Development 128, 4189-4201 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2746



# A morphogen gradient of Wnt/β-catenin signalling regulates anteroposterior neural patterning in *Xenopus*

**Clemens Kiecker and Christof Niehrs***

Division of Molecular Embryology, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
*Author for correspondence (e-mail: niehrs@dkfz-heidelberg.de)

*Accepted 2 August 2001*

## SUMMARY

**Anteroposterior (AP) patterning of the vertebrate neural plate is initiated during gastrulation and is regulated by Spemann's organizer and its derivatives. The prevailing model for AP patterning predicts a caudally increasing gradient of a 'transformer' which posteriorizes anteriorly specified neural cells. However, the molecular identity of the transforming gradient has remained elusive. We show that in *Xenopus* embryos (1) dose-dependent Wnt signalling is both necessary and sufficient for AP patterning of the neuraxis, (2) Wnt/β-catenin signalling occurs in a direct and long-range fashion within the ectoderm, and (3) that there is an endogenous AP gradient of Wnt/β-catenin signalling in the presumptive neural plate of the *Xenopus* gastrula. Our results indicate that an activity gradient of Wnt/β-catenin signalling acts as transforming morphogen to pattern the *Xenopus* central nervous system.**

Key words: Anteroposterior neuraxis, AP, β-catenin, Gastrulation, Head organizer, Long-range signalling, Morphogen gradient, Neural patterning, Neuroectoderm, Nieuwkoop, Spemann, Transformer, Wnt protein, *Xenopus*

## INTRODUCTION

The vertebrate central nervous system (CNS) is divided anteroposteriorly into forebrain, midbrain, hindbrain and spinal cord during early embryogenesis. Neural inducers and modifiers establish a crude anteroposterior (AP) pattern before and during gastrulation that becomes refined during later stages (Saha and Grainger, 1992; Lumsden and Krumlauf, 1996; Sasai and De Robertis, 1997; Chang and Hemmati-Brivanlou, 1998a; Beddington and Robertson, 1999; Gamse and Sive, 2000; Stern, 2001). In amphibia, classical experiments have established a central role for Spemann's organizer in this process. This organizer region does not only induce ectoderm to acquire neural fate, it also emits regionally specific inducers, with anterior mesendoderm inducing forebrain and posterior chordamesoderm inducing spinal cord (Gilbert and Saxén, 1993; Ruiz i Altaba, 1993; Gould and Grainger, 1997; Harland and Gerhart, 1997; Nieuwkoop, 1997). Similarly, classical experiments by Nieuwkoop, Saxén, Toivonen and colleagues have suggested a two-step model where a gradient of a posteriorizing factor ('transformer') confers progressive posterior identity to tissue which has initially been induced as anterior neuroectoderm by early involuting mesendoderm (Gilbert and Saxén, 1993; Doniach and Musci, 1995; Nieuwkoop, 1997; Sasai and De Robertis, 1997). The molecular identity of the transforming gradient has remained elusive, although a number of candidate posteriorizing signals have been proposed, notably fibroblast growth factors (FGFs), retinoic acid (RA) and Wnts (Durston et al., 1989; Ruiz i Altaba, 1993; Slack, 1994; Doniach, 1995; McGrew et al., 1995; Pownall et al., 1996; Kolm and Sive, 1997; McGrew et al., 1997; Moon et al., 1997; Isaacs et al., 1998; Pownall et al., 1998; Wodarz and Nusse, 1998; Holowacz and Sokol, 1999; Gavalas and Krumlauf, 2000; Ribisi et al., 2000; Gamse and Sive, 2001). Yet, for none of them has it been shown that (1) it is required for AP patterning, (2) it acts directly on neuroectoderm and (3) it functions endogenously in a graded fashion.

Among the candidate transformers, Wnts have recently been implicated as endogenous antagonists of the head organizer (Niehrs, 1999). Consistent with the distinct inducing activities of anterior mesendoderm and chordamesoderm noted by Spemann and Mangold, inhibitors of transforming growth factors β (TGFβs) and Wnts, which are thought to mediate inductions by the organizer, are differentially expressed along the AP axis of the organizer and its derivatives (Harland and Gerhart, 1997; De Robertis et al., 2000). Notably, Wnt inhibitors are expressed predominantly in the anterior mesendoderm (Niehrs, 1999; De Robertis et al., 2000; Kiecker and Niehrs, 2001). In line with these inhibitors regulating AP neural patterning, co-injection of Wnt and bone morphogenetic protein (BMP) inhibitors on the ventral side of *Xenopus* embryos induces ectopic heads including forebrain structures while BMP inhibitors only induce ectopic trunk including spinal cord (Glinka et al., 1997). Thus, a distinguishing feature of the head organizer is the repression of Wnt signalling (Niehrs, 1999).

Other data support the idea that Wnts antagonize the head

organizer and have posteriorizing properties. Overexpression of various Wnts (Christian and Moon, 1993; McGrew et al., 1995; Fredieu et al., 1997; McGrew et al., 1997; Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998b; McGrew et al., 1999; Gamse and Sive, 2001), the Wnt pathway components β-catenin and XTcf3 (McGrew et al., 1995; Darken and Wilson, 2001; Hamilton et al., 2001) or treatment with the artificial activator of the Wnt pathway lithium chloride (Fredieu et al., 1997) all lead to repression of anterior and concomittant induction of posterior neural markers in *Xenopus*. Conversely, overexpression of secreted Wnt antagonists anteriorizes *Xenopus* and zebrafish embryos (Itoh et al., 1995; Hoppler et al., 1996; Leyns et al., 1997; Deardorff et al., 1998; Glinka et al., 1998; Hsieh et al., 1999a; Fekany-Lee et al., 2000; Hashimoto et al., 2000; Shinya et al., 2000). A posteriorizing role for Wnts has also been suggested from studies on transgenic mice expressing *Wnt8* (Pöpperl et al., 1997). Furthermore, mice mutant for *Wnt3A* (Takada et al., 1994), the nuclear transducers of Wnt signalling *Lef1* and *Tcf1* (Galceran et al., 1999) and the Wnt co-receptor *LRP6* (Pinson et al., 2000) show posterior truncations, indicating a requirement for Wnt signalling in posterior structures. Finally, inhibition of Wnt signalling is required for formation of anterior neural structures as evidenced by interference with inhibitors of Wnt signalling: both inhibition of the secreted Wnt antagonist Dkk1 in *Xenopus* (Glinka et al., 1998; Kazanskaya et al., 2000), as well as inactivation of the Wnt repressors *tcf3* and *axin1* in the zebrafish *headless* and *masterblind* mutants, respectively, lead to microcephalic embryos (Kim et al., 2000; Heisenberg et al., 2001).

These properties make Wnts likely candidates for Nieuwkoop's posteriorizing factor. Thus, we investigated whether an endogenous Wnt signalling gradient could act as transformer in AP neural patterning during gastrulation, i.e. before the well-established function of Wnts during the pattern-refinement phase of neural development (Patapoutian and Reichardt, 2000; Wilson and Rubenstein, 2000). We show that dose-dependent Wnt signalling is both necessary and sufficient for AP patterning of the neuraxis, and that it occurs in a direct and long-range fashion. Furthermore, we reveal an AP activity gradient of Wnt/β-catenin signalling across the presumptive neural plate. Our results implicate a morphogen gradient of Wnt/β-catenin signalling as a component of Nieuwkoop's transformer.

## MATERIALS AND METHODS

### Recombinant proteins

XWnt8 and Fc-tagged MFz8-CRD proteins were produced as described (Hsieh et al., 1999b). XWnt8 protein concentration of conditioned media was determined by comparing its signal intensity produced in Western blot with signal intensities of known amounts of $^{35}$S-labelled, Myc-tagged XWnt8 protein synthesized by in vitro translation.

### Embryos and explants

Microinjections, conjugation and grafting of animal caps were performed in agarose-coated dishes containing 1× modified Barth's solution (MBS). Microsurgical operations were performed using eyebrow knives. For ectodermal grafts (Figs 5, 6), a small piece of ectoderm was cut from the pigmented donor embryo, and a hole of the same shape and size was cut into the presumptive neural plate of the albino host. The graft was positioned in the recipient's hole with gentle pressure and transferred to culture dishes after ~20 minutes of healing. Explants and operated embryos were cultured at 15°C in plastic dishes coated with HEMA (poly(2-hydroxyethylmethacrylate)) in 0.5× MBS. Explantation of early (stage 13) neuroectoderm for luciferase assays (Fig. 7C) was performed in 1× $Ca^{2+}$- and $Mg^{2+}$-free modified Barth's solution (CMFB). The presumptive neural plate was excised and cut into four AP slices together with the underlying tissue. After few minutes, the ectoderm disadhered and could easily be separated from the underlying material. Ectodermal explants were collected in 1× MBS and processed for luciferase assay.

### Treatment of dissociated ectodermal cells with protein

Concentrated XWnt8-conditioned and control media were dialyzed overnight at 4°C against 0.5× CMFB. Protein dilutions were prepared on ice using control medium, adjusted to 0.1 mg/ml heparin and kept at 15°C. For cell dissociation, 15 to 20 animal caps were collected per sample and transferred to a HEMA-coated tube containing 0.5 ml 1× CMFB + 2mM EDTA and incubated at room temperature for 5 minutes. Subsequently, the CMFB + EDTA was replaced by 0.2 ml of protein sample and the caps were resuspended after further incubation for 20 minutes by gentle pipetting using a HEMA-treated plastic tip. The tubes were incubated for 3 hours at room temperature in a horizontal position to prevent aggregation of the dissociated cells. Following treatment, the tubes were briefly centrifuged and the supernatant was replaced by 0.5 ml 0.5× MBS. The tubes were incubated in an upright position overnight at 18°C to allow reaggregation of the animal cap cells. When control siblings reached the desired stage, RNA was extracted from aggregates using Trizol (Gibco BRL).

### RT-PCR and luciferase assay

RT-PCR analysis was carried out as described previously (Gawantka et al., 1995) using gene-specific primer pairs for *Bf1* (Bourguignon et al., 1998), *En2* (Hemmati-Brivanlou et al., 1991), *H4*, *muscle actin*, *Otx2*, *sia*, *Xbra* (Glinka et al., 1998), *Krox20*, *NCAM2* (Hemmati-Brivanlou et al., 1994) and *Xnr3* (Smith et al., 1995). Reporter gene assays on embryonic explants were performed using the Dual-Luciferase Reporter Assay System (Promega).

### Constructs

The expression construct for the activated form of *Xenopus* β-catenin lacking the N-terminal 90 amino acids was cloned by oligonucleotide-mediated 'loop-out' mutagenesis (Sambrook et al., 1989), using pCS2+*Xβcat-GFP* (Miller and Moon, 1997) to yield the plasmid pCS2+*Xβcat*-GFP*-GFP*.

### In situ hybridization and β-catenin immunostaining

In situ hybridization was carried out using standard procedures (Gawantka et al., 1995; Hollemann et al., 1998b). All embryos and explants were photographed following immersion in 80% glycerol/phosphate-buffered saline (PBS). The embryos in Fig. 5D were bleached by illumination in 30% $H_2O_2$/methanol/water (1:1:1). β-catenin staining on mid-gastrula embryo halves was performed as reported previously (Schneider et al., 1996) using a rabbit β-catenin antibody (generous gift from H. Steinbeisser). Stained halves were treated with Murray's clear, mounted on glass slides and pictures were taken using a Zeiss Axioplan microscope, a Sony Power HAD 3CCD color video camera and the Adobe Photoshop software. Eight consecutive pictures were recorded along the AP axis within the presumptive neural plate, each window typically containing 25 to 40 cells within the analysed ectodermal layer. Grey values were determined for all nuclei using the NIH Image software, the grey value of the cytoplasmic background was subtracted and the resulting densities were averaged for individual pictures.

## RESULTS

### Wnts posteriorize anterior neuroectoderm in a concentration-dependent manner

An important experimental approach used to reveal the function of TGFβs as morphogens during dorsoventral (DV) mesoderm induction was the use of dissociated *Xenopus* ectodermal (animal cap) cells which were treated with different doses of Activin protein (Green and Smith, 1990). Previously, similar experiments using Wnts were difficult because they could not be obtained in soluble form. Recently though, various sources of soluble, bioactive vertebrate Wnts have been described (Shibamoto et al., 1998; Hsieh et al., 1999b; Piccolo et al., 1999). We used a soluble XWnt8 produced as conditioned medium from *Drosophila* S2 cells (Hsieh et al., 1999b) to test its dose-dependent effects on neuralized animal cap cells. Animal caps were explanted at late blastula stage and dissociated in $Ca^{2+}$- and $Mg^{2+}$-free medium. This treatment characteristically results in anterior neural induction presumably owing to dilution of anti-neuralizing BMPs (Chang and Hemmati-Brivanlou, 1998a). Cells were treated with XWnt8-conditioned medium until control embryos developed to late gastrula stage. After reaggregation, the cells were further incubated without XWnt8 and harvested at neural tube stage for analysis of marker gene expression by RT-PCR (Fig. 1A).

Dissociation of animal cap cells lead to induction of the pan-neural marker *NCAM2* (Kintner and Melton, 1987), the forebrain marker *Bf1* (Bourguignon et al., 1998) and *Otx2*, a marker of fore- and midbrain (Blitz and Cho, 1995; Pannese et al., 1995), indicative of anterior neural induction (Fig. 1B). Treatment with increasing doses of XWnt8 lead to a progressive posteriorization of the neural character of these cells. At 2 nM XWnt8, *Bf1* expression was repressed and *Otx2* was maintained, while the mid-hindbrain boundary marker *En2* (Hemmati-Brivanlou and Harland, 1989) became induced. At 20 nM XWnt8, these anterior neural markers were repressed and the hindbrain marker *Krox20* (Bradley et al., 1993) became induced.

To confirm that this posteriorization was due to XWnt8 in the conditioned medium, we also performed XWnt8 treatment in the presence of MFz8-CRD, a secreted form of the ligand-binding domain of mouse Frizzled8, which binds to XWnt8 and acts as Wnt antagonist (Deardorff et al., 1998; Hsieh et al., 1999b). MFz8-CRD abolished the ability of XWnt8 to induce *Krox20*, indicating that posteriorization was due to Wnt signalling (Fig. 1C). Furthermore, posteriorization by XWnt8 was not accompanied by mesoderm induction, as indicated by the absence of *muscle actin* expression, and hence occurred in a direct fashion (Fig. 1B,C).

These results show that Wnt protein can posteriorize anterior neuroectoderm in a dose-dependent manner, specifying with increasing dose forebrain (*Bf1*), midbrain (*Otx2*, not *Bf1*), mid-hindbrain (*En2*) and hindbrain (*Krox20*), respectively. The modulation of marker gene expression occurs over a 10-fold concentration range.

We tested if Wnts also elicit dose-dependent responses in solid tissue and whether the responses occur as a function of the distance from a local Wnt source. We performed animal cap sandwich experiments similar to those used by Gurdon and colleagues (Gurdon et al., 1994). Animal caps were taken from albino embryos injected with noggin mRNA (Smith and Harland, 1992) and were used as anterior neuralized responding tissue. These caps were conjugated with animal caps from pigmented embryos injected with *Xwnt3A* mRNA (Christian et al., 1991) or *Xwnt8* DNA as sources of posteriorizing Wnt protein (Fig. 2A). The conjugates were allowed to develop until control embryos reached neural plate stage and expression of neural markers was analysed by in situ hybridization. The use of albino and pigmented animal caps allowed easy identification of the Wnt-responding tissue. Cell mixing of pigmented with albino cells was negligible under these conditions, as shown previously (Gurdon et al., 1994), and this was also confirmed by lineage tracing with colloidal gold labelled caps (not shown).

Without Noggin, no neural induction occurred, even when *Xwnt3A* was expressed (Fig. 2B, a-a″). Noggin injection resulted in induction of *Bf1* throughout the responding albino cap but no expression of the posterior markers *En2* or *Krox20* was detectable (Fig. 2B, b-b″). When pigmented caps injected

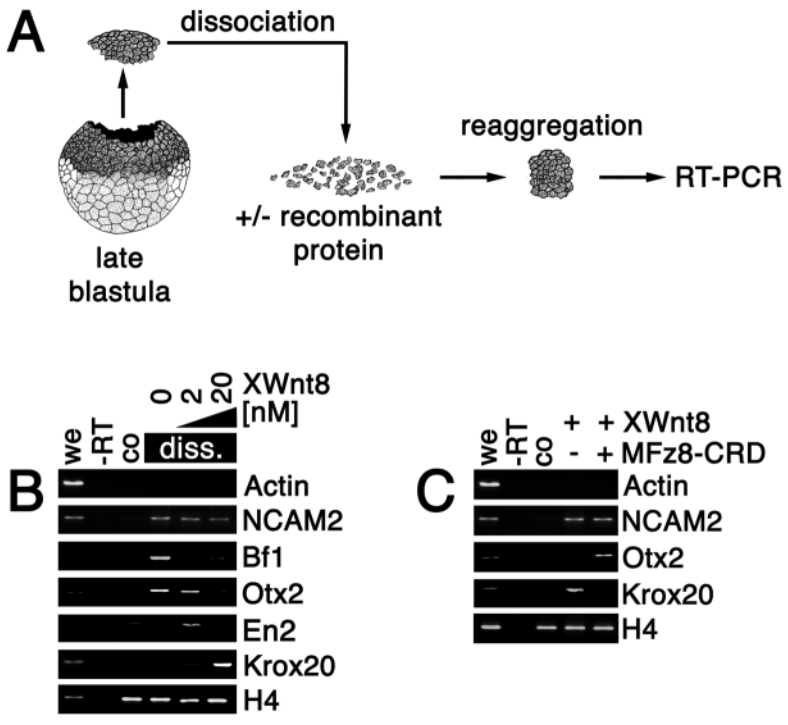


**Fig. 1.** Recombinant XWnt8 protein posteriorizes neuralized animal cap cells in a dose-dependent manner. (A) Experimental design. RT-PCR analyses were carried out when control embryos reached neural tube stage (stage 20). (B) RT-PCR analysis of whole embryos (we), intact control animal caps (co) and caps that were dissociated and reaggregated (diss.) as depicted in A and treated during dissociation with 0, 2 nM or 20 nM recombinant XWnt8. (C) RT-PCR analysis of whole embryos (we), intact animal caps (co) and caps that have been dissociated and reaggregated as depicted in A, and treated during dissociation with 25 nM XWnt8 (+) in the absence (–) or presence (+) of recombinant MFz8-CRD. H4, *histone4* for normalization; –RT, negative control without reverse transcriptase.

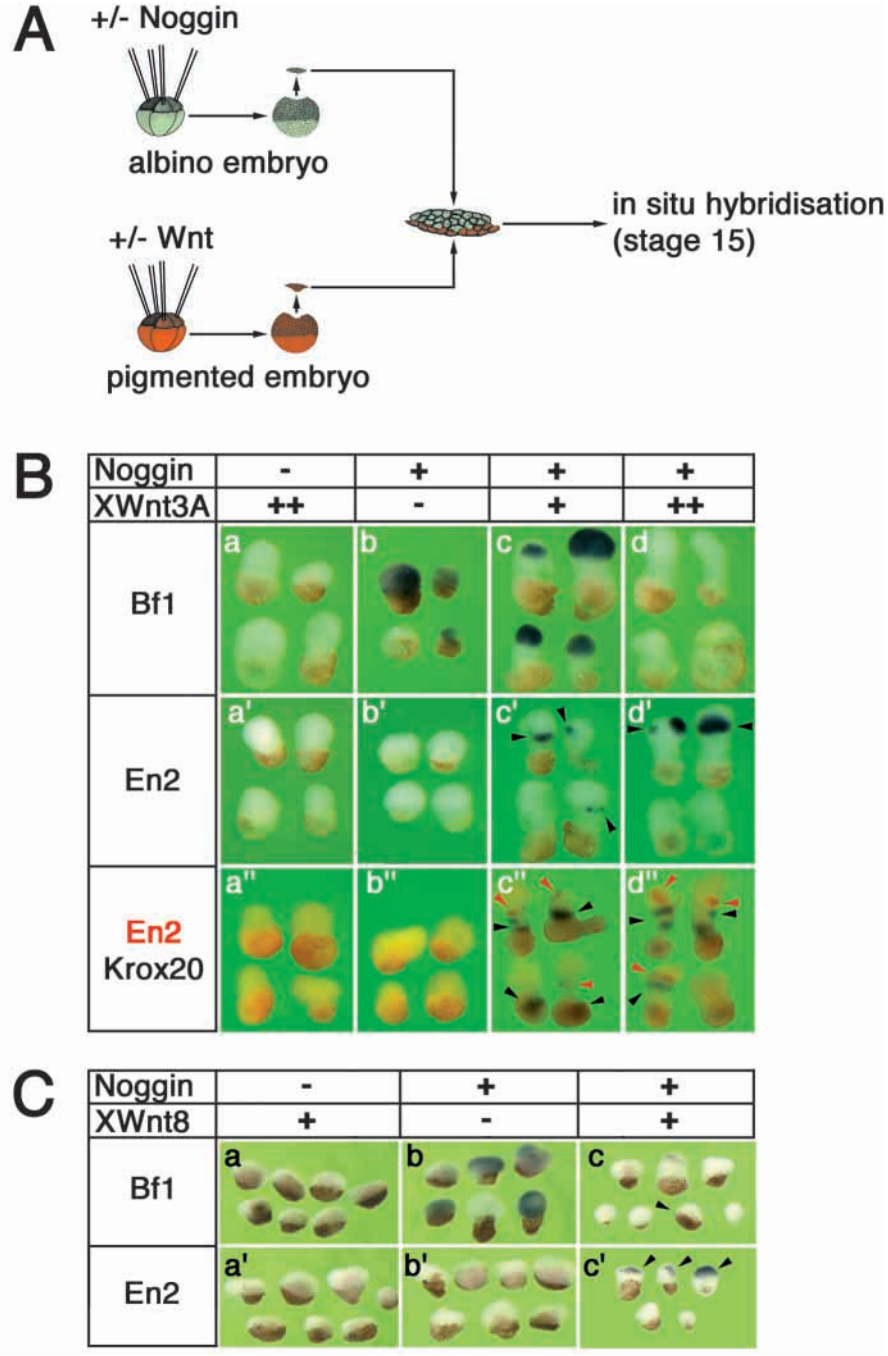


**Fig. 2.** Localized expression of Wnts induces a polar AP neural pattern at long range in animal caps. (A) Experimental design: at the eight-cell stage, albino embryos were injected with 0.25 ng/blastomere *noggin* mRNA and pigmented embryos were injected with *Xwnt3A* mRNA (B) or pCSKA-*Xwnt8* (C) into the four animal blastomeres. Animal caps were explanted at late blastula stage and sandwiched to yield pigmented/unpigmented conjugates. The conjugates were cultured until control siblings reached stage 15 and marker gene expression was analysed by in situ hybridization. Between 12-40 conjugates were analysed in B,C, of which representative samples are shown with albino halves of the conjugates always pointing to the top. (B) Animal cap conjugates from uninjected or Noggin-injected albino embryos and pigmented embryos injected with nil, 0.1 (+) or 1 ng/blastomere (++) *Xwnt3A* mRNA were analysed for the expression of *Bf1*, *En2* and *Krox20* as indicated. Note that in d″ an organized AP pattern is generated with the more posterior *Krox20* closer to the pigmented cap than *En2*. (C) Animal cap conjugates from uninjected or Noggin-injected albino embryos and pigmented embryos which were uninjected or injected with 0.05 ng/blastomere pCSKA-*Xwnt8* were analysed for the expression of *Bf1* and *En2* by in situ hybridization.

with a low XWnt3A dose were used as Wnt source, *Bf1* expression was typically restricted to the distal portion of the responding cap and was completely repressed at high XWnt3A dose (Fig. 2B, c,d). This was paralleled by induction of *En2* and *Krox20*. Importantly, double in situ hybridization revealed that the posterior marker *Krox20* was always expressed closer to the XWnt3A source than the more anterior marker *En2*, as would be expected if their expression requires different Wnt concentrations (Fig. 2B, c″,d″). Comparable results were obtained with injected *Xwnt8* DNA (Fig. 2C) and mRNA (not shown). The results indicate that a local Wnt source can induce different AP neural fates at long range as a function of the distance in solid tissue.

Next we tested the requirement for Wnt signalling during AP neural patterning in vivo. To modulate Wnt signalling, embryos were injected either with low or high doses of *Xdkk1* mRNA, encoding a secreted Wnt antagonist (Glinka et al., 1998), or with the plasmid pCSKA-*Xwnt8* (Christian and Moon, 1993) to drive Wnt expression after the midblastula transition (MBT; Fig. 3A). Early neural marker gene expression, including *Xanf1* (presumptive forebrain) (Zaraisky et al., 1992), *Otx2* (presumptive fore- and midbrain), *Gbx2* (presumptive hindbrain and anterior spinal cord) (von Bubnoff et al., 1996) and *HoxD1* (presumptive posterior hindbrain and spinal cord) (Kolm and Sive, 1997), was analysed at late gastrula stage when AP neural patterning is already overt. Expression of a low XDkk1 dose shifted the expression of *HoxD1* and *Gbx2* posteriorly, and expanded *Otx2* and *Xanf1* ventroposteriorly (Fig. 3A, compare b-b’’’ with c-c’’’). At high XDkk1 doses, *HoxD1* and *Gbx2* became repressed, *Otx2* remained expanded while *Xanf1* showed further ventral expansion (Fig. 3A, d-d’’’). Comparable results were obtained with the secreted Wnt antagonist NXFz8 (Deardorff et al., 1998; not shown). Conversely, injection of pCSKA-*Xwnt8* left *HoxD1* unchanged, expanded *Gbx2*, restricted *Otx2* anteriorly and repressed *Xanf1*. These results (summarized in Fig. 3B) show (1) that Wnt signalling is required for expression of

spinal cord and hindbrain markers already at gastrula stage, (2) that presumptive forebrain specification is inhibited by endogenous Wnt signalling in the ventroposterior, and (3) that endogenous Wnt signalling is not uniformly distributed but graded, with peak levels in the posterior (Fig. 3A, c) and lowest levels in the anterior (Fig. 3A, a''). Thus, the presumptive neural plate is under control of posteriorizing Wnt signalling at late gastrula stage.

To investigate how this patterning manifests itself at later stages of neurulation we analysed marker gene expression in embryos injected with *Xdkk1*, *NXfz8* or *Xfrzb1* (Leyns et al., 1997) mRNAs or pCSKA-*Xwnt8* at neural plate stage (stage 15; Fig. 4A). Overexpression of *Xwnt8* repressed *Bf1* and reduced *Otx2* expression, presumably to midbrain fate, indicating posteriorization of the neural plate (Fig. 4A, a,b). With injected Wnt antagonists, two transformation thresholds were observed with increasing doses. At low doses, expression of *Krox20* in rhombomere 5 was repressed but maintained in the more anterior rhombomere 3 (Fig. 4A, c-e). At high dose, both XDkk1 and NXFz8 repressed *Krox20* (Fig. 4A, c′,d′), while residual *Krox20* expression was observed with XFrzb1 (Fig. 4A, e′, arrowhead). The expression of *Bf1* and *Otx2* was expanded by all Wnt inhibitors. Interestingly, each Wnt antagonist altered the pattern of neural markers in a characteristic and reproducible manner. XDkk1 lead to a horseshoe-shaped expression of *Bf1* and *Otx2*, indicating a posterior expansion (Fig. 4A, c′). By contrast, XFrzb1 resulted in a lateral, semicircular expansion of expression (Fig. 4A, e′). These differences may relate to the inhibitory specificities of these Wnt antagonists. The results (summarized in Fig. 4B) confirm a dose-dependent requirement for Wnt signalling with at least two thresholds for AP neural patterning.

## Wnts posteriorize neuroectoderm in a direct and long-range fashion

In the previous experiments, whole embryos were microinjected with Wnts or Wnt antagonists which may affect Wnt signalling in all germ layers. This raises the question of whether the changes in AP neural patterning observed after Wnt modulation in vivo are due to a direct effect on neuroectoderm or if they occur indirectly via an effect on dorsal mesoderm. To specifically modulate Wnt signalling in neuroectoderm, we implanted small groups of animal cap cells from pigmented embryos injected with *Xwnt3A* or *Xdkk1* mRNAs into the presumptive neural plate of albino embryos. The host embryos were allowed to develop until neural plate stage (stage 15) and analysed for marker gene expression (Fig. 5A). The pigmentation of the transplanted animal cap cells allowed again easy identification of the grafted donor tissue. Implantation of *Xwnt3A*-expressing cells into the presumptive forebrain induced ectopic *Krox20* and *En2* at some distance from the graft and with inverted polarity compared to the primary AP neuraxis (Fig. 5B, a,b). The transplant also initiated the outgrowth of host tissue, presumably because convergent-extension movements characteristic of posterior neural plate morphogenesis were induced. Thus, a local Wnt source can induce an AP pattern in neuroectoderm at long range. When *Xdkk1*-expressing cells were implanted into the neural plate, *Bf1* expanded posteriorly in the vicinity of the grafted cells (Fig. 5C, a). Conversely, *Krox20* expression became downregulated and shifted posteriorly (Fig. 5C, b). It

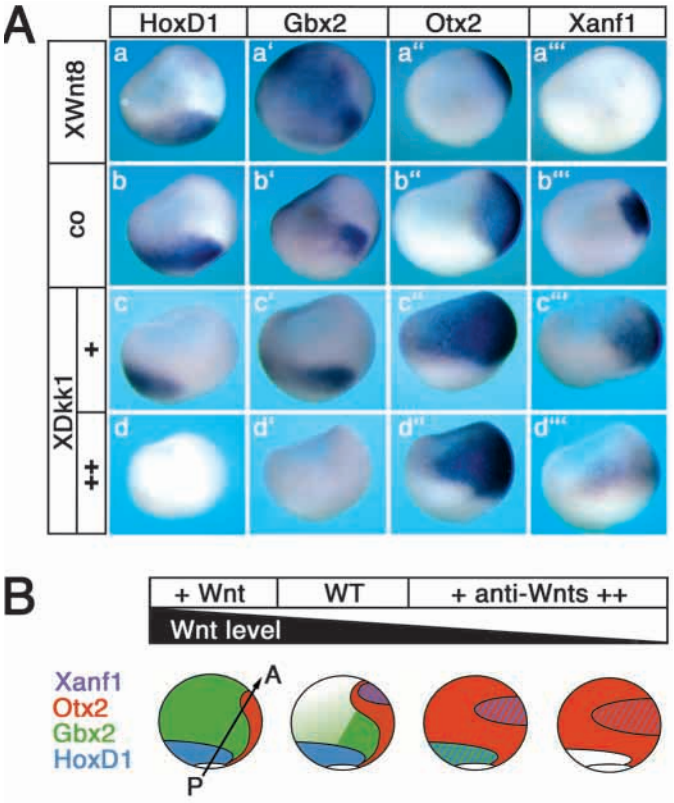


**Fig. 3.** Wnt signalling regulates AP patterning in gastrula ectoderm. (A) Albino embryos were uninjected (co) or injected at the eight-cell stage into the four animal blastomeres with 0.05 ng/blastomere pCSKA-*Xwnt8*, 0.05 ng/blastomere (+) or 0.2 ng/blastomere (++) *Xdkk1* mRNA. The embryos were cultured until late gastrula stage (stage 12) and analysed by in situ hybridization for the expression of *HoxD1*, *Gbx2*, *Otx2* and *Xanf1*. Lateral views are shown, dorsal points to the right. 12-18 embryos were analysed per sample in three independent experiments. (B) Summary of the results shown in A. Colour code of gene expression domains is on the left. The approximate orientation of the prospective ectodermal AP axis is indicated on the left (P→A). WT, wild-type embryo with normal Wnt levels.

is noteworthy that expression in rhombomere 5 was more sensitive to XDkk1 than in rhombomere 3 systematically, a phenomenon also observed in zebrafish (Shinya et al., 2000) and with other Wnt antagonists in *Xenopus* (Pera and De Robertis, 2000). This is consistent with the two thresholds of Wnt signalling defined for *Krox20* in whole embryo injections (Fig. 4). The results indicate a requirement for different concentrations of Wnt signalling within neuroectoderm.

In order to test if also more posterior marker gene expression requires Wnt signalling and to rule out the possibility that the effect of *Xdkk1*-expressing implants is due to an indirect effect via underlying mesoderm, we grafted cells expressing the intracellular Wnt inhibitor *Xgsk3β* (Itoh et al., 1995) into the presumptive posterior neural plate. Uninjected control grafts expressed *HoxD1* indicating that transplantation does not interfere with posterior neural gene expression per se (Fig. 5D, a,a′). By contrast, GSK3β grafts located in the presumptive spinal cord failed to express *HoxD1*, an effect best seen after bleaching of the pigment of the grafted cells (Fig. 5D, b′). This

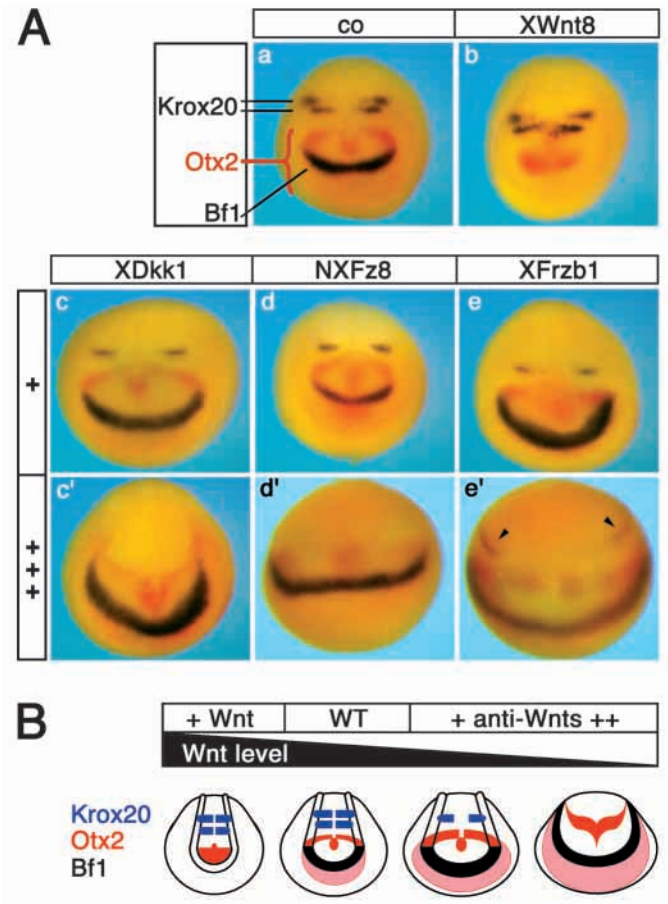


**Fig. 4.** Regulation of AP neural patterning by Wnt signalling in neurulae. (A) Albino embryos were uninjected or injected at the eight-cell stage into the four animal blastomeres with 0.05 ng/blastomere pCSKA-*Xwnt8*, 0.05 ng/blastomere or 0.2 ng/blastomere *Xdkk1* mRNA, 0.1 ng/blastomere or 0.4 ng/blastomere *NXfz8* mRNA, 0.8 ng/blastomere or 1.6 ng/blastomere *Xfrzb1* mRNA. Embryos were cultured until neural plate stage (stage 15) and analysed by triple in situ hybridization for the expression of *Krox20*, *Bf1* (black) and *Otx2* (red). Frontal views are shown, dorsal points towards the top. In a, the expression domains of *Bf1*, *Otx2* and *Krox20* (rhombomeres 3 and 5) are indicated. Note residual *Krox20* expression for highest doses of XFrzb1 (arrowheads in e′) but not XDkk1 (c′) or NXFz8 (d′). 25-35 embryos were analysed per sample in three independent experiments. (B) Summary of the results shown in A. Colour code of gene expression is on the left. WT, wild-type embryo with normal Wnt levels.

suggests that Wnt signalling is autonomously required for the specification of posterior (trunk) nervous system.

An important question raised by the preceding experiments is if the observed long-range effects (Figs 2B,C, 5B) are due to a direct action of diffusing Wnt protein acting as morphogen or if they occur via a relay mechanism. To distinguish between a direct and an indirect mechanism, we asked whether an implant expressing an intracellular component of the Wnt pathway is also able to affect neural patterning. We chose a constitutively active form of β-catenin (Xβcat*) (Barth et al., 1997) to activate the Wnt pathway in a cell-autonomous fashion. First, we titrated *Xβcat** and *Xwnt3A* mRNAs to activate Wnt/β-catenin signalling. In an axis duplication assay similar frequencies of induced secondary embryonic axes were achieved at an *Xβcat**/*Xwnt3A* mRNA ratio of 10:1 (Fig. 6A). When this mRNA ratio was used in animal cap assays, both mRNAs induced the immediate early Wnt target genes *siamois* (*sia*) (Brannon and Kimelman, 1996; Carnac et al., 1996) and *Xenopus nodal-related 3* (*Xnr3*) (McKendry et al., 1997) to similar levels by RT-PCR (Fig. 6B).

Implanted cells injected with XWnt3A repressed the forebrain marker *Bf1* (Fig. 6C, a,b) and induced the posterior markers *En2* and *Krox20* ectopically at some distance from the implant in the host neural plate (Fig. 6C, a′,b′,a″,b″). By contrast, cells expressing *Xβcat** induced no changes in marker gene expression in the host embryo (Fig. 6C, c-c″) even at highest doses of Xβcat* (20 ng/animal cap; not shown). We conclude that the long-range effect of XWnt3A on AP neural patterning is direct.

*Gbx2* and the posterior-dorsolateral neural marker *Pax3* (Bang et al., 1997) were expressed in both, *Xwnt3A*- and *Xβcat**-expressing grafts themselves, but not in control grafts (not shown). For *Xβcat**, *Gbx2* and *Pax3* expression were not detectable in adjacent albino tissue, indicating cell-autonomous posteriorization. Thus, both types of grafted cells can acquire comparable AP positional values.

### An AP gradient of Wnt/β-catenin signalling in the presumptive neural plate

We have shown that dose-dependent Wnt signalling is both necessary and sufficient for AP patterning of neural fates and that Wnts affect neuroectoderm in a direct, long-range fashion. This raises the crucial question of whether there is indeed an endogenous Wnt/β-catenin signalling gradient in the presumptive neural plate that can be directly detected. To analyse endogenous Wnt/β-catenin signalling, we made use of the Wnt-responsive luciferase reporter construct TOP-Flash, carrying Tcf-binding sites, which are directly activated by the Tcf/β-catenin complex (Korinek et al., 1997). The reporter was microinjected into the four animal blastomeres of eight-cell embryos, whose descendants populate predominantly ectoderm. Injected embryos were divided into four AP slices at the end of gastrulation. A gradient of reporter activation was detected for TOP-Flash, increasing 2.4-fold from the anterior towards the posterior of the late gastrula (Fig. 7A). We did not observe this gradient of reporter activation with FOP-Flash, an altered TOP-Flash construct in which the Tcf-binding sites are inactivated by point mutations (Korinek et al., 1997), suggesting that the graded activation of TOP-Flash was due to proper activation of the reporter via Lefs/Tcfs. Because the animal blastomeres of eight-cell stage *Xenopus* embryos do not give rise to ectoderm exclusively, the graded TOP-Flash activation could still reflect a gradient of Wnt signalling within the mesendoderm rather than in the ectoderm. Thus, we isolated AP slices of presumptive neural plates and performed the reporter experiment with these neuroectodermal explants. In line with the results using whole embryos, a graded activation of TOP-Flash could be observed with the explants, increasing approx. twofold from anterior to posterior (Fig. 7B). Some representative neuroectodermal explants were assayed by in situ hybridization for expression of the paraxial mesodermal marker *XmyoD* (Hopwood et al., 1989). No *XmyoD* expression was detected in the explants confirming the absence of mesodermal contamination (not shown).

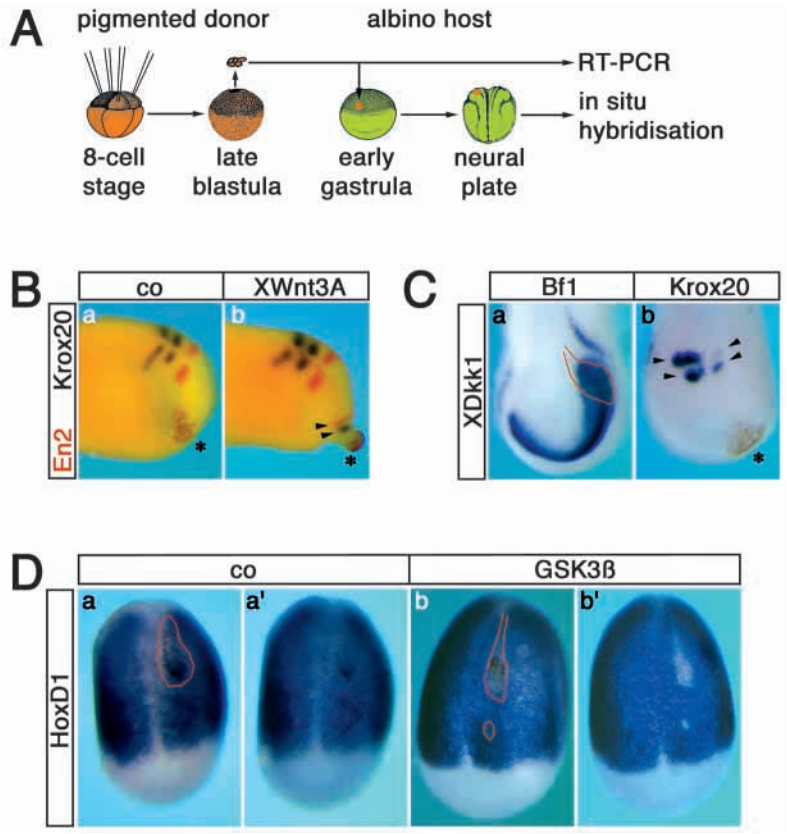


**Fig. 5.** Wnt signalling is necessary and sufficient to pattern neuroectoderm in a long-range fashion. (A) Experimental design: pigmented 8-cell stage embryos (donors) were injected into the four animal blastomeres with mRNAs encoding Wnt pathway activators or Wnt antagonists. At late blastula stage, small groups of pigmented animal cap cells were grafted into the presumptive neural plate of early gastrula albino embryos (hosts). The hosts were cultured until neurula stages and analysed for marker gene expression by in situ hybridization. (B) Grafted donor caps were uninjected (a) or injected with 0.25 ng/blastomere *Xwnt3A* mRNA (b) and placed in the presumptive anterior host neural plate. Embryos were analysed at neural plate stage for expression of *Krox20* (black) and *En2* (red) and are shown in lateral view, dorsal side upwards. Grafts are indicated by asterisks. Note ectopic induction of *En2* and *Krox20* with reversed AP polarity by the *Xwnt3A* expressing graft (arrowheads in b). (C) Grafted donors were injected with 0.25 ng/blastomere*Xdkk1* mRNA and placed in the presumptive posterior (a) or anterior (b) host neural plate, respectively. Embryos were analysed at neural plate stage for expression of *Bf1* (a) and *Krox20* (b) and are shown in dorsofrontal view. Grafted tissue is outlined in red in panel a. Note posterior expansion of *Bf1* expression in a and downregulation of *Krox20* (arrowheads in b). (D) Grafted donors were uninjected (a,a′) or injected with 1.5 ng/blastomere*Xgsk3β* mRNA (b,b′) and placed in the presumptive posterior host neural plate. Embryos were analysed at neural plate stage for expression of *HoxD1* and are shown in dorsal view, anterior down. Grafted tissue is outlined in red in panels a,b. The same embryos as in panels a,b are shown following bleaching in panels a′,b′. Note holes in *HoxD1* expression in panel b′ compared to continuous expression in panel a′.

However, measuring a Wnt signalling gradient by a reporter assay may not reflect acute levels of pathway activation because of the accumulation of luciferase protein between MBT and late gastrula stage. Thus, we analysed Wnt signalling in a more direct way. Upon Wnt pathway activation, β-catenin is known to translocate to the nucleus (Wodarz and Nusse, 1998). To visualize Wnt pathway activation in situ, we therefore detected nuclear β-catenin immunochemically in the presumptive neural plate of mid-gastrula embryos. Because there are few AP morphological landmarks at this stage, we cut gastrulae sagittally into left and right halves, and analysed them in parallel by in situ hybridization for the early posterior marker *Gbx2* and by β-catenin staining (Schneider et al., 1996; Rowning et al., 1997). Pictures of β-catenin staining were taken at eight consecutive AP levels within the presumptive neural plate (Fig. 8A). At the anterior-most levels (Fig. 8A,B, numbers 7 and 8), corresponding to future cement gland and forebrain territories, we detected no nuclear but only plasma membrane localization of β-catenin (Fig. 8B). Towards the posterior, β-catenin was seen increasingly localized to the nucleus. Densitometric analysis revealed a sixfold gradient of nuclear localization of β-catenin between the anterior- and posterior-most levels, with the region expressing *Gbx2* showing intermediate to peak levels which are maintained up to the blastopore (Fig. 8C). We conclude that there is an AP gradient of endogenous Wnt/β-catenin pathway activation in the presumptive neural plate of the *Xenopus* gastrula.

## DISCUSSION

Classical experiments have predicted gradients of posteriorizing factors involved in AP patterning of the early vertebrate neural plate. We provide evidence that the Wnt/β-catenin pathway constitutes one such signalling gradient. Our data show (1) that dose-dependent Wnt signalling is both necessary and sufficient for AP patterning of the neuraxis, (2) that Wnt/β-catenin signalling occurs in a direct and long-range fashion within the ectoderm, and (3) that there is an endogenous AP gradient of Wnt/β-catenin signalling in the presumptive neural plate of the *Xenopus* gastrula.

### Wnt/β-catenin signalling establishes a transforming gradient

We present three lines of evidence that different doses of Wnt signalling are sufficient to provide AP positional information to neural cells. First, experiments employing soluble protein show that different doses of XWnt8 can specify at least three distinct neural fates at increasing dose. Second, in animal cap conjugates an organized AP neural pattern is induced by a local Wnt source. Third, Wnt expressing grafts induce an ectopic AP neural pattern in the anterior neuroectoderm of host embryos in a direct fashion. Evidence that Wnts may have graded effects on neural patterning also comes from overexpression of the Wnt effector Dishevelled in *Xenopus* animal caps, which leads to induction of increasingly posterior neural markers with

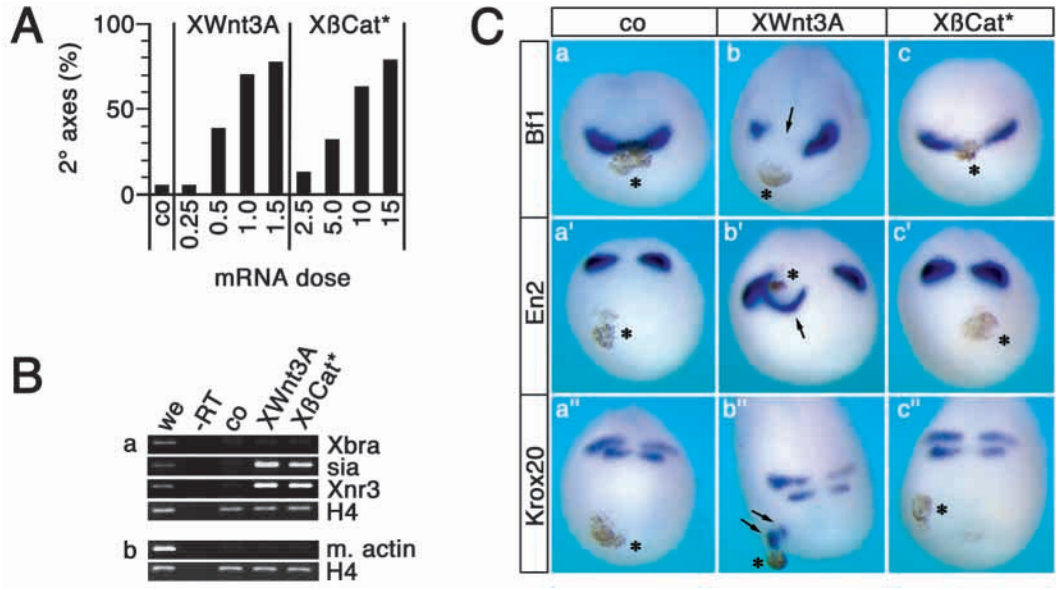


**Fig. 6.** Long-range patterning of neuroectoderm by XWnt3A is direct. (A) Titration of *Xwnt3A* and *Xβcat** mRNAs. For every mRNA concentration (in pg/nl), 15 embryos were injected at four- to eight-cell stages vegetally into two opposite blastomeres (2.5 nl/blastomere), cultured until tailbud stage and scored for the induction of secondary body axes. Comparable results were obtained in four independent experiments. (B) RT-PCR analysis of expression of the indicated marker genes at (a) early gastrula stage (stage 10+) and (b) neural plate stage (stage 15) in whole embryos (we) and animal caps cut from embryos injected at the eight-cell stage into the four animal blastomeres with no (co), 0.25 ng/blastomere *Xwnt3A* or 2.5 ng/blastomere *Xβcat** mRNAs. H4, *histone4* for normalization; –RT, negative control without reverse transcriptase. (C) Pigmented donors uninjected (co) or injected with 0.25 ng/blastomere *Xwnt3A* or 2.5 ng/ blastomere *Xβcat** mRNA were grafted as depicted in Fig. 5A into the presumptive host anterior neural plate. Embryos were analysed at neural plate stage (stage 15) for expression of *Bf1, En2* and *Krox20*. Frontal views are shown (dorsofrontal in b″,c″). Between 21 and 63 embryos were analysed for every type of transplant in six independent experiments. Note that Xβcat*-expressing grafts do not induce changes in host marker gene expression unlike *Xwnt3A* (arrows), even at 5 ng/blastomere (not shown).

increasing dose (Itoh and Sokol, 1997). Furthermore, we show by titrations with Wnt antagonists that different doses of Wnt signalling are required for AP neural patterning during gastrulation.

Soluble XWnt8 protein specifies forebrain, midbrain and hindbrain fates with increasing concentrations in animal cap cells neuralized by dissociation (Fig. 1). These inductions occur at 2-20 nM concentrations, which is consistent with the binding constant (~9 nM) of Wnt8 for Frizzled receptors (Bhanot et al., 1996; Hsieh et al., 1999b). To our knowledge, this is the first demonstration of dose-dependent Wnt signalling effects with any Wnt protein. It should be noted, however, that neural induction of animal cap cells by dissociation may not be comparable with neural induction in the embryo, which may involve instructive FGF and early Wnt/β-catenin signalling (Baker et al., 1999; Harland, 2000; Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001). Yet, similar to mammalian stem cells, dissociated animal cap cells may provide a powerful system to reconstitute neural differentiation in vitro by applying recombinant Wnt protein in combination with other growth factors.

Thresholds to different doses of Wnt/β-catenin signalling are also suggested by animal cap conjugate experiments (Fig. 2), where an organized AP pattern is induced as a function of the *Xwnt3A* mRNA dose and of the distance from the Wnt source.

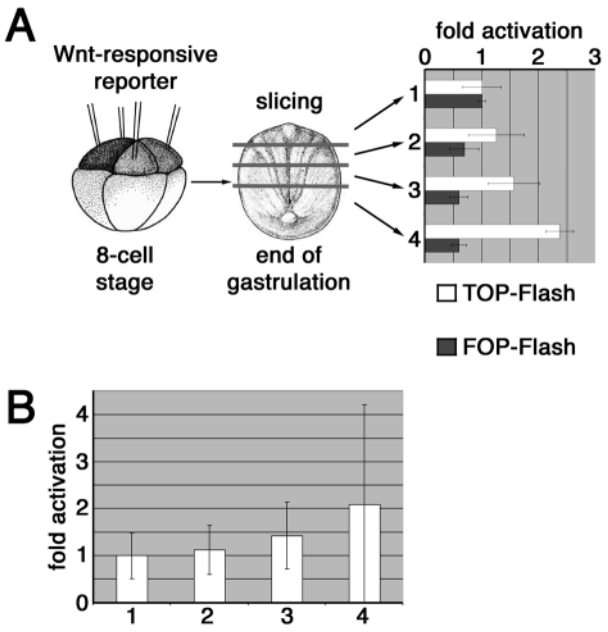


**Fig. 7.** An endogenous AP gradient of Wnt/β-catenin signalling in the late *Xenopus* gastrula. (A) Eight-cell stage embryos were injected into the four animal blastomeres with 25 pg/blastomere TOP-Flash or FOP-Flash (firefly luciferase) and 5 pg/blastomere pRL (Renilla luciferase) plasmids, cultured until early neurula stage (stage 13) and cut into four AP slices. Slices from three embryos per measurement were pooled, extracted and a double luciferase assay was performed. Firefly luciferase activity was normalized to Renilla luciferase activity. Relative TOP/FOP-Flash reporter activation is shown on the right. Note increasing AP activation for TOP- but not for FOP-Flash. (B) Eight-cell stage embryos were injected into the four animal blastomeres with 25 pg/blastomere TOP-Flash and 25 pg/blastomere pRL plasmids, cultured until early neurula stage (stage 13) and four AP slices of neuroectoderm were explanted from each embryo. Explants were extracted and a double luciferase assay was performed. Mean reporter activations are shown for three independent experiments (every column represents 90 ectodermal explants).

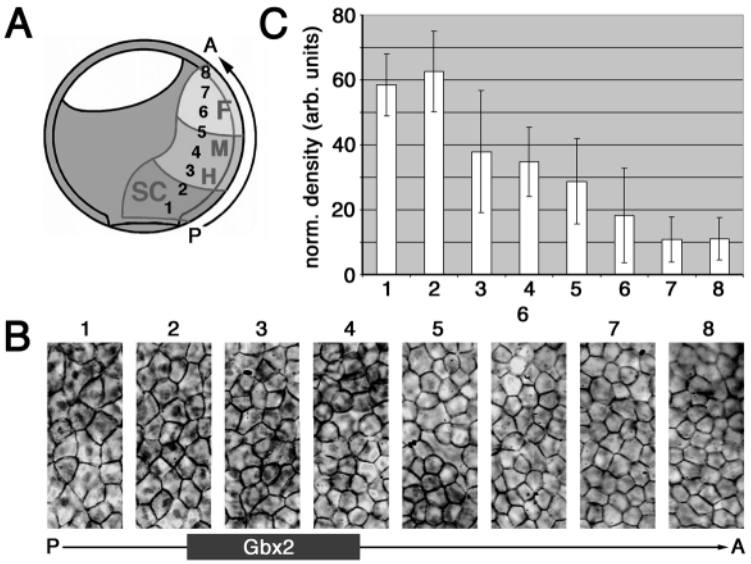


**Fig. 8.** An AP gradient of nuclear β-catenin in the presumptive neural plate during gastrulation. (A) Mid-gastrula embryos were cut sagittally and left and right halves were analysed by in situ hybridization for *Gbx2* expression and by immunostaining for β-catenin, respectively. Eight consecutive regions of the presumptive neuroectoderm of immunostained halves were selected from posterior to anterior positions, according to the gastrula fate map. Corresponding windows are shown in B for one representative embryo. Note prominent nuclear staining in windows 1 and 2, and absence of nuclear staining in 7 and 8. The AP axis is indicated (P→A) and the approximate position of the *Gbx2* expression domain is given in relation to the windows. Data in C represent mean normalized densities of the nuclear regions of all cells within a window.

XWnt3A and XWnt8 are both able to posteriorize neural fates in these conjugates at long range, suggesting that a variety of type I Wnts signalling via β-catenin (Wodarz and Nusse, 1998) are able to elicit these effects.

In vivo, we show that the presumptive neural plate of the late gastrula embryo is under control of a Wnt signalling gradient between future forebrain and spinal cord. This gradient is detected (1) by the pattern with which marker gene expression reacts after injection of Wnt antagonists and XWnt8 (Fig. 3), (2) by the graded activation of a Wnt-responsive reporter (Fig. 7), and (3) by the graded nuclear accumulation of β-catenin (Fig. 8). The orientation of the gradient, with peak levels in the posterior and lowest levels in the anterior, is consistent with the predicted properties of a transformer gradient proposed by Nieuwkoop and colleagues. In the late gastrula, the gradient appears to specify rather crude AP levels, because *Gbx2* and *HoxD1* are co-repressed at high XDkk1 doses. This crude pattern is refined at neurula stage when two Wnt thresholds for *Krox20* expression in rhombomeres 3 and 5 can be detected. A similar refinement of an initially crude response to a morphogen, which gives rise to distinct thresholds at later stages, has been described for Activin (Green et al., 1994; Wilson and Melton, 1994) and may depend on secondary cell-cell communication.

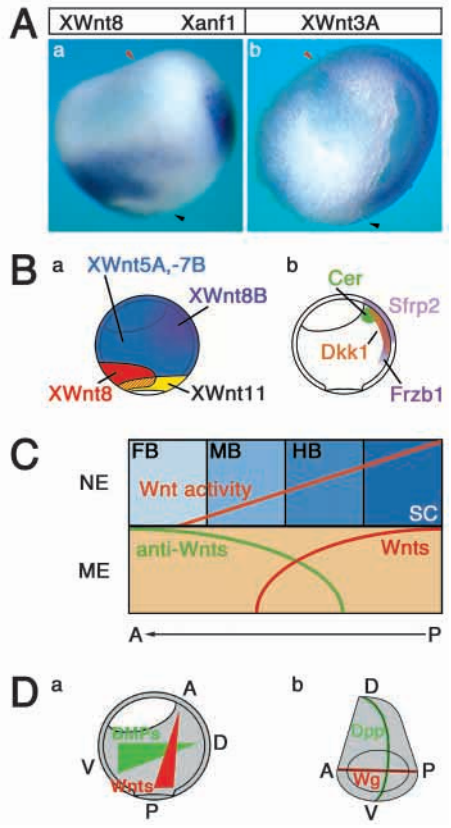


**Fig. 9.** Expression of various Wnts and Wnt antagonists establishes a Wnt activity gradient in the gastrulating *Xenopus* embryo. (A) Expression of *Xanf1*, *Xwnt8* (a) in whole-mount and *Xwnt3A* (b) in sagittal section at late gastrula stage by in situ hybridization. Black arrowheads, dorsal blastopore lip; red arrowheads, leading edge. Note expression of *Xwnt8* in proximity to presumptive posterior neuroectoderm (compare with fate map in Fig. 8A). *Xwnt3A* is expressed in chordamesoderm and in a ring surrounding the blastopore (not visible). (B) Expression domains in the late *Xenopus* gastrula of Wnts (a) and Wnt antagonists (b). Only ectodermal expressions are shown in a. The circumblastoporal expression of *Xwnt3A* is not depicted for simplicity. Note expression of Wnt antagonists in the anterior of the gastrulating embryo. (C) Simplified model for AP patterning of neuroectoderm by a Wnt activity gradient in *Xenopus*. Wnts (red) and Wnt antagonists (green) are expressed in the mesendoderm (ME, ochre) underlying the neuroectoderm (NE, blue). The expression of Wnts and Wnt antagonist in the neuroectoderm is not shown for simplicity (see B). Their combined activities result in the formation of a Wnt signalling gradient (dark orange) that patterns the AP neuraxis. The AP axis is indicated (A→P). Formation of the posterior nervous system also requires other factors (FGFs, RA) which are not shown here. (D) The AP and DV axes in the gastrulating *Xenopus* embryo are patterned by gradients of Wnts and BMPs, respectively (a). The *Drosophila* wing imaginal disc is patterned by gradients of Wingless (Wg) and Decapentaplegic (Dpp), which are secreted along the DV and AP compartment boundaries, respectively (b).

### Competence factors restrict AP patterning by Wnt signalling

Although the titrations with Wnt antagonists (Figs 4, 5) reveal a dose-dependent requirement for Wnt signalling, the gradual expansion of more anterior markers such as *Bf1* and *Otx2* is in contrast to the thresholds observed in dissociated animal cap cells responding to different doses of XWnt8 protein (Fig. 1). For example, in the in vitro experiments, populations of cells expressing only *Bf1* and *Otx2* or only *Krox20* are observed at different Wnt doses. Extrapolating from the in vitro results one might expect that in vivo the entire neural plate expresses *Krox20* when Wnts, or *Bf1* when Wnt antagonists are overexpressed. However, such a complete respecification of the neural plate is never observed in vivo, rather the changes in marker gene expression respect specific boundaries. This is most obvious for *Bf1* in Fig. 4, whose anterior stripe of expression only becomes broader but not significantly thicker with increasing doses of Wnt inhibitors. Similarly, after implantation of *Xdkk1*-expressing cells as in Fig. 5C (panel a), ectopic *Bf1* expression is never seen to extend into the midline but straddles the dorsal neural plate posteriorly. This suggests that factors in addition to Wnts restrict the competence of cells to express anterior markers. Obvious candidates for such modifiers are RA, Sonic Hedgehog, Nodals, BMPs and FGFs, which have been implicated in neural patterning (Lumsden and Krumlauf, 1996; Pownall et al., 1996; Sasai and De Robertis, 1997; Chang and Hemmati-Brivanlou, 1998a; Isaacs et al., 1998; Pownall et al., 1998; Gamse and Sive, 2000; Ribisi et al., 2000; Wilson and Rubenstein, 2000). To test if the inhibition of these factors abolishes this restriction of competence we implanted cells co-expressing *Xdkk1* and either mouse *Hip1*, *cerberus-short*, *noggin* or *FGF receptor-1* and *FGF receptor-4a* ectodomains into the neural plate, which encode inhibitors of Hedgehog, Nodal, BMP and FGF signalling, respectively (Zimmerman et al., 1996; Ye et al., 1998; Chuang and McMahon, 1999; Piccolo et al., 1999; Streit et al., 2000). None of these growth factor antagonists in conjunction with XDkk1 was able to expand *Bf1* expression into the entire neural plate (not shown). Similarly, inhibition of RA signalling by injecting the host embryos with *XCYP26* mRNA, encoding a RA degrading enzyme (Hollemann et al., 1998a), did not allow *Xdkk1*-expressing grafts to expand *Bf1* expression further, despite a posterior shift of hindbrain markers induced by *XCYP26* (not shown). The restriction of competence for anterior markers to become expressed posteriorly may occur via factors other than those tested. Alternatively, this/these factor(s) may be active before early gastrula, the stage of grafting. An ectodermal prepattern regulating the expression of *Otx2* has also been identified in the zebrafish central nervous system. This initial AP prepattern depends on a differential competence of the epiblast and is not imposed by organizer-derived signals (Koshida et al., 1998). We observed the same phenomenon in our experiments, where *Otx2* expression never expanded into the ventroposterior ectoderm even at high XDkk1 dose (Fig. 3A, d'').

Clearly then, a morphogen gradient of Wnt/β-catenin signalling is not sufficient to account for all AP patterning of the neural plate and indeed other signalling molecules, such as RA (Ruiz i Altaba, 1993; Kolm and Sive, 1997) and FGFs (Slack and Isaacs, 1994; Cox and Hemmati-Brivanlou, 1995; Doniach, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Holowacz and Sokol, 1999; Ribisi et al., 2000) have been implicated in posteriorization. FGFs are potent posteriorizing agents and FGF8 mediates effects of the isthmic organizer (Pownall et al., 1996; Isaacs et al., 1998; Pownall et al., 1998; Rhinn and Brand, 2001). However, the posteriorizing effects of FGFs can be reversed with Wnt antagonists, suggesting that they may be mediated through Wnts at least partially (McGrew et al., 1997; Kazanskaya et al., 2000). A direct requirement for RA signalling during early neural patterning has been demonstrated in the hindbrain but not in anterior head structures (Blumberg et al., 1997; Kolm et al., 1997; Hollemann et al., 1998a; Gavalas and Krumlauf, 2000; Niederreither et al., 2000; Dupé and Lumsden, 2001). Thus, hindbrain and spinal cord patterning are regulated by FGF, RA and Wnt signalling.

### An endogenous AP gradient of Wnt/β-catenin signalling in the presumptive neural plate

A major finding of our study is the discovery of a Wnt/β-catenin signalling gradient across the presumptive gastrula neural plate. How is this gradient established? Several Wnts are expressed in the *Xenopus* gastrula, including *Xwnt3A*, *Xwnt5A*, *Xwnt7B*, *Xwnt8*, *Xwnt8B* and *Xwnt11* (Fig. 9B, a). *Xwnt3A* and *Xwnt8* are expressed in chordamesoderm and lateroventral mesoderm, respectively (Fig. 9A) (Christian and Moon, 1993; McGrew et al., 1997; Bang et al., 1999), both of which harbour posteriorizing activity in *Xenopus*, zebrafish and chick (Hemmati-Brivanlou et al., 1990; Doniach and Musci, 1995; Bang et al., 1997; Muhr et al., 1997; Woo and Fraser, 1997). As we show that Wnts are able to signal at long range, XWnt3A and XWnt8 may diffuse from these sources to set up a concentration gradient. *Xwnt5A* and *Xwnt11*, although expressed in the gastrula, are unlikely to be posteriorizing because they do not appear to signal via β-catenin (Ku and Melton, 1993; Moon et al., 1993; Sokol, 2000). *Xwnt7B* and *Xwnt8B* are both expressed maternally and may contribute to posteriorization (Cui et al., 1995; Chang and Hemmati-Brivanlou, 1998b). Thus, the signalling gradient probably involves multiple Wnts. A Wnt signalling-free zone is maintained by Wnt antagonists secreted within the anterior neuroectoderm and from adjacent anterior mesendoderm, including Cerberus, Dkk1, Frzb1, Crescent/Frzb2 and Sfrp2 (Bouwmeester et al., 1996; Leyns et al., 1997; Glinka et al., 1998; Piccolo et al., 1999; Bradley et al., 2000; Pera and De Robertis, 2000; Fig. 9B, b). As these factors are potentially diffusible, they may also contribute to the establishment of a Wnt activity gradient. In conclusion, we suggest that the *Xenopus* AP neuraxis is patterned by a Wnt activity gradient formed in the presumptive neuroectoderm of the *Xenopus* gastrula by the interplay between posteriorizing Wnts and Wnt antagonists expressed in the anterior (Fig. 9C).

That multiple Wnts are involved in setting up the gradient is also suggested by the response of marker genes to different Wnt antagonists. Unlike XDkk1 and NXFz8, XFrzb1 failed to completely repress *Krox20* expression. In addition, the expansion of *Bf1* expression elicited by XDkk1 and XFrzb1 was reproducibly of different shapes, horseshoe versus semicircular, respectively (Fig. 4A). These differences probably reflect different specificities of the Wnt antagonists. For example, XFrzb1 is unable to inhibit signalling by

XWnt3A, unlike XDkk1 (Wang et al., 1997; Krupnik et al., 1999; Kazanskaya et al., 2000).

The formation of the Wnt/β-catenin signalling gradient resembles formation of the BMP morphogen gradient involved in DV patterning. *BMP2*, *BMP4*, *BMP7* and *BMP7R* are expressed in overlapping domains in the gastrula and an activity gradient is formed by the diffusion of multiple BMP antagonists secreted from Spemann's organizer (Harland and Gerhart, 1997; Barth et al., 1999; Dale and Wardle, 1999). Thus, the BMP and Wnt morphogen gradients regulate patterning in the *Xenopus* gastrula, providing DV and AP positional information, respectively (Fig. 9D, a). Interestingly, a system of orthogonal Decapentaplegic and Wingless morphogen gradients regulate AP and DV patterning of the *Drosophila* wing imaginal disc, respectively (Fig. 9D, b) (Strigini and Cohen, 1999). This raises the possibility that orthogonal BMP-Wnt gradients represent an evolutionarily conserved module that specifies bilateral symmetry.

We are indebted to Drs J. Nathans and H. Steinbeisser for generous gifts of XWnt8-expressing insect cells and β-catenin antibody, respectively, and for advice. Drs H. Clevers, M. Eilers, J. Gurdon, R. Harland, D. Kimelmann, P. Klein, J. Nathans and R. Moon kindly provided reagents. We thank C. Rumig and P. Steinmetz for support with in situ hybridization and RT-PCR, and C. Walter for skillful assistance in insect cell culture. We are grateful to Drs G. Davidson, O. Kazanskaya and J. Wittbrodt for critical reading of the manuscript. C. N. was supported by the DFG.

## REFERENCES

**Baker, J. C., Beddington, R. S. and Harland, R. M.** (1999). Wnt signaling in *Xenopus* embryos inhibits *bmp4* expression and activates neural development. *Genes Dev.* **13**, 3149-3159.

**Bang, A. G., Papalopulu, N., Kintner, C. and Goulding, M. D.** (1997). Expression of *Pax-3* is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm. *Development* **124**, 2075-2085.

**Bang, A. G., Papalopulu, N., Goulding, M. D. and Kintner, C.** (1999). Expression of *Pax-3* in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. *Dev. Biol.* **212**, 366-380.

**Barth, A. I., Pollack, A. L., Altschuler, Y., Mostov, K. E. and Nelson, W. J.** (1997). NH2-terminal deletion of β-catenin results in stable colocalization of mutant β-catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J. Cell Biol.* **136**, 693-706.

**Barth, K. A., Kishimoto, Y., Rohr, K. B., Seydler, C., Schulte-Merker, S. and Wilson, S. W.** (1999). Bmp activity establishes a gradient of positional information throughout the entire neural plate. *Development* **126**, 4977-4987.

**Beddington, R. S. P. and Robertson, E. J.** (1999). Axis development and early asymmetry in mammals. *Cell* **96**, 195-209.

**Bhanot, P., Brink, M., Samos, C. H., Hsieh, J.-C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R.** (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* **382**, 225-230.

**Blitz, I. L. and Cho, K. W. Y.** (1995). Anterior neuroectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development* **121**, 993-1004.

**Blumberg, B., Bolado, J. J., Moreno, T. A., Kintner, C., Evans, R. M. and Papalopulu, N.** (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

**Bourguignon, C., Li, J. and Papalopulu, N.** (1998). XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in *Xenopus* competent ectoderm. *Development* **125**, 4889-4900.

**Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and De Robertis, E. M.** (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

**Bradley, L. C., Snape, A., Bhatt, S. and Wilkinson, D. G.** (1993). The structure and expression of the *Xenopus Krox-20* gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech. Dev.* **40**, 73-84.

**Bradley, L., Sun, B., Collins-Racie, L., LaVallie, E., McCoy, J. and Sive, H.** (2000). Different activities of the frizzled-related proteins frzb2 and sizzled2 during *Xenopus* anteroposterior patterning. *Dev. Biol.* **227**, 118-132.

**Brannon, M. and Kimelman, D.** (1996). Activation of Siamois by the Wnt pathway. *Dev. Biol.* **180**, 344-347.

**Carnac, G., Kodjachbachian, L., Gurdon, J. B. and Lemaire, P.** (1996). The homeobox gene *siamois* is a target of the wnt dorsalization pathway and triggers organiser activity in the absence of mesoderm. *Development* **122**, 3055-3065.

**Chang, C. and Hemmati-Brivanlou, A.** (1998a). Cell fate determination in embryonic ectoderm. *J. Neurobiol.* **36**, 128-151.

**Chang, C. and Hemmati-Brivanlou, A.** (1998b). Neural crest induction by Xwnt7B in *Xenopus*. *Dev. Biol.* **194**, 129-134.

**Christian, J. L. and Moon, R. T.** (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev.* **7**, 13-28.

**Christian, J. L., Gavin, B. J., McMahon, A. P. and Moon, R. T.** (1991). Isolation of cDNAs partially encoding four *Xenopus* Wnt-1/int-1-related proteins and characterization of their transient expression during embryonic development. *Dev. Biol.* **143**, 230-234.

**Chuang, P. T. and McMahon, A. P.** (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621.

**Cox, W. G. and Hemmati-Brivanlou, B. A.** (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121**, 4349-4358.

**Cui, Y., Brown, J. D., Moon, R. T. and Christian, J. L.** (1995). *Xwnt-8b*: a maternally expressed *Xenopus Wnt* gene with a potential role in establishing the dorsoventral axis. *Development* **121**, 2177-2186.

**Dale, L. and Wardle, F. C.** (1999). A gradient of BMP activity specifies dorsal-ventral fates in early *Xenopus* embryos. *Semin. Cell Dev. Biol.* **10**, 319-326.

**Darken, R. S. and Wilson, P. A.** (2001). Axis induction by Wnt signaling: Target promoter responsiveness regulates competence. *Dev. Biol.* **234**, 42-54.

**De Robertis, E. M., Larrain, J., Oelgeschläger, M. and Wessely, O.** (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat. Rev. Genet.* **1**, 171-181.

**Deardorff, M. A., Tan, C., Conrad, L. J. and Klein, P. S.** (1998). *Frizzled-8* is expressed in the Spemann organizer and plays a role in early morphogenesis. *Development* **125**, 2687-2700.

**Doniach, T.** (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell* **83**, 1067-1070.

**Doniach, T. and Musci, T. J.** (1995). Induction of anteroposterior neural pattern in *Xenopus*: evidence for a quantitative mechanism. *Mech. Dev.* **53**, 403-413.

**Dupé, V. and Lumsden, A.** (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.

**Durston, A. J., Timmermans, J. P., Hage, W. J., Hendriks, H. F., de Vries, N. J., Heideveld, M. and Nieuwkoop, P. D.** (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340**, 140-144.

**Fekany-Lee, K., Gonzalez, E., Miller-Bertoglio, V. and Solnica-Krezel, L.** (2000). The homeobox gene *bozozok* promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. *Development* **127**, 2333-2345.

**Fredieu, J. R., Cui, Y., Maier, D., Danilchik, M. V. and Christian, J. L.** (1997). Xwnt-8 and lithium can act upon either dorsal mesodermal or neuroectodermal cells to cause a loss of forebrain in *Xenopus* embryos. *Dev. Biol.* **186**, 100-114.

**Galceran, J., Farinas, I., Depew, M. J., Clevers, H. and Grosschedl, R.** (1999). *Wnt3a*$^{-/-}$like phenotype and limb deficiency in *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice. *Genes Dev.* **13**, 709-717.

**Gamse, J. and Sive, H.** (2000). Vertebrate anteroposterior patterning: the *Xenopus* neurectoderm as a paradigm. *BioEssays* **22**, 976-986.

**Gamse, J. T. and Sive, H.** (2001). Early anteroposterior division of the presumptive neurectoderm in *Xenopus*. *Mech. Dev.* **104**, 21-36.

**Gavalas, A. and Krumlauf, R.** (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.

**Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C.**

(1995). Antagonizing the Spemann organizer: role of the homeobox gene *Xvent-1*. *EMBO J.* **14**, 6268-6279.

**Gilbert, S. F. and Saxén, L.** (1993). Spemann's organizer: models and molecules. *Mech. Dev.* **41**, 73-89.

**Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C. and Niehrs, C.** (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in *Xenopus*. *Nature* **389**, 517-519.

**Glinka, A., Wu, W., Delius, H., Monaghan, P. A., Blumenstock, C. and Niehrs, C.** (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

**Gould, S. E. and Grainger, R. M.** (1997). Neural induction and anteroposterior patterning in the amphibian embryo: past, present and future. *Cell. Mol. Life Sci.* **53**, 319-338.

**Green, J. B. and Smith, J. C.** (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347**, 391-394.

**Green, J. B. A., Smith, J. C. and Gerhard, J. C.** (1994). Slow emergence of a multithreshold response to activin requires cell-contact-dependent sharpening but not prepattern. *Development* **120**, 2271-2278.

**Gurdon, J. B., Harger, P., Mitchell, A. and Lemaire, P.** (1994). Activin signalling and response to a morphogen gradient. *Nature* **371**, 487-492.

**Hamilton, F. S., Wheeler, G. N. and Hoppler, S.** (2001). Difference in XTcf-3 dependency accounts for change in response to β-catenin-mediated Wnt signalling in *Xenopus* blastula. *Development* **128**, 2063-2073.

**Harland, R.** (2000). Neural induction. *Curr. Opin. Genet. Dev.* **10**, 357-362.

**Harland, R. M. and Gerhart, J.** (1997). Formation and function of Spemann's organizer. *Annu. Rev. Dev. Biol.* **13**, 611-667.

**Hashimoto, H., Itoh, M., Yamanaka, Y., Yamashita, S., Shimizu, T., Solnica-Krezel, L., Hibi, M. and Hirano, T.** (2000). Zebrafish Dkk1 Functions in Forebrain Specification and Axial Mesendoderm Formation. *Dev. Biol.* **217**, 138-152.

**Heisenberg, C. P., Houart, C., Take-Uchi, M., Rauch, G. J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M. L., Geisler, R., Dale, T. C., Wilson, S. W. and Stemple, D. L.** (2001). A mutation in the Gsk3-binding domain of zebrafish *Masterblind*/*Axin1* leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev.* **15**, 1427-1434.

**Hemmati-Brivanlou, A. and Harland, R. M.** (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

**Hemmati-Brivanlou, A., Stewart, R. M. and Harland, R. M.** (1990). Region-specific neural induction of an engrailed protein by anterior notochord in *Xenopus*. *Science* **250**, 800-802.

**Hemmati-Brivanlou, A., de la Torre, J. R., Holt, C. and Harland, R. M.** (1991). Cephalic expression and molecular characterization of *Xenopus* En-2. *Development* **111**, 715-724.

**Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A.** (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

**Hollemann, T., Chen, Y., Grunz, H. and Pieler, T.** (1998a). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17**, 7361-7372.

**Hollemann, T., Panitz, F. and Pieler, T.** (1998b). In situ hybridization techniques with *Xenopus* embryos. In *Comparative Methods Approach to the Study of Oocytes and Embryos* (ed. J. D. Richter), pp. 279-290. Oxford: Oxford University Press.

**Holowacz, T. and Sokol, S.** (1999). FGF is required for posterior neural patterning but not for neural induction. *Dev. Biol.* **205**, 296-308.

**Hoppler, S., Brown, J. D. and Moon, R. T.** (1996). Expression of a dominant-negative *wnt* blocks induction of MyoD in *Xenopus* embryos. *Genes Dev.* **10**, 2805-2817.

**Hopwood, N. D., Pluck, A. and Gurdon, J. B.** (1989). *MyoD* expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. *EMBO J.* **8**, 3409-3417.

**Hsieh, J. C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B. and Nathans, J.** (1999a). A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* **398**, 431-436.

**Hsieh, J. C., Rattner, A., Smallwood, P. M. and Nathans, J.** (1999b). Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. *Proc. Natl. Acad. Sci. USA* **96**, 3546-3551.

**Isaacs, H. V., Pownall, M. E. and Slack, J. M.** (1998). Regulation of *Hox* gene expression and posterior development by the *Xenopus caudal* homologue *Xcad3*. *EMBO J.* **17**, 3413-3427.

**Itoh, K. and Sokol, S. Y.** (1997). Graded amounts of *Xenopus* dishevelled specify discrete anteroposterior cell fates in prospective ectoderm. *Mech. Dev.* **61**, 113-125.

**Itoh, K., Tang, T. L., Neel, B. G. and Sokol, S. Y.** (1995). Specific modulation of ectodermal cell fates in *Xenopus* embryos by glycogen synthase kinase. *Development* **121**, 3979-3988.

**Kazanskaya, O., Glinka, A. and Niehrs, C.** (2000). The role of *Xenopus dickkopf1* in prechordal plate specification and neural patterning. *Development* **127**, 4981-4992.

**Kengaku, M. and Okamoto, H.** (1995). bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in *Xenopus*. *Development* **121**, 3121-3130.

**Kiecker, C. and Niehrs, C.** (2001). The role of prechordal mesendoderm in neural patterning. *Curr. Opin. Neurobiol.* **11**, 27-33.

**Kim, C.-H., Oda, T., Itoh, M., Jiang, D. I., Artinger, K., Chandrasekharappa, S. C., Driever, W. and Chitnis, A. B.** (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**, 916-920.

**Kintner, C. R. and Melton, D. A.** (1987). Expression of *Xenopus N-CAM* RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

**Kolm, P. J. and Sive, H. L.** (1997). Retinoids and posterior neural induction: a reevaluation of Nieuwkoop's two-step hypothesis. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 511-521.

**Kolm, P. J., Apekin, V. and Sive, H.** (1997). *Xenopus* hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

**Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W. and Vogelstein, B.** (1997). Constitutive transcriptional activation by a β-catenin-Tcf complexes in APC/colon carcinoma. *Science* **275**, 1784-1787.

**Koshida, S., Shinya, M., Mizuno, T., Kuroiwa, A. and Takeda, H.** (1998). Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast. *Development* **125**, 1957-1966.

**Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K., Chickering, T. W., Amaravadi, L., Brown, D. E., Guyot, D., Mays, G., Leiby, K. et al.** (1999). Functional and structural diversity of the human *Dickkopf* gene family. *Gene* **238**, 301-313.

**Ku, M. and Melton, D. A.** (1993). *Xwnt-11*: a maternally expressed *Xenopus wnt* gene. *Development* **119**, 1161-1173.

**Lamb, T. M. and Harland, R. M.** (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-3636.

**Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S. and De Robertis, E. M.** (1997). Frzb-1 is a secreted antagonist of wnt-signals expressed in the Spemann organizer. *Cell* **88**, 747-756.

**Lumsden, A. and Krumlauf, R.** (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

**McGrew, L. L., Lai, C. J. and Moon, R. T.** (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* **172**, 337-342.

**McGrew, L. L., Hoppler, S. and Moon, R. T.** (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus*. *Mech. Dev.* **69**, 105-114.

**McGrew, L. L., Takemaru, K., Bates, R. and Moon, R. T.** (1999). Direct regulation of the *Xenopus engrailed-2* promoter by the Wnt signaling pathway, and a molecular screen for Wnt-responsive genes, confirm a role for Wnt signaling during neural patterning in *Xenopus*. *Mech. Dev.* **87**, 21-32.

**McKendry, R., Hsu, S. C., Harland, R. M. and Grosschedl, R.** (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the *Xenopus nodal-related 3* promoter. *Dev. Biol.* **192**, 420-431.

**Miller, J. R. and Moon, R. T.** (1997). Analysis of the signaling activities of localization mutants of β-catenin during axis specification in *Xenopus*. *J. Cell Biol.* **139**, 229-243.

**Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. and Fraser, S.** (1993). Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. *Development* **119**, 97-111.

**Moon, R. T., Brown, J. D. and Torres, M.** (1997). Wnts modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**, 157-162.

**Muhr, J., Jessell, T. M. and Edlund, T.** (1997). Assignment of early caudal identity to neural plate cells by a signal from caudal paraxial mesoderm. *Neuron* **19**, 487-502.

**Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dollé, P.** (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.

**Niehrs, C.** (1999). Head in the Wnt – the molecular nature of Spemann's head organizer. *Trends Genet.* **15**, 314-319.

**Nieuwkoop, P. D.** (1997). Short historical survey of pattern formation in the endo-mesoderm and the neural anlage in the vertebrates: the role of vertical and planar inductive actions. *Cell. Mol. Life Sci.* **53**, 305-318.

**Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E.** (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**, 707-720.

**Patapoutian, A. and Reichardt, L. F.** (2000). Roles of Wnt proteins in neural development and maintenance. *Curr. Opin. Neurobiol.* **10**, 392-399.

**Pera, E. M. and De Robertis, E. M.** (2000). A direct screen for secreted proteins in *Xenopus* embryos identifies distinct activities for the Wnt antagonists Crescent and Frzb-1. *Mech. Dev.* **96**, 183-195.

**Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M.** (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.

**Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C.** (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538.

**Pöpperl, H., Schmidt, C., Wilson, V., Hume, C. R., Dodd, J., Krumlauf, R. and Beddington, R. S. P.** (1997). Misexpression of *Cwnt8c* in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm. *Development* **124**, 2997-3005.

**Pownall, M. E., Tucker, A. S., Slack, J. M. and Isaacs, H. V.** (1996). eFGF, *Xcad3* and *Hox* genes form a molecular pathway that establishes the anteroposterior axis in *Xenopus*. *Development* **122**, 3881-3892.

**Pownall, M. E., Isaacs, H. V. and Slack, J. M.** (1998). Two phases of *Hox* gene regulation during early *Xenopus* development. *Curr. Biol.* **8**, 673-676.

**Rhinn, M. and Brand, M.** (2001). The midbrain-hindbrain boundary organizer. *Curr. Opin. Neurobiol.* **11**, 34-42.

**Ribisi, S., Mariani, F. V., Aamar, E., Lamb, T. M., Frank, D. and Harland, R. M.** (2000). Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in *Xenopus laevis*. *Dev. Biol.* **227**, 183-196.

**Rowning, B. A., Wells, J., Wu, M., Gerhart, J. C., Moon, R. T. and Larabell, C. A.** (1997). Microtubule-mediated transport of organelles and localization of β-catenin to the future dorsal side of *Xenopus* eggs. *Proc. Natl. Acad. Sci. USA* **94**, 1224-1229.

**Ruiz i Altaba, A.** (1993). Induction and axial patterning of the neural plate: planar and vertical signals. *J. Neurobiol.* **24**, 1276-1304.

**Saha, M. S. and Grainger, R. M.** (1992). A labile period in the determination of the anterior-posterior axis during early neural development in *Xenopus*. *Neuron* **8**, 1003-1014.

**Saint-Jeannet, J.-P., He, X., Varmus, H. and Dawid, I. B.** (1997). Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc. Natl. Acad. Sci. USA* **94**, 13713-13718.

**Sambrook, J., Fritsch, E. F. and Maniatis, T.** (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

**Sasai, Y. and De Robertis, E. M.** (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5-20.

**Schneider, S., Steinbeisser, H., Warga, R. M. and Hausen, P.** (1996). β-catenin translocation into nuclei demracates the dorsalizing centers in frog and fish embryos. *Mech. Dev.* **57**, 191-198.

**Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M. and Takada, S.** (1998). Cytoskeletal reorganization by soluble Wnt-3a protein signalling. *Genes Cells* **3**, 659-670.

**Shinya, M., Eschbach, C., Clark, M., Lehrach, H. and Furutani-Seiki, M.** (2000). Zebrafish Dkk1, induced by the pre-MBT Wnt signaling, is secreted from the prechordal plate and patterns the anterior neural plate. *Mech. Dev.* **98**, 3-17.

**Slack, J. M.** (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol.* **4**, 116-126.

**Slack, J. M. and Isaacs, H. V.** (1994). The role of fibroblast growth factors in early *Xenopus* development. *Biochem. Soc. Trans.* **22**, 585-589.

**Smith, W. C. and Harland, R. M.** (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.

**Smith, W. C., McKendry, R., Ribisi, S. J. and Harland, R. M.** (1995). A *nodal-related* gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

**Sokol, S.** (2000). A role for Wnts in morpho-genesis and tissue polarity. *Nat. Cell Biol.* **2**, E124-E125.

**Stern, C. D.** (2001). Initial patterning of the central nervous system: How many organizers? *Nat. Rev. Neurosci.* **2**, 92-98.

**Streit, A., Berliner, A. J., Papanayotou, C., Sirulnik, A. and Stern, C. D.** (2000). Initiation of neural induction by FGF signalling before gastrulation. *Nature* **406**, 74-78.

**Strigini, M. and Cohen, S. M.** (1999). Formation of morphogen gradients in the Drosophila wing. *Semin. Cell Dev. Biol.* **10**, 335-344.

**Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A. and McMahon, A. P.** (1994). *Wnt-3a* regulates somite and tailbud formation in the mouse embryo. *Genes Dev.* **8**, 174-189.

**von Bubnoff, A., Schmidt, J. E. and Kimelman, D.** (1996). The *Xenopus laevis* homeobox gene *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm. *Mech. Dev.* **54**, 149-160.

**Wang, S., Krinks, M. and Moos, M. J.** (1997). Frzb-1, an antagonist of Wnt-1 and Wnt-8, does not block signaling by Wnts3A, -5A, or -11. *Biochem. Biophys. Res. Commun.* **236**, 502-504.

**Wilson, P. A. and Melton, D. A.** (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr. Biol.* **4**, 676-686.

**Wilson, S. I., Graziano, E., Harland, R., Jessell, T. M. and Edlund, T.** (2000). An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. *Curr. Biol.* **10**, 421-429.

**Wilson, S. W. and Rubenstein, J. L.** (2000). Induction and dorsoventral patterning of the telencephalon. *Neuron* **28**, 641-651.

**Wilson, S., Rydstrom, A., Trimborn, T., Willert, K., Nusse, R., Jessell, T. M. and Edlund, T.** (2001). The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. *Nature* **411**, 325-330.

**Wodarz, A. and Nusse, R.** (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

**Woo, K. and Fraser, S. E.** (1997). Specification of the zebrafish nervous system by nonaxial signals. *Science* **277**, 254-257.

**Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A.** (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.

**Zaraisky, A. G., Lukyanov, S. A., Vasiliev, O. L., Smirnov, Y. V., Belyavsky, A. V. and Kazanskaya, O. V.** (1992). A novel homeobox gene expressed in the anterior neural plate of the *Xenopus* embryo. *Dev. Biol.* **152**, 373-382.

**Zimmerman, L. B., De Jesús-Escobar, J.-E. and Harland, R. M.** (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein-4. *Cell* **86**, 599-606.