Posteriorization by FGF, Wnt, and Retinoic Acid Is Required for Neural Crest Induction

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The neural crest is a unique cell population induced at the lateral border of the neural plate. Neural crest is not produced at the anterior border of the neural plate, which is fated to become forebrain. Here, the roles of BMPs, FGFs, Wnts, and retinoic acid signaling in neural crest induction were analyzed by using an assay developed for investigating the posteriorization of the neural plate. Using specific markers for the anterior neural plate border and the neural crest, the posterior end of early neurula embryos was shown to be able to transform the anterior neural plate border into neural crest cells. In addition, tissue expressing anterior neural plate markers, induced by an intermediate level of BMP activity, was transformed into neural crest by posteriorizing signals. This transformation was mimicked by bFGF, Wnt-8, or retinoic acid treatment and was also inhibited by expression of the dominant negative forms of the FGF receptor, the retinoic acid receptor, and Wnt signaling molecules. The transformation of the anterior neural plate border into neural crest cells was also achieved in whole embryos, by retinoic acid treatment or by use of a constitutively active form of the retinoic acid receptor. By analyzing the expression of mesodermal markers and various graft experiments, the expression of the mutant retinoic acid receptor was shown to directly affect the ectoderm. We thereby propose a two-step model for neural crest induction. Initially, BMP levels intermediate to those required for neural plate and epidermal specification induce neural folds with an anterior character along the entire neural plate border. Subsequently, the most posterior region of this anterior neural plate border is transformed into the neural crest by the posteriorizing activity of FGFs, Wnts, and retinoic acid signals. We discuss a unifying model where lateralizing and posteriorizing signals are presented as two stages of the same inductive process required for neural crest induction.

Key Words: neural crest; Xenopus; BMP gradient; FGF; Wnts; retinoic acid; Xslug.

INTRODUCTION

The neural crest is a unique set of ectodermal-derived cells that undergo extensive and coordinated movements, giving rise to numerous and diverse cell types, including much of the peripheral nervous system, the craniofacial skeleton, and pigment cells (for reviews on neural crest development see Labonne and Bronner-Fraser, 1999; Mayor et al., 1999; Christiansen et al., 2000; Mayor and Aybar, 2001).

The neural crest originates at the border between the neural plate and the future epidermis. This border corresponds to the neural folds, which surround the entire neural plate, but only the medial and posterior portions of the folds give rise to neural crest cells, while the anterior neural fold differentiates as forebrain. Within the neural folds, presumptive neural crest cells are distinguishable from epidermal and neural plate precursors by virtue of their distinct patterns of gene expression. For example, the zinc finger and helix-loop-helix transcription factors Xsnail, Xslug, and Xtwist provide early markers for the region from which neural crest cells arise in frogs, fish, and chicks (Thiesen and Bach, 1993; Nieto et al., 1994; Mayor et al., 1995; Linker et al., 2000). The Xsnail gene is expressed initially at the midgastrula stage, in an arc above the dorsal marginal zone which can be precisely identified as a distinct band of cells surrounding the prospective neural plate cells (Mayor et al., 1993; Essex et al., 1993). At the end of gastrulation, the anterior transverse neural fold ceases to express Xsnail, which is then expressed exclusively in the medial and posterior portions of the neural folds, regions subsequently fated to become neural crest. On the other hand, the expression of Xslug and Xtwist begins at the end of gastru-
lation and does not occur in the anterior transverse neural fold. Consequently, this region does not form part of the neural crest but does contribute to the structure of the forebrain (Mayor et al., 1995; Hopwood et al., 1989). The loss of Xsnail expression in the anterior transverse neural fold is a consequence of gene down-regulation rather than cell movement (Linker et al., 2000). Taken together, these observations suggest that these neural fold marker genes are initially activated along the entire neural plate border, later becoming restricted to the medioposterior portion of the neural fold, which then generates prospective neural crest cells. It should be mentioned that Xsnail, Xtwist, and Xslug are not specific markers for the neural crest as they are also expressed in mesoderm (Linker et al., 2000; Mayor et al., 2000).

Several, but not necessarily substitutive, hypotheses have been proposed to explain how the neural crest is induced. Pioneering studies in Urodeles first suggested that the induction of the neural crest could be mediated by underlying tissue, independent from the neural plate (Raven and Kloos, 1945). More recent experiments show that the dorsolateral mesoderm, which underlies the prospective neural crest at the midgastrula stage, is able to induce neural crest markers in competent ectoderm, whereas Xenopus embryos, from which presumptive nonaxial mesoderm has been removed, fail to express neural crest markers (Bonstein et al., 1998; Marchant et al., 1998).

Several experiments also favor a second hypothesis which proposes that neural crest formation requires interactions between the neural plate and the epidermis (Moury and Jacobson, 1989, 1990; Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Mayor et al., 1995; Mancilla and Mayor, 1996). More recently, it has been suggested that levels of BMP signaling, intermediate to those that specify neural plate and epidermis, may play a role in determining the fate of the neural plate border as well as the neural crest (Wilson et al., 1997; Marchant et al., 1998). There is also evidence that this may be true in zebrafish (Navee et al., 1997; Nguyen et al., 1998). To date, it has been assumed that this BMP activity gradient is generated through the interaction of BMPs produced by the ectoderm and BMP-binding molecules produced by the Organizer (reviewed in Weinstein and Hemmati-Brivanlou, 1999). Consequently, this gradient should generate a maximum of BMP activity in the ventral ectoderm and a minimum in the most dorsal ectoderm, or dorsal midline, and should specify neural crest at an intermediate concentration. Although such a gradient could explain why the neural crest is induced at the border of the neural plate, this hypothesis does not account for the anterior–posterior localization of the crest. Indeed, the neural crest is not induced in the most anterior neural fold.

The anterior–posterior pattern of the neural plate has been the subject of many studies. Two models have been proposed to explain the specification of this anterior–posterior axis (reviewed in Slack and Tannahill, 1992; Gamse and Sive, 2000). The qualitative model proposes that the different anterior–posterior positions are determined by different signals arising from corresponding regions of the underlying mesoderm. On the other hand, the activation/transformation model (Nieuwkoop and Albers, 1990) suggests that two inducer factors act sequentially during gastrulation to induce the anterior–posterior specification. Initially, the dorsal ectoderm would receive a signal from the Organizer, determining the induction of the anterior neural plate. Subsequently, a posteriorizing factor would convert part of the previously induced anterior neural tissue to more posterior neural fates (transformation). Several lines of evidence favor this second model (Sive et al., 1989; Holowacz and Sokol, 1999; Blumberg et al., 1997; Papa-loulu and Kinter, 1996; McGrew et al., 1995). Much of the evidence that supports this model has come from the identification of neural inducers and molecules able to transform neuralized tissue. Several neural-inducing proteins, such as Noggin, Chordin, Follistatin, Xnr3, Cerberus, and Gremlin, bind to BMPs and induce the anterior neural plate (reviewed in Harland and Gerhart, 1997), functions which could correspond to the “activator” signal described in the activation/transformation model.

Various molecules have been proposed as putative mediators of the transformation phase. For instance, four members of the fibroblast growth factor (FGF) family, namely eFGF, FGF3, FGF8, and FGF9, expressed in the posterior dorsal, lateral, and ventral mesoderm of Xenopus embryos, are known to be involved in the posteriorization of the neural plate (Isaac, 1997; Holowacz and Sokol, 1999). Retinoic acid also appears to confer posterior positional information. Tissue culture experiments have suggested that retinoic acid acts in a graded manner to induce different anterior–posterior levels (Simeone et al., 1990).

Similarly, Wnt signaling induces posterior markers in Xenopus embryos during gastrulation (Wodarz and Nusse, 1998), at which stage, Xwnt3a and Xwnt8 are expressed in the posterior dorsal, lateral, and ventral mesoderm. Wnt3a can induce the expression of posterior marker genes in neuralized ectoderm (McGrew et al., 1995). Conversely, a truncated Xwnt8 that blocks Wnt signaling prevents the induction of posterior markers in neuralized ectoderm (Bang et al., 1999). In summary, there is strong evidence to support a role for FGFs, Wnts, and retinoic acid in the posteriorization of the neural plate. However, whether the neural crest is also posteriorized via a similar cellular or molecular mechanism has yet to be determined.

In this study, we show for the first time that the anterior neural fold, which does not express neural crest markers, can be transformed into a neural crest fate by the addition of posteriorizing signals. In order to study the anterior–posterior pattern of the neural plate border, a novel assay was employed. Pieces of anterior neural fold were dissected from late-gastrula embryos and either conjugated with the most posterior region of gastrula embryos or treated with FGF, Xwnt8, or retinoic acid. Under these conditions, the expression of anterior neural markers was inhibited and the induction of neural crest markers was observed. In addition, weakly neuralized animal caps, which expressed only ante-
rior neural markers due to the injection of a dominant negative form of the BMP4 receptor, were also transformed to express neural crest markers by conjugating with the posterior region of a late-gastrula embryo or by treating with posteriorizing molecules. Finally, the use of dominant negative mutants of the FGF receptor, a retinoic acid receptor (RAR), and a Wnt signaling molecule showed that each of these signals was required for the specification of neural crest cells. In this paper, we discuss a model whereby the mediolateral position of the neural crest is specified by a BMP signaling gradient, while the anterior-posterior pattern is specified by FGF, retinoic acid, and Wnt signaling.

**MATERIAL AND METHODS**

**Embryos and Micromanipulations**

Xenopus embryos were obtained by artificial fertilization, dejellied in 2% cysteine (Smith and Slack, 1983), reared in 10% normal amphibian medium (NAM; Slack, 1984), and staged according to Nieuwkoop and Faber (1967). Explants, conjugates, and dissections were carried out by using eyebrow knives, as described in Mancilla and Mayor (1996).

**RNA and RLDx Injection**

Depibilized one- or two-cell embryos were placed in 75% NAM with 5% Ficoll, injected with either differing amounts of RNA, as indicated, or with 10 nl of 25 mg/ml rhodamine dextran (RLDx, Molecular Probes), and subsequently reared at 14–16°C. Fluorescence was viewed and photographed by using epifluorescence optics (Zeiss).

Capped RNAs were synthesized from linearized plasmids by using an appropriate RNA polymerase (Boehringer Mannheim) in the presence of 500 mM 5'-MgpppG-3' cap analog. Injected RNAs were as follows: ΔBMPR, a dominant negative of the type II BMP receptor (Sasai et al., 1995); Xwnt-8 (Christian et al., 1991); a BMPR, a dominant negative of RARα1 (Blumberg et al., 1997); XFD, a dominant negative of FGF-1 receptor (Amaya et al., 1991); dnTCF-3, a dominant negative of TCF-3 (Molenaar et al., 1996); VP16-xRARα1, a constitutively active form of RARα1 (Blumberg et al., 1997); and β-galactosidase (β-gal) used as a lineage tracer.

**Treatment with FGF and Retinoic Acid**

Embryos or animal caps taken from stage 9.5 embryos or the anterior neural fold taken from stage 12.5 embryos were incubated with 0.1 ng/ml bFGF (Gibco) or several concentrations (10⁻² to 10⁻⁸ M) of all-trans-retinoic acid for 9–12 h, washed, and analyzed as described under Results. It should be mentioned that the minimal retinoic acid concentration able to induce neural crest in animal caps varied between retinoic acid batches.

**Whole-Mount in Situ Hybridization and β-Gal Staining**

Antisense RNA probes were prepared for Xslug (neural crest marker; Mayor et al., 1995); Xag (anterior neural fold marker; Blitz and Cho, 1995); Xbra (mesodermal marker; Smith et al., 1991); cpl-1 (anterior neural fold marker; Richter et al., 1998); goosecoid (anterior mesodermal marker; Blumberg et al., 1991); and Xsox-2 (pan neural plate marker; Dr. R. M. Grainger, personal communication). Specimens were prepared, hybridized, and stained by the method of Harland (1991) with the modifications described by Mancilla and Mayor (1996). Double in situ hybridization was performed as described by Lamb and Harland (1995), using the following alkaline phosphatase substrates: NBT/BCIP (Boehringer Mannheim), which produces a purple color; and BCIP, which produces a green color. β-Gal staining was developed by using X-gal, as described in Whiting et al. (1991).

**RESULTS**

**Posteriorizing Signals Induce Neural Crest Cells**

Previous studies have indicated that the induction of the neural crest occurs when a threshold concentration of BM P4 is reached in the ectoderm of Xenopus or zebrafish embryos (Marchant et al., 1998; Nguyen et al., 1998). Our own work in animal caps has shown that the expression of an intermediate level of ΔBMPR, a dominant negative form of the BMP receptor, induces the activation of neural crest markers, while neural plate markers are activated at higher levels of the same mutant receptor (Figs. 1A and 1B; Marchant et al., 1998). However, neural crest markers were usually found to be expressed in larger animal caps but not so in smaller ones. Whether the size of the animal caps was important in determining the expression of neural crest markers was therefore investigated. Embryos were injected at the one-cell stage with 35 pg of ΔBMPR mRNA, the threshold concentration found to induce neural crest markers in the absence of neural plate markers. Both large and small animal caps were dissected at stage 9.5 and cultured to the equivalent of stage 17 for the analysis of neural crest (Xslug) and neural plate (Xsox-2) markers and to stage 11 for the mesodermal marker Xbra. No mesodermal (0%, n = 38) or neural plate markers (Fig. 1E; 0%, n = 29) were found to be expressed in either large or small animal caps, confirming previous results (Marchant et al., 1998). However, Xslug expression was observed in 85% of large animal caps and 0% of small animal caps (Fig. 1C; n = 62). As a control that the number of cells was not the factor required to induce Xslug expression in the large animal caps, groups of three or four small animal caps were conjugated together in order to make a large animal cap, but no Xslug expression was detected (Fig. 1D; 0%, n = 6). These results suggest that, in order to induce neural crest cells, another factor is present and active in larger animal caps in addition to the BMP threshold.

In this context, we postulated that the application of this additional signal to small animal caps expressing the ΔBMPR threshold concentration could achieve the induction of neural crest markers. Here, we describe the posteriorizing signals in the context of the activation/transformation model; the initial analysis of these signals is summarized in Fig. 2A. Small animal caps from embryos injected at the one-cell stage with 35 pg of ΔBMPR mRNA
were dissected at stage 9.5 and cultured in isolation or combined with the most posterior fifth of late-gastrula embryos, until the caps reached the equivalent of stage 11 or 19 for the analysis of mesodermal or neural markers, respectively. Gastrula embryos were previously injected with RLDx as a lineage label, and the posterior segments

**FIG. 1.** Effect of the size of neuralized caps on neural crest induction. (A, B) Summary of previous results: intermediate levels of BMP signaling specify neural fold (NF) tissue, lower levels specify the neural plate (NP), and higher levels specify the epidermis (E). (C–E) 35 pg of ΔBMPR mRNA, corresponding to the neural crest threshold concentration, were injected at the one-cell-stage embryo. (C) Large and small animal caps were dissected at stage 9.5 and cultured to the equivalent of stage 17, when Xslug expression was analyzed: 85% of large caps expressed Xslug, while no small caps displayed Xslug expression (n = 62). (D) Small animal caps were dissected and conjugated in groups of three or four caps, cultured as in (C); no Xslug expression was observed (0%; n = 6). (E) Big animal caps as (C), analyzed for Xsox-2 expression (0%, n = 16).

**FIG. 2.** Posteriorization of nonneuralized ectoderm. (A) Embryos at the one-cell stage were injected with 35 pg of ΔBMPR mRNA. Animal caps were dissected at stage 9.5 and either cultured alone (B and C) or conjugated with the posterior fifth of stage 13 embryos injected at the one-cell stage with RLDx (E–H). (I–K) The neurula embryo segments were also cultured alone. The expression of Xag (B, E, I) and Xslug (C, F, J) was analyzed when caps reached the stage 19 equivalent or stage 11 for Xbra expression (D, G, K). (B–D) Injected caps expressed Xag (96%, n = 40) but no Xslug (0%, n = 42) or Xbra (0%, n = 26) expression was detected. (E, F) In conjugates, a down-regulation of Xag (20% of explants expressed Xag, n = 43) and an induction in the expression of Xslug (83% of explants expressed Xslug, n = 42) was observed. Although conjugates also expressed Xbra (G), the expression was originated from the neurula embryo (labeled tissue, dotted area in H). Note the quenching in the intensity of the fluorescence by the in situ signal. (I–J) Posterior pieces did not express Xag (0%, n = 33) or Xslug (0%, n = 38) but did express Xbra (100%, n = 42).
were cultured for the same time period as the animal caps. As expected, isolated caps expressed anterior neural plate markers such as Xag and cpl-1, but not neural crest markers such as Xslug or mesodermal markers such as Xbra (Figs. 2B–2D). Conversely, the isolated pieces of posterior gastrula expressed neither Xag nor Xslug, but as expected, expressed Xbra given their posterior mesoderm content (Fig. 2K).

**FIG. 3.** Neural crest markers induced by posteriorization of the anterior neural fold. (A) The anterior neural fold/plate was dissected from stage 12.5-embryos, avoiding the inclusion of mesoderm or epidermal tissue. The tissue was cultured alone (B, C) or conjugated with the posterior fifth of stage 13 embryos, injected with RLDx at the one-cell stage (D–G). (H and I) The posterior pieces of the neurula embryos were also cultured alone. The neural plate was also conjugated with the posterior fifth of stage 13 embryos (j, k). All explants were cultured to the stage 19 equivalent when the expression of Xag (B, D, and H), Xslug (C, E–G, I, K), and Xsox2 (J) was determined. (B, C) The anterior neural fold/plate expressed Xag (90%, n = 50) but no expression of Xslug (0%, n = 36) was detected. (D–G) In the conjugates, Xslug expression was observed in many cases (85% of Xslug expression, n = 45), while that of Xag was down-regulated (12% of conjugates expressed Xag, n = 48). Xslug-expressing cells came from the anterior neural fold/plate (unlabeled tissue; F, G), whereas the posterior neurula segments expressed neither Xag (H, 0%, n = 38) nor Xslug (I, 0%, n = 40). The conjugates of neural plate and the posterior fifth of a stage 13 embryo expressed Xsox2 (J, 100%, n = 25), but no induction of Xslug expression was observed (K, 0%, n = 25).

**FIG. 4.** Posteriorizing molecules involved in neural crest induction. (A) Embryos at the one-cell stage were injected with 35 pg of ΔBMPR mRNA. Animal caps were dissected at stage 9.5 and cultured in isolation (B) or with $10^{-6}$ M of retinoic acid (C), 0.1 ng/ml bFGF (D), or conjugated with stage 9.5 animal caps taken from embryos injected at the one-cell stage with 300 pg of pCSKA Xwnt-8 (E). Animal caps were then cultured to the stage 18 equivalent and the expression of Xslug was determined. (B) No Xslug expression was detected in injected but untreated animal caps (0%, n = 40), whereas Xslug expression was clearly observed in caps treated with bFGF (C, 50%, n = 30), retinoic acid (D, 45%, n = 22), or Xwnt-8 (E, 70%, n = 33). No expression of Xsox-2 or Xbra was observed in the caps. (F) The anterior neural fold was dissected from stage 12.5-embryos, treated with $10^{-6}$ M of retinoic acid (G) or with 0.1 ng/ml of bFGF (H), and cultured until the equivalent of stage 18, and the expression of Xslug was analyzed. Expression of Xslug was observed in 57% (G, n = 7) or 63% (H, n = 8) of the explants. Arrows indicate Xslug expression.

**FIG. 5.** Inhibition of posteriorizing signals blocks neural crest induction in vitro. (A) Embryos at the one-cell stage were coinjected with 35 pg of ΔBMPR mRNA and the dominant negatives for different posteriorizing signals, as indicated below. Animal caps were dissected at stage 9.5 and conjugated with the posterior fifth of stage 13 neurula embryos. Conjugates were cultured until the animal cap reached the equivalent of stage 19, when the expression of Xslug was analyzed. (B) Conjugates of control animal caps (ΔBMPR) displayed clear Xslug expression (85%, n = 32), while Xslug expression was inhibited in conjugate animal caps taken from embryos coinjected with 3 ng of RARa1 mRNA (C; 22% of explants expressed Xslug, n = 28), 0.2 ng of XFD mRNA (D; 21% of explants expressed Xslug, n = 31), or 0.5 ng of dnTCF-3 mRNA (E; 52% of explants expressed Xslug, n = 28).
However, the conjugation of injected animal caps with posterior or late gastrula down-regulated the expression of Xag (Fig. 2E, 20% of the explants expressed Xag) and induced the expression of Xslug (83% of the explants expressed Xslug, Fig. 2F). Moreover, expression of Xslug was never observed in the fluorescent part of the conjugates, indicating that the cells expressing Xslug originated from the animal cap.

In order to rule out the possibility that the posterior gastrula sections may induce the formation of mesoderm in the injected caps, leading to the induction of neural crest markers via the mesoderm, the following experiments were performed. First, conjugates expressing Xbra were analyzed under fluorescence microscope to identify the origin of the Xbra-expressing cells. No animal caps (nonfluorescent) were seen to express the mesodermal marker Xbra (Figs. 2G and 2H, 0%, n = 42). Second, analysis of Xbra was also carried out in animal caps conjugated with vegetal cells taken from stage 8 embryos and cultured until the caps reached the equivalent of stage 11. No Xbra-expressing cells were observed in these conjugates (not shown). These results indicate that animal caps taken at stage 9.5 were not competent to be responsive to the potential mesodermal inducers present in the posterior part of the late gastrula. Thus, the transformation of injected animal caps expressing anterior neural fold markers into caps expressing neural crest markers, achieved through the juxtaposition with posterior gastrula sections, suggests that neural crest induction is likely to require the presence of posteriorizing signals.

If the above conclusion is true, it should be possible to directly transform a segment of anterior neural fold, fated to become forebrain, into neural crest by conjugating it with the posteriorizing signal source. This experiment was carried out and is described in Fig. 3A. Sections of anterior neural fold were dissected from early neurula embryos, taking care to avoid including mesoderm or ventral epidermis in the explants. These pieces were conjugated with posterior portions of the same stage, embryos previously injected with RLDx (at the one-cell stage). As a control, anterior and posterior explants were cultured in isolation. As expected, anterior explants were found to express Xag but not Xslug, and posterior explants did not express either of these markers (Figs. 3B, 3C, 3H, and 3I). However, the expression of Xag was seen to be down-regulated in the conjugates (Fig. 3D; 12% of the explants expressed Xag n = 48), whereas a clear induction of Xslug was observed (Fig. 3E; 85% of Xslug n = 45). In order to determine the origin of the tissue expressing the neural crest marker, conjugates were examined under a fluorescence microscope. The Xslug-expressing cells were seen to originate from the anterior neural fold (unlabeled tissue). These findings show that the neural crest can be induced by posteriorization of the anterior neural fold. In order to analyze whether the induction of neural crest markers by the posterior region of a neurula was a specific transformation of the neural fold or was simply an effect of posteriorization of neural plate, which in turn expressed the neural crest markers, an additional experiment was carried out. The posterior part of a neurula embryo was conjugated with the anterior neural fold or with the neural plate, and the expression of Xslug and Xsox2 was analyzed (Figs. 3J and 3K).

Posteriorizing Molecules Induce Neural Crest Cells

As mentioned earlier, the involvement of three signaling molecules has been described in the posteriorization of the neural plate: FGF, Wnts, and retinoic acid. As the posteriorization of the anterior neural fold was found to contribute to the induction of the neural crest, the dependency of this process on the same signals that posteriorize the neural plate was subsequently analyzed.

Embryos were injected at the one-cell stage with 35 pg of ΔBMPR mRNA, and small animal caps were dissected at stage 9.5 and either cultured in 38% NAM or 38% NAM containing 0.1 ng/ml bFGF or 10⁻⁶ M retinoic acid or conjugated with caps taken from embryos injected with 300 pg pCSKAXwnt-8 DNA (Fig. 4A). The caps and conjugates were cultured to a stage 10 or 19 equivalent, after which the expression of Xslug, Xbra, and Xsox2 was determined. No Xbra or Xsox2 expression was detected, indicating the absence of mesodermal contamination in the cap explants and that the amount of ΔBMPR mRNA injected was insufficient to cause cap neuralization. Xslug expression was not detected in injected caps cultured alone (Fig. 4B). However, injected caps treated with FGF, retinoic acid, or Xwnt-8 showed a clear expression of Xslug (Figs. 4C–4E). An equivalent experiment, in which the segment of anterior neural fold described in Fig. 3A was treated with either FGF or retinoic acid, produced a similar expression of the neural crest marker Xslug (Figs. 4F–4H). These results show that any of these posteriorizing signals alone, under the present experimental conditions, is sufficient to induce neural crest in a tissue otherwise fated to become the anterior border of the neural plate.

As shown in Fig. 5A, the posteriorizing assay was then used to analyze whether a lack of these signals inhibited the induction of neural crest cells. Embryos were injected at the one-cell stage with either 35 pg ΔBMPR mRNA or the dominant negative forms of the FGF-1 receptor or the retinoic acid receptor, XFD (Amaya et al., 1991) and RARα1 dn (Blumberg et al., 1997), respectively, or with the dominant negative form of TCF3 (dnTCF3), a component of the Wnt signaling pathway (for a review, see Barker and Clevers, 2000). At the late blastula stage, animal caps were dissected and conjugated with the posterior part of early neurula-stage embryos, as described before. Conjugates were cultured until the equivalent of stage 19, after which the expression of Xslug was analyzed. As previously stated, conjugates containing ΔBMPR mRNA-injected caps ex-
pressed the neural crest marker Xslug (Fig. 5B, 83% of explants expressed Xslug; n = 34), while this expression was inhibited in animal caps coinjected with either RARα1\(^{405}\) (Fig. 5C, 22% of explants expressed Xslug; n = 28), XFD (Fig. 5D, 21% of explants expressed Xslug; n = 31), or dnTGF3 (Fig. 5E, 52% of explants expressed Xslug; n = 28). These results show that no neural crest cells are induced when each of the posteriorizing signals is inhibited, suggesting the involvement of all these signals in the induction process. It is important to note, however, that the inhibition achieved by blocking the Wnt signaling pathway was weaker than that observed for the absence of FGF or retinoic acid signaling; this will be commented on further under Discussion.

**Retinoic Acid Signaling in Neural Crest Induction**

Previous studies have proposed FGF and Wnt signals to be involved in neural crest induction (Mayer et al., 1995, 1997; Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998). However, those signals were not shown to function as posteriorizing agents and were instead proposed to act in conjunction with neural inducer molecules. In addition, the role of retinoic acid in neural crest induction has yet to be fully investigated. The results described in this study indicate that retinoic acid is not only sufficient but also necessary for the induction of neural crest cells, as determined by using the animal camp and posteriorizing assays. Further experiments using retinoic acid in whole embryos were therefore carried out.

Early-gastrula embryos treated with varying concentrations of retinoic acid for 3–6 h were cultured until stage 17. The expression of the anterior neural fold markers cpl-1 (Fig. 6A) and Xslug (Fig. 6D) was then analyzed. Several expression patterns were observed depending on the retinoic acid concentration employed. At 1 μM, the most frequent outcome was a complete absence of expression in the anterior neural fold and its localization to the most anterior part of the embryo (Fig. 6B). On the other hand, Xslug was up-regulated in the most anterior neural fold (asterisk in Fig. 6E). An anterior movement in the posterior limit of Xslug expression was also observed (Fig. 6E), and sometimes this concentration induced a complete absence of Xslug (white arrow in Fig. 6H). At 10 μM, complete inhibition of cpl-1 expression was observed (Fig. 6C), while Xslug was either detected at very low levels or not detected at all (Fig. 6F). These results can be interpreted to show retinoic acid functioning as a posteriorizing signal and determining the outcome of neural folds as anterior folds or neural crest. However, retinoic acid is also known to affect mesodermal patterning and the mesoderm is involved in neural crest induction.

In order to assess this alternative explanation, double in situ hybridization experiments were performed in treated embryos, using neural fold and mesodermal markers, to determine whether the embryos that displayed an affected pattern in the neural folds also possessed an abnormal pattern in the mesoderm. These experiments are summarized in Figs. 6G–6J. The most extreme phenotype observed for Xslug was the complete inhibition of its expression (Fig. 6H, 25% of explants expressed Xslug; n = 48). However, even in those cases, the position of the mesoderm was not affected, as shown by the normal localization of gsc in the same embryos (arrowheads in Figs. 6G and 6H). The same extreme inhibitory phenotype was obtained for cpl-1 (white arrow in Fig. 6J), in which case the position of the notochord was normal, as detected by Xbra expression (arrowhead in Figs. 6I and 6J). Taken together, these results suggest that retinoic acid is involved in the transformation of the anterior neural fold into neural crest cells without significant effects on the mesoderm. In order to test this conclusion further, several mutant forms of RARs were used.

Although only the results for RARα are described here, the dominant negative and constitutively active forms of both α and γ RAR were tested, as no difference was observed between these two kinds of retinoic acid receptors. One blastomere of two-cell-stage embryos was injected with 1 ng of xRARα1\(^{405}\) mRNA (the dominant negative form of RARα1 together with β-gal mRNA, as a marker for the injected blastomere). Embryos thus treated exhibited cpl-1 expression in a more posterior position (Fig. 7A), while the effect on Xslug expression was more complex. A clear movement toward the posterior end was observed at the posterior limit of Xslug expression, whereas no effect was observed at the anterior limit (Fig. 7C; 35%, n = 125). In normal embryos, the posterior border of cpl-1 expression corresponds with the anterior border of Xslug expression. If the embryos injected with xRARα1\(^{405}\) showed a posterior relocation in the expression of cpl-1 but no effect at the anterior border for the expression of Xslug, an overlapping in the expression of these genes should be expected; to test this possibility, double in situ hybridization was performed. A clear overlap in the expression of these two markers was observed at the injected side (Fig. 7E). These results show that inhibition of RAR is able to profoundly modify the anterior–posterior expression pattern of the neural folds and that the anterior neural fold and the posterior limit of the neural crest are repressed by RA activity.

Next, the effect of a constitutively active form of RAR (VP16-xRARα1) was assessed by injecting 500 pg of VP16-xRARα1 mRNA into one blastomere of two-cell-stage embryos and analyzing cpl-1 and Xslug expression at the neurula stage. The injection of VP16-xRARα1 produced an anterior movement in cpl-1 expression (Fig. 7B, 67%, n = 89) and sometimes complete inhibition. Upon analysis of Xslug expression, two phenotypes were most frequent: 75% of the injected embryos (n = 167) showed no Xslug expression in the injected side (Fig. 7D), whereas 16% displayed a shift toward the anterior fold. Expression of Xslug at the anterior neural fold was observed more frequently when the embryos were injected at the one-cell stage (Fig. 7F, 36%, n = 58). Thus, the most common phenotypes induced using VP16-xRARα1, an activated form of the RAR, were similar to those produced by direct treatment with retinoic acid.
Nonetheless, the effect was more localized by mRNA injection than by retinoic acid application in solution. As the results shown in Figs. 8G–8J suggest that the retinoic acid effect was not mesoderm-dependent, an additional experiment was performed to rule out this possibility. Embryos injected at the one-cell stage with 1 ng of either xRARα1405 or VP16-xRARα1 mRNA were cultured to stage 10 and a piece of ectoderm was taken and grafted onto...
normal embryos (Fig. 8A). Ectoderm taken from embryos injected with β-gal mRNA were used as controls. When control ectoderm was grafted onto the prospective neural crest region, no expression of cpl-1 was observed (Fig. 8B). However, when the graft contained xRARα1405, clear expression of cpl-1 was observed in the graft (Fig. 8C). Given that VP16-xRARα1 injection exerted an inhibitory effect on cpl-1 at the anterior neural fold (Fig. 7B), the graft expressing this construct was placed in the anterior neural fold. When control ectoderm was grafted onto that region, normal expression of cpl-1 was observed (Fig. 8D, 90%, n = 20). Grafting of VP16-xRARα1-expressing ectoderm, however,
caused an inhibition on cpl-1 expression in 80% of the grafts (Fig. 8E, n = 10), a result similar to that obtained by direct injection of VP16xRARα1 mRNA at the two-cell stage. This experiment shows that the effect of the mutant RAR forms was localized directly on the neural folds and was not dependent on a modification of the mesoderm, seen to be normal in the grafted embryos. Taken together, these results point to an important role for retinoic acid and its receptors for the correct anteroposterior specification of the neural crest cells.

DISCUSSION

Molecules Involved in Neural Crest Induction

The participation of several molecules in the induction of the neural crest has been previously reported. We have shown here that FGF, Wnts, and retinoic acid signals are involved in the induction of these tissues. BMPs have also been proposed for the dorsal specification of the neural tube via two different mechanisms. It has been shown that neural crest markers are induced by adding BMPs to neural plate explants (Liem et al., 1995). Recently, several independent reports have highlighted the importance of particular concentrations of BMPs in the specification of the neural plate border. Levels of BMP intermediate to those required to induce neural plate and epidermis have been shown to induce the anterior neural plate border (Wilson et al., 1997), as well as neural crest cells in Xenopus and in Zebrafish embryos (Morgan and Sargent, 1997; Marchant et al., 1998; LaBonne and Bronner Fraser, 1998; Nguyen et al., 1998; this report). Streit and Stern (1999) also showed that the neural plate border of chick embryos develops by a combination of signals where BMPs and their inhibitors are involved.

FGF has also been implicated in neural crest induction (Mayor et al., 1995, 1997; LaBonne and Bronner-Fraser, 1998). Addition of FGF to weakly neuralized animal caps has been shown to activate the expression of neural crest markers, whereas the use of a dominant negative form of the FGF receptor inhibited the induction of the neural crest in treated embryos. The role of FGF is nonetheless controversial, as it has also been implicated in neural plate induction (Launay et al., 1996). However, in Xenopus, the direct participation of FGF as a neural inducer was ruled out more recently (Holowacz and Sokol, 1999; Ribisi et al., 2000; Curran and Grainger, 2000). Wnts and some elements of the Wnt signaling pathway have also been implicated in neural crest induction (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998). Based on the timing of Wnt expression, Wnt factors were suggested to participate in the maintenance of the neural crest differentiation program, rather than its initial specification (Saint-Jeannet et al., 1997).

We have reported here that FGF and Wnt signaling participate in the neural crest induction process, confirming previous results. Evidence is also presented regarding the role of retinoic acid in neural crest induction. The latter is a novel finding, as this molecule has previously only been proposed to participate in neural crest differentiation (Baranova, 1989; Dupe et al., 1999). It has also been shown that treatment of Xenopus embryos with retinoic acid affects the development of cranial nerves derived from neural crest (Papalopulu et al., 1991). Additionally, it has been shown that, upon retinoic acid treatment, Pax-3, a gene expressed at the posterior border of the neural plate and within the neural plate, is induced in neuralized ectoderm (Bang et al., 1997). However, in the present study, a novel mechanism for the participation of these molecules in neural crest induction is explored.

Models for Neural Crest Induction

The model for neural crest induction currently accepted today relates to the interaction between neural plate and epidermis (review in Labonne and Bronner-Fraser, 1999). A different, but not necessarily substitutive model, is based on the induction of specific ectodermal tissues via a BMP gradient (reviewed in Mayor et al., 1999). Two independent reports have shown the difficulty of inducing neural crest markers by lowering BMP activity in the ectoderm, pointing to the need for an additional signal (Wilson et al., 1997; LaBonne and Bronner-Fraser, 1998). On the other hand, two other reports have shown that neural crest induction was possible under similar conditions (Morgan and Sargent, 1997; Marchant et al., 1998). In the latter case, the level of neural crest induction was not very high. The present study provides a possible explanation for this discrepancy, namely the size of the animal cap employed, as the neural crest is induced only when large animal caps are dissected from embryos treated with the anti-BMPs molecules. Consequently, another factor in addition to the BMP threshold concentration needs to be present in the animal cap for neural crest markers to be expressed.

Labonne and Bronner-Fraser (1998) analyzed an additional signal required for neural crest induction. These authors concluded that FGF and Wnt signals could induce the neural crest in weakly neuralized animal caps, and proposed a function for these molecules in what they named a “lateralization” process. We confirmed the participation of FGFs and Wnts’ signals on neural crest induction and added retinoic acid as another additional signal. We found that all these signals can work as posteriorizing signals based on an assay developed by Cox and Hemmati-Brivanlou (1995). Our results show that the anterior neural plate border, which expresses Xag, can be transformed by posteriorizing signals into a tissue that expressed neural crest markers. In addition, anterior neural plate markers, induced by an intermediate level of BMP activity, could also be transformed into neural crest by posteriorizing signals. Our evidence therefore indicates that the signals involved in the posteriorization of the neural plate exert a posteriorizing effect on the neural fold, such as FGF, Wnt-8, or retinoic acid, were sufficient to transform anterior neural fold into neural crest cells in weakly neuralized animal...
caps. Neural crest cells were also induced in pieces of the anterior neural fold treated with either FGF or retinoic acid. However, blocking any of these signals via dominant negative forms inhibited the induction of neural crest cells in the in vitro posteriorizing assay. Although each posteriorizing signal was able to induce the neural crest, the absence of a single agent inhibited the induction process. A possible explanation for this discrepancy could relate to the concentrations used in the assay, which are probably much above the levels reached in a normal embryo. It is possible that, in order to induce the neural crest, the participation of each posteriorizing signal must occur in an additive manner, so that independently or under the inhibition of one of the signals, these would be too weak to posteriorize the embryo. However, the addition in vitro of sufficiently elevated concentrations of each agent could rescue the embryo from the absence of other signals. Alternatively, FGF, retinoic acid, and Wnts signaling could not act independently. They could work serially; thus, blocking any of the signals would inhibit induction, but overexpression of any could hyperactivate the pathway leading to neural crest induction.

The outcomes using dominant negatives in whole embryos are also compatible with a role for these agents as posteriorizing signals, especially in the case of retinoic acid as analyzed here. The anterior neural fold was transformed into neural crest by addition of retinoic acid to the embryos or by expression of a constitutively active form of its receptor. Inhibition of retinoic acid signaling in dominant negative mutants caused the opposite result, whereby the region of the neural crest expressed markers for the anterior neural plate border, and the posterior limit of the neural crest moved to a more posterior position. It is interesting to note that the region of the neural crest least affected by both expression of the dominant negative form of RAR and retinoic acid application was the anterior border. This observation suggests that the different posteriorizing agents have different roles in the specification of different anterior-posterior positions in the neural plate border. Thus, retinoic acid may be more important in the specification of the posterior limit of the neural crest and the posterior limit of the anterior neural fold, while signals such as Wnts or FGF could play more prominent roles at the anterior limit of the neural crest cells. The use of a dominant negative form of the FGF receptor completely blocked the expression of neural crest markers, a finding which is concordant with a different role for FGF and retinoic acid in the posteriorization process of the neural plate border (Mayor et al., 1997). A similar inhibitory effect was also observed for the injection of a dominant negative mutant of Xwnt-8 in treated embryos (LaBonne and Bronner-Fraser, 1998).

A remaining question was the spatial localization of the FGFs, Wnts, and retinoic acid signaling molecules, which must be present in the right place in order to function as a posteriorizing agents. At the late-gastrula and early-neurula stages, several members of the FGF and Wnt families have been found to be expressed in the posterior region of the embryo (Isaac, 1997; McGrew et al., 1995; Christensen et al., 1991). Although it has been difficult to determine the distribution of retinoic acid in the embryo, RARs and converting enzymes have been found in the presumptive hindbrain and the posterior region of the embryo during Xenopus gastrulation (Kolm and Sive, 1997).

A Model of Neural Crest Induction

We present a unifying model of neural crest induction based on previous reports and on our observations described here (Fig. 9). At the early-gastrula stage, a gradient of BMP activity is established in the ectoderm, which specifies the neural plate, the neural plate border, and the epidermis at progressively higher concentrations of BMP (Fig. 9A). The neural plate border, induced at a precise location within the mediolateral axis of the ectoderm, has an anterior character (yellow in Fig. 9A). Later, between early and midgastrula stage, signals presumably originating from the ventrolateral mesoderm transform a region of the anterior neural plate border into prospective neural crest cells (Fig. 9B). A role for this mesoderm in neural crest induction has been shown (Bonstein et al., 1998; Marchant et al., 1998). The spread of these molecules from the mesoderm into the ectoderm (Fig. 9B) consequently locates them only in large animal caps, explaining why the neural crest was not induced when small animal caps were used. These signals could correspond to Wnt8 and eFGF, as it is known that they are expressed in the ventrolateral mesoderm, and could correspond to the lateralizing signals described by LaBonne and Bronner-Fraser (1998). However, the neural crest is not specified at this stage, as this does not occur until the end of gastrulation (Mayor et al., 1995; Mancilla and Mayor, 1996). Thus, additional signals are required for the final induction of the neural crest. Finally, as gastrulation proceeds, the ventrolateral mesoderm becomes localized to the posterior region of the embryo, where it continues to produce Wnt8, eFGF, and possibly retinoic acid, as well as other, as yet unknown, posteriorizing agent that generate an anterior-posterior gradient of these morphogens. This gradient would be required for the final specification of the neural crest in the most posterior region of the neural plate border (Fig. 9C). Thus, the lateral–posterior regions of the neural plate border receive the lateralizing/posteriorizing signals for an extended period of time, finally specifying them as neural crest. In contrast, the anterior neural plate border does not receive such signals or these are inhibited by other agents produced by the anterior regions of the embryo, such as cerberus or dkk1, two known Wnts inhibitors (Bouwmeester et al., 1996; Glinka et al., 1998), and, as a consequence, does not develop as neural crest cells. It is tempting to speculate that the anterior-posterior differences within the neural crest could be controlled by a similar mechanism.
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