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Neural Induction in the Absence of Mesoderm: β-Catenin-Dependent Expression of Secreted BMP Antagonists at the Blastula Stage in *Xenopus*

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A growing body of work indicates that neural induction may be initiated prior to the establishment of the gastrula mesodermal organizer. Here, we examine neural induction in *Xenopus* embryos in which mesoderm induction has been blocked by Cerberus-short, a reagent that specifically inhibits Nodal-related (Xnr) signals. We find that extensive neural structures with cyclopic eyes and brain tissue are formed despite the absence of mesoderm. This neural induction correlates with the expression of *chordin* and other BMP inhibitors—such as *noggin, follistatin,* and *Xnr3*—at the blastula stage, and requires β-Catenin signaling. Activation of the β-Catenin pathway by mRNA microinjections or by treatment with LiCl leads to differentiation of neurons, as well as neural crest, in ectodermal explants. Xnr signals are required for the maintenance, but not for the initiation, of BMP antagonist expression. Recent work has demonstrated a role for β-Catenin signaling in neural induction mediated by the transcriptional down-regulation of *BMP-4* expression. The present results suggest an additional function for β-Catenin, the early activation of expression of secreted BMP antagonists, such as Chordin, in a preorganizer region in the dorsal side of the *Xenopus* blastula.

***Key Words: Xenopus laevis*; neural induction; Spemann's organizer; β-Catenin; Chordin; Noggin; Follistatin; Xnr3; lefty; nodal-related.**

INTRODUCTION

Neural induction in early vertebrate development is a topic of considerable interest (Harland, 2000). Spemann and Mangold (1924) provided the initial insight showing that transplantation of dorsal lip mesoderm of the gastrulating amphibian embryo would induce an ectopic secondary axis that included a central nervous system (CNS). This led to the view that neural inducers emanate from dorsal mesoderm, a region also called Spemann's organizer. The molecular dissection of Spemann's organizer has led to the identification of multiple novel secreted proteins. Many of these were found to be antagonists that bind to growth factors in the extracellular space (reviewed by Harland and Gerhart, 1997; De Robertis *et al.*, 2000). Molecules such as Chordin, Noggin, and Cerberus bind bone morphogenetic proteins (BMPs) and prevent them from binding to their cognate receptors (Piccolo *et al.*, 1996, 1999; Zimmerman *et al.*, 1996). In the case of Follistatin/BMP complexes, receptor binding takes place but activation is inhibited (Iemura *et al.*, 1998). Xnr3 is a member of the TGF-β superfamily that also functions as an antagonist of BMP signaling, perhaps acting as a competitive inhibitor of BMP receptors (Smith *et al.*, 1995; Hansen *et al.*, 1997; Harland, 2000). The expression of this cocktail of BMP antagonists in the organizer has led to the view that neural induction by gastrula organizer grafts is in part mediated by inhibition of BMP signaling in the extracellular space (Harland and Gerhart, 1997; Sasai and De Robertis, 1997).

Evidence is also accumulating that neural tissue might be specified without an absolute requirement for the gastrula organizer. For example, in the mouse, HNF3-β mutants lack a morphological node and node derivatives, but still develop a neural plate (Klingensmith *et al.*, 1999). In ze-

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brafish, microsurgical deletion of the embryonic shield (gastrula organizer) has little effect on the development of the neural plate (Shih and Fraser, 1996; Saude *et al.*, 2000). In *Xenopus*, there is evidence that a predisposition for neural induction already exists on the dorsal side of the ectoderm prior to its interaction with the gastrula organizer (Sharpe *et al.*, 1987; London *et al.*, 1988). Therefore, the relationship between the undisputed neural-inducing activity emanating from the organizer at gastrula stage and the function of earlier signals in the formation of the neural plate is an area of intense interest (Harland, 2000).

An important advance has been the realization that the regulation of *BMP* expression at the transcriptional level plays an instrumental role in neural patterning. Activation of the β-catenin signaling pathway inhibits *BMP-4* transcription in *Xenopus* ectodermal explants at gastrula and results in the induction of neural markers (Baker *et al.*, 1999). Microinjection of an activated form of *β-catenin* into the ectoderm of developing embryos greatly expands the neural plate, whereas a dominant-repressive form of the β-Catenin cofactor XTcf-3 (ΔN-XTcf-3) reduces the neural plate (Baker *et al.*, 1999). In *Drosophila*, dTCF is known to regulate transcription of the BMP homologue *dpp* in the mesoderm (Yang *et al.*, 2000). In mouse, mutation of *β-catenin* results in embryos with severe anteroposterior defects that do not express the forebrain markers *Hesx1* and *Otx2* in the neuroectoderm (Huelsken *et al.*, 2000). In zebrafish, a mutation in Tcf3 (named *headless*) leads to the loss of forebrain and midbrain structures (Kim *et al.*, 2000). Zebrafish genetics also supports a function for transcriptional regulation of BMP expression in neurogenesis. The early homeobox gene *bozozok*, which shares sequence similarities with the organizer gene *goosecoid*, is activated by the β-Catenin pathway (Fekany *et al.*, 1999). In *bozozok* mutants, *BMP-2b* transcription is not repressed on the dorsal side of the embryo, leading to a moderate reduction of the CNS (Koos and Ho, 1999; Fekany-Lee *et al.*, 2000). In chick, transcriptional down-regulation of BMP expression appears to be mediated by a different signaling pathway. FGF-3 and -8 have been implicated in neural induction and are thought to act—at least partially—by inhibiting transcription of *BMP-4* and *-7* (Wilson *et al.*, 2000; Streit *et al.*, 2000; Harland, 2000).

Treatment of *Xenopus* embryos with LiCl leads to a dorsalized phenotype with greatly enhanced forebrain structures (Kao and Elinson, 1988). LiCl inhibits the activity of Glycogen Synthase Kinase-3β (GSK-3β), preventing the degradation of β-Catenin protein (Klein and Melton, 1996; Schneider *et al.*, 1996). The opposite effect, ventralization, is achieved by irradiation of *Xenopus* eggs with ultraviolet (UV) light. These ventralized embryos develop all three germ layers, but do not form a CNS, dorsal mesoderm, or Spemann's organizer (Harland and Gerhart, 1997; De Robertis *et al.*, 2000). UV treatment causes depolymerization of microtubule tracks required for the transport of dorsal determinant vesicles to the dorsal side of the embryo (Rowning *et al.*, 1997) and prevents accumulation of β-Catenin protein in cell nuclei of the future dorsal side of the embryo (Scharf and Gerhart, 1980; Schneider *et al.*, 1996; Larabell *et al.*, 1997). An intriguing aspect of the UV experiment is that dorsal development, including a complete CNS, can be restored by microinjection of a surprising variety of gene products, including members of the β-Catenin signaling pathway, Nodal-related proteins, and secreted BMP antagonists. This has led to the proposal that these diverse molecular players may be involved in a common dorsal specification pathway (De Robertis *et al.*, 2000).

In zebrafish, genetic studies have shown that Nodal-related factors are required for gastrula organizer formation. The loss of *cyclops* and *squint*, or of a cofactor required for Nodal signaling, one-eyed pinhead (*oep*), results in the lack of expression of the organizer gene *goosecoid* and in the absence of axial mesendodermal tissues. Surprisingly, embryos lacking Nodal-related signals still express *chordino* at early stages and later on develop an extensive CNS with a marked expansion of anterior brain located between the cyclopic eye and the auditory vesicle (Feldman *et al.*, 1998, 2000; Gritsman *et al.*, 1999; Shimizu *et al.*, 2000; Wilson and Rubenstein, 2000). Similarly, mouse *cripto* mutants, in which Nodal signaling is defective, develop extensive anterior neural tissue, resembling a head without a trunk (Ding *et al.*, 1998).

In *Xenopus*, five mesoderm-inducing Nodal-related molecules (Xnrs) have been described (Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000). Their activity can be blocked by overexpression of the Cer-S protein, the C-terminal portion of Cerberus (Bouwmeester *et al.*, 1996), which specifically binds to and inhibits Xnrs (Piccolo *et al.*, 1999; Takahashi *et al.*, 2000). In this paper, the term Xnrs refers specifically to mesoderm-inducing Xnrs (1, 2, 4, 5, 6) and not to Xnr3, which has neural-inducing activity, and is not blocked by Cer-S (Smith *et al.*, 1995; Agius *et al.*, 2000; Takahashi *et al.*, 2000). Microinjection of synthetic *cer-S* mRNA blocks the induction of both dorsal and ventral mesoderm in animal–vegetal Nieuwkoop-type tissue recombinants, indicating that mesoderm formation is mediated by a gradient of multiple Nodal-related signals released by endoderm at the blastula stage (Agius *et al.*, 2000).

The starting point for the present investigation was the observation that embryos injected with high doses of *cer-S* mRNA lacked all mesoderm, including Spemann's organizer markers at the gastrula stage, but still developed a CNS containing a cyclopic eye and extensive brain structures. This neural development was sensitive to UV treatment and required the β-Catenin pathway. A detailed reinvestigation of the expression of *chordin* revealed substantial expression on the dorsal side, including the animal cap, already at the blastula stage. This preorganizer expression includes other secreted molecules—such as *noggin*, *follistatin*, and *Xnr3*—that are later on also expressed in Spemann's organizer. Cer-S did not block the early expression of these BMP antagonists, but inhibited the maintenance of their expression in mesoderm of the gastrula

organizer. LiCl treatment or microinjection of *β-catenin* was sufficient to ectopically activate this early gene expression program in the animal cap. This preorganizer center may participate in neural induction by the early β-Catenin pathway.

MATERIALS AND METHODS

Embryo Manipulations

Xenopus embryos obtained by *in vitro* fertilization were maintained in 0.1× modified Barth medium (Sive *et al.*, 2000) and staged according to Nieuwkoop and Faber (1994). RNA injections were performed into each blastomere at the 4- or 8-cell stage. LiCl treatment and UV irradiation were performed as described (Fainsod *et al.*, 1994). In an effort to limit the perdurance of the LiCl signal on neural tissue, we treated embryos between 4-cell and 128-cell stages for 30 min followed by incubation in 1× Barth solution for 2 h to compete the effect of LiCl with NaCl. However, no treatments were found that reproducibly enhanced the neural-inducing activity of LiCl (measured by both RT-PCR for late neural markers as well as *in situ* hybridization for *β-neurotubulin*). Ectodermal explants were excised at stage 9 and cultured in 0.5× MMR saline until sibling embryos reached the required stage. *In situ* hybridization was performed on whole embryos or on paraplast sections as described (Lemaire and Gurdon, 1994; Belo *et al.*, 1997; Sive *et al.*, 2000; http://www.hhmi.ucla.edu/derobertis/).

RT-PCR Analysis and RNA Synthesis

Embryos and explants were processed for RT-PCR analysis as described (Sasai *et al.*, 1995). The following primer sets were used: *α-actin*, *α-globin*, *Brachyury* (*Xbra*), *dkk-1*, *EF1α*, *follistatin*, *frzb-1*, *gsc*, *NCAM*, *noggin*, *Ornithine decarboxylase* (*ODC*), and *Xnr3* (Agius *et al.*, 2000), *cerberus* (Bouwmeester *et al.*, 1996), *chordin* (Sasai *et al.*, 1994), *En-2*, *Krox-20*, and *Otx-2* (Sasai *et al.*, 1995). To generate synthetic mRNAs, the plasmids pCS2-*cer-S*, pCS2-*XtAlk4*, pCS2-*antivin/lefty*, pCS2-*dnGSK3*, pCS2-*β−catenin*, and pCS2-*caBR* were linearized with *Not*I, and pSP64-*XtBR* was linearized with *Eco*RI. In this study, *cer-S* was always injected at high doses (150 pg). At lower doses, residual Xnr activity causes cyclopia and anterior defects instead of the head-like structures analyzed here (Piccolo *et al.*, 1999). All mRNAs were transcribed with SP6 RNA polymerase as described (Piccolo *et al.*, 1999). The pCS2-*antivin/lefty* construct was cloned during a screen for proteins secreted at the gastrula stage (Pera and De Robertis, 2000), using a cDNA library in the $pCS2^{+}$ vector prepared from stage 11 *Xenopus* embryos treated with LiCl.

RESULTS

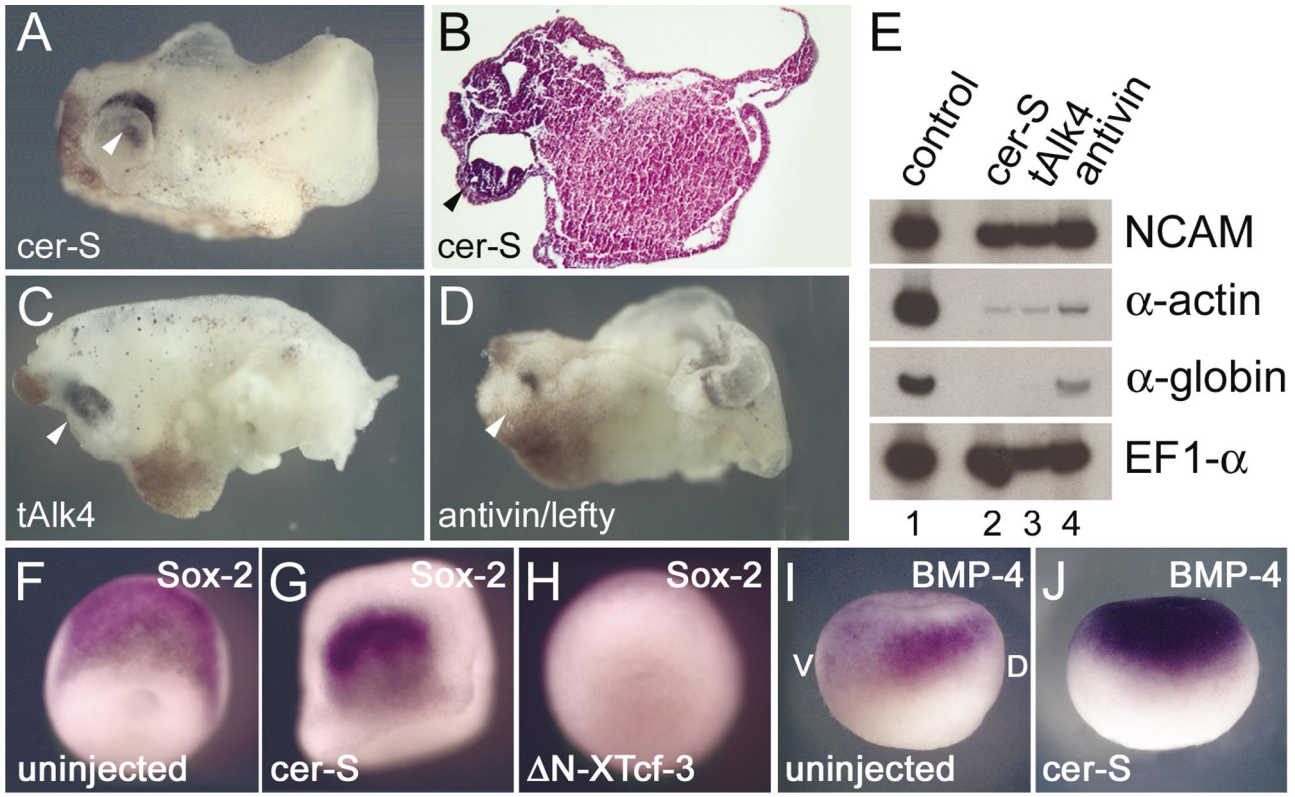
Embryos Lacking Mesoderm Develop a CNS

Embryos injected vegetally into each blastomere at the 4-cell stage with 150 pg of *cer-S* mRNA develop into head-like structures with a cyclopic eye and brain tissue that lack mesoderm, except for a small remaining tail-like structure (Figs. 1A and 1B). The presence of neural tissue was confirmed by RT-PCR analyses at stage 26, which showed expression of the pan-neural marker *NCAM*, and the absence of *α-actin* and *α-globin*, which mark dorsal and ventral mesoderm (Fig. 1E, lanes 1 and 2). The same phenotype was observed when two other mesoderm inhibitors were tested. A truncated version of a *Xenopus* Activin/Nodal receptor (*tAlk4*; Agius *et al.*, 2000) and Antivin/Lefty, an extracellular Activin/Nodal receptor antagonist (Cheng *et al.*, 2000), displayed a similar phenotype (CNS with cyclopic eye, decreased *α-actin*, and *α-globin* expression) when injected radially (Figs. 1C–1E).

In situ hybridization analyses showed that the pan-neural marker *Sox-2* was expressed on one side of the marginal zone in *cer-S*-injected embryos at the neural plate stage (Figs. 1F and 1G). Due to the lack of mesoderm, these embryos did not undergo epiboly but still formed a neural plate. To test whether *BMP-4* was regulated at the transcriptional level in *cer-S*-injected embryos, *in situ* hybridizations were performed at gastrula (stage 10.5). At this stage, *BMP-4* transcripts are expressed in the animal cap and ventral mesoderm (Fainsod *et al.*, 1994). In whole embryos in which mesoderm formation was blocked by *cer-S*, *BMP-4* expression was cleared from the entire marginal zone but was still present, at somewhat elevated levels, in animal cap ectoderm (Fig. 1J). We conclude that mesoderm formation and Xnr signaling are not required for neural induction in *Xenopus*.

Neural Induction in cer-S-Injected Embryos Requires β-Catenin Signaling

The asymmetric expression of *Sox-2* at the neurula stage in the marginal zone of *cer-S*-injected embryos (Fig. 1G) provided the first clue that dorsal β-Catenin signaling might be involved in neural induction in the absence of mesoderm. To test this, we examined whether neural induction would still take place in embryos in which cortical rotation of dorsal determinants was prevented by UV treatment. As shown in Fig. 2A, the neural markers *NCAM*, *Otx-2*, *Krox-20*, and *En-2* were expressed in *cer-S*-injected embryos at levels comparable to those of uninjected embryos (Fig. 2A, lanes 1 and 2), but were absent after UV irradiation (Fig. 2A, lanes 1–4). Suppression of neural plate formation was also observed when *ΔN-XTcf-3* mRNA (Molenaar *et al.*, 1996) was used to block transcriptional activation by β-Catenin (Fig. 1H). Importantly, *NCAM* and *Otx-2* expression could be restored in UV-treated embryos injected with *cer-S* and *β-catenin* mRNAs (Fig. 2A, lane 5). This indicates that β-Catenin is sufficient to restore, at least partially, neural differentiation. This effect of β-Catenin does not require the formation of dorsal mesoderm, since it takes place in *cer-S* embryos. In agreement with the prevailing view that inhibition of BMP signaling is required for neural induction, a dominant-negative version of the BMP receptor (*tBR*) also restored neural tissue when injected with *cer-S* together into UV-treated embryos (Fig. 2A, lane 6). In histological sections, the formation of cyclopic eyes and forebrain/hindbrain tissues in *cer-S*-



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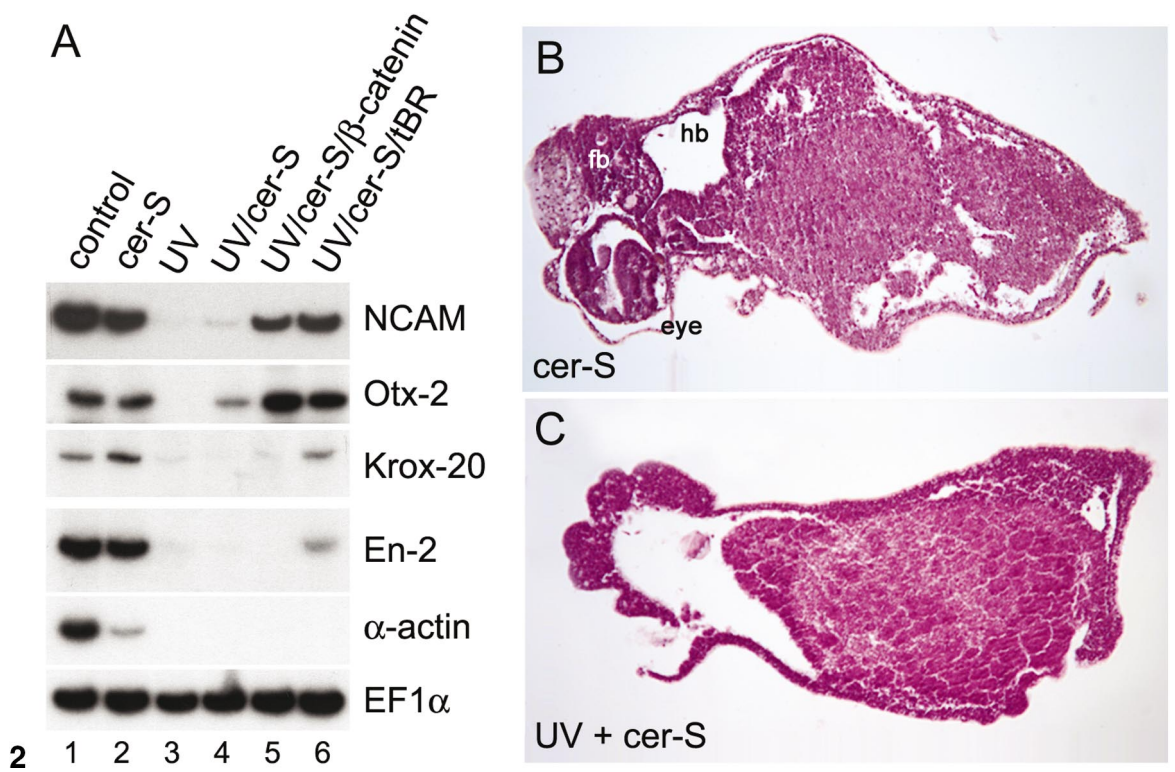

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injected embryos was prevented by UV treatment, confirming the molecular marker analyses (Figs. 2B and 2C). We conclude that neural induction in the absence of mesoderm is dependent on a functional β-Catenin dorsal signaling pathway.

A β-Catenin-Dependent Blastula Preorganizer

Neural induction in *cer-S*-injected embryos was puzzling, since we had observed that this treatment eliminated the expression of most organizer genes when embryos were examined at the gastrula stage 10.5 (Agius *et al.*, 2000). A helpful clue came from earlier work on blastula stage embryos. Smith and Harland (1992) had shown expression of *noggin* at the dorsal side of the stage 9 blastula. Expression of the neural inducer *Xnr3* had also been reported in the dorsal surface of stage 9 blastula embryos (Smith *et al.*, 1995). Similarly, expression of *chordin* had been noted before the start of gastrulation in *Xenopus* (Mizuseki *et al.*, 1998). In zebrafish, early *chordino* expression was observed even in Nodal signaling-deficient embryos (Grinblat *et al.*, 1998; Gritsman *et al.*, 1999; Shimizu *et al.*, 2000). We therefore reinvestigated the onset of *chordin* expression. Embryos were collected at 2-h intervals at stages 8, 9, 10, and 10.5 using pigmented embryos in order to time accurately the onset of dorsal lip formation (stage 10). As shown in Fig. 3D, a patch of zygotic *chordin* expression was detected on the animal cap and marginal zone 2 h before appearance of the blastopore lip. This early expression had been missed in our earlier studies (Sasai *et al.*, 1994). Expression intensifies with the onset of gastrulation and by stage 10.5 involutes with the mesoderm (Fig. 3J). *In situ* hybridization on paraffin sections showed that, at blastula (stage 9), *chordin* transcripts are expressed in the entire dorsal side, including deep cells of the animal cap, marginal zone, and vegetal regions (Fig. 4A). This pattern differs from that of *Xnr3* at blastula, which is localized in the surface layer (Smith *et al.*, 1995). Upon epiboly, *chordin* expression moved vegetally and by stage 10.5 was found in the involuting dorsal blastopore lip (Fig. 4B).

Microinjection experiments with *cer-S* or Δ*N-XTcf-3* showed that the early expression of *chordin* was independent of Xnr signaling, but dependent on an active β-Catenin pathway (Figs. 3D–3F). However, Xnr signaling was required for the maintenance of *chordin* expression in the mesoderm of Spemann's organizer at stage 10.5 (Figs. 3J–3L). To examine the full spectrum of genes affected by *cer-S* mRNA, embryos were injected and harvested at stages 9 and 10.5 by RT-PCR analyses. Interestingly, many organizer genes were expressed at early blastula stages even before the mesodermal marker *Xbra* was detectable (Fig. 4C, lane 1). Microinjection of *cer-S* mRNA did not affect the expression levels of *chordin*, *noggin*, *follistatin*, and *cerberus*, while the expression of *frzb-1* and *goosecoid*, and perhaps *dkk-1*, was decreased by inhibiting Xnr signaling (Fig. 4C, compare lanes 1 and 2). At the gastrula 10.5 stage, all organizer markers tested failed to be maintained in the presence of Cer-S (Fig. 4C, compare lanes 3 and 4). Having shown that *chordin* requires β-Catenin signaling for its expression at blastula, we next tested the wider spectrum of organizer genes that are dependent on this signaling pathway. As shown in Fig. 4D, the transcription of *chordin*, *noggin*, *follistatin*, *Xnr-3*, *goosecoid*, and *siamois* was inhibited by injection of Δ*N-XTcf-3* mRNA in whole embryos cultured until blastula (stage 9, 7.5 h postfertilization).

We conclude that BMP antagonists secreted by the mesoderm of Spemann's organizer at the gastrula stage, such as Chordin, Noggin, Follistatin, and Cerberus, are also expressed at the blastula stage. This expression takes place for at least 2 h before any external signs of blastopore formation are visible and before mesoderm, marked by *Xbra*, is formed. This preorganizer expression requires an active β-Catenin pathway. Xnr signaling is required for the maintenance of organizer-specific gene expression at gastrula, but not for its initiation.

The β-Catenin Pathway Is Sufficient to Induce Preorganizer Factors

We next investigated whether activation of the early β-Catenin pathway is sufficient to induce BMP antagonists at the blastula stage. To this end, *Xenopus* embryos were radially injected into the animal cap region at the 4-cell stage either with synthetic mRNA encoding *β-catenin*, or a

FIG. 1. Inhibition of Nodal signaling does not prevent CNS formation. (A–D) External and histological views of embryos injected radially into the vegetal pole of each blastomere at the 4-cell stage with 150 pg *cer-S* ($n = 167$), 1.5 ng *tAlk4* ($n = 21$), or 1.5 ng *antivin* mRNA ($n = 89$) at stage 32. The cyclopic eyes are indicated by arrowheads. (E) RT-PCR analysis of the same embryos showing expression of *NCAM*, but a decrease of the mesodermal markers *α-actin* and *α-globin* caused by the three anti-mesodermal agents. *EF1-α* serves as a loading control. (F–J) Whole-mount *in situ* hybridization analyses of control, *cer-S*-, and Δ*N-XTcf-3*-injected embryos with the neural plate marker *Sox-2* at stage 12.5 (F—H, dorsal view) and *BMP-4* at stage 10.5 (I and J, lateral view). D, dorsal; V, ventral.

FIG. 2. Neural induction is dependent on cortical rotation. (A) RT-PCR analysis of embryos that have been irradiated with UV light (lanes 3–6) and injected radially in the marginal zone at the 4-cell stage with *cer-S* mRNA (lanes 2 and 4), *cer-S* and *β-catenin* (150 pg each) mRNA (lane 5), or *cer-S* and *tBR* (1.5 ng) mRNA (lane 6). Note that expression of the neural markers *NCAM*, *Otx-2*, *Krox-20*, and *En-2* is inhibited by UV irradiation, but is restored after injection of either *β-catenin* or a dominant-negative BMP receptor (three embryos per sample; three independent analyses). (B, C) Histological analysis of embryos injected with *cer-S* mRNA at stage 42. UV treatment results in the loss of neural structures in *cer-S*-injected embryos ($n = 81$). fb, forebrain; hb, hindbrain.

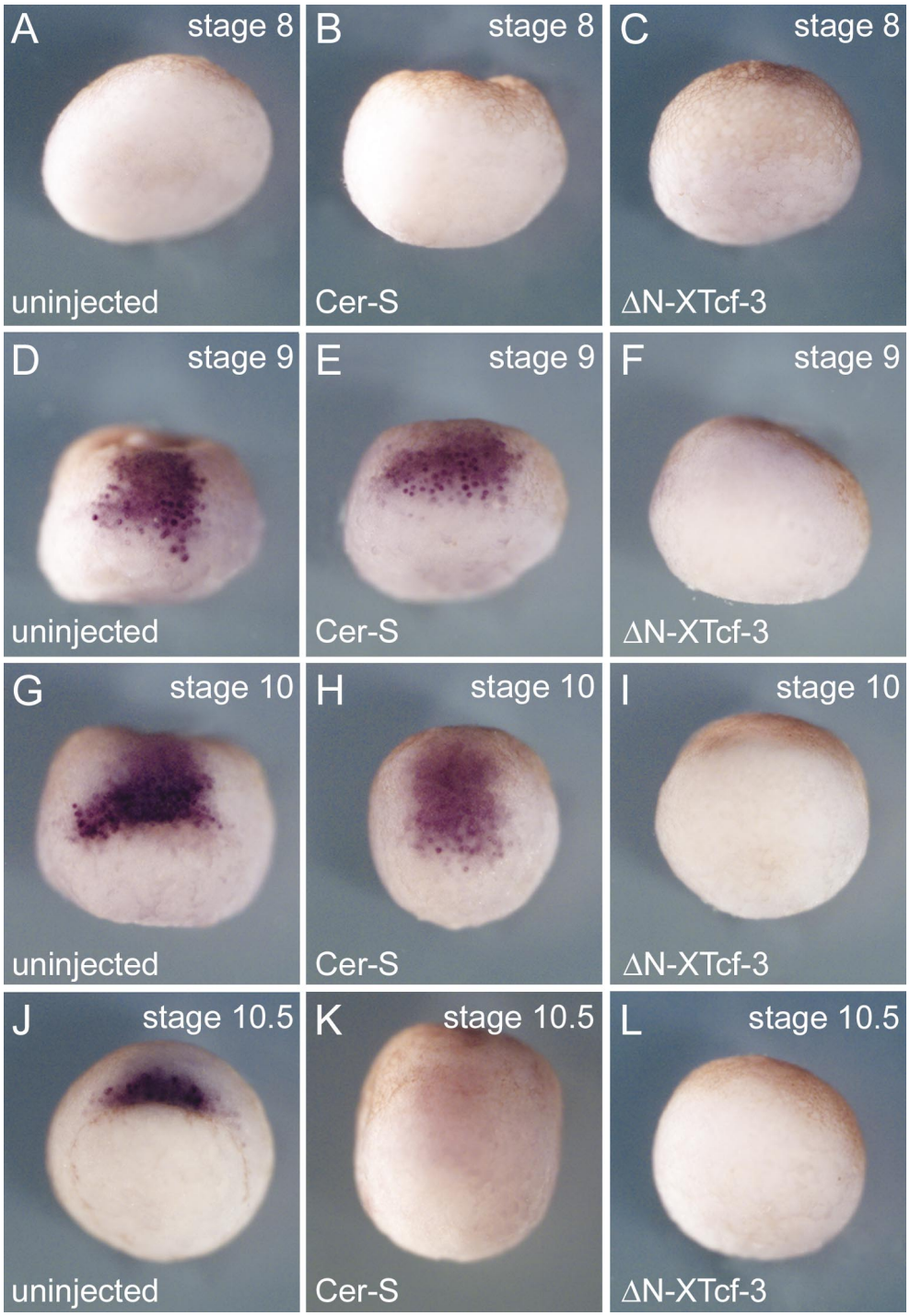
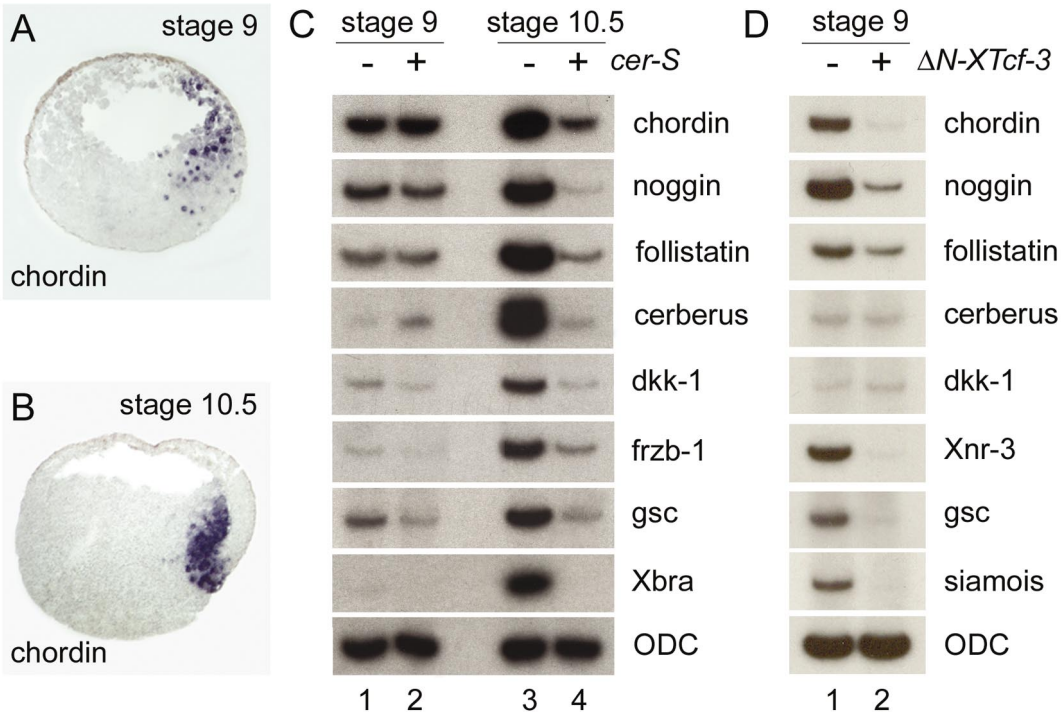


FIG. 3. Preorganizer expression of *chordin* requires the early *β*-Catenin pathway for its initiation, and Xnrs for its maintenance in the gastrula organizer. Whole-mount *in situ* hybridizations with *chordin* probe of embryos injected with *cer-S* (600 pg), Δ*N-XTcf-3* (600 pg), or uninjected controls at stages 8 (A–C), 9 (D–F), 10 (G–I), and 10.5 (J–L). A patch of *chordin* expression is detectable at least 2 h before the external dorsal lip is seen at stage 10 (three independent experiments). All embryos are shown in dorsal view; pigmented embryos with strong dorsal–ventral polarity were used in these experiments.



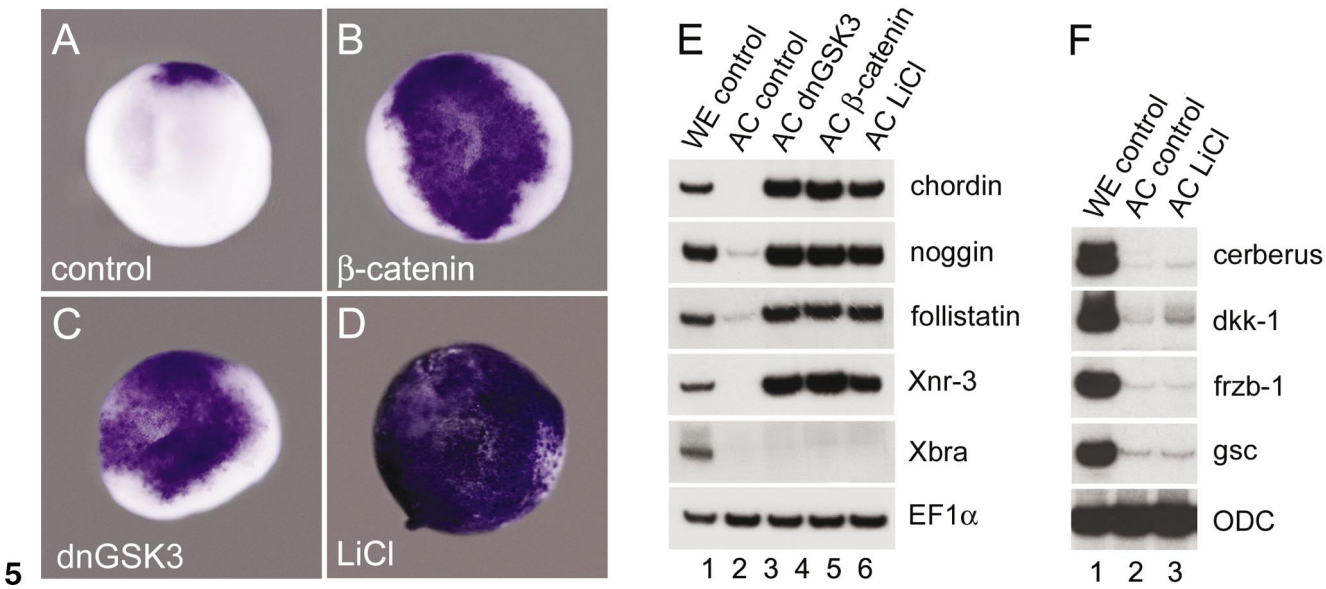


FIG. 4. Expression of organizer marker genes at the blastula and gastrula. (A, B) *In situ* hybridization on paraffin sections with a *chordin* probe at stages 9 and 10.5. Embryos were sectioned sagittally along the dorsal–ventral axis. Note the broad expression domain at stage 9, resembling the area of nuclear localization of β-Catenin (Schneider *et al.*, 1996). (C) RT-PCR analysis of embryos at stages 9 and 10.5 in the presence or absence of microinjected *cer-S* mRNA. Many classical organizer genes can be detected as early as stage 9 (lane 1) and *chordin*, *noggin*, *follistatin*, and *cerberus* continue to be expressed in the absence of Xnr signaling (lane 2). At stage 10.5, all organizer markers, as well as *Xbra*, are inhibited by the anti-Xnr reagent Cer-S (lanes 3 and 4). The lack of *Xbra* expression at stage 9 indicates that, at this early blastula stage, mesoderm induction has not yet taken place. (D) RT-PCR analysis of embryos injected with Δ*N-XTcf-3* (lane 2) and untreated controls (lane 1) at stage 9, showing that the induction of many organizer genes is dependent on an early β-Catenin signal. *ODC* serves as an RNA loading control.

FIG. 5. The β-Catenin pathway is sufficient to induce preorganizer gene expression program at the blastula stage. (A–D) Embryos were injected into the animal pole at 4-cell stage with synthetic mRNA for *β-catenin* (150 pg per blastomere), *dnGSK-3* (150 pg per blastomere), or were treated with LiCl and analyzed at stage 9 for *chordin* expression by *in situ* hybridization. The patch of expression of *chordin* in (A) marks the position of the preorganizer on the dorsal margin of blastula embryos. All embryos are shown in animal view. (E, F) Ectodermal explants of the embryos treated the same way as above were isolated at stage 8 and the expression levels of *chordin*, *noggin*, *follistatin*, and *Xnr3* (E) or *cerberus*, *dkk-1*, *frzb-1*, and *gsc* (F) were determined by RT-PCR at stage 9. *EF1α* and *ODC* serve as control for equal loading. Note that organizer gene markers in (E), but not those in (F), were induced by the β-Catenin pathway in ectodermal explants at blastula.

dominant-negative version of GSK3 (*dnGSK3*) that acts upstream of β-catenin preventing its degradation (He *et al.*, 1995). In addition, embryos were treated with LiCl, which leads to the accumulation and nuclear translocation of β-Catenin throughout the embryo (Schneider *et al.*, 1996). When these embryos were examined at stage 9 (7 h postfertilization), whole-mount *in situ* hybridization revealed abundant ectopic expression of *chordin* throughout the animal cap (Figs. 5A–5D). RT-PCR analysis of animal cap explants excised at stage 8 and harvested 2 h later at stage 9 showed a robust induction of the neural inducers *chordin*, *noggin*, *follistatin*, and *Xnr3* (Fig. 5E, compare to whole embryo controls in lane 1). This induction was specific, since not all organizer genes were induced in animal cap explants: LiCl treatment was unable to activate *cerberus*, *dkk-1*, *frzb-1*, and *gsc* at blastula (Fig. 5F). Up-regulation of β-Catenin in the presence of *cer-S* mRNA also led to the expression of *chordin*, *noggin*, and *follistatin* in animal cap explants, excluding a requirement for nodal signaling for this early phase of organizer gene expression (data not shown). The results suggest that activation of the β-Catenin pathway is sufficient to induce expression of multiple neural-inducing BMP antagonists already at the blastula stage.

CNS and Neural Crest Induction by the β-Catenin Pathway

Injection of synthetic *chordin* mRNA allows differentiation of mature neurons to occur in animal caps (Sasai *et al.*, 1995) and components of the β-catenin pathway induce neural marker genes such as *Nrp-1* (Baker *et al.*, 1999). To test whether mature neurons were formed by activating the β-Catenin pathway, animal cap explants from embryos injected with *dnGSK3* or *β-catenin* mRNA, or treated with LiCl, were cultured until stage 24. The pan-neural marker *NCAM* was induced in these caps in the absence of mesoderm, although at lower levels than those found in whole-embryo controls (Fig. 6A, lanes 1–5). This neuralization was prevented by microinjection of a constitutively active BMP receptor (Fig. 6A, lane 6), once again demonstrating the importance of the inhibition of BMP signaling in neural induction in *Xenopus*. To identify mature neurons, the β-neurotubulin marker (Richter *et al.*, 1988) was used in *in situ* hybridizations. Whereas microinjection of *chordin* leads to uniform and abundant neuronal differentiation in ectodermal explants at stage 25 (Sasai *et al.*, 1994), microinjection of *dnGSK3* or *β-catenin* mRNA leads to the appearance of isolated patches of neuronal cells (Figs. 6C–6E). Morphological and histological examination of the injected explants at stage 42 (Figs. 6F–6K) did not reveal the massive anterior neural induction observed when *chordin* is injected (Sasai *et al.*, 1995). The explants developed fluid-filled spaces (Fig. 6H) with patches of neural tissue surrounded by whorls of loose mesenchyme-containing melanocytes, i.e., tissue with the histological appearance of neural crest (Figs. 6J and 6K). The presence of neural crest in explants was confirmed by the expression of the neural crest marker *slug* in explants treated with LiCl (Fig. 6B). These data are in line with those of others showing that the Wnt signaling pathway promotes neural crest formation and CNS posterization (McGrew *et al.*, 1995; Saint-Jeannet *et al.*, 1997; LaBonne and Bronner-Fraser, 1998). Thus, the persistent activation of the β-Catenin pathway in our experimental conditions may cause some neural cells specified to become anterior neural tissue to subsequently adopt other fates such as neural crest. In conclusion, the results suggest a pathway in which β-Catenin activates the expression of BMP antagonists already at the blastula stage. These secreted factors, perhaps in concert with transcriptional down-regulation of BMPs, may participate in neural induction.

DISCUSSION

Neural induction and mesoderm formation have traditionally been thought to be associated during development (Sasai and De Robertis, 1997). However, recent findings have questioned this interpretation. Zebrafish and mouse mutants lacking mesendoderm develop with extensive anterior neural structures (Ding *et al.*, 1998; Gritsman *et al.*, 1999; Wilson and Rubenstein, 2000). We now show that *Xenopus* embryos injected with the Nodal-specific antagonist *cer-S*, that do not form mesoderm and lack Spemann's organizer at gastrula stage 10.5 (Agius *et al.*, 2000), develop extensive brain structures with large cyclopic eyes (Figs. 1A and 1B). Inhibition of CNS formation by UV irradiation or by the dominant-repressive Δ*N-XTcf-3* construct indicate that neural induction in the absence of mesoderm requires early signals mediated by the β-Catenin pathway in the ectoderm. We find that many of the organizer-specific BMP antagonists (Chordin, Noggin, Follistatin, and Cerberus) are already expressed at blastula stage 9, at least 2 h before the first sign of a dorsal lip appears at gastrula stage 10. This early phase of expression, in a region designated the blastula preorganizer, is Xnr-independent but requires an active β-Catenin pathway (Fig. 7). Thus, the β-Catenin signal could facilitate neural induction in part through secreted BMP antagonists.

The existence of a blastula organizer precursor has been suggested earlier in *Xenopus* (Gerhart *et al.*, 1991; Heasman, 1997), zebrafish (Grinblat *et al.*, 1998), and mouse (Tam and Steiner, 1999). We recently proposed a simplified pathway of dorsal development to explain how such diverse molecules as β-Catenin, Xnrs, and BMP antagonists can rescue the effect of UV irradiation in *Xenopus* (De Robertis *et al.*, 2000). In this model, β-Catenin (together with the endodermal determinants VegT and Vg1) would induce X*nr* expression in the endoderm. A gradient of multiple Xnrs would subsequently induce mesoderm and establish Spemann's organizer, which in turn secretes BMP and Wnt antagonists, promoting dorso-anterior cell fates. While the experimental data presented here still support this model

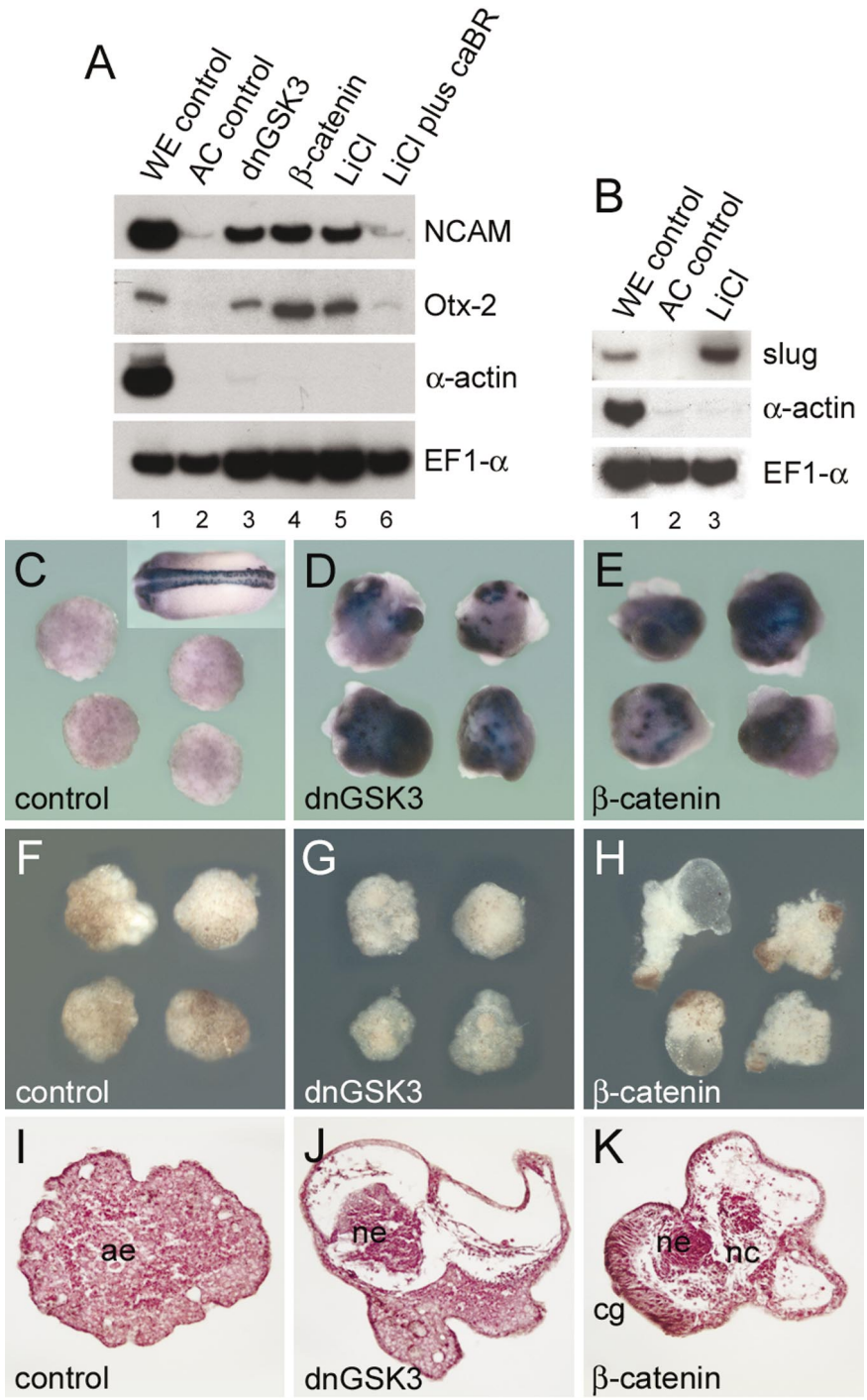


FIG. 6. *β*-Catenin signaling promotes neural differentiation and neural crest formation. (A) RT-PCR analysis of ectodermal explants injected with *dnGSK3* (lane 3; 150 pg/blastomere), *β-catenin* (lane 4; 150 pg/blastomere), treated with LiCl (lane 5; 120 mM in 0.1× Barth for 30 min at 32-cell stage), or injected with a constitutive active BMP receptor (*caBR*; 1 ng/blastomere) and treated with LiCl (lane 6) and harvested at stage 24. Note that induction of the neural marker genes *NCAM* and *Otx-2* is activated by the *β*-Catenin pathway and requires inhibition of BMP signaling. (B) Ectodermal explants of embryos treated with LiCl express the neural crest marker *Slug* by RT-PCR analysis. (C–K) Ectodermal explants microinjected with *dnGSK3* or *β−catenin* mRNA and analyzed by *in situ* hybridization using *β-neurotubulin* as a marker for differentiated neurons at stage 24 (C–E) or by morphological (F–H) and histological (I–K) criteria at stage 42. The inset in (C) shows the expression of *β-neurotubulin* in a control embryo. Note that, in explants of *dnGSK3* and *β-catenin*-injected embryos, *β-neurotubulin* expression is patchy and that, in histological sections, neural crest-like tissues with melanocytes are observed. ae, atypical epidermis; ne, neural tissue; nc, neural crest; cg, cement gland.

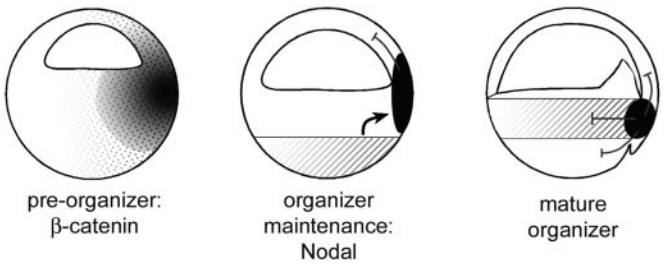


FIG. 7. Model for organizer induction. The diagram indicates three steps in the establishment of a dorsal signaling center. At the blastula stage, nuclear β-Catenin (dotted area) induces the early zygotic expression of organizer-specific genes (black), such as *chordin*, *noggin*, *follistatin*, and *Xnr3* in the preorganizer region. These BMP antagonists may participate in the predetermination of the neural plate. Later, Nodal signals originating from vegetal cells (hatched area) are required for the induction of mesoderm and for maintenance of organizer gene expression. At the gastrula stage, the same cocktail of factors secreted by the mature Spemann's organizer will pattern all three germ layers and is maintained by Nodal-related signals produced from within the mesoderm (hatched area).

for the patterning of the mesodermal germ layer, neural induction can take place in the ectoderm in the absence of Nodal signaling. Thus, the pathway should be modified for neural development, since it is not linear. The β-Catenin pathway, directly or indirectly, activates a blastula preorganizer region that expresses many neural-inducing secreted factors that are later on found in the mature organizer. As shown in Fig. 7, mesoderm induction is required for the maintenance of the expression of secreted BMP antagonists in Spemann's gastrula organizer. However, expression of these secreted antagonists is initiated via an earlier β-Catenin-dependent UV-sensitive pathway.

The expression domain of *chordin* in the preorganizer (Fig. 4A) encompasses a dorsal region that, in the animal cap, may include cells fated to become brain tissue (Dale and Slack, 1987; Bauer *et al.*, 1994). The expression of multiple BMP antagonists at blastula may contribute to the predisposition of dorsal ectoderm to neural induction (Sharpe *et al.*, 1987; London *et al.*, 1988). By the gastrula stage, expression of *chordin* (Fig. 4B) is found in mesendoderm caudal to the future forebrain region in *Xenopus* (Bauer *et al.*, 1994). An interesting modification of the Nieuwkoop activation/transformation model of neural patterning (reviewed by Sasai and De Robertis, 1997) has been proposed by Stern and colleagues (Foley *et al.*, 2000). In the chick, early signals would generate a proneural region that, although unable to differentiate by itself into forebrain, is later on stabilized by signals from underlying mesendoderm giving rise to the future forebrain. Hensen's node itself would serve as a source of caudalizing signals that posteriorize the CNS. The forebrain would escape caudalization due to morphogenetic movements that separate it from the gastrula organizer. Additional insulation of the forebrain from caudalization would be assured by inhibitory factors secreted by prechordal mesendoderm (Foley *et al.*, 2000). In zebrafish, Nodal-related factors emanating from the organizer and marginal zone have been proposed to play an important role in caudalizing the CNS (Thisse *et al.*, 2000). It is interesting to speculate that the preorganizer region of the *Xenopus* blastula could correspond to a region of neural predisposition that is subsequently maintained and patterned by signals from prechordal mesendoderm and organizer, as was proposed by Foley *et al.* (2000) for the chick embryo. Regardless of the early signals, the fact remains that a graft of dorsal mesodermal tissue at the gastrula stage, as in Spemann's experiment, can induce a complete CNS. It is interesting to note that the early and the late events share common molecules secreted by the blastula preorganizer and by the mature organizer. In both cases, a decrease in BMP signaling levels would facilitate the formation of a region in which neural induction and dorsal development can take place.

The existence of a β-Catenin-dependent preorganizer region may help understand not only neural formation in the absence of mesoderm, but also another unresolved issue in neural induction, the origin of planar signals. In *Xenopus* embryos, neural tissue can still be formed when the juxtaposition of mesoderm and ectoderm is prevented (Ruiz i Altaba, 1992). This has led to the proposal that neural-inducing factors do not only derive from the underlying mesoderm (vertical signals), but can also migrate in a planar fashion in the ectoderm. Since many anti-BMP factors are expressed at the blastula stage in CNS precursor cells (Bauer *et al.*, 1994), the early source of neural-inducing molecules may reside in the neural ectoderm itself.

Organizer Gene Expression in Xenopus and Zebrafish

We recently reported that, in *Xenopus* embryos, loss of mesoderm resulted in a loss of organizer markers at gastrula stage 10.5, including *chordin* and *goosecoid* (Agius *et al.*, 2000). However, in zebrafish mutants lacking axial mesoderm, such as *cyclops;squint* double homozygotes or maternal/zygotic one-eyed pinhead (MZ*oep*), expression of the organizer gene *chordino* could still be detected, whereas *goosecoid* was not (Feldman *et al.*, 1998; Gritsman *et al.*, 1999; Shimizu *et al.*, 2000). This implied that differences in the regulation of gene expression between these two vertebrates might exist. The concept of a blastula preorganizer may now help resolve this issue. In agreement with zebrafish, we show that, in *Xenopus*, *chordin*, as well as other BMP antagonists, are initially expressed independently of Xnr signaling at the blastula stage. Early activation of these secreted factors is initially dependent only on β-Catenin signaling, whereas *goosecoid* is expressed in mesendoderm and is strongly dependent on Nodal-related signaling.

Multiple Regulation of BMP Activity

The data presented here do not definitively prove that the BMP antagonists present in the preorganizer region in fact mediate CNS induction. This is a difficult issue to resolve because of the multiple factors involved, all of which would have to be inhibited simultaneously. The participation of multiple factors in neural induction has recently been underscored by the inactivation of the BMP antagonists *chordin* and *noggin* in mouse: neural plate induction takes place normally, but the forebrain fails to develop subsequently (Bachiller *et al.,* 2000). In *Xenopus* and zebrafish, inhibiting BMP activity seems to be a prerequisite for neural formation (Harland, 2000). Recent work indicates that this is achieved at two levels: by antagonizing BMP activity in the extracellular space and by repression of BMP transcription (Harland, 2000). In *Xenopus*, microinjections of a stabilized form of *β-catenin* or other components of the Wnt signaling pathway inhibit *BMP-4* transcription in gastrula ectodermal explants (Baker *et al.,* 1999). The down-regulation of *BMP-4* transcription cannot be mimicked by microinjection of *noggin* mRNA and may be a direct transcriptional effect (Baker *et al.,* 1999). However, we have shown here that *noggin*, *chordin*, *follistatin*, and *Xnr3* are all activated in the blastula preorganizer. Perhaps a combination of secreted BMP antagonists might be able to indirectly down-regulate *BMP-4* expression. In *cer-S*-injected embryos, the clearing of *BMP-4* transcripts, that is normally restricted to the dorsal side at the gastrula stage (Fainsod *et al.,* 1994), is extended to the entire marginal zone. The neural plate appears to develop on one side of this region free of *BMP-4* transcripts. Thus, *BMP-4* transcriptional down-regulation (Baker *et al.,* 1999) and asymmetric expression of BMP antagonists might cooperate in neural plate formation in the embryo.

Zebrafish genetics supports the idea that multiple inputs are required for neural plate development. Loss-of-function of *chordino*, the zebrafish homologue of Chordin, results in a reduced neural plate (Hammerschmidt *et al.,* 1996; Schulte-Merker *et al.,* 1997). The homeobox gene *bozozok*, a transcriptional repressor acting downstream of β-Catenin signaling, inhibits transcription of *BMP-2b/4* on the dorsal side of the embryo (Fekany *et al.,* 1999; Koos and Ho, 1999; Fekany-Lee *et al.,* 2000). Its mutation also causes a modest decrease of neural fates. Interestingly, in *chordino;bozozok* double-mutant embryos, synergistic effects are observed, resulting in a dramatic loss of head and trunk neuroectoderm (Gonzalez *et al.,* 2000). This strongly supports the view that BMP antagonism by Chordin and regulation of BMP transcription by the β-Catenin/bozozok pathway cooperate in neural development. However, the presence of a rudimentary tail argues that, even in double-mutant embryos, the anti-BMP function may not be completely eliminated and that additional pathways are involved in neural induction. The data from *Xenopus*, in which ΔN-XTcf-3 or UV treatment lead to a complete loss of neural structures, suggest that most of these pathways are triggered by the initial β-Catenin activation that takes place after fertilization.

In summary, our results suggest, but do not prove, that a blastula preorganizer dependent on the initial β-Catenin signal may participate in CNS specification. Taken together with previous investigations (Baker *et al.,* 1999), the results lend support to the emerging concept that neural induction may start very early in development with signals mediated by the β-Catenin pathway.

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