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Is Dependent on a *Wnt*-Mediated Signal from Posterior Nonaxial Mesoderm

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During early patterning of the vertebrate neuraxis, the expression of the paired-domain transcription factor *Pax-3* is induced in the lateral portions of the posterior neural plate via posteriorizing signals emanating from the late organizer and posterior nonaxial mesoderm. Using a dominant-negative approach, we show in explant assays that *Pax-3* inductive activities from the organizer do not depend on FGF, retinoic acid, or *XWnt-8,* either alone or in combination, suggesting that the organizer may produce an unknown posteriorizing factor. However, *Pax-3* inductive signals from posterior nonaxial mesoderm are *Wnt*-dependent. We show that *Pax-3* expression in the lateral neural plate expands in *XWnt-8*-injected embryos and is blocked by dominant-negative *XWnt-8.* Similarly, we show that the homeodomain transcription factor *Msx-1,* which like *Pax-3* is an early marker of the lateral neural plate, is induced by posterior nonaxial mesoderm and blocked by dominant-negative *XWnt-8.* Finally, we show that Rohon–Beard primary neurons, a cell type that develops within the lateral neural plate, are also blocked *in vivo* by dominant-negative *Xwnt-8.* Together these data support a model in which patterning of the lateral neural plate by *Wnt*-mediated signals is an early event that establishes a posteriolateral domain, marked by *Pax-3* and *Msx-1* expression, from which Rohon–Beard cells and neural crest will subsequently arise.

Key Words: Pax-3; Msx-1;* posteriorization; *Wnt;* primary neurons; *Xenopus.

INTRODUCTION

The development of the vertebrate nervous system is initiated during gastrulation when Spemann's organizer induces dorsal ectoderm to assume a neural fate. Organizer signals also act to pattern the CNS along its anterior–posterior (A–P) axis, a process which has been proposed to occur in two steps. In the first step, activation signals from the early organizer induce ectoderm to become neural tissue with an anterior or forebrain fate, and in the second, the anterior neural tissue is posteriorized into midbrain, hindbrain, and spinal cord fates by transforming signals. As predicted by this model, the organizer-derived factors *noggin, follistatin,* and *chordin* can convert explants of naive ectoderm (animal caps) from *Xenopus* blastula-stage embryos into neural tissue with an anterior, forebrain-like fate (reviewed in Lumsden and Krumlauf, 1996). Moreover, anterior neuroectoderm can be transformed into posterior neural tissue by signals emanating from the late organizer as well as more lateral mesoderm. Recently, fibroblast growth factor (FGF), retinoic acid (RA), and members of the *Wnt* family have been identified as putative transforming signals because of their ability to repress expression of anterior neural markers and induce posterior neural markers in anterior neuroectoderm (reviewed in Lumsden and Krumlauf, 1996; McGrew *et al.,* 1995, 1997). While experiments in *Xenopus* have shown that FGF, RA, and *Wnt* exhibit overlapping as well as distinct posteriorizing activities, it remains unclear how these signals are coordinated and their inputs integrated to give rise to a regionalized posterior nervous system.

The transcription factor *Pax-3,* a member of the paired family of homeodomain proteins, is a unique marker with which to study posteriorization of the neuraxis. At neural plate stages in both chick and *Xenopus, Pax-3* expression is

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restricted posteriorly as well as laterally, thus marking the prospective dorsal hindbrain and spinal cord. Since regionally restricted *Pax-3* expression occurs extremely early during neural plate formation, its regulation is likely to represent a primary response to A–P patterning signals. Previously, we proposed that *Pax-3* expression is initiated by signals that posteriorize the neuraxis (Bang *et al.*, 1997). This model is based on several lines of evidence. First, in chick and *Xenopus* gastrulae the onset of *Pax-3* expression occurs in regions fated to become posterior CNS. Second, *Pax-3* expression is induced in *noggin*-treated *Xenopus* animal cap explants by the putative posteriorizing factors RA and bFGF. Third, the late, but not the early, organizer and posterior nonaxial mesoderm (PNM), two sources of posteriorizing signals, are capable of inducing ectopic *Pax-3* expression in anterior neural tissue. These results led us to propose a model in which early expression of *Pax-3* is established by signals produced by the organizer and the PNM that act to posteriorize the neuraxis. In this model, once this domain is established, the dorsal restriction of *Pax-3* expression would be maintained at least in part through *sonic hedgehog* (*shh*)-mediated signals arising from the floorplate and notochord that act to repress *Pax-3* in the ventral neural tube and positively acting signals like *bone morphogenetic protein-4* (*BMP-4*) from epidermal ectoderm that would upregulate *Pax-3* dorsally (reviewed in Tanabe and Jessell, 1996).

Of the two potential sources of *Pax-3* inductive signals, PNM is a particularly attractive candidate because it underlies the posteriolateral neural plate, suggesting that the posteriolateral restriction of *Pax-3* expression in the early neural plate might be due to the localized action of PNM-derived signals. Indeed, the PNM has been found in several studies to be a source of signals capable of conferring posterior fates on presumptive anterior neural tissue in *Xenopus*, chicks, and zebrafish (Bang *et al.*, 1997; Muhr *et al.*, 1997; Kolm *et al.*, 1997; Woo and Fraser, 1997). In addition, later acting signals arising from somitic mesoderm control patterning of the developing hindbrain and spinal cord, regulating Hox gene expression, *Pax-6* expression, and motor neuron columnar subtype identity (Itasaki *et al.*, 1996; Ensini *et al.*, 1998; Grapin-Botton *et al.*, 1997; Pituello *et al.*, 1999). Together these studies suggest that PNM and its derivatives may produce multiple, temporally and functionally distinct signals that act to pattern the posterior CNS. However, the nature of PNM-derived posteriorizing factor(s) remains unknown.

Recently, members of the *Wnt* family of signaling molecules have been proposed to pattern the posterior neuraxis in *Xenopus* embryos: overexpression of *XWnt-3A* induces expression of the posterior markers *Krox-20* and *En-2* in neuralized animal caps from *noggin*-injected embryos, whereas overexpression *in vivo* of a dominant-negative form of *XWnt-8* (dn *XWnt-8*) blocks expression of these markers (Hoppler *et al.*, 1996; McGrew *et al.*, 1995, 1997). In addition, it has been shown that *Wnt*-signaling induces markers of neural crest, a cell type that arises within the *Pax-3* expression domain in the posteriolateral neural plate (LaBonne and Bronner-Fraser, 1998; Chiang and Hemmati-Brivanlou, 1998; Saint-Jeannet *et al.*, 1998). These observations led us to ask whether a *Wnt* signal could play a role in the induction of *Pax-3* expression by the PNM or the organizer. Using dominant-negative constructs, we show in explant assays that *Pax-3* inductive activities from the organizer do not depend on FGF, RA, or *XWnt-8*, either alone or in combination, suggesting that this effect may be mediated by an unknown posteriorizing factor. However, *Pax-3* inductive signals from PNM are *Wnt*-dependent. Results from *in vivo* experiments support this conclusion, as expression of *Pax-3* in the lateral neural plate expands in embryos injected with *XWnt-8* and is blocked in embryos injected with dominant-negative *XWnt-8*. Similar results were obtained with the homeodomain transcription factor *Msx-1*, another early marker of the lateral neural plate: *XWnt-8* induces *Msx-1* expression in *noggin* animal caps, *Msx-1* inductive signals from PNM are *Wnt*-dependent, and finally, *Msx-1* expression is blocked in dominant-negative *XWnt-8*-injected embryos. The results obtained with *Pax-3* and *Msx-1* indicate that prior to neural crest formation *Wnt*-signaling is required to establish a posteriolateral domain during early patterning of the neural plate. Consistent with this finding, we show that Rohon–Beard primary neurons, a cell type which, like neural crest, arises from the lateral neural plate within the *Pax-3* expression domain, are also blocked *in vivo* by dominant-negative *XWnt-8*. We propose that a *Wnt* signal from PNM induces *Pax-3* and *Msx-1* expression in the lateral neural plate during early patterning of the neural plate, establishing a posteriolateral region from which neural crest cells and Rohon–Beard primary neurons will develop.

MATERIALS AND METHODS

Embryos

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg-laying and *in vitro* fertilization using standard methods. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967). White Leghorn hens' eggs and quail eggs were incubated at 38°C in a humidified, forced-draft incubator. Avian embryos were staged according to Hamburger and Hamilton (1951).

In Situ Hybridization

Whole-mount *in situ* hybridization of *Xenopus* embryos was performed according to Harland (1991) with modifications described by Knecht *et al.* (1995) using digoxigenin-labeled antisense RNA probes for *Xenopus Pax-3* (Espeseth *et al.*, 1995), *Xwnt-8* (Christian and Moon, 1993), *MyoD* (Hopwood *et al.*, 1992), *Xnrgr-1* (Ma *et al.*, 1996), *β-tubulin* (Chitnis *et al.*, 1995), and *Msx-1* (Suzuki *et al.*, 1997). Templates for *slug* and *elrC* probes corresponding to full-length coding regions were isolated using RT-PCR, based on published sequences (Good, 1995; Mayor *et al.*, 1995).

Isolation, Treatment, and Culturing of Xenopus Embryos and Animal Caps

For animal caps, *Xenopus* embryos at the two-cell stage were injected in the animal region of each blastomere with capped synthetic RNAs encoding *noggin* (0.5 ng; Lamb *et al.*, 1993); *XWnt-8* (0.5 ng; Christian and Moon, 1993); dominant-negative *XWnt-8* (0.5 ng; Hoppler *et al.*, 1996); *XFD* (0.5 ng; Amaya *et al.*, 1991); dominant-negative *ras* (0.5 ng, *ras* p21 (Asn-17)$^{Ha\text{-}ras}$; Feig and Cooper, 1988), as described in Bhushan *et al.* (1994); or dominant-negative *xRARγ-1* (1 ng; dn *xRARγ-1* was a generous gift from Dr. Bruce Blumberg and was constructed as described for the dominant-negative form of *xRARα-1* in Blumberg *et al.*, 1997). Animal caps were dissected at stage 9. Some caps were treated with 2×10^{-6} M RA, diluted in 0.5× MMR, or 100 ng/ml bFGF (Boehringer Mannheim) in 0.5× MMR, 0.1% BSA, immediately after dissection. Some animal caps were combined with chick tissues as described below. Animal caps were cultured on agarose-coated petri dishes in 0.5× MMR containing penicillin/streptomycin until sibling controls reached the stage noted.

For analysis of whole embryos, *XWnt-8* DNA (200 pg CSKA-X8; Christian and Moon, 1993) was co-injected with *β-galactosidase* mRNA as a tracer at the 2-cell stage in the animal region of a single blastomere. The dn *XWnt-8* RNA (0.5 ng) was co-injected with *β-galactosidase* mRNA at the 8- to 16-cell stage in the animal region in a single blastomere. Injected embryos were allowed to develop to stage 14–16, stained for *β-galactosidase* activity with X-gal to localize the tracer, and then analyzed by whole-mount *in situ* hybridization.

Xenopus/Chick Explant Recombinant Cultures

Dissections of avian tissue explants were performed as described in Bang *et al.* (1997). Hensen's node or PNM explants were "sandwiched" between two pieces of *Xenopus* animal cap tissue (Fig. 1A). Animal cap/chick tissue recombinants were cultured in 0.5× MMR at room temperature as described above.

RNase Protection

RNA was isolated and analyzed by RNase protection assay (RPA), using ^{32}P-labeled antisense RNA probes, as previously described (Melton *et al.*, 1984; Kintner and Melton, 1987). The probes used to detect *Pax-3*, *N-CAM*, *EF-1α*, *Otx-2*, *β-tubulin*, and *Msx-1* RNAs have been described previously (Kintner and Melton, 1987; Ferreiro *et al.*, 1994; Papalopulu and Kintner, 1996; Bang *et al.*, 1997; Suzuki *et al.*, 1997). RNA samples isolated from 10 animal caps or 5 animal cap/chick tissue recombinants were analyzed simultaneously with several probes. *EF-1α* expression was used as a loading control.

RESULTS

Pax-3 Induction by PNM and Hensen's Node

In a previous study we demonstrated that two sources of posteriorization signals, the organizer and the PNM, can induce ectopic *Pax-3* expression in anterior neural tissue (Bang *et al.*, 1997). In addition, we showed that signals from these tissues can be mimicked by the putative posteriorizing factors bFGF and RA. To examine the possibility that *Wnt* signaling may also play a role in the induction of *Pax-3*, *Xenopus* stage 9 (blastula) animal caps from embryos co-injected with *noggin* and *XWnt-8* RNAs were allowed to develop until sibling embryos reached stage 16 (early neurula), and then were analyzed by RPA (Fig. 1). *Noggin* animal caps have an anterior neural fate and do not express *Pax-3* (Lamb *et al.*, 1993; Bang *et al.*, 1997). However, *Pax-3* is induced in animal caps from embryos co-injected with *noggin* and *XWnt-8* RNA, suggesting that *Wnt* signaling could act to induce *Pax-3* expression in the posterior neural plate *in vivo*. Similar results were obtained when an *XWnt-8* DNA expression construct was co-injected with *noggin*, showing that induction of *Pax-3* occurs independent of any dorsalizing effects caused by *Xwnt-8* RNA injections (data not shown).

To test whether the PNM-mediated induction of *Pax-3* is dependent on a *Wnt* signal, explant sandwiches were made in which PNM from Hamburger–Hamilton (HH) stage 6 chicks was combined with *Xenopus* blastula stage animal caps from embryos co-injected with *noggin* and dn *XWnt-8* (Fig. 1A). This heterospecies assay offers a number of advantages (see also Kintner and Dodd, 1991). First, it is conducted at room temperature, at which *Xenopus* develops, but growth and differentiation of the chick tissue is arrested. Thus, signals arising from the explanted chick tissues are likely to reflect properties of the stage at which they were isolated. Second, expression of genes in the responding versus the inducing tissue can be easily distinguished. And finally, it is possible to perform very accurate dissections of early mesoderm from chicks. The chick/*Xenopus* sandwiches were incubated until *Xenopus* stage 16 and then analyzed by RPA. This experiment revealed that dn *XWnt-8* blocks *Pax-3* inductive signals from stage 6 PNM (Fig. 1B). In contrast, we showed previously that although bFGF and RA can induce *Pax-3* expression in *noggin* animal caps, stage 6 PNM induction of *Pax-3* in *noggin* animal caps is not blocked by a dominant-negative FGF receptor (XFD), a dn *ras*, or a dn *xRAR-γ*) (Amaya *et al.*, 1991; Bang *et al.*, 1997; Blumberg *et al.*, 1997; Feig and Cooper 1988). Thus, PNM-mediated induction of *Pax-3* expression occurs independent of RA and FGF (or other *ras*-mediated signals); however, it does require a *Wnt*-dependent signal.

In our previous study HH stage 6 Hensen's node (HN) from chick was also identified as a source of *Pax-3* inductive signals. Similar to PNM, explants of stage 6 HN induced *Pax-3* expression in *noggin* animal caps, independent of RA or FGF signaling, as induction was not blocked by XFD, dn *ras*, or dn *RAR-γ*. To test whether HN-mediated induction of *Pax-3* also depends on a *Wnt* signal, stage 6 HN was combined with animal caps from embryos co-injected with *noggin* and dn *XWnt-8*. Analysis of these recombinants revealed that, in contrast to PNM, *Pax-3* inductive signals from HN are not *Wnt*-dependent (Fig. 1B). To address the question of whether multiple, redundant posteriorization signals emanate from the organizer, and thus it is insufficient to block

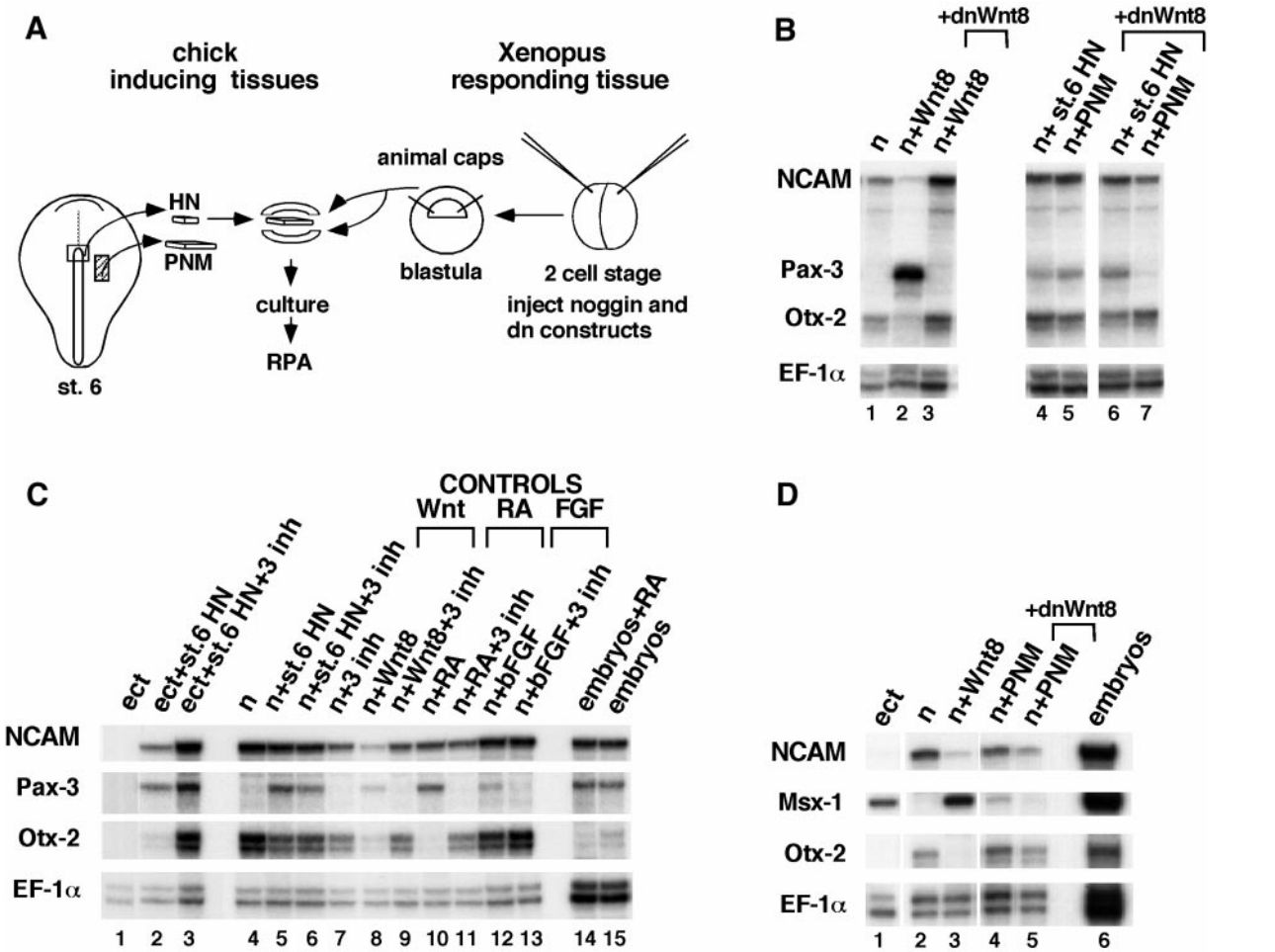


FIG. 1. *Pax-3* inductive activities from PNM are *Wnt*-dependent; however, Hensen's node induction of *Pax-3* does not depend on FGF, RA, or *Wnt,* either alone or in combination. (A) Experimental design. (B) Analysis of chick PNM/*Xenopus* dn *XWnt-8* and *noggin* animal cap recombinants. Animal caps from *Xenopus* blastulae were injected with only *noggin* (lanes 1, 4, 5) or co-injected with *noggin* and *XWnt-8* (lane2) or with *noggin* and *dn XWnt-8* (lanes 3, 6, 7). Lanes 1–3 show that *Xwnt-8* strongly induces *Pax-3* in *noggin* animal caps and that dn *XWnt-8* efficiently blocks this induction. Lanes 4–7 show that both stage 6 HN (lane 4) and PNM (lane 5) induce *Pax-3* expression in *noggin* animal caps; however, dn *XWnt-8* blocks *Pax-3* inductive signals from PNM (lane 7), but not HN (lane 6). Animal caps were analyzed by RPA when sibling controls were stage 16. (C) RPA analysis of chick HN/*Xenopus XFD,* dn *ras,* dn *RARg-1,* and *noggin* animal cap recombinants. Animal caps from blastulae co-injected with all three inhibitors dn *Ras,* dn *RARg-1,* and dn *XWnt-8,* in the absence (lane 3) or presence (lane 6) of *noggin,* were recombined with stage 6 HN. In both ectoderm and *noggin* animal caps, the combination of the three inhibitors did not block HN-mediated induction of *Pax-3* expression. Interestingly, repression of the anterior marker *Otx-2* by HN in lane 3 is blocked in the presence of the three inhibitors. Control lanes 8–13 show that each of the inhibitors can still function to block *Pax-3* expression in the presence of the other inhibitors when *XWnt-8* RNA is co-injected (lanes 8 and 9) or in the presence of 2×10^{-6} M RA (lanes 10 and 11) or in the presence of 100 ng/ml bFGF (lanes 12 and 13). ect, ectoderm; n, *noggin;* st.6 Hn, stage 6 Hensen's node; 3 inh, three inhibitors. (D) Analysis of *Msx-1* expression in chick PNM/*Xenopus* dn *XWnt-8* and *noggin* animal cap recombinants. Animal caps from *Xenopus* blastulae were injected with only *noggin* (lanes 2 and 4) or co-injected with *noggin* and *XWnt-8* (lane 3) or co-injected with *noggin* and dn *XWnt-8* (lane 5) or not injected (lane 1). Lane 1 shows that *Msx-1* is expressed in ectoderm alone, presumably the BMP-dependent component of the *Msx-1* expression pattern. *Msx-1* is not expressed in *noggin* animal caps (lane 2); however, *Msx-1* is strongly induced by *XWnt-8* in *noggin* animal caps (lane 3). Lanes 4 and 5 show that PNM induces *Msx-1* expression in *noggin* animal caps (lane 4); however, dn *XWnt-8* blocks this induction (lane 5).

only one, animal caps isolated from embryos co-injected with *noggin,* dn *XWnt-8,* dn *ras,* and dn *xRAR-γ* were combined with stage 6 HN explants. Analysis of these recombinants showed that HN explants still induce *Pax-3* expression even when FGF, RA, and *Wnt* signaling are simutaneously blocked (Fig. 1C). These results suggest that stage 6 HN produces a yet to be identified factor that can induce *Pax-3* expression.

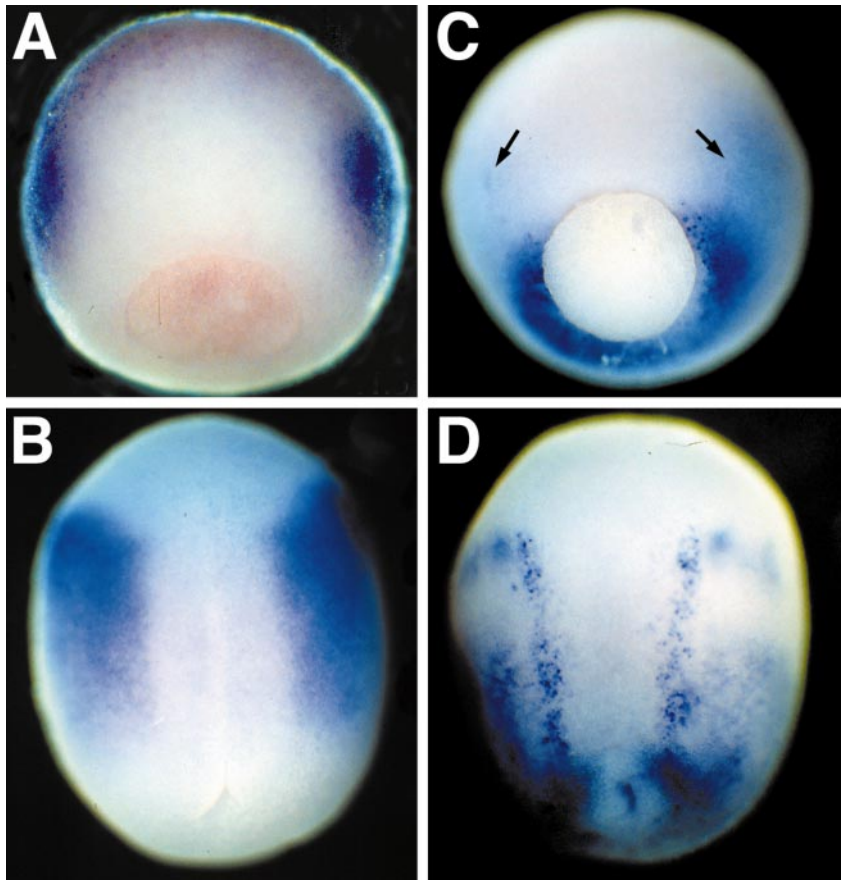


FIG. 2. Whole-mount *in situ* hybridization analysis of *Pax-3* expression in *Xenopus* embryos. (A) Dorsovegetal view at stage 11.5 showing that *Pax-3* is expressed in distinct lateral domains of the presumptive neural plate. (B) Dorsal view at stage 13 showing the refinement of *Pax-3* expression to lateral domains of the neural plate during convergence and extension. (C) Dorsovegetal view at stage 11.5 showing that *XWnt-8* is expressed in involuting ventral-lateral mesoderm. "Wings" of involuted mesoderm that underlie the prospective lateral neural plate are indicated by arrows. (D) Dorsal view at stage 13 showing X*Wnt*-8 expression in the lateral neural plate.

The Pattern of XWnt-8 Expression Is Consistent with a Role in the Regulation of Pax-3

The results above indicate that a *Wnt* signal produced by the PMN induces *Pax-3* expression during posteriorization of the neural plate. Since the dn *XWnt-8* construct used in this study blocks the activities of *XWnt-1*, *-3A*, and *-8*, we next considered whether the spatiotemporal expression of any of these *Wnt*-family members is consistent with that expected for an early *Pax-3* inducing activity. The earliest expression of *Pax-3* is detected in broad domains in the posterior and lateral neural plate in *Xenopus* stage 11 embryos (gastrula) (Fig. 2A, and data not shown). These broad domains become progressively refined to the neural folds with a rostral limit at the caudal midbrain, consistent with our proposal that an early signal associated with posteriorization of the neural plate initiates *Pax-3* expression, which is then refined further by D–V patterning signals (Fig. 2B; Bang *et al.*, 1997). Comparison of the expression patterns of *XWnt-1*, *-3A*, and *-8* with that of *Pax-3* indicates that *XWnt-8* is the most likely candidate to play a role in initiation and/or maintenance of *Pax-3* expression at gastrula stages. *XWnt-3A* is expressed in the lateral margins of the presumptive neural plate at stage 13, well after the onset of *Pax-3* expression, and *XWnt-1* expression is not detected in the dorsal neural tube until tailbud stages (McGrew *et al.*, 1997; Wolda *et al.*, 1993). However, *Xwnt-8* is restricted to ventral-lateral mesoderm during gastrulation, suggesting that it could act to induce or maintain *Pax-3* expression in a laterally restricted domain of the neuroectoderm (Fig. 2C, see also Smith and Harland, 1991; Christian *et al.*, 1991). Moreover, *CWnt-8C*, a chicken gene that is homologous to *XWnt-8*, is expressed in nonaxial mesoderm in gastrulating chick embryos and may therefore represent the PNM-derived *Wnt* signal we detect in our chick/*Xenopus* explant recombinant assay (Hume and Dodd, 1993). If *XWnt-8* were to play a role in initiating

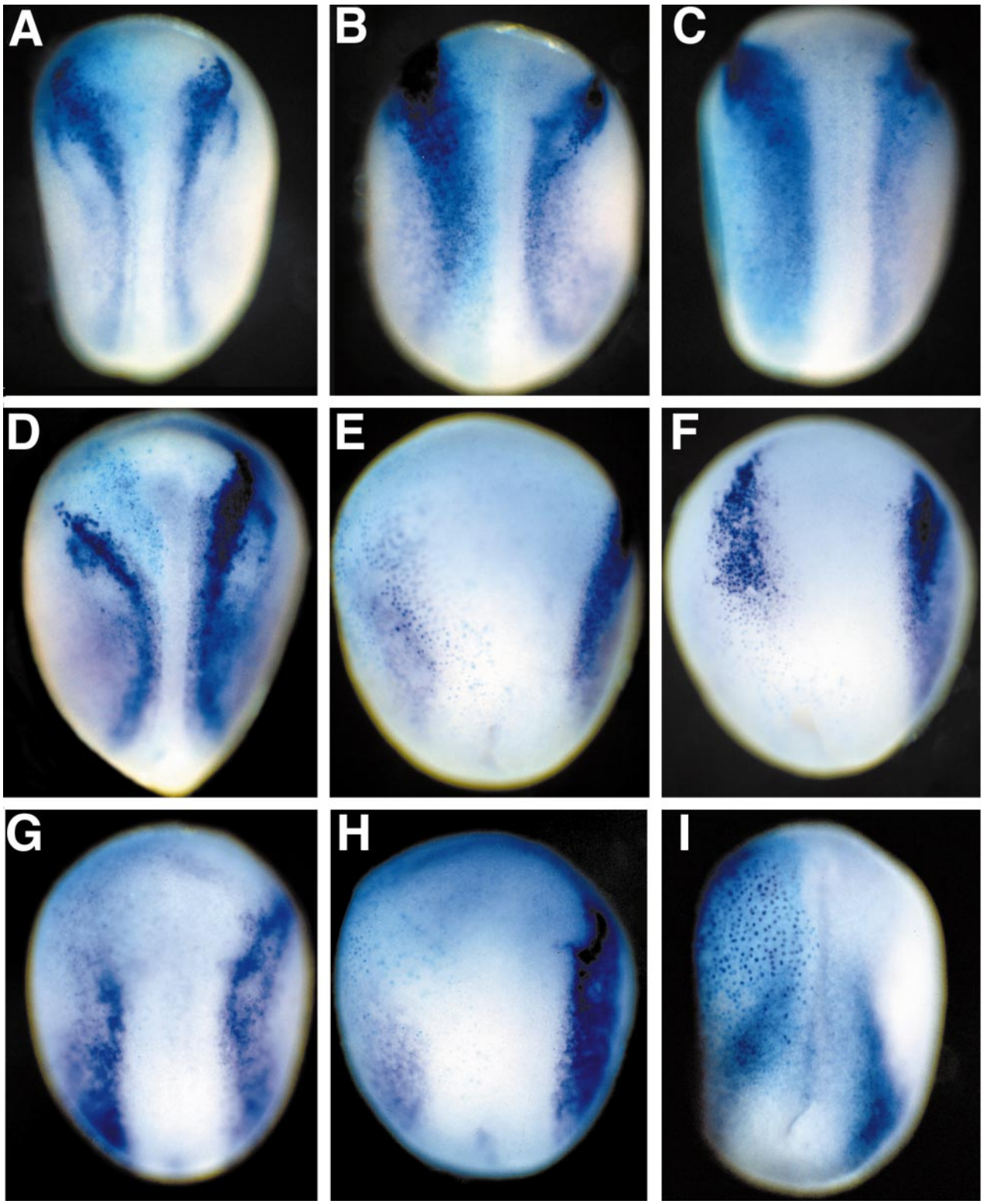


FIG. 3. Effects of gain and loss of *Wnt* function on the expression of *Pax-3* and *Msx-1*. Embryos were injected with *β-galactosidase* RNA alone (A) or co-injected with *β-galactosidase* and *XWnt-8* DNA (B and C), or *β-galactosidase* and dn *Xwnt-8* RNA (D–I). Dorsal views are shown in all images (anterior is at the top) with the injected side always on the left. Injected embryos were analyzed by whole-mount *in situ* hybridization for expression of *Pax-3* (A–F), *Msx-1* (G, H), or *myoD* (I).

Pax-3, it is unclear whether it would act in a planar or vertical fashion as there is very little *XWnt-8*-expressing lateral mesoderm that has involuted at the onset of *Pax-3* expression (see Discussion). However, by stage 11.5 the involuted "wings" of *XWnt-8*-expressing lateral mesoderm could play a role in maintaining *Pax-3* expression (Fig. 2C). Interestingly, *XWnt-8* is also detected in posteriolateral neuroectoderm commencing at stage 13 (early neural plate) in a pattern also consistent with it playing a role in the maintenance of *Pax-3* expression (Fig. 2D).

Expression of Pax-3 in the Lateral Neural Plate Expands in Embryos Injected with XWnt-8 and Is Blocked in Embryos Injected with Dominant-Negative XWnt-8

To further test our hypothesis that *Pax-3* is regulated by a *Wnt*-mediated signal, we examined the consequences of gain and loss of *XWnt-8* function on the pattern of *Pax-3* expression *in vivo*. To perform the gain-of-function experiment we injected an *XWnt-8* DNA expression construct into one cell of a two-cell-stage *Xenopus* embryo. *β-galactosidase* RNA was co-injected as a lineage tracer. An *XWnt-8* DNA construct was used because it would not be expressed until the midblastula transition, thus secondary dorsal-axis induction resulting from *XWnt-8* RNA injections could be avoided. Injected embryos were allowed to develop until neurula stages 14–16. Embryos were then stained for β-galactosidase activity using X-gal and analyzed for *Pax-3* expression by *in situ* hybridization. Comparison of the injected and uninjected sides of the embryos revealed an expansion of the *Pax-3* expression domain and an apparent upregulation of *Pax-3* transcript levels that colocalizes with the *β-galactosidase* lineage tracer (Figs. 3B and 3C, Table 1). The *XWnt-8*-induced expansion of the *Pax-3* expression domain primarily occurred laterally, with ectopic *Pax-3* expression not detected in more medial domains. This suggests that there are strong inhibitors medially that block the effects of ectopic *XWnt-8* expression or, alternatively, that there are laterally localized cofactors that are required for *Wnt-8*-mediated induction of *Pax-3* expression.

Next we asked whether blocking *Wnt* signaling *in vivo* using the dn *XWnt-8* construct would affect the early pattern of *Pax-3* expression. It has been shown that injection of dn *XWnt-8* RNA into the marginal zone of 2- to 4-cell-stage embryos inhibits formation of ventral-lateral mesoderm and blocks induction of *MyoD* expression (Hoppler *et al.*, 1996). To avoid disturbing mesoderm development, dn *XWnt-8* RNA was injected into single blastomeres in the animal region of 8- to 16-cell-stage embryos. In dn *XWnt-8*-injected embryos *Pax-3* expression was eliminated or strongly downregulated in areas that colocalized with the *β-galactosidase* tracer (Figs. 3D–3F, Table 1). This inhibition included the early broad domain associated with the formation of the posteriolateral neural plate and not just that associated with early neural crest formation in the cranial region. To confirm that it is unlikely that this phenotype results indirectly from an effect of dn *XWnt-8* on underlying mesoderm, *MyoD* expression was also examined and found to be unaltered (Fig. 3I, Table 1). These results reveal that either initiation and/or maintenance of *Pax-3* expression in the lateral neural plate requires a *Wnt*-mediated signal that is blocked by dn *XWnt-8*. Furthermore, the chick/*Xenopus* explant recombinant experiments described above suggest that this *Wnt*-meditated signal may arise from posteriolateral mesoderm.

To examine whether our results showing that *Pax-3* expression is *Wnt*-dependent reflect a more general requirement for *Wnt* signaling in patterning of the posteriolateral neural plate, we also analyzed expression of the transcription factor *Msx-1* in dn *XWnt-8*-injected embryos. The expression pattern of *Msx-1* in the presumptive neural plate of both chicks and *Xenopus* is strikingly similar to that of *Pax-3* (Suzuki *et al.*, 1997; Muhr *et al.*, 1997). In *Xenopus*, the onset of *Msx-1* expression occurs in the presumptive posteriolateral neural plate at gastrula stage 11, and this broad domain of expression subsequently becomes refined to the neural folds (Suzuki *et al.*, 1997). Similar to *Pax-3*, *Msx-1* is not expressed in *noggin* animal caps, but it can be induced by co-injection with *XWnt-8* or by recombination with PNM explants from chicks (Fig. 1D). In addition, PNM-mediated induction of *Msx-1* is blocked by dominant-negative *XWnt-8*. We note that in contrast to *Pax-3*, *Msx-1* is also expressed outside the neural plate in the presumptive epidermal ectoderm (Suzuki *et al.*, 1997). The epidermal component of *Msx-1* expression is dependent on *BMP-4;* however, the observation that *Msx-1* expression can be induced in *noggin* animal caps suggests that initiation of the neural component of *Msx-1* expression may be *BMP*-independent (Fig. 1D; Suzuki *et al.*, 1997). In dn *XWnt-8*-injected embryos *Msx-1* expression in the neural plate was eliminated or strongly downregulated in areas that colocalized with the *β-galactosidase* tracer, suggesting that similar to *Pax-3*, initiation and/or maintenance of *Msx-1* expression in the neural plate depends on a *Wnt*-mediated signal (Figs. 3G and 3H and Table 1). The epidermal component of *Msx-1* expression was unaffected in the dominant-negative *XWnt-8*-injected embryos (data not shown). We attempted to analyze the effect of *XWnt-8* overexpression on *Msx-1;* however, this proved to be difficult. Since *Msx-1* is also expressed in epidermal ectoderm that flanks the neural plate it was not possible to unambiguously discern whether the neural *Msx-1* expression domain was expanded (data not shown).

Dominant-Negative XWnt-8 Blocks Development of Rohon–Beard Cells

The expression domain of *Pax-3* in the early neural plate encompasses a rather broad region from which both neural crest and primary sensory neurons or Rohon–Beard cells will arise. Recently, it has been demonstrated that expression of the neural crest marker *slug* expands in embryos injected with *XWnt-8* and is blocked in embryos injected

TABLE 1

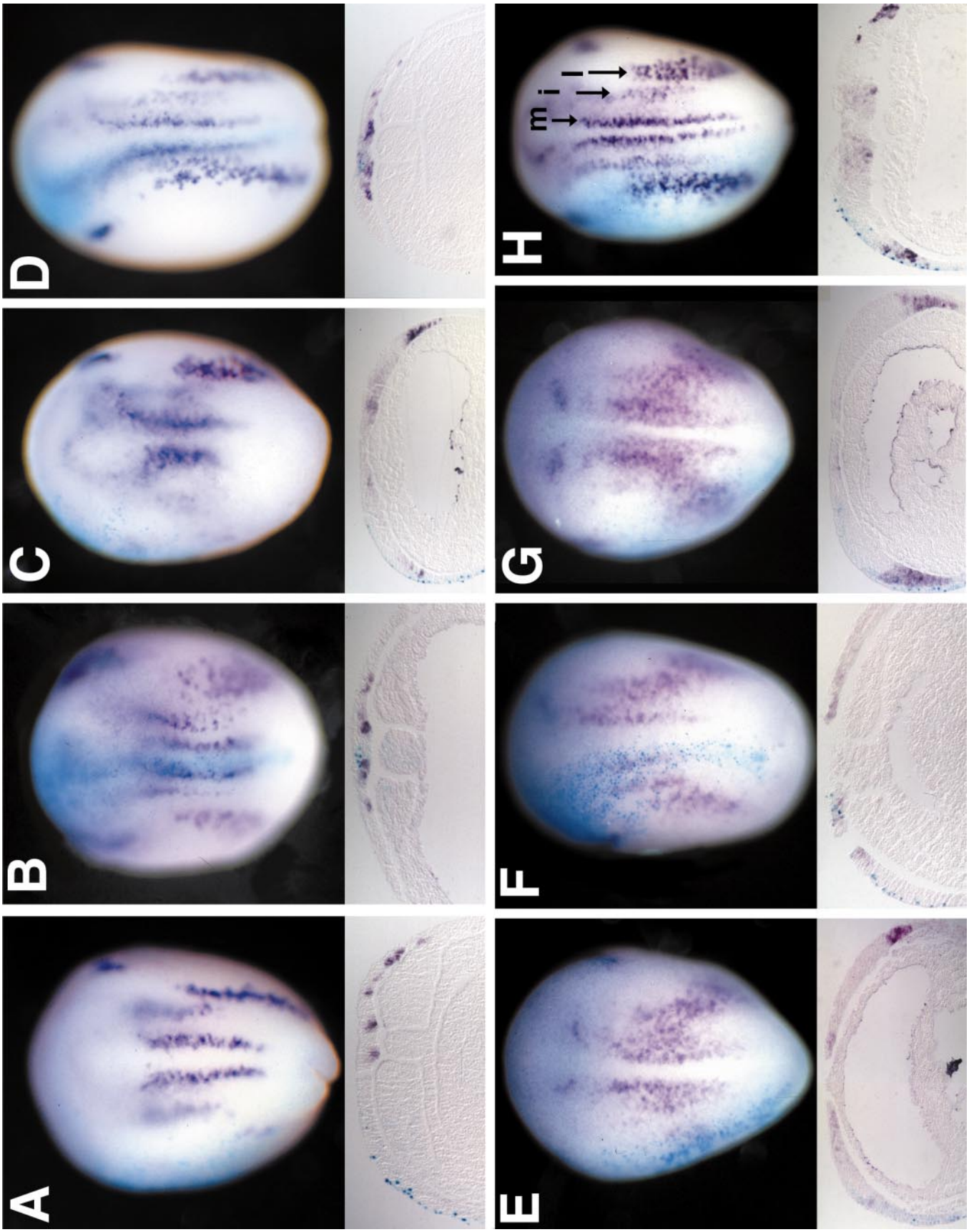
Injection	Probe	*n*	Eliminated or reduced	Mild phenotype or unaffected	Expanded
dn *Xwnt-8*	*Pax-3*	53	46 (86%)	5 (10%)	2 (4%)
dn *Xwnt-8*	*myoD*	42	2 (4%)	40 (96%)	0
dn *Xwnt-8*	*Msx-1*	45	32 (71%)	13 (29%)	0
dn *Xwnt-8*	*tubulin*	23 lat.	20 (87%)	3 (13%)	0
		25 med.	1 (4%)	24 (96%)	0
dn *Xwnt-8*	*elrC*	59 lat.	50 (85%)	9 (15%)	0
		36 med.	0	36 (100%)	0
dn *Xwnt-8*	*Xnrgr-1*	51 lat.	43 (84%)	8 (16%)	0
		21 med.	4 (20%)	17 (80%)	0
Xwnt-8	*Pax-3*	47	3 (7%)	5 (10%)	39 (83%)

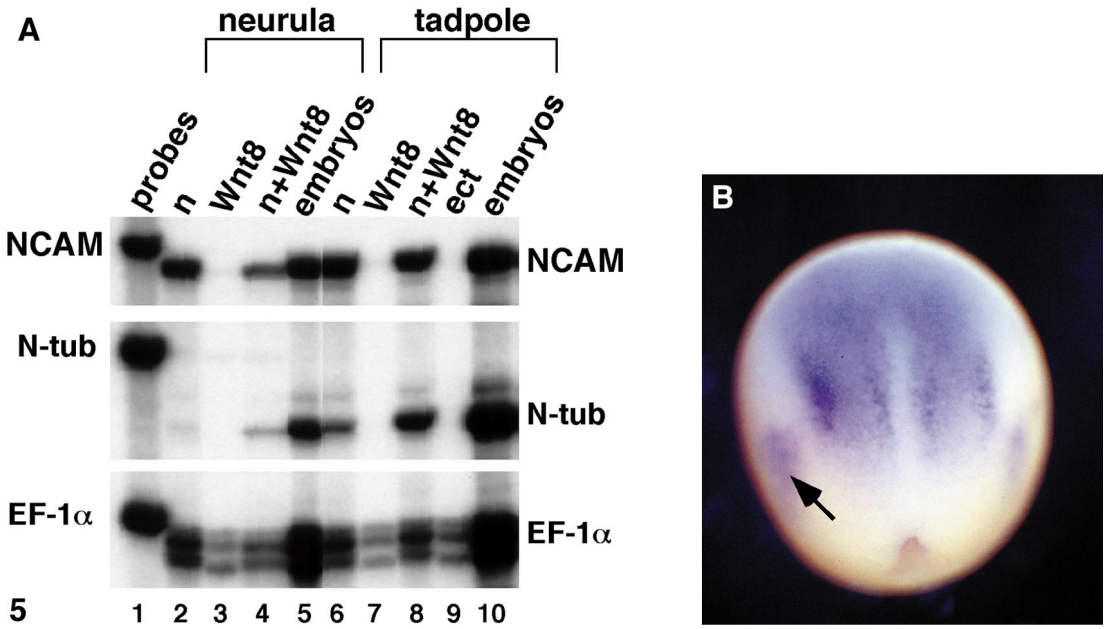
Note. Injected embryos were scored for either reduction or expansion of expression of the above genes. *β-tubulin, elrC,* and *Xnrgr-1* were scored for Rohon–Beard (lat.) and motor neuron (med.) domains.

with dominant-negative *XWnt-8* as we have shown for *Pax-3* (La Bonne and Bronner-Fraser, 1998). Thus, we were interested in determining whether dn *Xwnt-8* blocks only neural-crest-cell markers or whether Rohon–Beard sensory neurons are also *Wnt*-dependent. dn *XWnt-8* RNA was injected into single blastomeres in the animal region of 8- to 16-cell-stage embryos. The injected embryos were analyzed at neural plate stages for expression of the primary neuron markers *elrC* and *neural specific β-tubulin* (Good, 1995; Chitnis *et al.,* 1995). The Rohon–Beard cells, as detected by both *elrC* and *β-tubulin* expression, were either eliminated or greatly reduced in number when the *β-galactosidase* tracer was localized to the lateral domain of the neural plate in dn *XWnt-8*-injected embryos (Figs. 4A–4D, Table 1). In contrast, when the *β-galactosidase* tracer was localized to the medial (motor neuron) domain of primary neurons, *elrC* and *β-tubulin* expression was unaffected.

A possible explanation for these observations is that differentiation of Rohon–Beard cells, but not primary motor neurons, is *Wnt*-dependent. Alternatively, the loss of *elrC* and *β-tubulin* expression in dn *XWnt-8*-injected embryos could reflect an earlier defect, in that a domain competent to undergo neurogenesis fails to be established in the lateral neural plate. To distinguish these possibilities we examined expression of an earlier marker, *Xenopus neurogenin related-1* (*Xnrgr-1*), which marks the precursor cells from which primary neurons arise (Ma *et al.,* 1996). In dn *XWnt-8*-injected embryos *Xnrgr-1* expression in the medial domain was unaffected or mildly repressed, while in the lateral neural plate *Xnrgr-1* expression was eliminated or strongly downregulated, suggesting that a lateral domain of neuronal competence is not specified (Figs. 4E–4G, Table 1). Although we did observe some repression of *Xnrgr-1* in the medial domain it was relatively mild in comparison to the strong repression we observed in the lateral domain. Interestingly, whereas *Xnrgr-1, elrC,* and *β-tubulin* expression was always reduced in the Rohon–Beard domain of dn *XWnt-8*-injected embryos, the expression of these genes in the trigeminal ganglia was reduced in some embryos but expanded in others (data not shown). We did not investigate this phenotype further; however, it implies that *Wnt*-dependent patterning of neurogenesis in the trigeminal ganglia may be more complex than that of Rohon–Beard cells. We note that Marcus *et al.* (1998) showed that overexpression in *Xenopus* embryos of *glycogen synthase kinase-3* (*GSK-3*), an inhibitor of *Wnt*-signaling, represses *β-tubulin* expression in both sensory and motor domains, but does not repress *NeuroD* expression. In addition, they observed that *GSK-3* could block the ability of *Xnrgr-1* to induce ectopic *β-tubulin*-expressing cells, leading to a model in which *GSK-3* is required downstream of *NeuroD* for the differentiation of primary neurons. However, we found that dn *XWnt-8* does not block *Xnrgr-1*-induced ectopic neurogenesis, nor does it block the differentiation of primary motor neurons, suggesting that the activity of *GSK-3* described by Marcus *et al.* (1998) may affect a pathway different from that affected by the dn *XWnt-8* construct (Fig. 4 and data not shown).

In gain-of-function experiments we observed that injection of *XWnt-8* DNA *in vivo* did not reliably expand or alter the pattern of *β-tubulin* expression, suggesting that a *Wnt* signal, while necessary, is not sufficient for the development of Rohon–Beard cells or that there are inhibitors present *in vivo* that block the effects of ectopic *XWnt-8* expression (data not shown). The latter possibility is supported by our observation that expression of *XWnt-8* is sufficient to upregulate *β-tubulin* expression *in vitro* in *noggin* animal caps. Stage 9 (blastula) animal caps from embryos co-injected with *noggin* RNA and *XWnt-8* DNA were allowed to develop until sibling embryos reached stage 16 (early neurula) or 25 (early tadpole) and then analyzed by RPA (Fig. 5A). At both stages, levels of *β-tubulin* expression increased when *Xwnt-8* was co-injected with *noggin*. However, the observed increase in *β-tubulin* expression is lower than expected compared with control embryos, suggesting that *Wnt*-dependent neurogenesis may require other factors





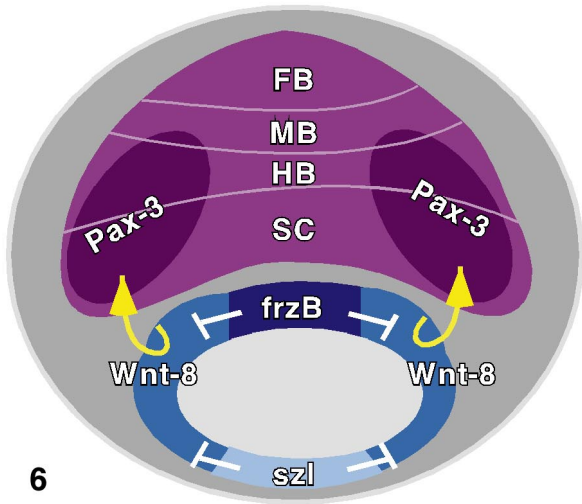


FIG. 5. (A) *XWnt-8* upregulates *β-tubulin* expression in *noggin* animal caps. Animal caps from *Xenopus* blastulae were injected with *noggin* RNA (lanes 2 and 6) or with *XWnt-8* DNA (lanes 3 and 7) or were co-injected with *noggin* RNA and *XWnt-8* DNA (lanes 4 and 8). Lanes 4 and 8 show that *Xwnt-8* upregulates *β-tubulin* expression in *noggin* animal caps. Note that in lane 4 NCAM expression is decreased (see also Fig. 1B, lane 2, 1C, lane 8, and 1D, lane 3). Animal caps were analyzed by RPA when sibling controls were stage 16 (neurula) and stage 25 (early tadpole). ect, ectoderm; n, *noggin;* N-tub, *neural specific β-tubulin*. (B) Whole-mount *in situ* hybridization analysis of a stage 12.5 embryo showing that the Rohon–Beard cells, marked by *elrC* (arrow, punctate staining), lie outside the central portion of the neural plate, marked by NCAM (diffuse staining). Both probes were detected with NBT/BCIP.

FIG. 6. Model for induction of *Pax-3* expression in the lateral neural plate. A *Wnt*-mediated posteriorizing activity arising from PNM would act to initiate and/or maintain *Pax-3* expression in the posteriolateral neural plate. The secreted inhibitors of *Wnt* signaling, *frzB* and *szl*, which pattern the mesoderm, could also play a role in restricting the activity of *Wnt* signaling in the neural plate, leading to a refinement of the *Pax-3* expression domain. Furthermore, the *Wnt*-dependent induction of a *Pax-3*-positive domain would establish spatial information necessary for appropriate specification of later arising cell types such as Rohon–Beard primary neurons and neural crest.

FIG. 4. Effects of loss of *Wnt* function on the expression of *elrC*, *β-tubulin*, and *Xnrgr-1*. Embryos were co-injected with *β-galactosidase* and dn *Xwnt-8* RNA (A–F) or injected with *β-galactosidase* RNA alone (G, H). Dorsal views are shown in all images (anterior is at the top) with the injected side always on the left. 10-μm transverse paraffin sections showing that the *β-galactosidase* tracer colocalizes with the affected domains of primary neurons are shown immediately beneath the corresponding whole-mounted embryo. Injected embryos were analyzed by whole-mount *in situ* hybridization for expression of *β-tubulin* (A, B), *elrC* (C, D, H), and *Xnrgr-1* (E–G). The lateral (l), intermediate (i), and medial (m) domains of *elrC*-expressing primary neurons are indicated by arrows in H.

not present in the *noggin* cap and/or that inhibitors are present. Interestingly, this increase of *β-tubulin* expression was accompanied by a decrease in NCAM expression (Figs. 5A, 1B lane 2, 1C lane 8, and 1D lane 3; see also Cunliffe and Smith, 1994). NCAM marks the medial portion of the neural plate, excluding the Rohon–Beard domain (Fig. 5B; see also Sasai and De Robertis, 1997). Thus, the observed decrease in NCAM expression suggests that *XWnt-8* acts to confer a posteriolateral fate to *noggin* animal caps.

Together these data support a model in which patterning of the lateral neural plate by *Wnt*-mediated signals is an early event that establishes a domain, marked by *Pax-3* and *Msx-1* expression, from which Rohon–Beard cells, as well as neural crest, will develop.

DISCUSSION

Expression of Pax-3 in the Neural Plate Depends on a Wnt-Dependent Signal from PNM

Several studies have shown recently that signals from PNM are capable of posteriorizing the neuraxis. In zebrafish, transplantation studies have demonstrated that transforming signals that are not mimicked by FGF arise from the mesoderm and endoderm lateral to the shield (organizer) (Woo and Fraser, 1997). In *Xenopus,* lateral mesoderm was shown to produce an RA-dependent signal that induces expression of *HoxD1* (Kolm *et al.,* 1997). In chicks, gastrula stage PNM is capable of inducing *Pax-3* expression in anterior neural tissue both *in vitro* and *in vivo,* as well as in *Xenopus noggin* animal caps in a manner independent of RA- or FGF-mediated signaling (Bang *et al.,* 1997). Finally, Muhr *et al.* (1997) identified a PNM-derived activity from gastrula-stage chicks, distinct from RA and FGF, that can caudalize anterior neural plate explants. Together these studies indicate that PNM acts to pattern the posterior CNS, but whether this involves multiple posteriorizing activities, with redundant overlapping functions, remains unclear.

Four lines of evidence presented here suggest strongly that one of the activities produced by PNM is a *Wnt*-mediated signal that acts during early gastrula stages to induce *Pax-3* expression in the posteriolateral neural plate. First, a *Pax-3* inductive signal from chick PNM is blocked by dn *XWnt-8* in chick/*Xenopus* explant recombinants. Second, blocking *Wnt*-signaling *in vivo* by injecting dn *XWnt-8* into *Xenopus* embryos results in loss of *Pax-3* expression. Third, ectopic expression of *XWnt-8* via DNA injections results in an expansion of *Pax-3* expression laterally, although not medially. Finally, the spatial and temporal requirements for the early pattern of *Pax-3* expression are more consistent with a *Wnt*-mediated signal arising from mesoderm rather than from neuroectoderm. We note that in using chick/*Xenopus* explant recombinants in this study we have assumed that a potential role for PNM in the early patterning of the lateral neural plate has been evolutionarily conserved between avians and amphibians. It is clear that there will be differences in mechanisms that pattern the *Xenopus* and chick neural plates. For instance, unlike in *Xenopus,* blocking BMP signaling with *chordin* is not sufficient to induce neural tissue in chicks (Streit *et al.,* 1998). It is certainly possible that chick PNM is a biologically irrelevant source of a *Wnt*-dependent signal that induces *Pax-3* in *Xenopus* animal caps. However, the following observations support the hypothesis that PNM-mediated activities may be conserved. First, *Pax-3* and *Msx-1* are very early markers of the lateral neural plate in both chicks and *Xenopus* that can be induced by PNM in both chick and *Xenopus* neural explants (Bang *et al.,* 1997, Muhr *et al.,* 1997, A.G.B. and M.D.G., unpublished observations; this study). Second, as discussed below, both *Xenopus* and chick PNM have been shown to play roles in neural crest induction. Indeed, *Xenopus* dorsolateral mesoderm has been shown to induce *slug* expression in *chordin* neuralized animal caps, similar to our observation that chick PNM can induce *Pax-3* and *Msx-1* expression in *noggin* animal caps (La Bonne and Bronner-Fraser, 1998).

Of the known *Wnt* genes whose activities are blocked by dn *XWnt-8, XWnt-8* which is expressed in lateral mesoderm is the best candidate both spatially and temporally for the induction and/or maintenance of the early *Pax-3* expression. *XWnt-8* and *XWnt-3A* are not detected in the neuroectoderm until stage 13, well after the onset of *Pax-3* expression and in a domain that is significantly more narrow than that of *Pax-3.* In contrast, expression of *XWnt-8* in lateral mesoderm at gastrula stages is temporally consistent with the onset of *Pax-3* expression. However, if *XWnt-8* were to play a role in initiating *Pax-3* expression it is unclear whether it would act in a planar or vertical fashion since very little *XWnt-8*-expressing lateral mesoderm has involuted at the onset of *Pax-3* expression at stage 11. *Pax-3* is expressed in Keller explant "giant sandwiches" made at stage 10.5, suggesting that, although vertical contacts may play a role prior to stage 10.5, after this time planar signals are sufficient to maintain most *Pax-3* expression. However, neither *Pax-3* nor *slug* expression is completely normal in these sandwiches as *Pax-3* is reduced and *slug* is absent in the cranial masses, suggesting that vertical signals may be important in this region (A.G.B., C.K., and Raymond Keller, unpublished observations; Poznanski *et al.,* 1997). While these observations suggest strongly that a *Wnt*-dependent signal from the PNM in early gastrulae plays a role in either inducing or maintaining a lateral domain of *Pax-3* expression in the posterior neural plate, it is also possible that *Wnt* signaling may be necessary only for the neural plate to be competent to form posteriolateral tissue. *XWnt-7B* is expressed uniformly in the animal half and marginal zone of gastrula-stage embryos, and even though it is not spatially localized, it could play a role in patterning the lateral neural plate as its overexpression causes an expansion of expression of the neural crest marker *twist* (Chang and Hemmati-Brivanlou, 1998). For instance, it is possible that *XWnt-7B,* or a yet to be

identified *Wnt*, could make the ectoderm competent to respond to the *Pax-3* inducing signal from PNM.

The observation that *XWnt-8* is required for specification of ventral and somitic mesoderm, coupled with our findings that an early *Wnt* signal is required for patterning the lateral neural plate, suggests that the roles of *Wnt* signaling in the mesoderm and early neural plate may be coordinated. For instance, inhibitors of *Wnt* signaling that pattern the mesoderm could also play a role in restricting the activity of *Wnt* signaling in the neural plate, leading to a refinement of the *Pax-3* expression domain. *frzB* and *sizzled* (*szl*), which encode secreted proteins that act as antagonists of *XWnt-8* signaling, are expressed during gastrulation in dorsal and ventral mesodermal domains, respectively (Leyns *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997). Thus, the highest levels of *XWnt-8* signaling would occur in lateral mesoderm, correlating well with restricted expression of *Pax-3* in lateral neuroectoderm. The expression of *frzB* and *szl* also provides an explanation for our observation that although injection of *XWnt-8* into *Xenopus* embryos induces an expansion of the *Pax-3* expression domain, it does not result in ectopic *Pax-3* expression in the medial neural plate, or in ventral ectoderm, regions that could be influenced by these *Wnt* antagonists from the mesoderm (Fig. 6).

Although these results emphasize an early role of *Wnt* signaling at neural plate stages, there is likely to be a role for *Wnt* signaling in the dorsal neural tube at late stages as well. For example, examination of mice that are compound null mutants for *Wnt-1* and *Wnt-3A* revealed reduced expression of *Pax-3* in the dorsal hindbrain, a phenotype attributed to failure of expansion of dorsal neural precursors (Ikeya *et al.*, 1997). While such a proliferation defect is unlikely to underlie the loss of *Pax-3* expression that we observed in *dn XWnt*-8-injected embryos at neural plate stages, is it possible that *XWnt-1* and *XWnt-3A* could play a similar role in *Xenopus* by promoting the expansion of dorsal precursors in the neural tube at later stages. It has also been proposed that *Wnt* signaling acts to posteriorize other aspects of the *Xenopus* neuraxis. McGrew *et al.* (1998) have shown that the expression of the posterior neural markers *En-2* (midbrain–hindbrain) and *Krox-20* (rhombomeres 3 and 5) is blocked in *Xenopus* embryos by the dn *XWnt-8* mutant. However, it should be emphasized that the requirement for *Wnt* signaling for expression of *En-2* and *Krox-20* may not be the same as that for *Pax-3*, given that *Wnt*s are likely to have multiples roles in neural patterning, all of which could be targeted by the dn *XWnt-8* mutant. Thus, the observed *Wnt* dependence of *Krox-20* and *En-2*, which are expressed relatively late during neural plate development in very specific subdomains, may reflect secondary patterning interactions, rather than a primary response to A–P patterning signals as we suggest for *Pax-3*.

Is Wnt-Signaling Required to Establish a Posteriolateral Domain That Is Competent to Give Rise to Neural Crest and Rohon–Beard Cells?

The loss of neural-crest-cell derivatives in *Splotch* mice, which carry loss-of-function mutations in the *Pax-3* gene, suggests that the induction of *Pax-3* is an important step in the differentiation of neural crest cells from the lateral neural plate (Franz, 1990; Epstein *et al.*, 1991 and references therein). The observation that the onset of *Pax-3* expression is at stage 11 (midgastrula), whereas *slug* expression is not detected until stage 12 (late gastrula), in the cranial region, and stage 16 (neural plate), at more caudal levels, implies that establishment of a *Pax-3*-positive region in the lateral neural plate may be an important event upstream of the initiation of *slug* expression in the neural crest (Mayor *et al.*, 1995; Bang *et al.*, 1997). One possibility is that *Wnt* signaling induces neural crest cells directly. Alternatively, it could be required even earlier to pattern the neural plate, thus generating a posteriolateral domain where neural crest cells arise. The latter possibility is supported by the observation that *Pax-3* and *Msx-1* are expressed considerably earlier and in a broader domain than *slug*. We propose that the *Wnt* requirement exhibited by *slug* reflects a *Wnt*-dependent posteriorizing signal from PNM that establishes a posteriolateral domain, marked by *Pax-3* expression, that gives rise to neural crest cells. This model is consistent with studies in *Xenopus* embryos that used explant conjugates to demonstrate that either dorsal or lateral marginal zone can induce *slug* expression in ectoderm, leading to the proposal that an early signal from lateral mesoderm may "prime" the neural plate, enabling it to respond later to neural-crest inducing signals from the epidermal ectoderm (Mayor *et al.*, 1995; Bonstein *et al.*, 1998). Similarly, Muhr *et al.* (1997) showed in chicks that a PNM-derived signal(s) acts to caudalize the early neural plate, rendering it competent to express *slug* in response to BMP-4, suggesting that specification of neural crest cells is linked to posteriorization of the neuraxis. This idea is consistent with the observation that neural crest cells arise from posterior neural tissue at levels caudal to the caudal diencephalon and are not generated from more rostral forebrain (Muhr *et al.*, 1997; Couly and Le Douarin, 1987).

Current models suggest that a cascade of transcription factors acts to regulate neurogenesis, such that "determination genes" like *Xnrgr-1* confer neuronal potential to a domain of cells and "differentiation genes" like *NeuroD* stably induce neuronal fate in individual cells within those domains (see Ma *et al.*, 1996). However, little is known about how the different domains of neuronal potential from which primary neurons arise are established during development of the *Xenopus* neural plate. Similar to models of neural crest development discussed above, it has been proposed that neurogenesis in the *Xenopus* embryo is also linked to A–P patterning of the neuraxis (Papalopulu and Kintner, 1996). Thus, in this model the first wave of neurogenesis that occurs in the neural plate to give rise to

primary neurons would take place only in neural tissue with a posterior fate. Based on our observation that *Xwnt-8* upregulates *β-tubulin* expression in *noggin* animal caps, and that dn *XWnt-8* blocks neurogenesis in the Rohon–Beard domain, we propose that posteriolateralization of the neural plate by a *Wnt*-dependent signal is a necessary first step in the development of Rohon–Beard primary neurons. Moreover, our observation that dn *XWnt-8* specifically blocks *Xnrgr-1* expression in the Rohon–Beard domain suggests that *XWnt-8* may act to set up a region of neuronal potential in the lateral neural plate. Indeed, such a mechanism may be conserved in evolution as some proneural domains in the developing *Drosophila* adult peripheral nervous system are dependent on *wingless* (Phillips and Whittle, 1993).

The observation that both neural crest and Rohon–Beard neurons are *Wnt*-dependent supports a model in which a *Wnt* signal acts extremely early during neural plate development to confer a posteriolateral identity, setting up a *Pax-3*-positive domain from which these cell types arise.

A Pax-3 Inductive Signal That Is Independent of FGF, RA, and Wnt Arises from HN

In addition to PNM, we had also identified chick stage 6, but not stage 4, HN as a source of *Pax-3* inductive activity, consistent with studies showing that late, but not early, organizer produces posteriorizing signals. Using chick/*Xenopus* recombinants we have found that the *Pax-3* inductive activity from stage 6 HN does not depend on *XWnt-8*, or FGF and RA, either alone or in combination, suggesting, not surprisingly, that the organizer may produce additional, unknown posteriorizing factor(s). This result is consistent with *in vivo* experiments showing that *XFD* transgenic *Xenopus* embryos exhibit relatively normal expression of *Pax-3* and that ectopic expression of a dominant-negative form of the *RAR-α1* does not alter the *Pax-3* expression pattern (Kroll and Amaya, 1996; N.P., unpublished observation).

If there are *Pax-3* inductive signals localized medially (from the organizer) then why are *Pax-3* transcripts detected only laterally in the early neural plate? One possibility is that *in vivo* there are medial inhibitors of *Pax-3* expression that would override *Pax-3* inductive signals coming from the organizer. Indeed, *Pax-3* appears to be actively repressed in the ventral neural tube by midline signals, as loss of the notochord or of *shh* results in a ventral expansion of *Pax-3* expression in *Xenopus*, chicks, and mice (Chiang *et al.*, 1996; Espeseth *et al.*, 1995; Liem *et al.*, 1995; Goulding, *et al.*, 1993). Thus, the laterally restricted expression of *Pax-3* may depend on the lateral restriction of *XWnt-8* in the lateral mesoderm in combination with a medial signal that would act to repress inductive signals from the organizer.

Moreover, our observation that there are qualitative differences in *Pax-3* inductive signals arising from medial (the organizer) versus lateral (PNM) tissues suggests that such differences may contribute to patterning the posterior neural plate along its medial–lateral axis. Thus, there may be different transformation signals that specify posteriomedial versus posteriolateral regions of the neuraxis. In addition, competence factors within the neuroectoderm itself may regulate its response to patterning signals. Indeed, the zinc-finger protein *opl* and the translation initiation factor *eIF4AII* have both been shown to induce *Pax-3* and *slug* expression in animal caps (Kuo *et al.*, 1998; Morgan and Sargent, 1997). Interestingly, both *opl* and *eIF4AII* alter the sensitivity of animal caps to neuralization by *noggin*, suggesting that they may be competence factors that mediate the response of neural tissue to signals that pattern the neural plate. Indeed, Kuo *et al.* (1998) suggest that *opl* may either regulate *Wnt*-signaling or be downstream of a *Wnt* signal based on the similarities of *Wnt* and *opl* gain-of-function phenotypes in animal caps and embryos.

In conclusion, we have investigated the tissue interactions and molecular signals that underlie induction of *Pax-3* expression in the *Xenopus* posteriolateral neural plate. The onset of *Pax-3* expression in a restricted posteriolateral domain occurs quite early during neural plate formation and thus represents a "primary" patterning event. We propose a model in which a *Wnt*-mediated posteriorizing activity arising from PNM acts to initiate and/or maintain *Pax-3* expression in the posteriolateral neural plate. This activity appears to be required more generally as expression of *Msx-1* in the early lateral neural plate is also *Wnt*-dependent. Moreover, our data suggest that this PNM-derived *Wnt* activity is a transformation signal in that it is able to induce expression of *Pax-3* and *Msx-1* in *noggin* animal caps which have an anterior neural fate. We suggest that this *Wnt*-dependent induction of a *Pax-3*- and *Msx-1*-positive domain establishes, in part, the spatial information necessary for the appropriate specification of later arising cell types such as Rohon–Beard cells and neural crest.

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