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Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo

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SUMMARY

The dorsal ectoderm of the vertebrate gastrula was proposed by Nieuwkoop to be specified towards an anterior neural fate by an activation signal, with its subsequent regionalization along the anteroposterior (AP) axis regulated by a graded transforming activity, leading to a properly patterned forebrain, midbrain, hindbrain and spinal cord. The activation phase involves inhibition of BMP signals by dorsal antagonists, but the later caudalization process is much more poorly characterized. Explant and overexpression studies in chick, *Xenopus*, mouse and zebrafish implicate lateral/paraxial mesoderm in supplying the transforming influence, which is largely speculated to be a Wnt family member.

We have analyzed the requirement for the specific ventrolaterally expressed Wnt8 ligand in the posteriorization of neural tissue in zebrafish wild-type and Nodal-deficient embryos (Antivin overexpressing or *cyclops;squint* double mutants), which show extensive AP brain patterning in the absence of dorsal mesoderm. In different genetic situations that vary the extent of mesodermal precursor formation, the presence of lateral *wnt8*-expressing cells correlates with the establishment of AP brain pattern. Cell tracing experiments show that the neuroectoderm of Nodal-deficient embryos undergoes a rapid anterior-to-posterior transformation in vivo during a short period at the end of the gastrula stage. Moreover, in both wild-type and Nodal-deficient embryos, inactivation of Wnt8 function by morpholino (MO^{wnt8}) translational interference dose-dependently abrogates formation of spinal cord and posterior brain fates, without blocking ventrolateral mesoderm formation. MO^{wnt8} also suppresses the forebrain deficiency in *bozozok* mutants, in which inactivation of a homeobox gene causes ectopic *wnt8* expression. In addition, the *bozozok* forebrain reduction is suppressed in *bozozok;squint;cyclops* triple mutants, and is associated with reduced *wnt8* expression, as seen in *cyclops;squint* mutants. Hence, whereas *boz* and Nodal signaling largely cooperate in gastrula organizer formation, they have opposing roles in regulating *wnt8* expression and forebrain specification. Our findings provide strong support for a model of neural transformation in which a planar gastrula-stage Wnt8 signal, promoted by Nodal signaling and dorsally limited by Bozozok, acts on anterior neuroectoderm from the lateral mesoderm to produce the AP regional patterning of the CNS.

Key words: Zebrafish, *cyclops*, *squint*, *bozozok*, *wnt8*, Neuroectoderm, *nodal*, Morpholino

INTRODUCTION

The prevalent model for neural AP patterning in vertebrate embryos invokes a two-step mechanism first proposed by Nieuwkoop. In this model, neural patterning is accomplished via two factors: (1) an activation signal that separates non-neural from neural ectoderm, resulting in neuroectoderm of anterior character; and (2) a subsequent transforming signal that acts in a graded fashion to specify posterior brain and spinal cord fates in the induced neuroectoderm (Nieuwkoop, 1952).

Good evidence to support the activation phase of Nieuwkoop's model comes from explant analyses in *Xenopus*. Inhibition of bone morphogenetic proteins (BMPs) in animal cap explants by the potent organizer-derived antagonists Chordin, Noggin or Follistatin induce expression of anterior neural markers of both cement gland and forebrain character (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1995). These findings suggest that removal of BMP signaling from the dorsal ectoderm is sufficient for anterior neural induction. More recently, it has been postulated that a cleavage-stage Wnt signal, mediated by β-catenin, contributes to an early repression of BMP4 on the dorsal side of the embryo, and thereby sensitizes the ectoderm to respond to the neural-inducing signals from the organizer described above (Baker et al., 1999). In the chick embryo, neural induction is proposed to be initiated before the onset of gastrulation by a fibroblast growth factor (FGF) signal emanating from

organizer precursor cells (Streit et al., 2000). In summary, the current model of neural activation involves the action of Wnt and/or FGF signaling pathways before the onset of gastrulation, followed by the inhibition of BMP activity by factors released from the gastrula organizer.

A wealth of experimental support has been generated for the subsequent posteriorization step by various explant and heterospecific tissue recombination studies. When chick cells fated to populate the caudal neural tube are explanted early in development and grown in culture, they express neural markers of anterior character (Muhr et al., 1999). Additionally, prospective neuroectoderm explanted from early gastrula stage *Xenopus* embryos initially expresses anterior neural markers, with more posterior markers becoming activated in late gastrula explants (Kolm et al., 1997). In zebrafish, transplanted prospective mid-, but not early, gastrulation stage hindbrain progenitors express the rhombomere 3 and 5 marker, *krx20*, thus revealing the time of commitment to hindbrain fate (Woo and Fraser, 1998). Direct lineage tracing evidence showing that the proposed anterior-to-posterior neural fate transformation occurs in the context of the whole embryo is, however, lacking.

Much controversy has centered on the origin of posteriorizing influences in the vertebrate embryo. Some studies in *Xenopus* suggest that organizer-derived axial chordamesoderm can provide transforming influences (reviewed by Niehrs, 1999). Similarly, late organizer/node cells from chick have the ability to induce expression of the posterior-lateral neural markers *pax3* and *msx1*, in neuralized *Xenopus* animal cap explants (Bang et al., 1999). By contrast, mouse and zebrafish mutants that lack the entire axial mesoderm still form posterior neural tissues, indicating that in these animals the transforming signals can arise from elsewhere (Ang et al., 1994; Weinstein et al., 1994; Fekany et al., 1999).

There is evidence implicating the lateral/paraxial mesodermal precursors as the source of endogenous transformers. In zebrafish, transplantation of non-axial, but not axial, marginal tissue into the prospective forebrain region could induce ectopic expression of the hindbrain marker, *krx20* (Woo and Fraser, 1997). In chick and *Xenopus*, both pre- and post-segmented caudal paraxial mesodermal explants exert posteriorizing influences on rostral neural tissue (Bang et al., 1999; Bang et al., 1997; Gould et al., 1998; Kolm et al., 1997; Muhr et al., 1997). Additional experiments that directly link induction of lateral mesoderm to neural posteriorization have come from studies of the potent Nodal antagonist, Antivin/Lefty1 (Atv/Lft1). Overexpression of Atv in dose-dependent manner blocks mesendoderm induction and development of posterior neural fates (Thisse et al., 2000). Collectively, these data implicate tissues outside the organizer, specifically the paraxial/lateral mesoderm, as the source of endogenous posteriorizing factors.

At the molecular level, FGFs, Wnts and retinoids (RAs) can all demonstrate properties of neural transformers by overexpression and dominant negative studies in *Xenopus* (Bang et al., 1999; Doniach, 1995; Lumsden and Krumlauf, 1996; McGrew et al., 1997; McGrew et al., 1995). However, in heterospecific tissue recombination experiments posterior-lateral neural markers can be induced in frog animal caps independently of RA- and FGF-mediated signaling (Bang et al., 1997). Additionally, in chick, a posterior non-axial mesodermal activity, distinct from FGFs and RAs, can caudalize anterior neural plate explants (Muhr et al., 1997; Muhr et al., 1999). Similarly, in zebrafish, basic FGF cannot mimic the posteriorizing effect of transplanted non-axial marginal tissue (Woo and Fraser, 1997), and neither immersion nor local application of RAs leads to transformation of forebrain into hindbrain fates (Holder and Hill, 1991; Hyatt et al., 1996). Moreover, a dominant-inhibitory form of the FGF receptor, XDF (Amaya et al., 1991), does not block AP neural patterning in *Xenopus* transgenic assays (Kroll and Amaya, 1996).

Recent evidence points to the key role of Wnt signaling in posteriorizing the neuroectoderm. Overexpression of Xwnt3a induces posterior and suppresses anterior marker expression in neuralized *Xenopus* animal caps (McGrew et al., 1995). Functionally blocking the axis-inducing subset of Wnt ligands by overexpression of a dominant negative Wnt8 (dnXwnt-8) results in loss of posterior neural fates in both *Xenopus* and zebrafish (Bang et al., 1999; Fekany-Lee et al., 2000; McGrew et al., 1997). The first genetic evidence that a Wnt signaling pathway is involved in neural transformation comes from the zebrafish *headless* mutant, which shows severe anterior defects caused by an inactivation of T-cell factor 3 (TCF3), a repressor of Wnt target genes (Kim et al., 2000). Additional support implicating Wnt ligands in neural posteriorization comes from the findings that the most anterior axial mesodermal tissue, the prechordal plate, protects the presumptive forebrain from posteriorizing influences by producing a Wnt-antagonist Dickkopf 1 (Dkk1; Glinka et al., 1998; Kazanskaya et al., 2000; Shinya et al., 2000). Previous to the identification of this ‘anteriorizing’ Wnt antagonist, grafts of either chick or mouse prechordal mesoderm had been shown to anteriorize prospective hindbrain (Ang and Rossant, 1993; Dale et al., 1997; Foley et al., 1997). Finally, inhibition of both the BMP and Wnt pathways is sufficient for head development (Glinka et al., 1997), whereas Cerberus, which can bind and antagonize the activities of BMP, Wnt and Nodal ligands, is a potent head inducer (Piccolo et al., 1999).

Despite this wealth of information, it is still unclear whether and when overt posteriorization occurs in vivo, or which endogenous ligand exerts the transforming influence from lateral mesodermal cells. Overexpression, dominant negative and expression pattern analyses suggest that Wnt8, produced by the chick posterior non-axial mesoderm, acts as a neural transformer in vivo (Bang et al., 1999). Additionally, in zebrafish *bozozok* (*boz*) mutants, ectopic expression of *wnt8* in the dorsal mesoderm correlates with excessive posteriorization of the neuroectoderm (Fekany-Lee et al., 2000). This phenotype can be suppressed by overexpressing dnXwnt-8 (Fekany-Lee et al., 2000) or the Wnt antagonist, Dkk1 (Hashimoto et al., 2000 and Shinya et al., 2000).

We have analyzed the requirement for Wnt8 in posteriorization of the neural tissue in zebrafish wild-type and Nodal-deficient embryos. Using two different genetic situations that vary the extent of mesodermal precursor formation, we find that lateral *wnt8* expression correlates with the degree of neural AP patterning. By lineage labeling dorsal neuroectodermal cells in Nodal-deficient embryos, we also show that a rapid anterior-to-posterior transformation occurs in vivo and that the timing of this event is restricted to a short period at the end of gastrulation. Furthermore, specific

inactivation of Wnt8 function inhibits formation of spinal cord, hindbrain and the midbrain-hindbrain boundary (MHB) dose dependently, without disrupting ventrolateral mesoderm formation. In addition, we show that the homeoprotein Boz and the Nodal signaling ligands have opposing roles in regulation of *wnt8*, with *boz* being required in the presence of Nodal signaling to negatively regulate *wnt8* expression in order to promote forebrain specification. Therefore, we conclude that a planar Wnt8 signal, arising from lateral mesodermal precursors and shaped by the opposing functions of the Boz and Nodal pathways, is required for endogenous process of neural posteriorization.

MATERIALS AND METHODS

Fish maintenance and strains

Fish were maintained as described (Solnica-Krezel et al., 1994). Homozygous *cyc*^{m294};*sqt*^{cz35} (Feldman et al., 1998) or *cyc*^{m294};*sqt*^{cz35} *enh* embryos were obtained by intercrossing heterozygous *cyc*^{m294};*sqt*^{cz3} or *cyc*^{m294};*sqt*^{cz35} *enh* adults, respectively. The *cyc;sqt enh* phenotype was noted when these mutations, normally maintained in a AB genetic background, were outcrossed to a TL genetic background. Because the genetic modifiers of this phenotype, enhancers or suppressors, are unknown, we refer to the newly isolated line simply as *cyc;sqt enh*. *boz*$^{m168/m168}$ embryos used for the morpholino (MO) MO^{wnt8} injection experiments were obtained by crossing homozygous mutant parents (Fekany et al., 1999). Heterozygous triple mutant lines for *boz;cyc;sqt* were obtained by crossing a homozygous *boz* female to a heterozygous double *cyc;sqt* male. When needed, embryos were genotyped by PCR after in situ analysis and photographed as described (Feldman et al., 2000; Sampath et al., 1998; Fekany et al., 1999).

Oligonucleotides and mRNAs

The antisense morpholino (Summerton et al., 1997) 5′-ACG-CAAAAATCTGGCAAGGGTTCAT-3′ (which recognizes *wnt8* nucleotides +1 to +25; sequence complementary to starting ATG is underlined) was dissolved in water and diluted with 1× Danieau Medium/Phenol Red (Saúde et al., 2000) and 5, 10 or 15 ng of MO^{wnt8} were injected. Silent nucleotide substitutions (lower case) were introduced by PCR into the *wnt8* construct (*wnt8*^{mut}): 5′-ATGAAtCCaTGtCAGATTTTTGCGT-3′ (+1 to +25), without changing other aspects of the construct. Three to four mismatches are usually sufficient to abolish binding of an antisense morpholino nucleotide to its target sequence (Gene Tools). RNAs were synthesized from zebrafish *antivin* (*atv*/*lft1*, Thisse and Thisse, 1999), *Xenopus dnXwnt-8* (Hoppler et al., 1996), zebrafish *wnt8* (Kelly et al., 1995) and *wnt8*^{mut} constructs using mMESSAGE mMACHINE™ (Ambion), and quantified by agarose gel electrophoresis. Doses of RNA injected were 60 pg of *atv* RNA to mimic the *cyc;sqt* phenotype, 400 pg of *dnXwnt-8* RNA or 5-10 ng of MO^{wnt8} to block Wnt8 activity, and 50 pg of *wnt8* or *wnt8*^{mut} to check for MO^{wnt8} specificity. MO and RNA were injected through the chorion into the yolk of one to four-cell stage embryos using a pneumatic picopump (WPI).

Heat shock experiments

One to two-cell stage wild-type embryos were injected with 5 and 10 pg of an hsp-*wnt8* (J. Topczewski, unpublished) or hsp-*wnt8*^{mut} (was derived from hsp-*wnt8* by replacing a *Hin*dIII/*Sal*I fragment bearing the mutated part from the *wnt8* construct) construct both alone and together with 5 ng of MO^{wnt8}, and cultured in 30% Danieau medium (Saúde et al., 2000) at 23°C. Injected embryos were heat shocked at 37°C in 30% Danieau medium for 1 hour starting around onset of gastrulation (50% epiboly) and then further incubated at 28.5°C (100% of untreated control embryos survived the heat shock treatment and developed normally). Small numbers of injected embryos were fixed immediately after heat-shock treatment (60-65% epiboly) to confirm stress-induced ectopic *wnt8* expression (injected embryos not subjected to heat shock treatment served as controls). The remaining embryos were monitored for development of paraxial/axial mesoderm, fixed at the six-somite stage and applied to in situ hybridization using *six3*, *pax2.1*, *krx20* and *huC* as region-specific markers.

In situ hybridization

In situ hybridization was performed as described (Thisse et al., 1993). Digoxigenin-labeled RNA antisense probes represented *huC* (Kim et al., 1996), *isl-1* (Inoue et al., 1994), *ephA4* (also known as *rtk1*; Durbin et al., 1998), *alpha-tropomyosin* (Ohara et al., 1989), *otx1* (Li et al., 1994), *ntl* (Schulte-Merker et al., 1992), *wnt8* (Kelly et al., 1995), *gsc* (Stachel et al., 1993), *six3* (Kobayashi et al., 1998), *pax2.1* (Krauss et al., 1991), *krx20* (*krx-20*; Oxtoby and Jowett, 1993) and *papc* (Yamamoto et al., 1998). Fluorescein-labeled RNA antisense *ntl* and *pax2.1* probes were used in double in situ hybridization experiments.

Uncaging fluorescein

Caged-fluorescein (0.5%, 1 nl; Kozlowski et al., 1997) with 60 pg *antivin* were injected into the yolk of one to four cell zebrafish embryos. Uncaging at 70% epiboly, in situ hybridization and immunostaining with anti-fluorescein antibody were as described (Sepich et al., 2000).

RESULTS

During early blastula and gastrula stages, standard *cyc*^{m294};*sqt*^{cz35} mutants and Atv-induced phenocopies lack dorsal mesoderm (Feldman et al., 1998; Thisse et al., 2000; Thisse and Thisse, 1999), as marked by the pan-mesodermal marker, *no tail* (*ntl;* Schulte-Merker et al., 1992), but retain

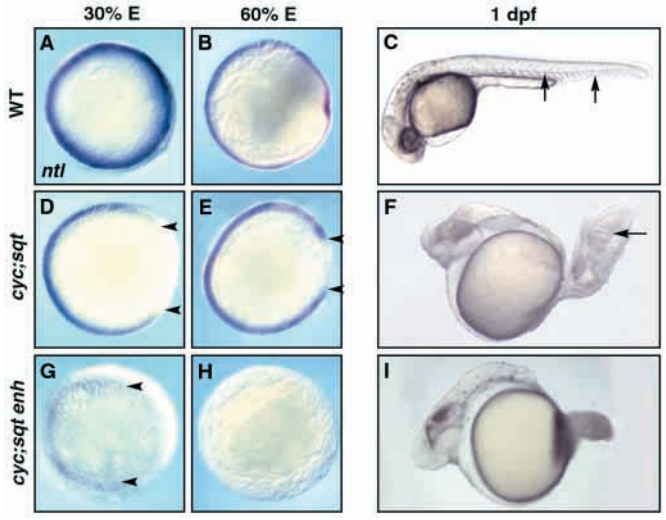


Fig. 1. Mesoderm induction in Nodal signaling-deficient embryos. Animal view. In situ hybridization of *ntl* expression in wild type (A,B), *cyc;sqt* (D,E) and *cyc;sqt enh* (G,H) embryos at 30% (A,D,G) and 60% (B,E,H) epiboly. Arrowheads indicate *ntl* boundaries. Bright field images (C,F,I) show posterior tail somite formation (arrows) in wild type (C) and *cyc;sqt* (F), but not *cyc;sqt enh* (I).

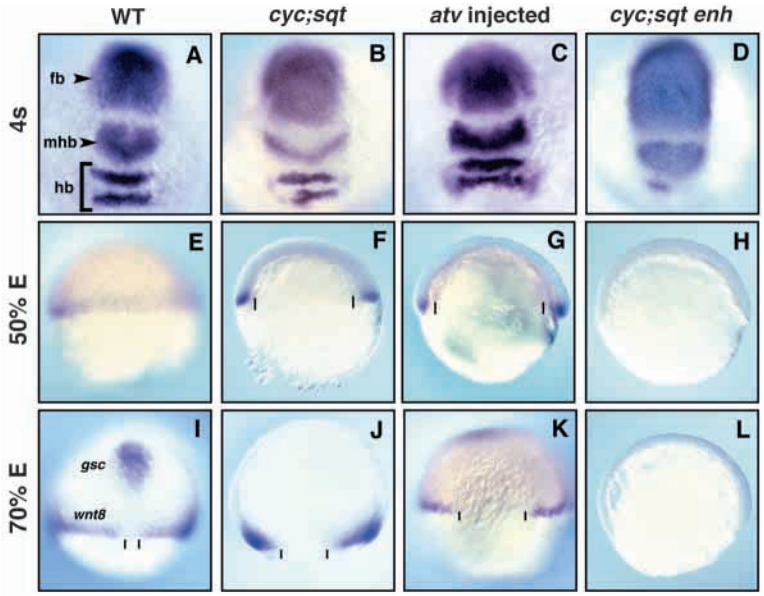


Fig. 2. Proper AP brain regionalization correlates with *wnt8* expression. Dorsal view; anterior is upwards. In situ hybridization for *six3*, *pax2.1* and *krox20* expression in the brain anlagen of wild type (A), *cyc;sqt* (B), *atv* RNA-injected (C) and *cyc;sqt enh* (D) embryos at the four-somite stage. In situ hybridization for *wnt8* at 50% (E-H) and 70% (I-L) epiboly. Dorsal view. Black lines denote dorsal gap in *wnt8* expression (F,G,I-K). Embryos were co-hybridized with *gsc* probe (I-L) to phenotypically distinguish double mutant embryos from wild-type or single mutant embryos before genotyping, and to monitor the dose of *atv* RNA injected. fb, forebrain; mhb, midbrain-hindbrain boundary; hb, hindbrain; 4s, four-somite stage.

ventrolateral mesendodermal precursors (Fig. 1D, *n*=7; 1E, *n*=6). At 1 day post fertilization (dpf), these *cyc;sqt* mutants and Atv-overexpressing phenocopies show loss of head and trunk mesendoderm, but retain tail somites (Fig. 1F; *n*=9; Feldman et al., 1998; Thisse et al., 1999; Meno et al., 1999).

We also identifed an enhanced phenotype (see Materials and Methods), $cyc^{m294};sqt^{cz35}$ *enh*, observed when these mutations were introduced into the TL genetic background. The enhanced phenotype was characterized by almost completely abrogated mesoderm induction, similar to that seen in embryos overexpressing high levels of Atv (Thisse et al., 2000). In this genetic situation, ventrolateral *ntl* expression was only very transiently detected at low levels before (Fig. 1G, *n*=35), but not during (Fig. 1H, *n*=27), gastrulation. Consequently, *cyc;sqt enh* embryos failed to form trunk or tail somites (*n*=17; compare Fig. 1F-I), or to express trunk/tail mesodermal markers such as *alpha-tropomyosin* (Ohara et al., 1989) at 1 dpf (not shown). Both phenotypic classes of *cyc;sqt* compound mutants proved informative in our analyses of the relationship between lateral mesoderm formation and neural patterning.

Lateral *wnt8* expression correlates with properly patterned brain anlagen

Consistent with the findings that lateral/paraxial mesoderm can elicit posteriorizing effects in several vertebrate species (Bang et al., 1999; Bang et al., 1997; Gould et al., 1998; Itasaki et al., 1996; Kolm et al., 1997; Muhr et al., 1997; Woo and Fraser, 1997), standard *cyc;sqt* mutants and Atv-overexpressing phenocopies, both of which retain *ntl*-expressing ventrolateral mesodermal precursors, have relatively well-patterned brain anlagen and posterior spinal cord, but show slightly enlarged telencephalon and eyes, and lack anterior spinal cord (Feldman et al., 2000; Masai et al., 2000; Sirotkin et al., 2000; Thisse et al., 2000). At the four-somite stage, both types of embryos showed spatially ordered expression of markers of the definitive forebrain (*six3;* Kobayashi et al., 1998), the midbrain-hindbrain boundary domain (MHB) (*pax2.1;* Krauss et al., 1991) and the hindbrain (rhombomeres 3 and 5; *krx20;* Fig. 2B, *n*=16; Fig. 2C, *n*=45). By contrast, *cyc;sqt enh* embryos showed severe loss of posterior brain fates with a concomitant large-scale expansion of the anterior markers expression domains (Fig. 2D, *n*=13). A similar loss of posterior brain fates was observed in wild-type embryos injected with higher *atv* RNA doses (not shown; Thisse et al., 2000). Consistent with the progressive loss of posterior neural fates generated by increased doses of *atv* RNA (Thisse et al., 2000), we conclude that ventrolateral mesoderm precursors are not only sufficient, but essential for the development of normal AP brain patterning.

We then asked what signal derived from the ventrolateral mesoderm could account for the posteriorizing activity of this tissue. In early zebrafish embryos, a previously implicated Wnt ligand-encoding gene, *wnt8* (Kelly et al., 1995), shows the appropriate spatial and temporal expression pattern. *wnt8* is

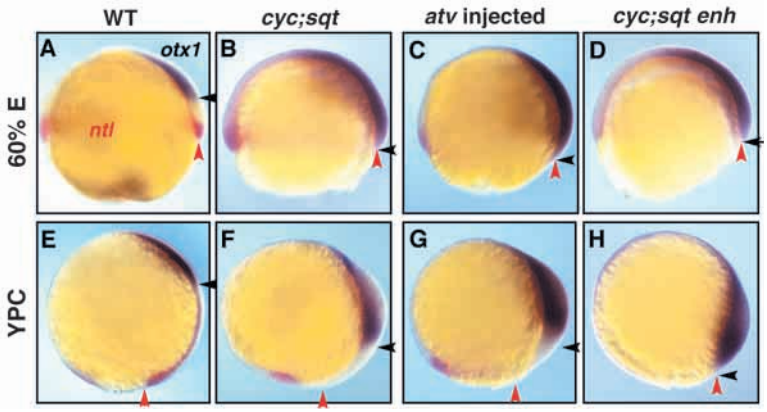


Fig. 3. Loss of *wnt8* expression is associated with expansion of anterior neural fates during gastrulation. Lateral view; dorsal is towards the right. Wild-type (A,E), *cyc;sqt* (B,F), *atv* RNA-injected (C,G) and *cyc;sqt enh* (D,H) embryos at 60-65% epiboly (A-D) and YPC (E-H). *ntl* (red); *otx1* (blue). Black arrowheads indicate the posterior *otx1* boundary. Red arrowheads indicate the margin.

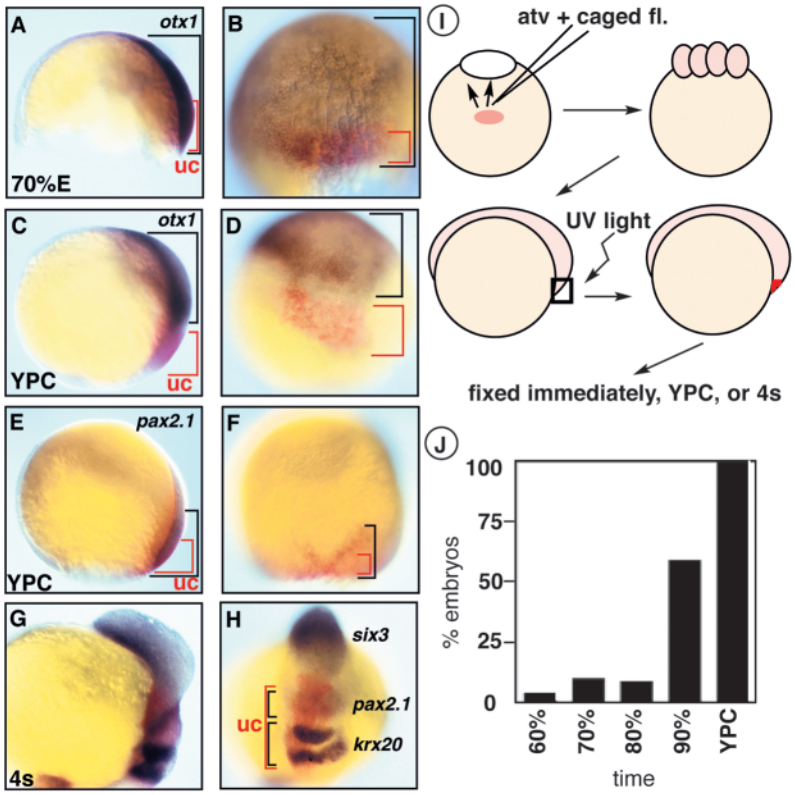


Fig. 4. (A-H) Neuroectoderm of Nodal-deficient embryos is posteriorized between 80% epiboly and YPC. *atv* RNA/caged-fluorescein co-injected embryos at indicated stages. Dorsal is towards the right in A,C,E,G. B,D,F,H are dorsal views. Black brackets indicate in situ probe signal. Red brackets indicate uncaged cells. (I) Cartoon depicting the co-injection experiment in (A-H). (J) *atv* RNA-injected embryos were collected over a gastrulation timecourse (60%, 70%, 80%, 90% and 100% (YPC) epiboly), and analyzed for the number of embryos showing anterior displacement of the *otx1* expression domain (*n*=2/55 (4%); *n*=4/42 (10%); *n*=2/23 (9%); *n*=23/39 (59%); and *n*=27/27 (100%), respectively).

initially expressed in the yolk syncytial layer (YSL; Ho et al., 1999), and at early gastrulation stages (50% epiboly) in the entire blastoderm margin (Fig. 2E; Kelly et al., 1995). By mid-gastrulation (70% epiboly), however, *wnt8* is locally downregulated at the dorsal midline, but persists ventrolaterally (Fig. 2I; Ho et al., 1999; Kelly et al., 1995). We have noted previously that persistent and increased expression of *wnt8* around the entire blastoderm margin of *boz* mutants was correlated with excessive posteriorization of the neuroectoderm (Fekany-Lee et al., 2000).

Based on these observations, we wanted to determine whether *wnt8* expression was maintained in the ventrolateral mesoderm of Atv-overexpressing and *cyc;sqt* mutant gastrulae. In standard *cyc;sqt* mutants (Fig. 2F, *n*=6; Fig. 2J, *n*=7) or Atv-overexpressing embryos (Fig. 2G, *n*=18; Fig. 2K, *n*=32), *wnt8* expression at both 50% and 70% epiboly was observed in ventrolateral mesodermal precursors, but showed a larger dorsal clearing compared with wild type. In *cyc;sqt enh* mutants, however, *wnt8* expression was strongly reduced or not detected at either stage (Fig. 2H, *n*=16; Fig. 2L, *n*=9). This dramatic reduction or absence of *wnt8* expression was also observed in embryos that overexpressed higher doses of Atv (not shown), which, like *cyc;sqt enh* mutants, showed loss of *ntl* expression and posterior brain fates (Thisse et al., 2000). These data provide circumstantial evidence that absence of *wnt8* expression is associated with loss of posterior neural fates in *cyc;sqt enh* mutants and embryos overexpressing high levels of Atv, thus making Wnt8 an excellent candidate for an endogenous neural transformer in zebrafish.

Loss of *wnt8* expression is associated with expansion of anterior neural fates

We next analyzed how the brain regionalization observed at early segmentation is connected to patterning processes occurring during gastrulation. In both wild-type and Nodal-deficient embryos, the initially induced neural tissue, including mid- and hindbrain progenitors (Grinblat et al., 1998; Kolm et al., 1997; Lumsden and Krumlauf, 1996; McGrew et al., 1997; McGrew et al., 1995), is proposed to express first general or anterior neural markers, such as *otx1* (Fig. 3A; Li et al., 1994). Normally, the posterior boundary of the neuroectoderm is set by the presence of mesendodermal precursors in the dorsal marginal region. The lack of dorsal mesoderm in Nodal-deficient embryos, however, allowed the encroachment of *otx1* expression into the blastoderm margin at ~60% epiboly (Fig. 3B, *n*=20; Fig. 3C, *n*=53; Fig. 3D, *n*=20). Consistent with these observations, the fate-mapping of dorsal marginal cells in standard *cyc;sqt* embryos revealed that they inappropriately adopt neural fates, instead of endodermal and dorsal mesodermal fates (Feldman et al., 2000). Moreover, a similar expansion of the presumptive brain marker *AS11* has been reported in Atv-overexpressing embryos (Thisse and Thisse, 1999). Even in embryos with an almost complete loss of presumptive mesoderm (such as *cyc;sqt enh*), the exclusion of the neuroectoderm from the lateral margin presumably reflects the action of early non-Nodal-dependent pathways that affect dorsoventral (DV) patterning (Baker et al., 1999; Fekany-Lee et al., 2000; Harland, 2000; Sirotkin et al., 2000).

Analysis of *cyc;sqt* mutants and Atv-overexpressing embryos at the end of gastrulation (yolk plug closure; YPC) showed that the *otx1* expression domain was now well separated from the dorsal margin (Fig. 3F, *n*=11; Fig. 3G, *n*=43), indicating that perhaps more posterior genes were induced. By contrast, *cyc;sqt enh* mutants (Fig. 3H; *n*=12), as well as embryos overexpressing high levels of Atv (not shown), did not show this displacement, suggesting that the induced neuroectoderm remained unpatterned or retained anterior character. Consistent with this notion, posterior brain fates were lost in these late gastrulae as revealed by the absence of the hindbrain marker *krx20* during early segmentation (Fig. 2C,D).

Anterior neural cells become posteriorized in Nodal-deficient embryos

The observed animalward displacement of the posterior boundary of the *otx1* expression domain in *cyc;sqt* and Atv-

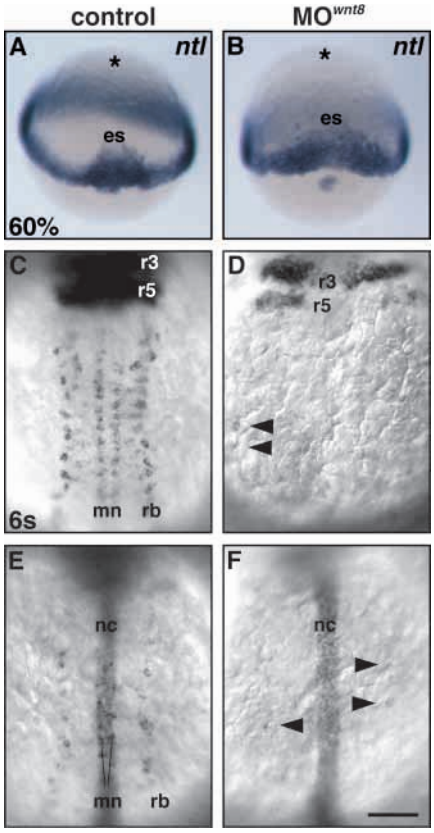


Fig. 5. Downregulation of Wnt8 function results in loss of spinal cord primary neurons. (A,B) Expression of *ntl* at 60% epiboly. (C-F) Expression of *krx20*/*huC* and *ephA4*/*isl1* at six-somite stage. (A,C,E) Untreated wild-type embryos; (B,D,F) MO^{wnt8}-injected wild-type embryos (class I). (A,B) Dorsal view, animal pole is upwards (asterisks mark animal pole position). (C-F) Dorsal view, anterior is upwards. The number of *huC*- or *isl1*-expressing Rohon-Beard neurons (rb) and motoneurons (mn) of the developing spinal cord was dramatically reduced after injection of MO^{wnt8} (146/219 (66.7%); arrowheads in D,F). By contrast, expression of the mesodermal marker *ntl* (B) and of *ephA4* in the notochord (E,F; nc, not in focus) was not affected. General dorsalization of injected embryos is manifested by mediolateral enlargement of rhombomeres (marked by *krx20* and *ephA4*, D) and notochord (F). Embryonic shield (es), rhombomere 3/5 (r3/5); Scale bar: 100 μm.

overexpressing phenocopies could have been caused by the posteriorization of marginal *otx1*-expressing cells or by their translocation as a result of gastrulation movements. Nodal-deficient embryos undergo epiboly and convergence, but not wild-type involution (Feldman et al., 2000) or extension movements. These properties, together with the encroachment of the neuroectoderm to the blastoderm margin, allowed alterations in the expression of regional neural markers in whole Nodal-deficient embryos to be assessed in reference to the distinct morphological landmark of the margin. This allowed us to distinguish experimentally whether the animalward displacement of *otx1* expression was caused by posteriorization or cell movement.

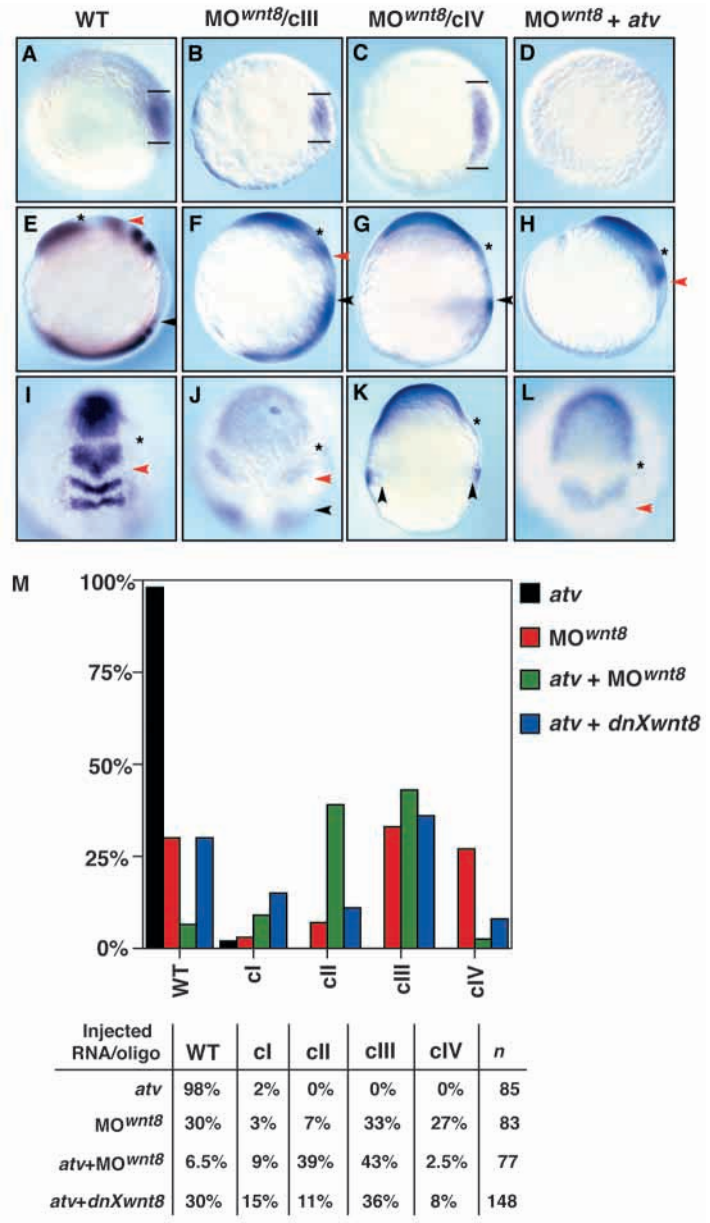
To track dorsal marginal neuroectodermal cells in Nodal-signaling-deficient embryos, we co-injected *atv* RNA (at doses that phenocopy standard *cyc;sqt* double mutants) with a caged-fluorescein lineage tracer into the yolk of one to four cell embryos, thereby loading the entire embryo with the inactive tracer (Fig. 4I; Kozlowski et al., 1997). Because involution is abrogated and the morphological embryonic shield is absent in Nodal-deficient embryos (Feldman et al., 2000), we used the thickening of the dorsal margin, which becomes apparent at mid-gastrulation (70% epiboly, 7.5 hours post fertilization, hpf), to locate the dorsal region of the embryo. At this stage, we photoactivated the lineage tracer in a cluster of dorsal marginal cells by focused UV irradiation and fixed the embryos immediately, at the end of gastrula period (YPC, 9.5 hpf), or during early somitogenesis (four-somite stage, 11.3 hpf).

In situ hybridization analysis for region-specific markers was performed on fixed embryos, followed by anti-fluorescein antibody staining to detect cells in which the lineage tracer had been activated. As expected, we found that, at 70% epiboly, the labeled dorsal marginal cells expressed *otx1* (Fig. 4A,B; *n*=10/10). By the end of gastrulation (YPC), however, equivalent lineage tracer-containing marginal cells no longer expressed *otx1* (Fig. 4C,D; *n*=6/9). These results indicated that early *otx1*-expressing cells had not simply been displaced animally by ongoing gastrulation movements, but rather had downregulated expression of this presumptive anterior neural marker.

To assay if more posterior neuroectodermal genes were induced in equivalently labeled cells that downregulated *otx1* at the end of gastrulation, we first analyzed expression of the MHB marker, *pax2.1*. We found that photoactivated marginal cells had switched to expressing *pax2.1* (Fig. 4E,F; *n*=32/32), showing that posteriorization of more anterior neural fates had occurred in the Nodal-deficient embryos. During early somitogenesis, lineage-labeled cells were found in the MHB and hindbrain anlagen, where they expressed the region-specific markers *pax2.1* and *krx20*, respectively (Fig. 4G,H; *n*=10/10). Furthermore, the initially broad rectangular domain of lineage labeling at gastrulation became narrowed mediolaterally and lengthened along the AP axis by segmentation stages. Hence, like *cyc;sqt* mutants (Feldman et al., 2000), the dorsal marginal cells of Atv-overexpressing embryos underwent epiboly, convergence and reduced extension movements, but remained at the margin rather than involuting. As the onset of *pax2.1* and *krx20* expression is comparable between Nodal-deficient and wild-type embryos, these lineage-labeling experiments directly demonstrate the posteriorization of initially anterior-type neuroectodermal cells in the context of whole embryo, probably reflecting the equivalent process in the wild-type condition.

Overt transformation occurs at the late gastrula stage in Nodal-signaling-deficient embryos

Next, we addressed the timing of overt transformation in Nodal-signaling-deficient embryos. We injected *atv* RNA to generate *cyc;sqt* mutant phenocopies and monitored the position of the *otx1* expression domain relative to the blastoderm margin. The posterior boundary of *otx1* expression shifted anteriorly, away from the margin, between 80% and 90% epiboly (8.5-9.0 hours post fertilization, hpf) in most embryos, with all embryos showing the displacement by YPC (Fig. 4J). Therefore, we concluded that overt posteriorization of the brain anlage occurs rapidly in Nodal-signaling deficient embryos within a 1 hour time window between 80% epiboly (8.5 hpf) and YPC (9.6 hpf).



Injected RNA/oligo	WT	cI	cII	cIII	cIV	*n*
atv	98%	2%	0%	0%	0%	85
MO^{wnt8}	30%	3%	7%	33%	27%	83
atv+MO^{wnt8}	6.5%	9%	39%	43%	2.5%	77
atv+*dnXwnt8*	30%	15%	11%	36%	8%	148

Fig. 6. (A-D) MO^{wnt8} blocks neural posteriorization. In situ analysis of *goosecoid* (*gsc*) expression at 50% epiboly. Animal view, dorsal right. Black lines show lateral *gsc* borders. (E-L) *six3*, *pax2.1*, *krox20* and *papC* expression, four-somite stage. Lateral view, anterior is towards the left and upwards, posterior is downwards. *, *six3* posterior border; red arrowhead, *pax2.1* posterior border; black arrowhead, anterior *papC* border. (M) Graph shows phenotypic distribution of injected embryos, recorded in the table below. Class I, loss of spinal cord *huC* expression and partial loss of rhombomere 5 (r5) *krox20* expression; Class II, loss of r5 and partial loss of rhombomere 3 (r3) *krox20* expression; Class III, loss of all *krox20* expression; Class IV, loss of *krox20* and *pax2.1*, altered embryonic morphology.

Previously, transplantation analyses have shown that presumptive zebrafish hindbrain cells acquire regional identity as a group at 80% epiboly (Woo and Fraser, 1998). Hence, the overt transformation in Nodal-signaling-deficient embryos, reported here, occurs at or immediately after wild-type cells become committed to a hindbrain fate. The similarity between the time of overt posteriorization and hindbrain progenitor commitment makes it likely that the transformation occurs equivalently in both Nodal-deficient and wild-type embryos.

Wnt8 is required for neural posteriorization

Next, we asked whether specific loss of Wnt8 signaling affected AP brain patterning in wild-type and Nodal-deficient embryos. Although *dnXwnt8* overexpression analyses in frog and fish have provided valuable advances towards identifying the axis-inducing class of Wnt ligands as crucial mediators of posteriorization (Fekany-Lee et al., 2000; Hoppler et al., 1996; McGrew et al., 1997), our studies differ in beginning to distinguish between the requirement for different Wnt signals. As *wnt8* mutations are not available, we adopted a different loss-of-function approach – morpholino (MO) inhibition – in which stable antisense oligonucleotides specifically block the translation of selected mRNAs (Heasman et al., 2000; Nasevicius and Ekker, 2000; Summerton et al., 1997).

At all MO^{wnt8} doses tested, expression of *ntl* (*n*=79) and *wnt8* (*n*=14) was observed in the blastoderm margin during gastrulation stages (Fig. 5B and data not shown, respectively), and morphologically distinct somites formed in all injected embryos (*n*=219, not shown). These results suggest that inhibition of Wnt8 activity did not affect mesoderm specification or its early development. However, injection of a range of MO^{wnt8} (5, 10 or 15 ng/embryo) revealed a dose-dependent requirement for Wnt8 function for the process of neural transformation, which we divided into classes I-IV (Fig. 6M; summarized in Table 1).

At low doses, class I embryos displayed dramatic loss of presumptive spinal cord fates, including lateral Rohon-Beard sensory neurons and medial motoneurons, as revealed by the neuronal markers, *huC* (Fig. 5D; *n*=60/117 (51.3%); Kim et al., 1996) and *isl1* (Fig. 5F; *n*=86/102 (84.3%); Inoue et al., 1994). These embryos also showed dramatic reduction of *krx20* (Fig. 5D) or *ephA4/rtk1* (Xu et al., 1995; Durbin et al., 1998; not visible in 5F) expression in rhombomere 5. Low numbers of embryos showed only minor reduction in *huC*- or *isl1*-positive cells and, in addition, maintenance of the full rhombomere 5

Table 1. Dose-dependent effect of MO^{wnt8} on neural posteriorization

Amount injected	% Wild type	% Class I	% Class II	% Class III	% Class IV	Not scorable	*n*
5 ng	87	7	4.5	1.5	0	0	131
10 ng	30	4	7	33	27	0	83
15 ng	19	1	2	5	14	61	103

See legend to Fig. 6 for class description

krx20 or e*phA4/rtk1* expression (20/117; 17.1% and 7/102; 6.9%), respectively. At slightly elevated MO^{wnt8} doses, while gastrulation still appeared grossly normal and organizer-type mesoderm was not expanded (Fig. 6B; *n*=23/32), most embryos lost all *krx20* expression, falling into class III (Fig. 6F,J; Table in Fig. 6). Additionally, MO-injected embryos showed expression of the paraxial mesodermal marker, *papc* (*protocadherin C*; Yamamoto et al., 1998; Fig. 6 F,G,J,K), supporting the notion that although paraxial Wnt8 function was abrogated, specification and maintenance of this tissue was not affected. Therefore, interference with Wnt8 function can specifically inhibit neural posteriorization without expanding organizer-derived axial mesoderm or preventing specification of the paraxial somitic mesoderm.

Higher doses of MO^{wnt8} resulted in embryonic dorsalization and severely impaired epiboly. This phenotype is an expected consequence of global Wnt8 inhibition, as Wnt signaling at the blastula stage is involved in restricting formation of the organizer in frog and fish, and is an endogenous global ventralizing molecule (Fekany-Lee et al., 2000; Hoppler et al., 1996). These embryos (class IV) displayed slightly enlarged organizer tissue (Fig. 6c; *n*=9/32), as marked by expression of *goosecoid* (*gsc;* Stachel et al., 1993). During somitogenesis, the expression domain of *papc* was ventrally expanded and displayed an abnormally wide dorsal gap (Fig. 6G,K; *n*=22/22); a typical dorsalized phenotype. In addition, these embryos displayed loss of *krx20* and *pax2.1* expression plus posterior and ventral expansion of *six3*. Although these embryos displayed many features similar to dorsalized, BMP-signaling-deficient mutants (Mullins et al., 1996), they differed by the additional loss of posterior neural brain fates. These MO^{wnt8} inhibition experiments provide strong support for Wnt8 being an endogenous neural transformer emanating from the ventrolateral blastoderm margin of zebrafish gastrulae.

Wnt8 is required for neural posteriorization in Nodal-deficient embryos

Atv-overexpressing embryos phenocopy standard *cyc;sqt* double mutants and lack axial/paraxial mesendoderm, but have relatively normal ventrolateral *wnt8* expression and AP brain patterning (Fig. 2C,G,K). To test whether the residual expression of *wnt8* in ventrolateral blastoderm margin of Nodal-deficient embryos can account for specification of posterior brain structures, MO^{wnt8} was co-injected with *atv* RNA into wild-type embryos. As expected, embryos co-injected with MO^{wnt8} and *atv* RNA lacked both axial and paraxial marker expression (*gsc*, Fig. 6D, *n*=15/15; *papc*, Fig. 6H,L; *n*=19/19). Like MO^{wnt8}-injected wild-type embryos, most co-injected embryos lost hindbrain *krx20* expression, typically falling into class III (Fig. 6D,H,L,M). Additional evidence for involvement of Wnt signaling during neural posteriorization events in Nodal-deficient embryos came from co-injecting *atv* RNA with *dnXwnt-8* RNA, which caused a similar phenotypic array to that of *atv* RNA/MO^{wnt8} co-injection (Fig. 6M). These findings are consistent with the observation that overexpression of the Wnt antagonist Dkk1 promotes anterior neuroectodermal development in the absence of Nodal signaling (Hashimoto et al., 2000).

To address the issue of whether MO^{wnt8} produced a Wnt8-specific effect, we introduced three silent nucleotide substitutions within the 5′ region of the *wnt8* cDNA sequence

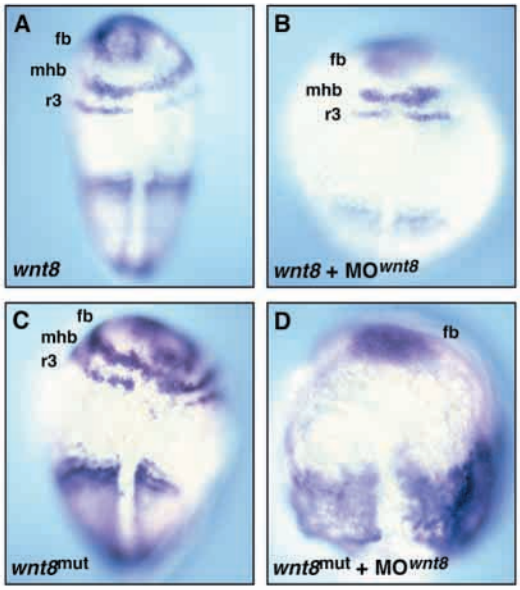


Fig. 7. MO^{wnt8} blocks effects of Wnt8 in a highly specific manner. Dorsal view, anterior is upwards. (A) *wnt8* RNA-injected, (B) *wnt8* RNA + MO^{wnt8}-injected, (C) *wnt8*^{mut} RNA-injected and (D) *wnt8*^{mut} + MO^{wnt8}-injected embryos. In situ analysis of four-somite stage embryos with markers *six3*, *pax2.1*, *krox20* and *papC*.

(see Materials and Methods, *wnt8*^{mut}) targeted by MO^{wnt8}. Injection of both *wnt8* (Kelly et al., 1995) or *wnt8*^{mut} RNA, as expected, activated the β-catenin pathway, as observed previously (Schneider et al., 1996; Kelly et al., 1995), and dorsalized the embryos (Fig. 7A, *n*=14, 86% dorsalized; 7C, *n*=10, 100% dorsalized). Loss of rhombomere 5 *krx20* expression in both *wnt8* and *wnt8*^{mut} RNA injected embryos could be caused either by early dorsalization of a subset of the paraxial mesendoderm and concomitantly less endogenous Wnt8 activity, or repressed embryogenesis (Fig. 7A,C). Co-injection of MO^{wnt8} curtailed the dorsalizing effect caused by *wnt8* RNA injection (Fig. 7B; *n*=20; 25% dorsalized), showing that MO^{wnt8} suppresses the gain-of-function *wnt8* phenotype. MO^{wnt8} could not, by contrast, decrease the dorsalizing effect of *wnt8*^{mut} (Fig. 7D; *n*=28; 93% dorsalized). Instead, the co-injected embryos became even more dorsalized, and in the forming CNS only expressed the forebrain marker *six3* (Fig. 7D). Potentially, this extreme phenotype results from the early translation of *wnt8*^{mut} RNA causing dorsalization (see Fig. 7C) and from MO^{wnt8} blocking the remaining endogenous ventralizing Wnt8 function. These results indicate that MO^{wnt8} binds in a sequence-specific manner to mRNA.

We also performed rescue experiments to define the specificity of the MO^{wnt8} effect on neural patterning. To circumvent the dorsalizing effect of early ectopic *wnt8* expression (such as that caused by *wnt8* RNA), we co-injected MO^{wnt8} with a plasmid carrying *wnt8* under the control of the *hsp70-4* heat shock promoter (hsp-*wnt8*), and induced *wnt8* expression by mild heat shock treatment at the onset of gastrulation (see Materials and Methods). Control hsp-*wnt8*-injected embryos subjected to heat shock showed dorsalization and strong reduction of *six3* forebrain expression at six somites, consistent with the induction of excessive Wnt8 signaling during gastrulation (data not shown). Control

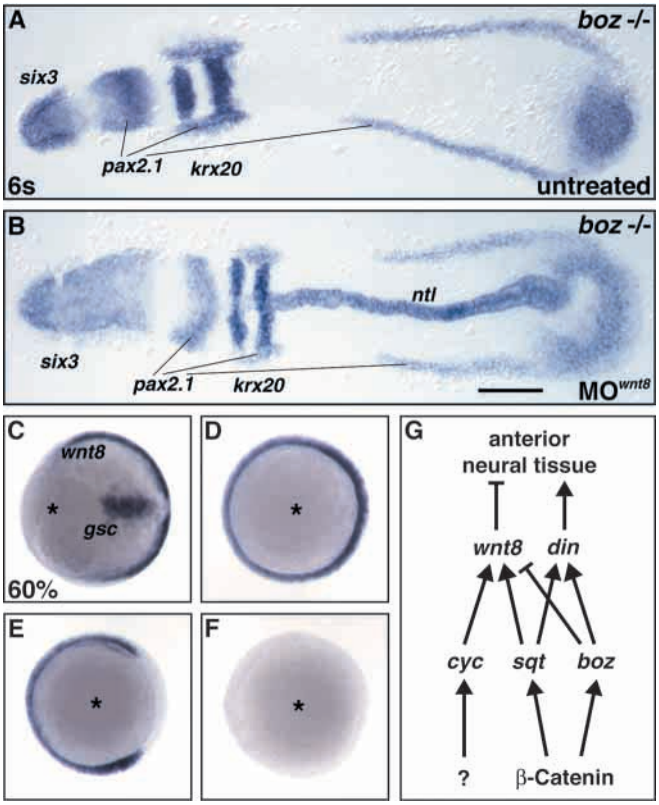


Fig. 8. *boz* and Nodal signaling have opposing roles in regulation of *wnt8* expression and forebrain specification. Expression of *six3*, *pax2*.1, *krx20* and *ntl* in *boz* mutant embryos at the six-somite stage. (A) Untreated, (B) after MO^{wnt8} injection; dorsal views, anterior is upwards. Expression of *wnt8* is upregulated in *boz* mutant embryos (D), but downregulated or completely lost in *cyc*;*sqt* (E) and *boz*;*cyc*;*sqt* (F) mutant embryos. (C) Wild type. Embryos (D-F) have been genotyped by PCR after in situ hybridization (for details, see text). The mesodermal marker *gsc* was used for better identification of phenotypic classes in triple mutant background (C-F). (G) Model illustrating the opposing roles of *boz*, *cyc* and *sqt* in *wnt8* regulation. Scale bar: 100 μm.

MO^{wnt8}-injected embryos displayed anteriorized phenotypes (enlargement of *six3* expression, and loss or reduction of *krx20* rhombomere 5 and spinal cord-specific *huC* expression). The majority of MO^{wnt8}/hsp-*wnt8* co-injected embryos had a normal-sized *six3* domain with only a minor expansion of axial mesoderm (Table 2). This experiment essentially shows that gastrulation-stage *wnt8* expression can titrate away the anteriorizing effect of MO^{wnt8}. However, to show that MO^{wnt8} specifically blocks Wnt8 function, rather than inactivating translation from other RNAs, we tested if the neural anteriorization caused by MO^{wnt8} could be rescued by heat shock of embryos co-injected with hsp-*wnt8*^{mut}, which produces a modified transcript that cannot bind the morpholino (see Materials and Methods). Whereas hsp-*wnt8*^{mut} injected non-heat shocked embryos generally did not exhibit ectopic *wnt8* expression (0/18 embryos injected with 5 pg hsp-*wnt8*^{mut}, 1/8 embryos injected with 10 pg hsp-*wnt8*^{mut}), most of the injected embryos subjected to a 1 hour heat shock treatment showed several small patches of ectopic *wnt8* RNA distributed randomly throughout the blastoderm (8/9 and 12/13 embryos at the 5 pg and 10 pg hsp-*wnt8*^{mut} doses, respectively). Heat-shocked hsp-*wnt8*^{mut}-injected embryos showed strong reduction of *six3* expression at the six-somite stage (Table 2). MO^{wnt8}-injected control embryos had an anteriorized phenotype with relative enlargement of the *six3* forebrain domain and reduction of *krx20* rhombomere 5 and spinal cord-specific *huC* expression (Table 2; 19%; *n*=63). By contrast, only a very small number of heat-shocked MO^{wnt8}/hsp-*wnt8*^{mut}-co-injected embryos showed such neural anteriorization (3%; Table 2). 36% of MO^{wnt8}/hsp-*wnt8*^{mut}-co-injected embryos were dorsalized and showed lateral expansion of neuroectoderm, perhaps because of low levels of *wnt8*^{mut} expression before heat shock. These embryos, however, showed normal expression of *krx20* in the prospective hindbrain, in contrast to the great reduction or loss of *krx20* and *pax2* expression in MO^{wnt8}-anteriorized embryos (Fig. 6F,G). This ability of late-onset *wnt8*^{mut} expression to suppress the anteriorizing effects of MO^{wnt8} injection provides a bona fide control for MO^{wnt8} specificity.

Reduction of Wnt8 activity suppresses the forebrain and notochord deficiency of *boz* mutant embryos

In the absence of the homeoprotein Bozozok, the intensity of *wnt8* expression in the presumptive non-axial mesoderm is increased, and the dorsal-most downregulation of *wnt8* expression does not occur. This increased *wnt8* expression in *boz* mutants has been correlated with the excessive neural posteriorization and forebrain deficiency, a phenotype that can be suppressed by overexpression of Wnt inhibitors (Fekany-Lee et al., 2000; Hashimoto et al., 2000). Furthermore, misexpression of *boz* itself results in strong or complete loss of *wnt8* expression in presumptive axial and paraxial mesoderm (Fekany-Lee et al., 2000). Together, these results indicate that one function of *boz* is to downregulate, directly or indirectly, *wnt8* expression.

Further evidence that Wnt8 is an endogenous neural transformer was obtained by injecting MO^{wnt8} into *boz* homozygous embryos. In 93% (*n*=40/43) of the untreated *boz* mutant embryos, the forebrain marker *six3* was significantly reduced and the *ntl* notochord expression domain was missing, as previously reported (Fig. 8A; Solnica-Krezel et al., 1996; Fekany-Lee et al., 2000). In contrast, 100% of the MO^{wnt8}-injected *boz* mutant embryos (*n*=83/83) exhibited slightly broader *ntl* notochord expression domains, most probably owing to the general dorsalizing effect of MO^{wnt8} described above. In addition, the *six3* expression domain was enlarged compared with control *boz* mutant embryos (Fig. 8B). These studies provide direct evidence that the reduction of axial

Table 2. Effect of late *wnt8* and *wnt8*^{mut} expression on MO-dependent anteriorization

Injected RNA	Amount	Reduced *six3*	Enlarged *six3*	*n*
hsp-*wnt8*	10 pg	100%	0	17
MO^{wnt8}	5 ng	0	49%	33
hsp-*wnt8*/MO^{wnt8}	10 pg/5 ng	35%	14%	43
hsp-*wnt8*^{mut}	5 pg	16%	0	58
hsp-*wnt8*^{mut}	10 pg	50%	0	76
MO^{wnt8}	5 ng	0	19%	63
hsp-*wnt8*^{mut}/MO^{wnt8}	5 pg/5 ng	0	3%	40
hsp-*wnt8*^{mut}/MO^{wnt8}	10 pg/5 ng	22%	4%	102

hsp, heat shock protein

mesoderm and forebrain in *boz* mutants is due to increased and ectopic *wnt8* expression and function (Fig. 8; Fekany-Lee et al., 2000).

boz and *cyc;sqt* have opposing functions in *wnt8* regulation and forebrain specification

While *boz* function is necessary to downregulate *wnt8* expression in the blastoderm margin (Fekany-Lee et al., 2000), Nodal signaling is crucial for the maintenance of dorsolateral *wnt8* expression (Fig. 2F,J). Thus, *boz* and *cyc;sqt* perform opposing roles in the regulation of *wnt8* in the blastoderm margin of the zebrafish gastrula. Previously, it has been demonstrated that the loss of anterior neuroectoderm (forebrain) in *boz* mutants is exacerbated in *boz;sqt* double mutants (Sirotkin et al., 2000) and *boz;cyc* double mutants (Shimizu et al., 2000), but suppressed in *boz;cyc;sqt* triple mutants (Sirotkin et al., 2000). As *boz* and *cyc;sqt* have opposite functions in regulating *wnt8* expression in the dorsolateral mesoderm, we hypothesized that the recovery of the forebrain observed in *boz;cyc;sqt* triple mutants might be due to downregulation of ectopic dorsal *wnt8* expression caused by *cyc;sqt* being epistatic to *boz*. To test this hypothesis, we compared *wnt8* expression and AP neural regionalization of *boz;cyc;sqt* triple mutants with that of *cyc;sqt* double mutants obtained at the same time from crosses between *boz*;*cyc*;*sqt* triple heterozygous animals.

The *boz* single mutants, as previously reported, exhibited increased and ectopic *wnt8* expression around the blastoderm margin compared with wild type (Fig. 8C,D; *n*=62/343, this number is slightly lower than 25% because of the variable penetrance and expressivity of the boz^{m168} mutation: Fekany et al., 1999; Fekany-Lee et al., 2000). By contrast, *cyc;sqt* double mutant siblings showed reduced or dramatically reduced *wnt8* expression ventrolaterally, and an absence of expression dorsolaterally (Fig. 8E,F; *n*=45/795, expected 50). By the four-somite stage, *cyc;sqt* double mutants showed loss of the rhombomere 5 *krx20* expression domain (not shown). Therefore, in this particular genetic line, the *cyc;sqt* double mutant phenotype was more severe than normal double mutants (Fig. 2B,F,J), but less severe than *cyc;sqt enh* embryos (Fig. 2D,H,L). *boz;cyc;sqt* triple mutant siblings were indistinguishable from *cyc;sqt* double mutants, showing dramatically reduced *wnt8* expression ventrolaterally and absence of expression in the dorsolateral margin (Fig. 8F; *n*=9/759, expected 12). Consistent with previous reports, *boz;cyc;sqt* triple mutants at the four-somite stage showed a milder forebrain deficiency than *boz* and *boz;sqt* and *boz;cyc* embryos (Sirotkin et al., 2000; *n*=16/274; expected 17; not shown). Hence, simultaneous loss of *cyc* and *sqt* function is epistatic to *boz* inactivation: ectopic *wnt8* expression is lost in *boz;cyc;sqt* triple mutants, thereby allowing forebrain fates to be maintained. Collectively, we conclude that *boz* and Nodal signaling have opposing functions with respect to *wnt8* regulation in the ventrolateral margin and forebrain specification (Fig. 8B-G).

DISCUSSION

In the present study, we took advantage of *cyc;sqt* mutants and embryos overexpressing Atv to analyze in vivo Nieuwkoop's theory of neural transformation and its timing, and to identify the endogenous ligand involved in orchestrating this process. The main phenotypic aspects of Nodal-deficient embryos that allows these questions to be addressed in the context of the whole embryo include both the ability to control the extent of ventrolateral mesoderm formation and the vegetalward expansion of the initially induced anterior neuroectoderm into the dorsal marginal region that provides a distinct landmark. Our cell tracing experiments showed that initially anterior-type neural cells become posteriorized in vivo during a short period at the end of the gastrula stage. Additionally, these studies point to Wnt8 as a ligand whose ventrolateral expression pattern correlates with AP neural regionalization in both wild-type and mutant conditions. Using MO^{wnt8} as a tool to specifically abrogate Wnt8 function, we found that Wnt8 is necessary for transformation of anterior neural fates, but is not essential for paraxial mesoderm specification. Therefore, we propose that Wnt8 acts from the lateral mesoderm to dose-dependently posteriorize neuroectodermal cells of anterior character.

Induced anterior neuorectodermal cells become caudalized in vivo

Several explant and tissue recombination studies (i.e. Bang et al., 1999; Bang et al., 1997; Gould et al., 1998; Itasaki et al., 1996; Kolm et al., 1997; Muhr et al., 1997) provide information pertinent to Nieuwkoop's activation/transformation model. However, in vivo cell tracing evidence supporting this hypothesis has so far not been reported. Our studies took advantage of the dorsal expansion of the anterior neuroectoderm in embryos overexpressing low doses of Atv to address this issue in the context of a whole embryo. We found that lineage-labeled dorsal marginal cells initially express a marker associated with anterior neural fates, which is then downregulated to be replaced by expression of posterior markers later in development. Although these experiments provide direct evidence for Nieuwkoop's two-step model in vivo, we cannot completely rule out the possibility that neural induction and transformation in Nodal-signaling deficient embryos is not equivalent to that of wild type. It is noteworthy, however, that regionalization of the brain anlage in Nodal-deficient embryos directly mirrors that of wild type, with equivalent timing of MHB and hindbrain marker gene expression. Additionally, our estimation of the timing of overt posteriorization in these embryos (80-100% epiboly) agrees well with the time that wild-type hindbrain progenitors acquire regional identity, as revealed by transplantation studies (Woo and Fraser, 1998). We therefore favor the idea that transformation occurs equivalently in Nodal-deficient and wild-type embryos.

Wnt8 is required for neural posteriorization

Overexpression and dominant-negative approaches first implicated Wnt signaling as a candidate pathway executing the neural transformation and A-P patterning process in vivo (Bang et al., 1999; Fekany-Lee et al., 2000; Kelly et al., 1995; McGrew et al., 1997; McGrew et al., 1995; McGrew et al., 1999). Recent genetic evidence broadly supports this view. A downstream repressive effector of the Wnt cascade, TCF3, is required for maintenance of anterior neural fates (Kim et al., 2000). These studies, however, leave unresolved the identity of the endogenous transforming signal. Several new lines of

evidence from our studies presented here specifically implicate Wnt8 as the caudalizing factor in zebrafish. We have shown that as MO*wnt8* doses are increased, embryos display a progressive loss of posterior neural fates with concomitant expansion of more anterior ones. The lowest doses of MO*wnt8* abrogated the development of only the posteriormost CNS, including the Rohon-Beard sensory neurons and primary motoneurons that occupy the lateral and medial regions of the developing spinal cord, respectively. Although loss of lateral Rohon-Beard neurons has previously been observed in embryos overexpressing dnXwnt-8 (Bang et al., 1999), our analyses differ in showing that the specification of medially located motoneurons is also Wnt8 dependent. Successively higher doses of MO*wnt8* caused the progressive loss of hindbrain and midbrain fates. Therefore, the simplest interpretation of these results is that the highest levels of Wnt8 signaling are required for specification of the entire (not only lateral) spinal cord, with lower levels being sufficient for specification of the hindbrain and midbrain.

Our analyses also uncouple a Wnt8 function in neural patterning from its putative role in ventrolateral mesoderm specification. Embryos injected with MO*wnt8* that showed severe anteriorization continued to express *wnt8* or *ntl* mesodermal markers in the lateral margin of gastrula stage embryos, and *papc* in the paraxial mesoderm of early segmentation stage embryos. Taken together with the *dnXwnt-8* loss-of-function studies (Bang et al., 1999; Fekany-Lee et al., 2000; Hoppler et al., 1996; McGrew et al., 1997) and the observation that *wnt8* is normally enriched in ventrolateral mesendodermal precursors (Christian et al., 1991; Ho et al., 1999; Kelly et al., 1995), these new loss-of-function studies establish a role for endogenous Wnt8 in the AP regionalization of the induced neuroectoderm.

Model for Wnt8 signaling

Combining our results with both the neuroectodermal fate map (Woo and Fraser, 1995) and the transplantation data from zebrafish (Woo and Fraser, 1997; Woo and Fraser, 1998), a model emerges in which lateral mesendodermal precursors in the gastrula produce a posteriorizing signal that is transmitted in a planar fashion to proximate 'anterior' neuroectoderm. Furthermore, this signal is Wnt8 itself or is crucially Wnt8 dependent. It is possible that a morphogenetic gradient of Wnt8 signaling regulates AP regionalization of the brain anlage and spinal cord. The specification of forebrain progenitors in dorsal animal regions of the embryo might require high levels of Wnt8 inhibition by downregulation of *wnt8* expression through organizer region-derived Wnt antagonists, such as Dkk1 or Cerberus (Bouwmeester et al., 1996; Glinka et al., 1998; Glinka et al., 1997; Hashimoto et al., 2000; Piccolo et al., 1999; Shinya et al., 2000) and the homeoprotein Boz (Fekany-Lee et al., 2000). Furthermore, the separation between these progenitors and the ventrolateral margin, followed by their further displacement during gastrulation (Woo and Fraser, 1995), results in a minimal exposure to Wnt8, and maintenance of forebrain identity. By contrast, neuroectodermal cells that arise and remain in proximity to the ventrolateral margin would develop progressively more posterior neural fates dependent on the dose of the Wnt8 signal they experience.

This model, with our observation that *cyc;sqt* double mutants have an expanded dorsal gap in *wnt8* expression, provides a plausible explanation for the loss of anterior trunk spinal cord and enlargement of telencephalon and eyes in Nodal-signaling-deficient embryos (Feldman et al., 2000; Gritsman et al., 1999; Masai et al., 2000). Similarly, our demonstration that increasing doses of *atv* RNA cause a progressive loss of *wnt8* expression might also explain the progressive loss of posterior neural fates in Atv-overexpressing embryos (Thisse et al., 2000; Thisse and Thisse, 1999).

Also consistent with the proposed mechanism of Wnt8 action are the neural patterning phenotypes produced by *boz*, *boz;sqt*, *boz;cyc* and *boz;sqt;cyc* mutants (Fekany-Lee et al., 2000; Sirotkin et al., 2000; Shimizu et al., 2000). For *boz*, we previously suggested that the loss of anterior neural fates is caused in part by neural over-posteriorization as a result of ectopic *wnt8* expression (present study and Fekany-Lee et al., 2000). Our demonstration here that MO*wnt8* suppresses the forebrain deficiency in *boz* mutants provides direct experimental support for this idea. The forebrain deficiency of *boz* mutants is exacerbated in *boz;sqt* (Sirotkin et al., 2000) and *boz;cyc* double mutants (Shimizu et al., 2000). By contrast, *boz;cyc;sqt* triple mutants exhibit a more complete development of anterior neuroectoderm fates (Sirotkin et al., 2000). These genetic interactions indicate that in zebrafish embryos, Nodal signaling normally acts to repress anterior neural fate development. Similarly, appropriately timed inhibition of Nodal signaling has been proposed to be required for anterior fate specification in *Xenopus* embryos (Piccolo et al., 1999). We propose that Nodal signaling affects AP patterning indirectly, in part by regulating *wnt8* expression. The simultaneous loss of *cyc* and *sqt* is epistatic to *boz* with respect to *wnt8* gene expression. Triple *boz;cyc;sqt* mutants probably lose dorsolateral mesendodermal cell fates and, concomitantly, express less *wnt8* in the dorsal and lateral blastoderm margin, as is observed in *cyc;sqt* double mutants. The loss of dorsolateral mesendodermal cells would suppress the excess of Wnt8 signaling observed in *boz* single mutants, thereby partially restoring anterior neural fates. Our studies add to the current understanding of the complex genetic network regulating organizer formation and forebrain specification in zebrafish, in which *boz* and the *nodal*-related genes act in a cooperative manner (e.g. *chordin* (*din*) and *gsc* positive regulation; Shimizu et al., 2000; Sirotkin et al., 2000), but also perform opposing functions (*wnt8* regulation).

In principle, similarities in embryonic topology suggest that similar patterning mechanisms might regulate neural patterning in *Xenopus*. In chick, mouse and zebrafish, embryological manipulations and genetic studies show that neural transformation does not require axial mesendoderm (Fekany-Lee et al., 2000; Foley et al., 2000; Klingensmith et al., 1999; Muhr et al., 1997). The transformation step of AP neural patterning in all vertebrate embryos may therefore be driven by planar-type signaling processes similar to those described here, involving the intermediacy of Wnt8-like ligands.

Note added in proof

While this report was in press, Lekven et al. (Lekven et al., 2001) described genetic and morpholino functional analysis of a bicistronic zebrafish *wnt8* locus, encoding Wnt8-like proteins 1 and 2. Removing the entire *wnt8* locus causes interference with dorsoventral mesoderm and AP neural patterning,

consistent with our analysis of *wnt8* function reported here. Lekven et al. report that morpholino oligonucleotides that target the Wnt8-like protein 1 (MO1) had no effect, whereas a Wnt8-like 2-specific morpholino (MO2), injected alone to together with MO1 produced phenotypes. In our case, neural anteriorization and partial dorsalization of mesoderm were observed with a *wnt8*-specific morpholino that is identical in sequence to MO1 of Lekven et al.; the reason for this discrepancy is unknown.

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