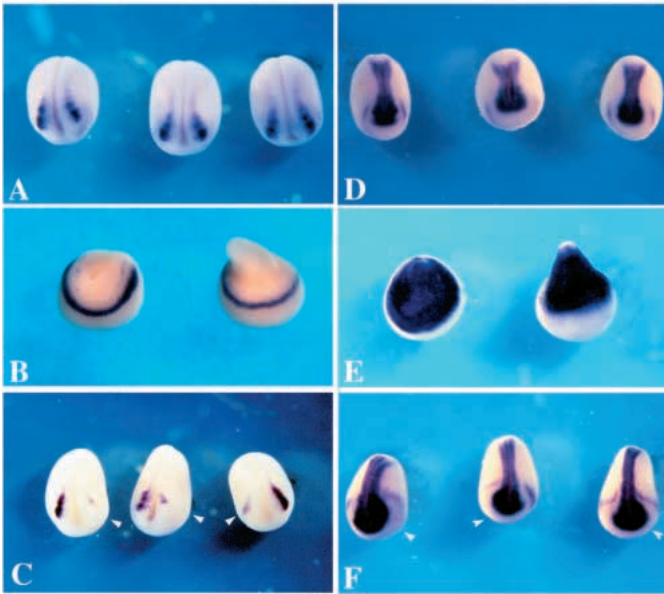
Similar results were obtained using either noggin or a dominant negative BMP receptor to inhibit BMP signaling (data not shown). This suggests that, while the regional level of BMP signaling in the ectoderm may play a role in the specification of the neural crest, this alone is insufficient to account for neural crest induction in vivo. Consistent with this, no dose of chordin was found that could induce melanocytes, a neural crest derivative, in ectodermal explants (not shown). In contrast, when chordin-expressing explants were conjugated with dorsolateral marginal zone tissue (dlmz, prospective somitic mesoderm), increased levels of neural crest markers were induced and melanocytes formed (Fig. 2 and not shown).

A second signal is required for neural crest induction

As chordin overexpression alone was insufficient to induce robust expression of neural crest markers, we asked what signal might be able to cooperate with chordin to induce neural crest. Recently, it has been demonstrated that FGF and Wnt family

Fig. 3. Chordin-expressing ectoderm can be lateralized to neural crest fates by members of the FGF and Wnt families. (A) Schematic of the assay. Conjugates were made by combining explants from control (C) or chordin (CHD)-injected embryos with explants expressing XeFGF or XWnt-8 at late stage 9. Explants were isolated at stage 17 for analysis by in situ hybridization or RT-PCR. (B) In situ hybridization showing *Xslug* expression in conjugates. *Xslug* expression is found in CHD/XWnt-8 and CHD/XeFGF conjugates but not in conjugates of control ectoderm with CHD, XeFGF or XWnt-8. (C) Section through a CHD/Wnt conjugate *slug* expression is found throughout the conjugate except for a crenelated epithelial-like outer layer. (D) In situ hybridization showing expression of the neural plate marker *Xsox2* in stage 17 embryos and in conjugates. *Xsox2* expression is found throughout C/CHD conjugates, but is diminished in CHD/XWnt-8 conjugates. (E) RT-PCR analysis of gene expression in conjugates. Controls and primers are the same as in Fig. 1. (F) Co-expression of a dominant inhibitory Wnt (dnWnt) inhibits the induction of neural crest markers in CHD/FGF conjugates.

Fig. 4. *Xslug* expression is altered in response to changes in Wnt activity. In situ hybridization showing *Xslug* (A-C) and *Xsox2* (D-F) expression in control embryos (A,D) or embryos injected in one cell at the 2-cell stage with 200 pg XWnt-8 mRNA (B,E) or 1 ng of dominant negative XWnt-8 mRNA (D,F). Arrowhead denotes the injected side of embryos in C and F.

members can alter the anterioposterior character of neural tissue induced by noggin, another BMP antagonist (McGrew et al., 1995; Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995). We therefore asked whether these factors could alter the mediolateral character of neural tissue induced by chordin. To address this, we used an assay system which allows prospective neural tissue to be exposed to potential modifying signals at stages when neural crest induction is likely to be occurring in vivo (Fig. 3A). Control animal caps, or animal caps expressing eFGF or XWnt-8, were explanted at late stage 9 and conjugated with animal caps expressing chordin. These conjugates were cultured to stage 17 and assayed for neural crest gene expression by in situ hybridization or RT-PCR.

Conjugates composed of chordin-expressing ectoderm and control ectoderm did not express *Xslug* to levels detectable by in situ hybridization, consistent with a model in which inhibition of BMP signaling is insufficient to specify neural crest cell fates. When chordin-expressing explants were conjugated with explants expressing XWnt-8, however, significant levels of *Xslug* expression were induced (Fig. 3B). Similar results were obtained using conjugates of chordin and eFGF (Fig. 3B), confirming previous observations of neural-crest-forming interactions between bFGF and noggin (Mayor et al., 1995). The observed *Xslug* induction could be secondary to mesoderm induced in eFGF-expressing explants, as these explants express muscle-specific actin (Fig. 3E). We found that XWnt-8 was a better inducer of *Xslug* than was eFGF, although the extent of induction by both was dose-dependent. As FGF is capable of inducing XWnt-8 expression in mesoderm induction assays, is seemed possible that the ability of FGF to induce neural crest markers in our conjugate assay was

secondary to its ability to induce Wnt expression. To address this possibility, the ability of eFGF to cooperate with chordin to induce neural crest markers was assayed in the presence and the absence of a dominant inhibitory mutant of XWnt-8 that has been shown to inhibit XWnt-8 signaling in whole embryos (Hoppler et al., 1996). Co-expression of the dominant inhibitory Xwnt-8 significantly inhibited the ability of eFGF to induce neural crest markers in chordin-expressing ectoderm, suggesting that this ability may be indirect (Fig. 3F).

As neither chordin nor XWnt-8 are mesoderm-inducing factors, this induction appears to be mesoderm-independent. Consistent with this, no muscle actin expression was observed in these conjugates (Fig. 3E). Interestingly, chordin/XWnt-8 conjugates can give rise to solid blocks of *Xslug*-expressing tissue at stage 17 (Fig. 3B,C). As *Xslug* is expressed on both sides of these conjugates, this implies either that XWnt-8 is directly inducing *Xslug* expression in cells where BMP signaling has been inhibited by chordin, or alternatively, if the effects are indirect, that they are mediated by another soluble factor. Xnr3, a soluble TGF-β-related growth factor that can be induced by Xwnt-8 does not induce neural crest markers in this assay (unpublished observation). Neither eFGF nor XWnt-8 alone induced *Xslug* to levels detectable by in situ hybridization, although low levels of expression could sometimes be detected by RT-PCR (Fig. 3E). Lateralization of chordin-induced neural tissue by XWnt-8 was accompanied by loss of expression of the neural plate marker *Xsox2* and resulted in the induction of significant numbers of melanocytes (Fig. 3D and not shown).
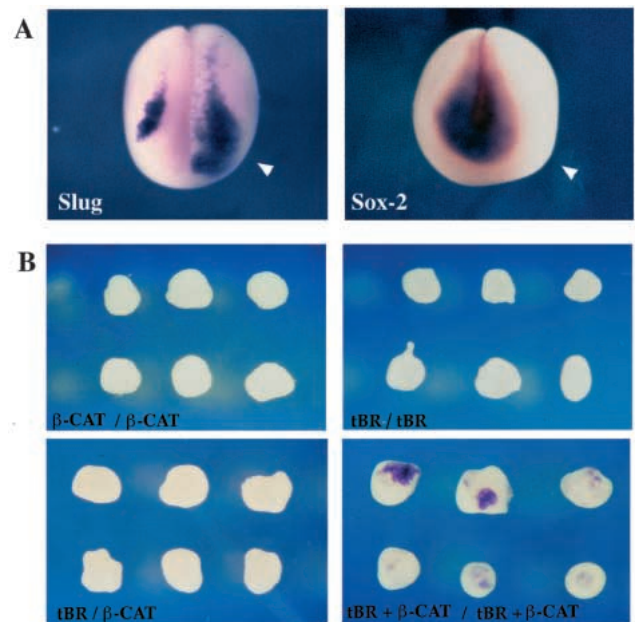

Fig. 5. Effects of β-catenin overexpression in whole embryos and conjugates. (A) in situ hybridization showing expression of *Xslug* and *Xsox-2* in embryos injected in one cell at the 2-cell stage with 100 pg β-catenin. Expanded expression of the neural crest marker and diminished expression of the neural plate marker is seen on the injected side (white arrowheads). (B) In situ hybridization of conjugates which tBR and β-catenin were each expressed in only one half of each conjugate, or were coexpressed in both halves of each conjugate. *Xslug* expression is found only in conjugates in which tBR and β-catenin are coexpresed in the same cells.

Fig. 6. The effects of Wnt signaling on neural crest induction are direct. (A) Section through the midbrain region of a stage 18 embryo coinjected in one cell at stage 2 with β-catenin and β-galactosidase mRNAs. Embryos were stained for β-galactosidase activity (light blue) and processed for in situ hybridization using a *Xslug* probe (purple). Ectopic *Xslug* expression is restricted to cells expressing β-galactosidase. (B) Section through the rostral trunk region of a similarly treated embryo. β-galactosidase expression is seen exclusively in cells of ectodermal lineage. β-catenin overexpression induced ectopic *Xslug* expression lateral to endogenous expression domains, at the expense of epidermis. (C) Section through the caudal hindbrain region of a stage 16 embryo coinjected with dnWNT and β-galactosidase mRNAs. Expression of the injected mRNAs is exclusively ectodermal and mesodermal derivatives appear normal. (D) Closeup of a region of residual *Xslug* expression. Inhibition of *Xslug* expression was noted only in or directly adjacent to cells which expressed high levels of β-galactosidase activity.
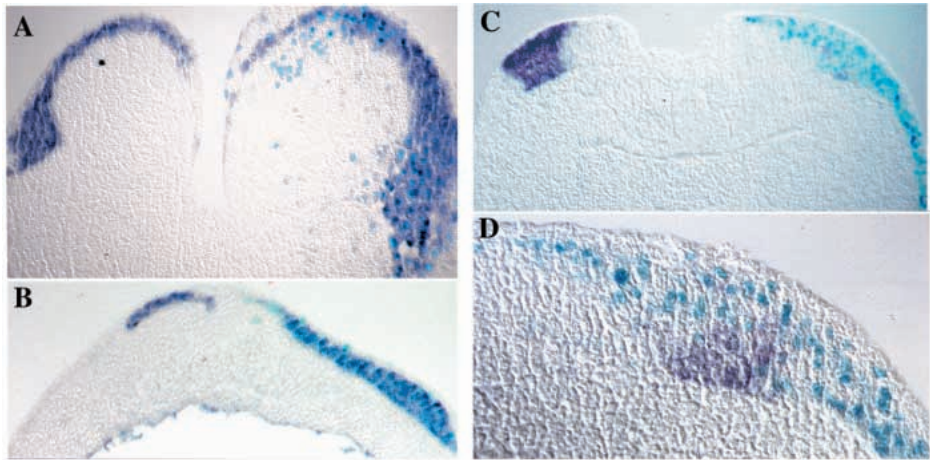
Changes in Wnt expression perturb neural crest induction

As XWnt-8-expressing tissue can cooperate with chordin-expressing tissue to induce neural crest markers in vitro, we examined the effects of modulating Wnt activity in whole embryos. Embryos were injected in the animal pole of one cell at the 2-cell stage with 200 pg XWnt-8 mRNA. Overexpression of XWnt-8 resulted in embryos (n=43) that were hyperdorsalized to varying degrees. *Xslug* expression was found in a band around radially symmetric embryos, or partially around embryos retaining a recognizable neural plate (Fig. 4B). In both cases, *Xslug* expression appears to reflect the boundary of neural plate and non-neural ectoderm, as seen by comparing the expression patterns of *Xslug* and the neural plate marker *Xsox2* (Fig. 4B versus E).

In order to determine if Wnt signaling is required for neural crest formation in vivo, we investigated the effects of decreasing Wnt activity in whole embryos. 1 ng of dominant negative XWnt-8 mRNA was injected in the animal pole of one cell of 2-cell-stage embryos. A significant loss of *Xslug* expression was found on the injected side of these embryos, demonstrating a requirement for a Wnt signal for neural crest induction. (Fig. 4C). This loss of *Xslug* expression appears to be accompanied by a slight increase in the domain of *Xsox2* expression (Fig. 4F).

The effects of Wnt signaling on neural crest formation are direct

The dorsalized phenotype of XWnt-8-injected embryos made it difficult to evaluate any direct effect of ectopically expressed XWnt-8 on *Xslug* expression. Although expression of *Xslug* was expanded, this was likely secondary to the expanded neural plates in these embryos. In order to more clearly investigate the effects of increased Wnt signaling on neural crest formation in vivo, we overexpressed β-catenin, a downstream mediator of Wnt signals (Fagotto et al., 1997). As β-catenin functions in the cytosol and nucleus during Wnt signaling, its effects should be cell autonomous. This makes it easier to control what cells receive ectopic Wnt signals and assess whether any observed effects on neural crest formation are direct or indirect.

100 pg β-catenin mRNA was injected into one cell of 2-cell embryos. At stage 17, these embryos were harvested for in situ hybridization using probes for *Xslug* and *Xsox-2*. β-catenin overexpression resulted in a dramatic increase in *Xslug* expression on the injected side. (Fig. 5A) Similar to what was observed for chordin overexpression, the neural-crest-forming regions were expanded into more lateral and posterior regions of the prospective ectoderm. This was accompanied by a
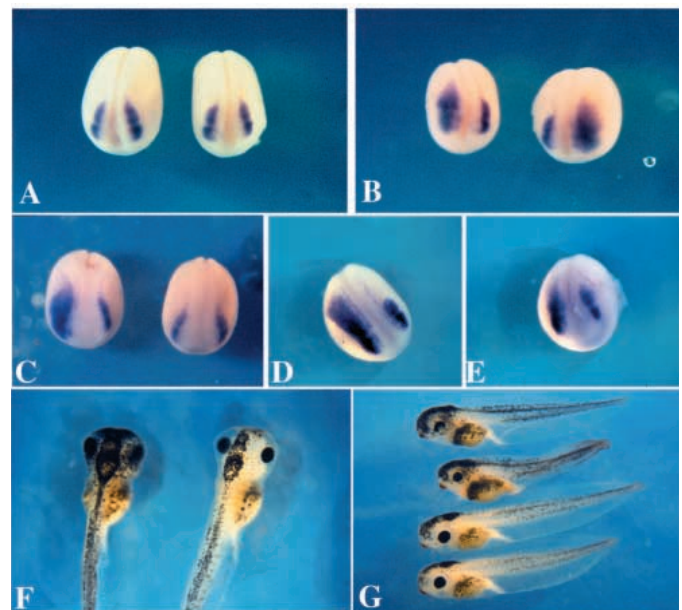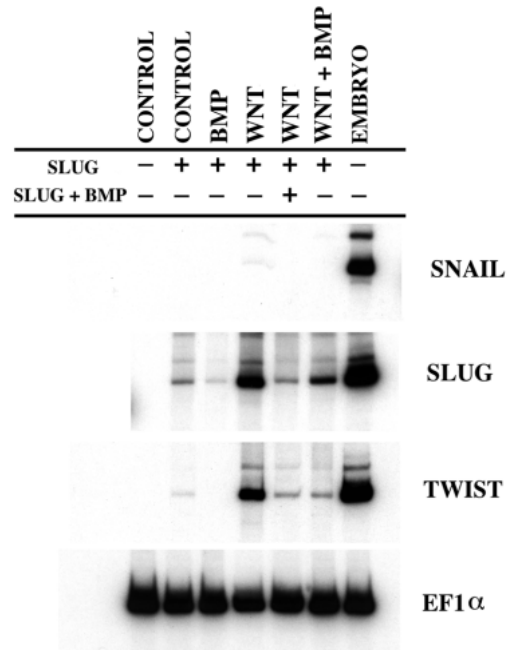

Fig. 7. Effects of Slug overexpression in whole embryos. In situ hybridization showing expression of *Xtwi* (A,B) and *Xslug* (C-E) in control embryos (A, both embryos; C, right embryo) and embryos injected in one cell at the 2-cell stage with 0.5 ng Slug mRNA. Expanded expression of neural crest markers is seen on the injected side. Increased numbers of melanocytes are found when such embryos are allowed to develop until stage 41. (F) Left embryo is injected, right embryo is a sibling control. (G) Top two embryos are injected, bottom two are sibling controls.
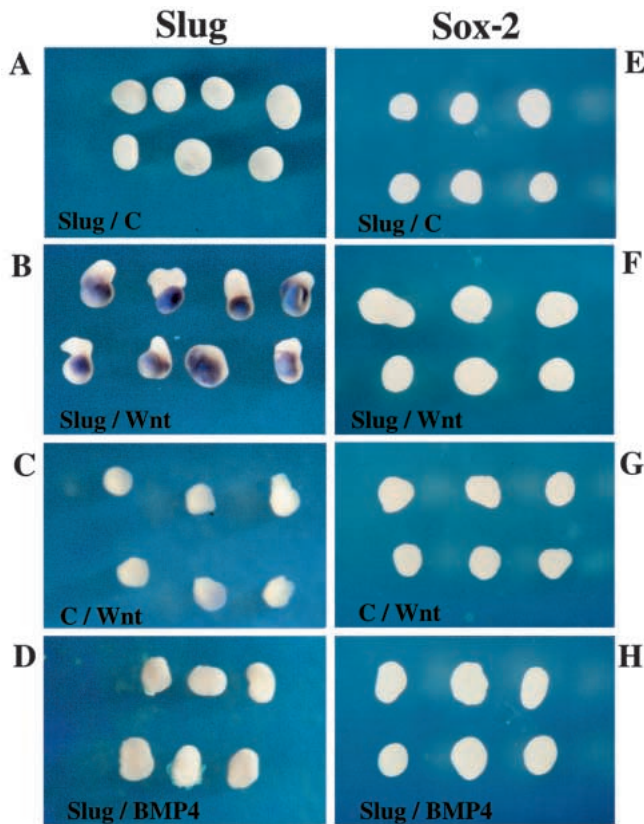
Fig. 8. Neural crest markers, but not neural markers, are induced in Slug/Wnt conjugates. (A-D) In situ hybridization showing expression of *Xslug* in conjugates of control or Slug-expressing explants with explants expressing Xwnt-8 or BMP4. Expression of *Xslug* is seen only in Slug-expressing explants exposed to a Wnt signal. (E-H) *Xsox-2* expression is not seen in Slug-expressing conjugates.

Fig. 9. Neural crest induction in Slug-expressing conjugates is BMP-sensitive. RT-PCR analysis of gene expression in Slug-expressing conjugates in the presence and absence of increased BMP levels. BMP is co-expressed in either the Slug-expressing or Wnt-expressing halves of the conjugate. *Xtwi* is a neural crest marker. Other primers and controls are the same as in Fig. 1.

decrease in the levels of *Xsox-2* expression on the injected side. A more dramatic effect on the *Xsox-2* expression domain was not necessarily expected as the expression patterns of *Xsox-2* and *Xslug* are overlapping in vivo (unpublished observation.)

As β-catenin overexpression resulted in an expansion of neural-crest-forming regions, and as it acts in a cell autonomous fashion, it provides a tool for determining whether the effects of Wnt signaling on neural crest formation in vitro are direct or indirect. In these experiments, a dominant inhibitory BMP receptor (tBR) was used in place of chordin as the means of inhibiting BMP signaling (Graff et al., 1994). This allowed us to set up an assay in which the modulation of both Wnt and BMP signals is cell autonomous. Conjugate assays were set up in which tBR and β-catenin were each expressed in only one half of each conjugate, or were coexpressed in both halves of each conjugate. The conjugates were cultured until stage 17 and harvested for in situ hybridization. Significant *Xslug* expression was detected in conjugates in which tBR and β-catenin were coexpressed, but not in conjugates in which their expression was non-overlapping (Fig. 5B). This strongly suggests that the effects of both molecules on neural crest formation are direct. It should be noted that high doses of β-catenin were capable of inducing *Xslug* expression in the absence of tBR-mediated BMP inhibition at late blastula stages. The levels of β-catenin mRNA

injected for conjugate assays was well below that required for tBR-independent *Xslug* induction (Fig. 5B and not shown.) These low levels of mRNA, together with the cell-autonomous nature of both tBR and β-catenin, most likely explain the rather patchy *Xslug* induction achieved in these experiments.

Since the effects of Wnt signaling on neural crest induction in vitro appeared to be direct, we next wished to determine whether the effects on neural crest formation in vivo were also direct. 100 pg β-catenin mRNA or 250 pg dnWNT mRNA was coinjected with β-galactosidase mRNA in one cell of 2-cell embryos. Embryos were cultured to neurula stages, stained for β-galactosidase activity and processed for in situ hybridization using a *Xslug* probe. The embryos were then sectioned to determine the extent of overlap between the β-galactosidase-expressing cells and the effects on *Xslug* expression. The effects of β-catenin overexpression on ectopic neural crest formation were found to be highly cell autonomous. Ectopic *Xslug* expression appeared to be restricted to cells expressing the injected mRNAs (Fig. 6A,B). β-galactosidase expression was often found exclusively in cells of ectodermal lineage, ruling out secondary effects from the mesoderm. Interestingly, β-catenin overexpression induced ectopic *Xslug* expression lateral and posterior to endogenous expression domains, largely at the expense of epidermis. This is similar to the phenotype observed following chordin overexpression. When the injected mRNA was inherited by cells in more medial regions of the neural plate, ectopic *Xslug* expression was not observed (Fig. 6A and not shown).

Similar to what was found for β-catenin overexpression, the effects of dnWnt overexpression on neural crest formation was

observed to be highly cell autonomous. Inhibition of *Xslug* expression was noted only in or directly adjacent to cells that expressed high levels of β-galactosidase activity (Fig. 6C,D). Where there was residual *slug* expression, little or no β-galactosidase activity was observed (Fig. 6D). Expression of the injected mRNAs was often exclusively ectodermal; mesodermal derivatives appeared to develop normally in these embryos. Thus the inhibition of *Xslug* expression appears to reflect a requirement for Wnt signaling in the ectoderm fated to give rise to neural crest and is not a secondary consequence of defects in the underlying mesoderm.

Role of the transcription factor Slug

The above experiments suggest a model for neural crest induction in which inhibiting BMP signaling specifies a tissue that expresses very low levels of early neural crest markers, and which is competent to respond to lateralizing signals that enhance and maintain neural crest gene expression. Members of the Wnt and FGF family are sufficient to provide such a lateralizing signal, although the ability of FGF to do so may be indirect. We wished to know whether, once robust levels of early neural crest markers had been induced, subsequent neural crest ontogeny required either continued inhibition of BMP signals or the presence of a lateralizing signal.

To address this question, we assayed the effects of overexpressing the zinc finger transcription factor Slug in whole embryos and explants. We chose to overexpress Slug as it is one of the earliest neural crest markers, and as up until stage 17 it is expressed exclusively in the prospective neural crest. This is in contrast to other early neural crest markers such as Snail (*Xsna*) (Essex et al., 1993), a related zinc finger transcription factor, and Twist (*Xtwi*) (Hopwood et al., 1989), a helix-loop-helix containing factor, which are also expressed in the mesoderm at early stages. mRNA containing the coding region of Slug was injected in the animal pole of one cell of 2-cell-stage embryos. The embryos were cultured to stage 17 and processed for in situ hybridization using antisense probes for *Xtwi* or for the 3′ UTR of *Xslug*. The latter probe will recognize endogenous *Xslug*, but not the injected Slug message.

The external morphology of injected embryos was fairly normal through swimming tadpole stages, although headfolds were sometimes slightly enlarged on the injected side at neurula stages. In situ hybridization demonstrated that at these stages, expression of *Xtwi* (Fig. 7B) and *Xslug* (Fig. 7C-E) was enhanced on the injected side of the embryo. Similar to what was seen following chordin or β-catenin overexpression, the expression of neural crest markers was expanded laterally at the expense of non-neural ectoderm, rather than into the neural plate proper (Fig. 7B-E). Expression of neural crest markers was sometimes enhanced posteriorly on the injected side, extending into posterior hindbrain and spinal cord regions where neural crest induction is generally less massive (Fig. 7D). Expression of neural crest markers was increased to some degree in 87% of injected embryos (*n*=130). The observation that Slug can enhance its own expression, in addition to that of later neural crest markers such as *Xtwi*, suggests that endogenous Slug may play a role in the maintenance of its own expression.

In order to examine further the effects of Slug overexpression, some injected embryos were allowed to develop to swimming tadpole stages, when they were scored for quantitative or spatial changes in the formation of neural-crest-derived melanocytes. Slug-injected embryos (*n*=91) displayed a pronounced increase in the number of melanocytes formed relative to control embryos (*n*=102, Fig. 7F,G). These supernumerary melanocytes had a normal dendritic morphology, were light-responsive and populated characteristic regions of the embryo, albeit in larger numbers. Injection of β-galactosidase mRNA caused no noticeable increase in the number of melanaocytes relative to uninjected embryos. These results indicate that Slug overexpression leads not only to increased expression of early neural crest markers, but also to increased numbers of at least one neural crest derivative.

Slug overexpression in ectodermal explants

The observation that, following Slug overexpression, ectopic expression of neural crest markers is limited to areas contiguous with endogenous neural-crest-forming regions suggested that Slug expression itself was insufficient to direct a program of neural crest ontogeny. Instead, it appears that neural crest formation additionally requires signals present either laterally, in the non-neural ectoderm, or in the underlying mesoderm.

In order to examine further the neural-crest-forming potential of Slug-expressing ectoderm, we performed explant experiments similar to those described in Fig. 3. Slug mRNA was injected into the animal poles of 2-cell-stage embryos, and animal pole explants were isolated at late blastula stages and cultured until stage 17. Slug-expressing explants cultured in isolation, or conjugated with control explants, failed to express endogenous *Xslug* or *Xtwi* to levels detectable by in situ hybridization (Fig. 8A and not shown) although, when explants were isolated at early gastrula stages, small foci of faint *Xslug* expression were sometimes noted (not shown). These data suggested that Slug overexpression itself is insufficient to elicit neural crest gene expression. We next asked if XWnt-8, which is sufficient to lateralize chordin-induced neural tissue to neural crest fates, could also induce neural crest gene expression in Slug-expressing ectoderm. Neural crest-specific markers could be induced in all or most of the Slug-expressing cells in Slug/XWnt-8 conjugates but not in the Wnt-expressing cells (Fig. 8B), indicating that, unlike chordin, the effects of Slug on neural crest induction were cell-autonomous. eFGF was able to induce expression of neural crest markers in Slug-expressing ectoderm, although it was a less potent inducer than XWnt-8 (not shown). BMP4 was unable to induce neural crest markers in Slug-expressing ectoderm (Fig. 8D). Induction of neural crest markers in Slug-expressing ectoderm was not dependent upon the expression of neural plate markers such as *Xsox-2* (Fig. 8E-H).

In order to analyse more sensitively the ability of Slug to induce neural crest gene expression, similar experiments were carried out and analyzed by RT-PCR. In these experiments, Slug overexpression was sufficient to induce very low level expression of *Xslug* itself and of *Xtwi*. The significance of this induction, which appears to be below the level detectable by in situ hybridization, is unclear. It is reminiscent, however, of the low-level expression of these markers noted following chordin overexpression. Slug overexpression does not appear to induce any detectable expression of *Xsna*, which is

expressed prior to *Xslug* in whole embryos. Similarly, both Slug/XWnt-8 conjugates and Slug/eFGF conjugates expressed robust levels of *Xslug* and *Xtwi*, but expressed *Xsna* weakly if at all (Fig. 9).

If neural crest induction requires both a suppression of BMP-mediated epidermal fate, together with a lateralizing signal that can be provided by XWnt-8 or eFGF, the above experiments suggest that Slug overexpression is sufficient to bypass the need to inhibit BMP signaling. We therefore reasoned that neural crest induction in Slug/XWnt-8 conjugates should be insensitive to changes in BMP levels. To test this, BMP4 mRNA was co-expressed in either the Slug or XWnt-8-expressing portions of such conjugates and the effects on neural crest marker expression was assayed by RT-PCR. Surprisingly, BMP4 expression was able to repress expression of *Xtwi* and *Xslug* in these conjugates (Fig. 9), indicating that some aspect of this process remains sensitive to increases in BMP signaling.

DISCUSSION

BMP as a morphogen

Accumulating evidence supports the idea that, at least in *Xenopus*, BMPs mediate a choice between neural and epidermal fates in the prospective ectoderm (Wilson and Hemmati-Brivanlou, 1995; Piccolo et al., 1996; Zimmerman et al., 1996). More recently, our knowledge of the concentration-dependent patterning abilities of BMP4 has been expanded to include induction of the cement gland (Wilson et al., 1997), a highly specialized tissue that is induced at the anterior-most junction of neural and non-neural ectoderm. Indeed, as has been found in *Drosophila* (Ferguson and Anderson, 1992), BMP4 appears to be capable of acting as a morphogen, evoking distinct cell fate responses at different concentrations. A simple model for induction of the neural crest, a cell type that also arises at the junction between neural and non-neural ectoderm, would be that, like cement gland, neural crest is specified at levels of BMP signaling intermediate to those that induce neural plate and epidermis.

Our experiments using chordin as a BMP antagonist suggest, however, that this model is overly simplistic. We find no level of chordin overexpression capable of inducing early neural crest markers to levels consistent with their expression in vivo, although it remains possible that other neural inducers may be capable of doing so (Morgan and Sargent, 1997). Indeed, under a model in which the absolute level of BMP signaling was the dominant factor influencing the formation of neural crest, we would expect that, if the BMP concentration was raised locally, on one side of the prospective ectoderm, neural crest formation would tend to be lost on that side of the embryo. Instead, while such a local increase in BMP levels seems to lead to a reduction in the size of the neural plate, the neural crest itself is, to a surprising degree, unaffected. Expression of neural crest markers continues at the border between the prospective epidermis and the diminishing neural plate until BMP levels are achieved that eliminate the neural plate altogether. These experiments suggest that it is the border region itself that is critical for neural crest formation, rather than the absolute level of BMP signaling at the border. One caveat to these findings

is that the timing of the BMP signal may be crucial to realizing its effect. BMP signaling levels do appear to play an important role in the formation of the border region, as seen by the expanded expression of neural crest markers following chordin overexpression.

Lateralizing versus posteriorizing influences

If a distinct level of BMP signaling is by itself insufficient for neural crest formation, what additional signals are required? Recent work addressing the anterior-posterior patterning of the neural plate demonstrates that members of the FGF and Wnt families of growth factors can caudalize or 'transform' prospective forebrain tissue to hindbrain and spinal cord fates (McGrew et al., 1995; Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995). This transforming activity was first suggested by the experiments of Nieuwkoop (Nieuwkoop and Albers, 1990). It appears that these same families of growth factors, as demonstrated here using XWnt-8 and eFGF, are able to lateralize prospective neural tissue as well, inducing neural crest formation. Alternatively, these factors may be exerting a posteriorizing influence, not on anterior neural plate, but rather on anterior border cell types such as cement gland.

The latter model might explain the varying degrees of neural crest induction that can be achieved in chordin/Wnt conjugates. Recently, it has been demonstrated that, at some concentrations of the BMP antagonist noggin, ectodermal explants are induced to express multiple neural plate markers, with some degree of dorsoventral pattern (Knecht and Harland, 1997). In addition, some concentrations of noggin will induce predominantly neural plate markers or predominantly cement gland markers (Wilson et al., 1997). If only prospective cement gland can be posteriorized to neural crest fates, then the degree of cement gland induction at a particular dose of BMP antagonist would limit the degree of neural crest formation in chordin/Wnt conjugates. Experiments are currently underway to address this possibility. Two experiments in the current study argue against this model, however. In the two halves of chordin/Wnt conjugates there is likely to be a gradation in the degree of BMP inhibition, with lower levels of BMP activity found on the chordin-expressing side. Nevertheless, induction of neural crest markers can be achieved throughout the conjugate. In addition, in mildly ventralized embryos, anterior-most fates such as cement gland are among the first to be lost. If only this anterior border cell type can be posteriorized to neural crest fates, one might expect neural crest cells to be absent in such embryos. Expression of neural crest markers is not lost in these embryos, however, but instead continues to reflect the boundary between neural plate and epidermis.

Role of Wnts and FGFs in neural crest induction

The importance of a Wnt signal for neural crest formation is demonstrated by the loss of expression of neural crest markers following overexpression of a dominant negative XWnt-8. Recently, it has also been demonstrated that overexpression of a dominant inhibitory FGF receptor has a similar effect (Mayor et al., 1997). As ventrolateral mesoderm is a potent neural crest inducer, and as dnWnt has been shown to inhibit somite formation when expressed in the mesoderm, it was possible that the effects of Wnt inhibition on the neural crest were secondary to effects on the mesoderm. However, as a dramatic

inhibition of neural crest induction is noted in embryos in which dnWNT is expressed exclusively in the ectoderm, it appears that the requirement for Wnt signaling in the neural crest is independent of any role it may also play in the mesoderm. Consistent with this finding, when Wnt signaling is increased by overexpressing β-catenin, the effects on ectopic *Xslug* expression are cell autonomous and largely confined to more lateral and posterior region of the ectoderm.

As Wnt signaling has also been implicated in the posteriorization of anterior neural tissue (McGrew et al., 1995), it was possible that the effects of Wnt signaling on neural crest formation could be secondary to this posteriorization, and thus indirect. Several observations suggest that this is not the case, however. Wnt signaling can induce neural crest markers in Slug-expressing ectoderm. As *Xslug* is never expressed in anterior neural tissue and as Slug overexpression does not induce expression of neural plate markers such as *Xsox-2*, the neural-crest-inducing abilities of Wnt in this assay must be independent of its ability to posteriorize the neurectoderm. In addition, the ability of Wnt signals to cooperate with weak neuralizing signals to induce neural crest in ectodermal conjugates is cell-autonomous, and thus reflects a direct requirement for a Wnt signal during neural crest formation.

Consistent with this finding, the effects of up- or down-regulating Wnt signaling on neural crest formation in vivo is also highly cell-autonomous. In addition, the ability of β-catenin overexpression to induce ectopic *Xslug* expression in cells normally fated to become epidermis is not consistent with a model in which the effects of Wnt signaling on the neural crest are secondary to the posteriorization of anterior neural tissue. Indeed, this result suggests that formation of the boundary between the neural and non-neural ectoderm is highly dynamic. Experiments are currently underway to address the mechanisms underlying the regulation of this boundary formation.

Are members of the FGF and Wnt families present at a time and place consistent with a role in this process? At gastrula stages, eFGF and XWnt-8 are present in the ventral and lateral marginal zone, and could participate in early planar patterning events. *Xsna* expression, the earliest known marker of neural crest induction, can be detected by in situ hybridization at late gastrula stages (Essex et al., 1993), and it has recently been demonstrated that its expression requires only planar inductive signals (Poznanski et al., 1987). In addition, FGF receptor-dependent MAP kinase activity can be detected in the prospective ectoderm throughout gastrulation (LaBonne and Whitman, 1997). *Wnt-1* and *Wnt-3a* are expressed in the dorsal neural tube of chick and mouse embryos, making them attractive candidates for a role in this process. Their expression is preceded by that of *Slug,* however, precluding a role for these Wnts in the earliest aspects of the inductive process. Interestingly, a recent murine Wnt-1/Wnt-3a double knockout displays a marked deficiency in neural crest derivatives (Ikeya et al., 1997), indicating that these molecules are most likely required for the maintenance and proliferation of the neural crest in the mouse. Recently, expression of *Xenopus* Wnt-3a has been described at the lateral edges of the open neural plate (McGrew et al., 1997). This raises the possibility that Wnt3A may be involved in earlier steps of neural crest induction in this system. Wnt 3A has been shown to induce expression of neural crest markers in noggin-neuralized ectoderm (Saint-Jennet et

al., 1997). In addition, a novel Wnt family member, XWnt7B is present throughout the ectoderm during neural-crest-forming stages and could also play a role in this process (Chang and Hemmati-Brivanlou, 1998). Two avian Wnts are also present in the ectoderm bounding the open neural plate (C. Marcelle and M. B.-F., unpublished data). The role of these Wnts in neural crest induction can be addressed once their *Xenopus* homologues have been cloned.

Role of the transcription factor Slug

Expression of the zinc finger transcription factor *Slug* in the neural crest was first described in avian embryos, where it is expressed in the closing neural folds prior to the onset of neural crest migration (Nieto et al., 1994). Antisense experiments suggested that Slug may play a role in the migratory ability of the neural crest (Nieto et al., 1994). More recently, it has been demonstrated that *Slug* expression can promote migratory ability in other cell types (Savagne et al., 1997). In contrast, the neural crest-specific expression of *Xslug* commences at late gastrula stages (Mayor et al., 1995), long before any role in neural crest migration would dictate. The earliest migration of neural crest in Xenopus, in the prospective hindbrain region, does not occur until stage 19 when the neural tube has closed. The experiments described here suggest that *Xslug*, in addition to any role in migratory ability, also plays a role in earlier aspects of neural crest formation. Although Slug overexpression alone is insufficient to direct neural crest formation in ectodermal explants, it can do so in cooperation with a Wnt or FGF signal. Similarly, Slug overexpression causes an expansion of the neural crest domain and an increase in melanocyte formation in whole embryos. The inability of Slug overexpression to elicit such responses in ectodermal explants suggests either that Slug must act in cooperation with other transcriptional regulators, or that Slug requires post-translational modification in order to effect changes in gene expression. It is interesting to note that Slug may play a role in regulating its own expression. Such an autoregulatory loop could be an important component in the maintenance of neural crest cell fate from its specification at late gastrula stages until migration commences at late neurula stages.

Neural crest induction in avians and amphibians

Several lines of evidence suggest, at first glance, that key aspects of neural crest induction may be different in avian and amphibian embryos. Why, for example, does expression of *Slug* commence at late gastrula stages in *Xenopus*, but not until the neural folds are closing in avian embryos? Does this reflect differing roles for Slug in the two systems, or a later specification of neural crest cell fate in avian embryos? It is possible that differences in the timing of *Slug* expression in avian versus amphibian embryos reflect a difference in the time that prospective neural plate/dorsal neural tube is exposed to the second lateralizing (or in the case of avian embryos, dorsalizing) signal. One possible reason why neural crest may be specified earlier in *Xenopus* is the different behavior of hindbrain neural crest in these embryos. In *Xenopus*, the cranial neural crest masses segregate from the neural tube prior to its closure and never participate in the movements of neurulation (Schroeder, 1970; Hall and Hörstadius, 1988). In contrast, the neural crest does not emerge from the dorsal neural tube of avian embryos until after neural tube closure (Hall and

Hörstadius, 1988). Indeed, it has been demonstrated that until this time, single avian dorsal neural tube cells can give rise to epidermal, neural tube and neural crest derivatives (Selleck and Bronner-Fraser, 1995).

A second apparent difference between the two systems involves the requirement for a second, non-BMP signal during neural crest induction in *Xenopus*. Upon closer examination, however, this may prove to be true in avians as well. In both avian and amphibian embryos, a signal derived from the epidermis is sufficient to lateralize/dorsalize prospective neural tissue and evoke neural crest cell formation. The ability of BMPs to mimic this signal and induce neural crest from avian neural plate explants seems, at first glance, to preclude the need for additional modifying signals. It is possible, however, that the induction of neural crest in these explants is indirect. BMP treatment may result in the epidermalizing of a portion of the neural plate explant. This newly induced epidermis may then lateralize/dorsalize the remaining neural tissue, generating neural crest.

The current work suggests that, at least in *Xenopus*, endogenous BMP signaling must initially be inhibited to levels that can specify the neural plate border region; this tissue is then competent to respond to Wnt-like lateralizing signals that enhance and maintain neural crest induction. It remains possible that these interactions at the neural plate border lead to a subsequent upregulation of BMP activity in this region, and that this upregulation is also required for neural crest formation. Interestingly, expression of *BMP4* and *BMP7* is upregulated at the neural plate border region in *Xenopus* (Hemmati-Brivanlou and Thomsen, 1995). The necessity of this upregulation for neural crest formation is currently being investigated.

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