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\textsubscript{wnt8}) translational interference dose-dependently abrogates formation of spinal cord and posterior brain fates, without blocking ventrolateral mesoderm formation. MO\textsubscript{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 *pax*3 and *msx*1, 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 (Thissle 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., 1997; 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 Cereberus, 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 *wnt*8 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 *wnt*8 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
Wnt8 is required for neural posteriorization

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 cycm294;sqtc35 (Feldman et al., 1998) or cycm294;sqtc35 enh embryos were obtained by intercrossing heterozygous cycm294;sqtc35 or cycm294;sqtc35 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. bozm168bm168 embryos used for the morpholino (MO) MOwnt8 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 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'-ACGCAAAATCCTGGCAAGGGTTCA-3' (which recognizes wnt8 nucleotides +1 to +25; sequence complementary to starting A is underlined) was dissolved in water and diluted with 1X Danieau Medium/Phenol Red (Saúde et al., 2000) and 5, 10 or 15 ng of MOwnt8 were injected. Silent nucleotide substitutions (lower case) were introduced by PCR into the wnt8 construct (MOwnt8): 5'-ATGAACCTTGTCAGGGTTCCGT-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 to its target sequence (Gene Tools). RNAs were synthesized from zebrafish antivin (atvfl1. Thiese and Thiese, 1999), Xenopus dnXwnt8-8 (Hoppler et al., 1996), zebrafish wnt8 (Kelly et al., 1995) and wnt8mut 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 dnXwnt8-8 RNA or 5-10 ng of MOwnt8 to block Wnt8 activity, and 50 pg of wnt8 or wnt8mut to check for MOwnt8 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-wnt8mut (was derived from hsp-wnt8 by replacing a HindIII/StuI fragment bearing the mutated part from the wnt8 construct) construct both alone and together with 5 ng of MOwnt8, 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 (Thiese 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., 1996), 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 Lovett, 1993) and pape (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-fluorescin antibody were as described (Sepich et al., 2000).

RESULTS

During early blastula and gastrula stages, standard cycm294, sqtc35 mutants and Atv-induced phenocopies lack dorsal mesoderm (Feldman et al., 1998; Thiese et al., 2000; Thiese and Thiese, 1999), as marked by the pan-mesodermal marker, no tail (ntl; Schulte-Merker et al., 1992), but retain
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; Feldman et al., 1998; Thisse et al., 1999; Meno et al., 1999).

We also identified 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; Thisset 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; Thisset et al., 2000). Consistent with the progressive loss of posterior neural fates generated by increased doses of atv RNA (Thisset 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
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, \( \text{ntl} \) 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 \( \text{wnt8} \) around the entire blastoderm margin of \( \text{boz} \) mutants was correlated with excessive posteriorization of the neuroectoderm (Fekany-Lee et al., 2000).

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

**Loss of \( \text{wnt8} \) expression is associated with expansion of anterior neural fates**

We next analyzed how the brain regionalization observed at early gastrulation 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 \( \text{otx1} \) (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 \( \text{otx1} \) 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 \( \text{cyc} \cdot \text{sqt} \) 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 \( \text{AS11} \) has been reported in Atv-overexpressing embryos (Thissle and Thissle, 1999). Even in embryos with an almost complete loss of presumptive mesoderm (such as \( \text{cyc} \cdot \text{sqt enh} \)), 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 \( \text{cyc} \cdot \text{sqt} \) mutants and Atv-overexpressing embryos at the end of gastrulation (yolk plug closure; YPC) showed that the \( \text{otx1} \) 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, \( \text{cyc} \cdot \text{sqt enh} \) 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 \( \text{krx20} \) 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 \( \text{otx1} \) expression domain in \( \text{cyc} \cdot \text{sqt} \) and Atv-
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 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).

**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).
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 MOwnt8 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 MOwnt8 (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 krox20 (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 expression.

### Table 1. Dose-dependent effect of MOwnt8 on neural posteriorization

<table>
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<th>Amount injected</th>
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<td>14</td>
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See legend to Fig. 6 for class description.
krx20 or ephA4/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 a markedly elongated organizer tissue (Fig. 6c; n=19/19). Like MO\(^{wnt8}\)-injected wild-type embryos, as expected, embryos co-injected with MO\(^{wnt8}\) RNA + MO\(^{dpC}\) RNA, which caused a severe defect in mesodermal differentiation (see Materials and Methods, papc targeted by MO\(^{wnt8}\)), dorsalized (Fig. 6G,K). These embryos (class IV) displayed slightly enlarged mesoderm 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;slt 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 (see Materials and Methods, wnt8mut targeted by MO\(^{wnt8}\)). Injection of both wnt8 (Kelly et al., 1995) or wnt8mut RNA, as expected, activated the \(\beta\)-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 wnt8mut 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 wnt8mut (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 wnt8mut 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-wnt8mut-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
MO<sup>wnt8</sup>-injected embryos displayed anteriorized phenotypes (enlargement of <i>six3</i> expression, and loss or reduction of <i>krx20</i> rhombomere 5 and spinal cord-specific <i>huC</i> expression). The majority of MO<sup>wnt8</sup>/hsp-wnt8 co-injected embryos had a normal-sized <i>six3</i> domain with only a minor expansion of axial mesoderm (Table 2). This experiment essentially shows that gastrulation-stage <i>wnt8</i> expression can titrate away the anteriorizing effect of MO<sup>wnt8</sup>. However, to show that MO<sup>wnt8</sup> specifically blocks Wnt8 function, rather than inactivating translation from other RNAs, we tested if the neural anteriorization caused by MO<sup>wnt8</sup> could be rescued by heat shock of embryos co-injected with hsp-wnt8<sup>mut</sup>, which produces a modified transcript that cannot bind the morpholino (see Materials and Methods). Whereas hsp-wnt8<sup>mut</sup> injected non-heat shocked embryos generally did not exhibit ectopic <i>wnt8</i> expression (0/18 embryos injected with 5 pg hsp-wnt8<sup>mut</sup>, 1/8 embryos injected with 10 pg hsp-wnt8<sup>mut</sup>), most of the injected embryos subjected to a 1 hour heat shock treatment showed several small patches of ectopic <i>wnt8</i> RNA distributed randomly throughout the blastoderm (8/9 and 12/13 embryos at the 5 pg and 10 pg hsp-wnt8<sup>mut</sup> doses, respectively). Heat-shocked hsp-wnt8<sup>mut</sup>-injected embryos showed strong reduction of <i>six3</i> expression at the six-somite stage (Table 2). MO<sup>wnt8</sup>-injected control embryos had an anteriorized phenotype with relative enlargement of the <i>six3</i> forebrain domain and reduction of <i>krx20</i> rhombomere 5 and spinal cord-specific <i>huC</i> expression (Table 2; 19%; <i>n</i>=63). By contrast, only a very small number of heat-shocked MO<sup>wnt8</sup>/hsp-wnt8<sup>mut</sup>-co-injected embryos showed such neural anteriorization (3%; Table 2). 36% of MO<sup>wnt8</sup>/hsp-wnt8<sup>mut</sup>-co-injected embryos were dorsalized and showed lateral expansion of neuroectoderm, perhaps because of low levels of <i>wnt8</i><sup>mut</sup> expression before heat shock. These embryos, however, showed normal expression of <i>krx20</i> in the prospective hindbrain, in contrast to the great reduction or loss of <i>krx20</i> and <i>palx2</i> expression in MO<sup>wnt8</sup>-anteriorized embryos (Fig. 6F,G). This ability of late-onset <i>wnt8</i><sup>mut</sup> expression to suppress the anteriorizing effects of MO<sup>wnt8</sup> injection provides a bona fide control for MO<sup>wnt8</sup> specificity.

**Reduction of Wnt8 activity suppresses the forebrain and notochord deficiency of <i>boz</i> mutant embryos**

In the absence of the homeoprotein Bozozok, the intensity of <i>wnt8</i> expression in the presumptive non-axial mesoderm is increased, and the dorsal-most downregulation of <i>wnt8</i> expression does not occur. This increased <i>wnt8</i> expression in <i>boz</i> 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 <i>boz</i> itself results in strong or complete loss of <i>wnt8</i> expression in presumptive axial and paraxial mesoderm (Fekany-Lee et al., 2000). Together, these results indicate that one function of <i>boz</i> is to downregulate, directly or indirectly, <i>wnt8</i> expression.

Further evidence that Wnt8 is an endogenous neural transformer was obtained by injecting MO<sup>wnt8</sup> into <i>boz</i> homozygous embryos. In 93% (<i>n</i>=40/43) of the untreated <i>boz</i> mutant embryos, the forebrain marker <i>six3</i> was significantly reduced and the <i>ntl</i> 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<sup>wnt8</sup>-injected <i>boz</i> mutant embryos (<i>n</i>=83/83) exhibited slightly broader <i>ntl</i> notochord expression domains, most probably owing to the general dorsalizing effect of MO<sup>wnt8</sup> described above. In addition, the <i>six3</i> expression domain was enlarged compared with control <i>boz</i> mutant embryos (Fig. 8B). These studies provide direct evidence that the reduction of axial

**Table 2. Effect of late <i>wnt8</i> and <i>wnt8</i><sup>mut</sup> expression on MO-dependent anteriorization**

<table>
<thead>
<tr>
<th>Injected RNA</th>
<th>Amount</th>
<th>Reduced &lt;i&gt;six3&lt;/i&gt;</th>
<th>Enlarged &lt;i&gt;six3&lt;/i&gt;</th>
<th>&lt;i&gt;n&lt;/i&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp-wnt8</td>
<td>10 pg</td>
<td>100%</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>MO&lt;sup&gt;wnt8&lt;/sup&gt;</td>
<td>5 ng</td>
<td>0</td>
<td>49%</td>
<td>33</td>
</tr>
<tr>
<td>hsp-wnt8/MO&lt;sup&gt;wnt8&lt;/sup&gt;</td>
<td>10 pg/5 ng</td>
<td>35%</td>
<td>14%</td>
<td>43</td>
</tr>
<tr>
<td>hsp-wnt8&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>5 pg</td>
<td>16%</td>
<td>0</td>
<td>58</td>
</tr>
<tr>
<td>hsp-wnt8&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>10 pg</td>
<td>50%</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>MO&lt;sup&gt;wnt8&lt;/sup&gt;</td>
<td>5 ng</td>
<td>0</td>
<td>19%</td>
<td>63</td>
</tr>
<tr>
<td>hsp-wnt8&lt;sup&gt;mut&lt;/sup&gt;/MO&lt;sup&gt;wnt8&lt;/sup&gt;</td>
<td>5 pg/5 ng</td>
<td>3%</td>
<td>4%</td>
<td>40</td>
</tr>
<tr>
<td>hsp-wnt8&lt;sup&gt;mut&lt;/sup&gt;/MO&lt;sup&gt;wnt8&lt;/sup&gt;</td>
<td>10 pg/5 ng</td>
<td>22%</td>
<td>4%</td>
<td>102</td>
</tr>
</tbody>
</table>

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 having 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. 2FJ). 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 bozm168 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,I). 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 Atp 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 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 vegetal expression pattern correlates with AP neural regionalization in both wild-type and mutant conditions. Using MQwt8 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 neuroectodermal 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; Itasaka 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 Atp 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<sup>wnt8</sup> doses are increased, embryos display a progressive loss of posterior neural fates with concomitant expansion of more anterior ones. The lowest doses of MO<sup>wnt8</sup> 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<sup>wnt8</sup> 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<sup>wnt8</sup> that showed severe anteriorization continued to express wnt8 or ntl mesodermal markers in the lateral margin of gastrula stage embryos, and pappc 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 (Thissee et al., 2000; Thisse and Thissee, 1999).

Also consistent with the proposed mechanism of Wnt8 action are the neural patterning phenotypes produced by boz, boz;sqt, boz;cyce and boz;sqt;cyce 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<sup>wnt8</sup> 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;cyce 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 intermediary 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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REFERENCES


Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B. and Kawakami, K. 3582 C. E. Erter and others
Wnt8 is required for neural posteriorization


