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Specification of ectoderm restricts the size of the animal plate and patterns neurogenesis in sea urchin embryos

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The animal plate of the sea urchin embryo becomes the apical organ, a sensory structure of the larva. In the absence of vegetal signaling, an expanded and unpatterned apical organ forms. To investigate the signaling that restricts the size of the animal plate and patterns neurogenesis, we have expressed molecules that regulate specification of ectoderm in embryos and chimeras. Enhancing oral ectoderm suppresses serotonergic neuron differentiation, whereas enhancing aboral or ciliary band ectoderm increases differentiation of serotonergic neurons. In embryos in which vegetal signaling is blocked, Nodal expression does not reduce the size of the thickened animal plate; however, almost no neurons form. Expression of BMP in the absence of vegetal signaling also does not restrict the size of the animal plate, but abundant serotonergic neurons form. In chimeras in which vegetal signaling is blocked in the entire embryo, and one half of the embryo expresses Nodal, serotonergic neuron formation is suppressed in both halves. In similar chimeras in which vegetal signaling is blocked and one half of the embryo expresses Goosecoid (Gsc), serotonergic neurons form only in the half of the embryo not expressing Gsc. We propose that neurogenesis is specified by a maternal program that is restricted to the animal pole by signaling that is dependent on nuclearization of β-catenin and specifies ciliary band ectoderm. Subsequently, neurogenesis in the animal plate is patterned by suppression of serotonergic neuron formation by Nodal. Like other metazoans, echinoderms appear to have a phase of neural development during which the specification of ectoderm restricts and patterns neurogenesis.

KEY WORDS: Neural development, Cell fate specification, Sea urchin, Animal plate, Ectoderm, Cellular signaling

INTRODUCTION

As the animal plate forms one pole of the animal-vegetal axis of the sea urchin embryo, it has figured prominently in studies of cell fate specification. Early investigators established that there are opposing influences within the embryo operating along the animal-vegetal axis (Horstadius, 1973). Animal plate ectoderm has limited potential, as the animal halves of eggs, or animal quartets of blastomeres, differentiate into permanent blastulae containing only ectoderm (Angerer and Angerer, 2003). Conversely, vegetal halves of eggs or embryos undergo extensive regulation and nearly normal embryos can result (Horstadius, 1973; Maruyama et al., 1985). However, the animal plate is a robust region that resists conversion to other cell types. With only a few exceptions, expression of transcription factors or signaling molecules alters cell fate in all cells except a small thickened region at the animal pole (Wikramanayake and Klein, 1995; Angerer et al., 2000; Angerer et al., 2001, Amore et al., 2003; Duboc et al., 2004; Bradham and McClay, 2006). In the absence of vegetal signaling, a permanent blastula forms in which animal plate ectoderm expands, forming a hemisphere or more of the embryo. Many authors consider the expanded ectoderm to be undifferentiated or partially differentiated (Angerer and Angerer, 2003). Some ectodermal markers are expressed, but the thickened ectoderm does not express crucial indicators of either oral or aboral ectoderm (Logan, 1999; Wikramanayake and Klein, 1995; Wikramanayake et al., 1998; Takacs et al., 2004). The region is thought to be determined by a set of maternally derived transcription factors that endow the region with what is termed an animalizing influence (Angerer et al., 1998; Angerer and Angerer, 2000; Angerer and Angerer, 2003; Ettensohn and Sweet, 2000). The opposing, vegetalizing influence is known to be a series of short-range signaling events initiated by activation of canonical Wnt signaling in micromeres (Davidson et al., 2002a; Davidson et al., 2002b; Ettensohn and Sweet, 2000; Angerer and Angerer, 2003). The vegetal pole initiates progressive specification of tiers of blastomeres to mesodermal, endodermal and ectodermal fates. However, there is no satisfactory molecular explanation for the animalizing influence centered at the opposite pole.

The larval nervous system is an array of neurons that control swimming and feeding. There are several clusters of neurons with associated neuropil that are thought to function as ganglia. The best know of these is the apical organ. In the early larva it is an elliptical patch containing three types of cells: four to six bilaterally positioned serotonergic neurons, a central cluster of 10-12 non-serotonergic neurons and several non-neural support cells (Nakajima, 1986; Burke, 1983; Nakajima et al., 1993; Nakajima et al., 2004). The neurons of the apical organ differentiate late in gastrulation and have been revealed with antibodies to serotonin, probes for tryptophan hydroxylase and antibodies to synaptotagmin (Bisgrove and Burke, 1986; Bisgrove and Burke, 1987; Nakajima et al., 1993; Yaguchi and Katow, 2003; Nakajima et al., 2004). Although we lack detailed lineage studies, topology of sequential stages indicates that the apical organ is derived from the animal plate (Burke, 1983; Nakajima et al., 1993). In gastrulae this region expresses the transcription factor SpNK2.1 and the serotonergic cells form at the interface of the SpNK2.1 domain and the aboral ectoderm (Tackacs et al., 2004; Nakajima et al., 2004). Thus, the apical organ arises as a precisely patterned neural structure from a defined ectodermal domain and provides an opportunity to examine neural specification in sea urchin embryos.

We report here that the animal plate ectoderm that forms in permanent blastulae when vegetal signaling is blocked differentiates as neural tissue forming an expanded and unpatterned apical organ.

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Using expression of components of the pathways that specify oral, aboral and ciliary band ectoderm in embryos and chimeras in which vegetal signaling is blocked, we have investigated the signaling pathways that restrict the expansion of the animal plate and pattern neurogenesis. We propose that maternal determinants specify the animal plate as neurogenic and that it is restricted in size and patterned by specification of ectoderm.

MATERIALS AND METHODS

Embryos

Strongylocentrotus purpuratus were collected locally, and embryos cultured using standard procedure at 12-14°C. The inhibitor SB431542 (10 μM) was applied at 19 hours and SB203580 (20 μM) was applied at 12 hours.

Microinjection of RNAs

De-jellied eggs were arrayed in rows on a plastic culture dish coated with 1% protamine sulfate. RNA was synthesized with mMessage mMachine kit (Ambion), quantified by gel-electrophoresis, diluted in 22.5% glycerol, filtered and loaded into injection needles. We used the following plasmids: Δ-cadherin (Logan et al., 1999); stabilized β-catenin (XBC69) (Yost et al., 1996); normal and kinase dead-Gsk3β SU-G31, SU-G32 (Emily-Fenouil et al., 1998); Antivin, Nodal (Duboc et al., 2004); BMP (Angerer et al., 2000); Noggin (Lamb et al., 1993; Angerer et al., 2000); and Gsc (Angerer et al., 2001). Concentrations of RNA use in the injection needles were: Δ-cadherin (0.5 μg/μl), XBC69 stabilized β-catenin (100 ng/μl), Gsk3β (2.0 μg/μl), dominant-negative Gsk3β (1.0 μg/μl), Antivin (40 ng/μl), BMP (2.0 μg/μl), Nodal (200 ng/μl), Noggin (1.0 μg/μl), Gsc (100 ng/μl) and about 1% of the egg volume was injected. In all instances, previously reported phenotypes were obtained. In some experiments, eggs were injected with Δ-cadherin, and after cleavage, one blastomere was re-injected with a second RNA mixed with RNA encoding six iterations of the 9E10 myc epitope (Evan et al., 1985). To inject one blastomere of an eight-cell embryo, eggs were inseminated in filtered seawater (FSW) containing 1 mM 3-amino1,2,4-triazol (ATA) and rinsed with FSW after 30 minutes. Eight-cell embryos were put in $Ca^{2+}Mg^{2+}$-free artificial seawater (CMFASW, 10 minutes), injected and cultured in FSW.

Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde in FSW for 10 minutes and rinsed with phosphate-buffered saline with Tween [PBST; 0.8 mM Na_2HPO_4-$12H_2O$, 0.15 mM KH_2PO_4, 14 mM NaCl, 0.27 mM KCl, 0.1% Tween-20 (pH 7.0)] three times, blocked with 5.0% lamb serum in PBST (1 hour) and incubated with rabbit anti-serotonin antibody (Sigma) or rabbit anti-SpNk2.1 antibody (Takacs et al., 2004), and mouse anti-synaptotagmin (1E11) (Nakajima et al., 2004) or mouse anti-myc antibody (9E10) (Evan et al., 1985) overnight (4°C). After rinsing, specimens were incubated in secondary antibodies conjugated with Alexa 488 or Alexa 568 (Molecular Probes) in PBST (2 hours). Specimens were examined with a confocal laser-scanning microscope (Zeiss) or a epifluorescence microscope (Zeiss or Leica). Serotonergic cells were counted in through focus confocal stacks. A one-way analysis of variance (ANOVA) was used with Tukey-Kramer or Dunnett multiple comparison post tests (Instat 3.0) to analyze data.

Exogastrulated embryos

To make mechanically derived exogastrulae, hatched blastulae (33 hours) were trapped under a coverslip with clay spacers and pressed until the blastocoel was no longer visible. Embryos were left under the coverslip until 65 hours when exogastrulae had formed.

Whole-mount in situ hybridization

Embryos were prepared following the method of Yaguchi and Katow (Yaguchi and Katow, 2003). Digoxigenin (DIG: Roche)-labeled SpHnf6 antisense RNA probes were synthesized from linearized plasmids. After proteinase K treatment (2 ng/μl) and post-fix with 4% paraformaldehyde, overnight hybridization (47.5°C) in 50% formamide, 5×Denhardt solution, 0.1 M MOPS (pH 7.0), 0.5 M NaCl, 1 mg/ml bovine serum albumin (BSA), 0.1% Tween-20 with 0.1 ng/μl probes.

For combined immunolocalization and in situ hybridization, specimens prepared for in situ localization were rinsed in PBST, blocked with 5.0% lamb serum in PBST (1 hour), and incubated in anti-SpNk2.1 overnight at 4°C.

Chimeric embryos

Eggs co-injected with Δ-cadherin and myc RNA and normal eggs were inseminated in ATA-FSW, transferred to 1% agarose-coated dishes, and incubated to 16 cells. Embryos were transferred to CMFASW (10 minutes). With fine glass needles, the mesomere tier was removed from an RNA-injected embryo and grafted to macromere and micromere halves of a normal embryo. Chimeras were cultured in small indentations in an agarose-coated dish.

RESULTS

Without vegetal signaling an expanded and unpatterned apical organ forms

Injection of eggs with Δ-cadherin RNA prevents nuclear localization of β-catenin and interferes with activation of the endomesodermal gene regulatory network (Logan et al., 1999; Davidson et al., 2002b). Embryos injected with Δ-cadherin RNA form permanent blastulae with a thickened epithelium in the animal hemisphere and a thin epithelium at the vegetal pole (Fig. 1A). Preparations of 72-hour embryos with neuron-specific antibodies reveal permanent blastulae contain 50 or more serotonergic neurons scattered throughout the thickened epithelium of the animal pole (Fig. 1B-D). The cells are flask-shaped and have a short, apical dendritic pole and neurites projecting basally. The neurites form a basiepithelial plexus surrounding the blastocoel. By contrast, embryos injected with glycerol have an apical organ with four to six serotonergic neurons (Fig. 1E). The serotonergic cells of the apical organ normally appear after 55 hours; however, in Δ-cadherin-injected embryos, they appear as early as 48 hours (47% of 111 embryos). In 120-hour permanent blastulae labeled with anti-synaptotagmin and anti-serotonin, all the serotonergic cells co-localize with anti-synaptotagmin. However, there are cells that are only synaptotagmin immunoreactive, which are similar in form to the serotonergic cells but outnumber them by 2:1 (Fig. 1F). This is similar to 4- to 5-day-old plutei, where there are non-serotonergic neurons in the apical organ.

Injection of RNA encoding Gsk3β (Gsk) is thought to interfere with vegetal signaling by enhancing the degradation of endogenous β-catenin (Emily-Fenouil et al., 1998). Gsk RNA injection produces permanent blastulae similar to Δ-cadherin RNA injected embryos (Fig. 1G). In preparations with neuron-specific antibodies of 72-hour permanent blastulae injected with Gsk RNA there are numerous serotonergic neurons throughout the expanded thickened ectoderm (Fig. 1G; 68% of 321 embryos). The thickened ectoderm of Gsk RNA-injected embryos is smaller than that of Δ-cadherin-injected embryos and has fewer serotonergic neurons. An alternative means to produce permanent blastulae is to isolate the animal half of eight-cell or 16-cell embryos (Wikramanayake and Klein, 1995). As with permanent blastulae produced by RNA injection, these embryos contain large numbers of serotonergic cells scattered throughout the thickened epithelium (Fig. 1H, 100% of 21 embryos).

Takacs et al. (Takacs et al., 2004) showed that the apical organ serotonergic neurons differentiate at the edge of a region of ectoderm that expresses SpNk2.1 where it contacts aboral ectoderm. Otim et al. (Otim et al., 2004) showed that SpHnf6 transcripts accumulate in cells of the ciliary band; a strip of cells at the interface of oral and aboral ectoderm. The animal plate ectoderm is the only region that co-expresses these two

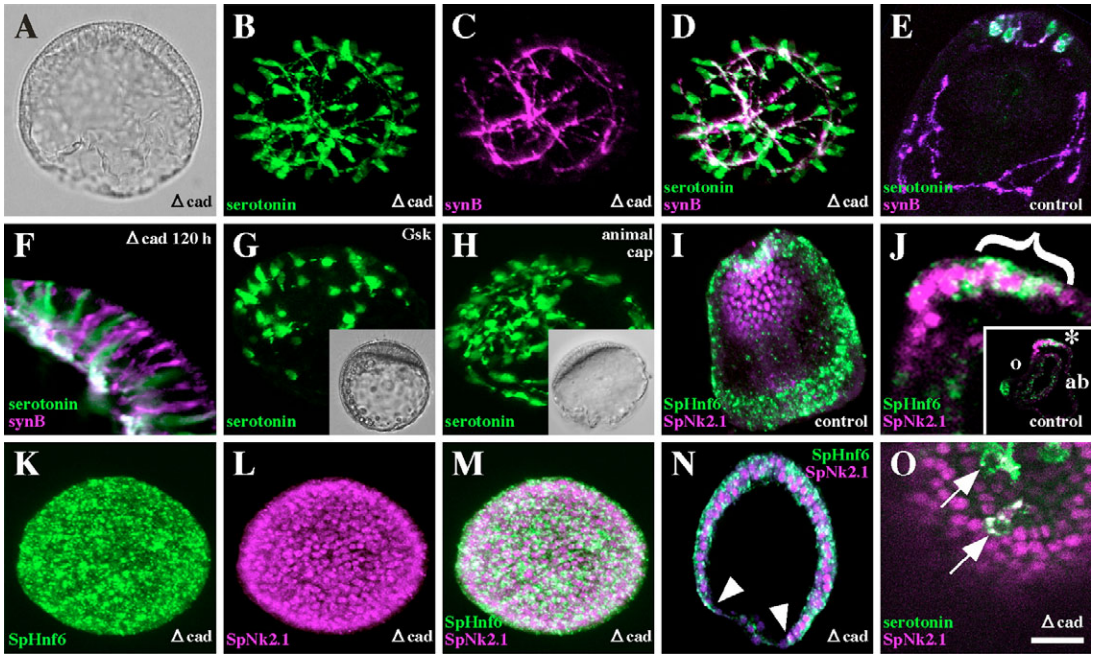

Fig. 1. Blocking vegetal signaling produces permanent blastulae with an expanded animal plate that differentiates as unpatterned neural tissue. (A-D,F) Embryos injected with Δ-cadherin RNA. (**A**) DIC image of embryo (72 hour) injected with Δ-cadherin RNA. (**B**) Anti-serotonin immunoreactive cells in a Δ-cadherin-injected embryo. Confocal stack of the animal pole. (**C**) Anti-synaptotagmin immunoreactivity (SpSyn B) is principally in neurites of the serotonergic cells. (**D**) Merged image of B,C. (**E**) Merged image of control embryo (72 hours); serotonin (green) and synB (magenta) co-localize in apical organ neurons and synB identifies ciliary band neurons. (**F**) Detail of serotonergic and non-serotonergic neurons of the animal plate of a Δ-cadherin RNA injected embryo (120 hours). (**G**) Anti-serotonin immunoreactive cells in embryo expressing Gsk. Inset is DIC image. (**H**) Permanent blastula from animal cap blastomeres with abundant unpatterned anti-serotonin immunoreactive cells. Inset is DIC image. (**I**) Uninjected embryo (72 hours) expressing SpHnf6 (green) in ciliary band and SpNk2.1 protein (magenta). Only the animal plate co-expresses these transcription factors. (**J**) A single confocal, optical section at the midline showing a lateral view of the region co-expressing SpHnf6 and SpNk2.1 (bracket). Enlargement of the region shown with asterisk in inset. o, oral ectoderm; ab, aboral ectoderm. (**K-M**) Recombined confocal image of Δ-cadherin RNA injected embryo from animal pole. All of the thickened epithelial cells express SpHnf6 (K) and SpNk2.1 (L). (M) Merged image of (K,L). (**N**) Lateral view of a Δ-cadherin RNA-injected embryo labeled with in situ RNA hybridization of SpHnf6 and immunolocalization of SpNk2.1. Arrowheads indicate the border between thick and thin epithelium. (**O**) Single confocal optical section of Δ-cadherin RNA-injected embryo. Serotonergic cells (green) express SpNk2.1 (magenta), but SpNk2.1 is cytoplasmic rather than nuclear in these cells (arrows). Scale bar: 20 μm.

transcription factors (Fig. 1I,J). In Δ-cadherin-injected embryos prepared for in situ hybridization and immunolocalization, SpHnf6 mRNA and SpNK2.1 protein are present throughout the thickened epithelium (Fig. 1K-M). Thus, treatments that block or interfere with vegetal signaling result in permanent blastulae in which the thickened ectoderm in the animal hemisphere differentiates as neurons. The markers expressed indicate that this is an expansion of the animal plate ectoderm that normally gives rise to the apical organ.

Stabilized β-catenin is resistant to proteolysis and in sea urchins enhances vegetal signaling (Yost et al., 1996; Wikramanayake et al., 1998). Embryos injected with stabilized β-catenin form exogastrulae with most cells converted to endomesodermal lineages. We did not detect neurons in the small remaining region of ectoderm (Fig. 2A-C; 78% of 45 embryos). Control embryos injected with 22.5% glycerol form normal prisms with four to six serotonergic neurons (Fig. 2J-L). Similarly, a kinase-dead form of Gsk3β (dnGsk) acts as a dominant negative and produces exogastrulae with excess vegetal lineage cells and reduced ectoderm (Emily-Fenouil et al., 1998). Neither serotonin immunoreactive cells nor anti-synaptotagmin immunoreactive cells are detected in these embryos (Fig. 2D-F; 98% of 104 embryos). To ensure that neurogenesis is not in some way dependant on gastrulation, we made exogastrulae with physical pressure. These exogastrulae have a normal complement of four to six serotonergic neurons, indicating it is not internalizing the archenteron that is necessary for neurogenesis (Fig. 2G-I). In stabilized β-catenin injected embryos, SpNk2.1 protein is not detected in the ectoderm (Fig. 2M-O). We conclude that enhanced vegetal signaling suppresses formation of the animal plate.

Vegetal signaling acts indirectly to restrict the size of the animal plate

Vegetal signaling is initiated by micromeres at the vegetal pole and the embryo is patterned by a series of short-range signaling events that sequentially specify adjacent tiers of blastomeres (Davidson et al., 1998). Although β-catenin has not been detected in the nuclei of animal blastomeres, a direct role for this factor in suppressing neurogenesis in animal blastomeres can not be excluded. To distinguish between direct and indirect signaling, we prepared chimeric embryos. Mesomeres of a 16-cell embryo injected with Δ-cadherin and myc RNA as a lineage marker were grafted onto the vegetal half of an uninjected embryo (Fig. 3A). Chimeras developed into embryos in which the archenteron and mesenchyme cells have no myc signal and ectoderm that is anti-myc immunoreactive (Fig. 3C-E). In all the chimeras, serotonergic neurons are detected that are identical in number and position to control embryos, or embryos injected with myc RNA alone (4 embryos). We further tested this by injection of Δ-cadherin and myc RNA into one cell of an eight-cell embryo (Fig. 3B). Embryos in which Δ-cadherin and the myc lineage marker were introduced into a vegetal octomere had deficiencies in

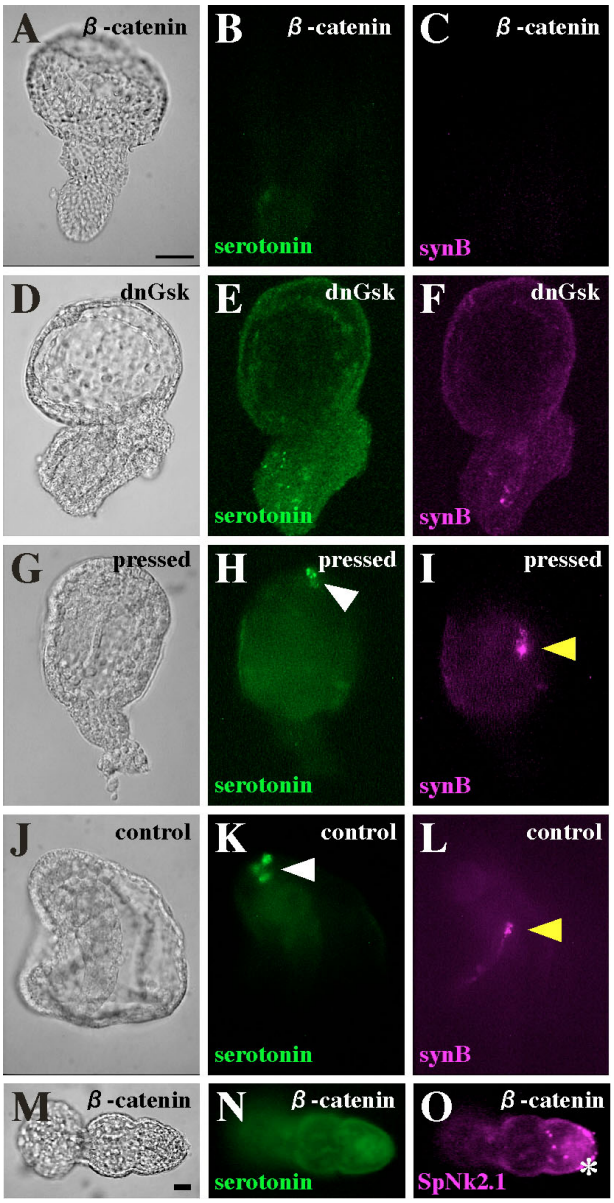

Fig. 2. Neurons do not differentiate in exogastrulated embryos injected with RNA encoding stabilized β-catenin (XBC69) or a dominant-negative Gsk3β (dnGsk). (**A**) Embryo (72 hours) injected with stabilized β-catenin RNA. (**B**,**C**) Epifluorescent images of embryos labeled with anti-serotonin (B) and anti-synaptotagmin (SpSynB) (C). Neither neural marker is detected in exogastrulae. (**D**) Embryo injected with RNA encoding dnGsk. Neither serotonin (**E**) nor synaptotagmin (SpSynB) (**F**) can be detected in exogastrulae expressing dnGsk. (**G-I**) Exogastrula prepared by compressing embryos under a coverslip. (G) Exogastrula. (H,I) Epifluorescent images of G. Although the archenteron is not internalized, normal serotonergic neurons form in the apical organ (H, white arrowhead) and anti-synaptotagmin immunoreactive cells (I, yellow arrowhead) in mechanical exogastrula (72 hours). (**J**) Glycerol injected, control prism at 72 hours. (**K**,**L**) Epifluorescent images of J. (K) Serotonergic cells are immunochemically detected at the apical organ (white arrowhead). (L) Anti-synaptotagmin (Sp SynB) immunoreactive cells in the ciliary band (yellow arrowhead). (**M-O**) SpNk2.1 expression in embryos injected with RNA encoding stabilized β-catenin. (M) The ectoderm is on the left of image. Neither anti-serotonin immunoreactive cells (N) nor anti-SpNk2.1 immunoreactive cells (O) are detected. SpNk2.1 localizes to the tip of the archenteron (O, asterisk). Scale bars: 20 μm.

their morphology (data not shown; 33% of 46 embryos had defects). In some octomere-injected embryos 1/4 of the ectoderm contained Δ-cadherin and the myc lineage marker (Fig. 3F-H). These embryos appear normal, express SpNK2.1 normally, and have four to six neurons in the apical organ (Fig. 3F-K, 67% of 46 embryos). We conclude that vegetal signaling has an indirect effect on differentiation of the animal pole, most probably through its role in specification of ectoderm.

Enhancing aboral or ciliary band specification increases serotonergic neurons

To investigate the effect of ectoderm specification on animal plate ectoderm, we employed several reagents that alter specification of oral, aboral or ciliary band ectoderm. Embryos injected with Antivin RNA have suppressed oral and aboral ectoderm and are radially symmetric with thick ciliary band ectoderm surrounding the animal plate (Duboc et al., 2004). Expression of SpNK2.1 is restricted to the central region of the thickened ectoderm and SpHnf6 is expressed throughout the entire thickened ectoderm (Fig. 4A,B; 91% of 299 embryos). Serotonergic neurons form a ring of cells at the periphery of the SpNK2.1 domain (Fig. 4C). The number of serotonergic neurons formed in 72-hour-old embryos is similar to that of control embryos, but by 96 hours, there are significantly more serotonergic neurons than in uninjected controls (Fig. 6). Ciliary band associated neurons revealed by anti-synaptotagmin (SpSynB) are spread throughout the thickened ectoderm (Fig. 4D).

SB203580 is a p38 kinase inhibitor that has been shown to interfere with Nodal expression and specification of ectoderm (Bradham and McClay, 2006). Embryos treated with SB203580 are radially symmetric and have significantly more serotonergic neurons than control plutei (Fig. 4E, Fig. 6). The serotonergic neurons lack bilateral symmetry.

SB431542 treatment is thought to block the Nodal receptor (Duboc et al., 2005) and in sea urchins produces embryos that are similar to Antivin RNA-injected embryos. In embryos treated with SB431542 from hatching, more serotonergic neurons form than in untreated controls and the serotonergic neurons form around the edge of the SpNk2.1-expressing cells of the animal plate (Fig. 4F,G; Fig. 6).

BMP RNA injection produces radially symmetric embryos that have a small animal plate that bulges outward (Angerer et al., 2000). Expression of SpNK2.1 is most intense in the small region of thickened ectoderm, but there are cells adjacent that have nuclear SpNK2.1 protein (Fig. 4H). SpHnf6 expression is restricted to the thickened ectoderm (Fig. 4I). Serotonergic cells are scattered throughout the thickened ectoderm (Fig. 4J; 86% of 79 embryos). The number of serotonergic neurons in BMP-injected embryos is similar to control embryos at 72 hours, but by 96 hours, the number of neurons is almost twice that of controls (Fig. 6).

Enhancing oral ectoderm specification reduces serotonergic neurons

Several reagents have been identified that enhance oral ectoderm specification. Injection of eggs with RNA encoding Nodal produces radialized embryos with the distinctive shape of a top (Duboc et al., 2004). SpNK2.1 is expressed in the narrowed pole region of these embryos and SpHnf6 expression is restricted to a subset of these cells at the pole (Fig. 4K-M). The region expressing both markers corresponds to the animal plate cells that are surrounded by a region corresponding to the region of oral ectoderm that expresses

SpNK2.1 (Takacs et al., 2004). In Nodal-injected embryos, serotonergic cells rarely differentiate (Fig. 4M; 94% of 123 embryos lack serotonergic neurons).

Noggin, which is thought to antagonize BMP specification of aboral ectoderm produces radially symmetric embryos with a small animal plate and lack aboral ectoderm markers (Angerer et al., 2000). In these embryos, SpNK2.1 protein is in the nuclei of cells in the small region of thickened animal plate ectoderm (Fig. 4N,O; 100% of 146 embryos). In 96-hour embryos injected with Noggin RNA, significantly fewer serotonergic cells form at either 72 or 96 hours (Fig. 4O, Fig. 6).

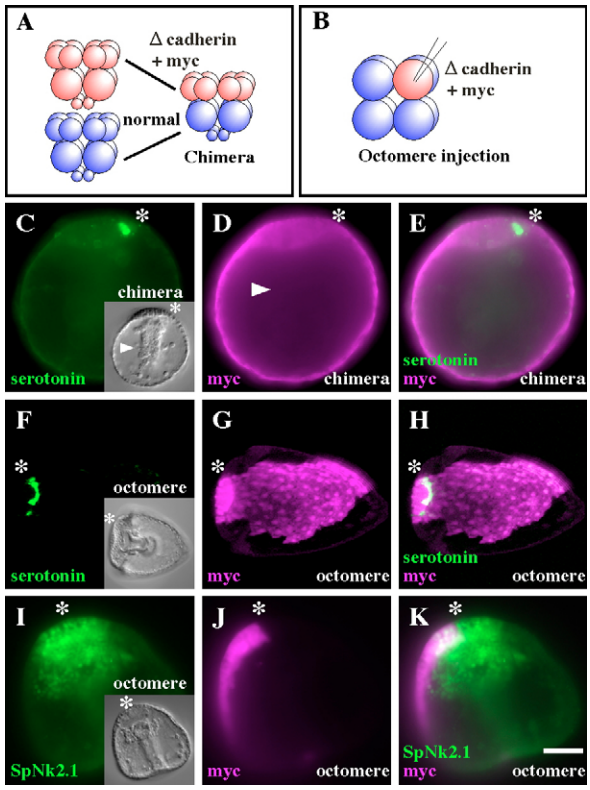

Fig. 3. Chimeric embryos and single blastomere injections indicate that signaling restricting the size of the animal plate is indirectly dependent on vegetal signaling. (**A**) The scheme for chimeras with animal half derived from a Δ-cadherin RNA-injected embryo and vegetal half uninjected. (**B**) Scheme for octomere injections. (**C-E**) Epifluorescence of chimera labeled with anti-serotonin and anti-myc. (C) A normal number and pattern of serotonergic neurons form in the chimera. Inset is DIC of chimera (72 hours) the animal plate is marked with an asterisk. Normal archenteron forms in chimeras (arrowhead). (D) The recombinant embryo has co-injected myc protein lineage marker in ectoderm. Arrowhead indicates the position of endoderm and the asterisk is beside the animal plate. (E) Merged image of C,D. (**F-H**) Recombined confocal stack of an embryo in which one cell of eight-cell embryo was co-injected with RNA encoding stabilized β-catenin and myc as a lineage marker. (F) Normally patterned apical organ with four serotonergic neurons differentiates in an octomere injected chimera. Inset is DIC, an asterisk marks the animal plate. (G) The lineage marker Myc and Δ-cadherin is in ectoderm from injected octomere. (H) Merge of F,G. (**I-K**) Epifluorescent images of octomere-injected embryo. (I) SpNk2.1 protein is like uninjected embryos, indicating no direct effect of Δ-cadherin. Inset is DIC image, asterisk indicates the animal plate. (J) Myc immunoreactive cells. (K) Merge of I,J. Scale bar: 20 μm.

Goosecoid (SpGsc) is a transcription factor thought to function as a repressor and is expressed in oral ectoderm in response to Nodal signaling (Angerer et al., 2001; Duboc et al., 2004). Embryos expressing SpGsc form blastulae that lack a thickened animal plate ectoderm and express the oral ectoderm marker EctoV (Angerer et al., 2001). In these permanent blastulae, SpNk2.1 is expressed throughout embryos injected with SpGsc and there are no serotonergic cells formed (Fig. 5J-L; 100% of 150 embryos). We are unable to detect SpHnf6 mRNA in SpGsc-injected embryos (Fig. 5K). Thus, enhancing SpGsc expression produces embryos that have features of the region of oral ectoderm adjacent to the animal plate that expresses SpNk2.1.

With the exception of SpGsc, all treatments that respecify ectoderm produce embryos in which an animal plate, defined by co-expression of SpHnf6 and SpNK2.1, is formed. When the animal plate is surrounded by ciliary band or aboral ectoderm, more serotonergic neurons are formed, but bilateral patterning is lost. This response is not clear until 96 hours and may be an indirect effect. When the thickened animal plate cells are surrounded by oral ectoderm, fewer serotonergic neurons form.

Nodal inhibits formation of serotonergic neurons

Treatments that enhance oral or aboral specification are carried out in embryos in which normal vegetal specification occurs and there is a background of endogenous signaling. To isolate the effects of treatments, we have produced embryos in which vegetal signaling is blocked by co-injection of Δ-cadherin RNA. Embryos in which Antivin or BMP is co-expressed with Δ-cadherin form permanent blastulae with a thickened animal plate. These embryos are like the Δ-cadherin-only controls; SpNK2.1 is expressed in the thickened ectoderm and up to 50 serotonergic neurons differentiate (Fig. 5A-C; Antivin, 82% of 106 embryos; BMP, 90% of 104 embryos with phenotype). Co-expression of Nodal with Δ-cadherin produces permanent blastulae that have a broad, thickened animal plate. The animal plate ectoderm expresses SpNK2.1 and SpHnf6, as in embryos injected with Δ-cadherin alone (Fig. 5D,E). However, there are only a few serotonergic cells that differentiate (Fig. 5F; 99% of 156 embryos with this phenotype). In chimeras in which one half of the embryo expresses Δ-cadherin, and other half expresses Δ-cadherin and Nodal, serotonergic neurons form in animal plate of the half expressing Δ-cadherin only. However, the serotonergic neurons that form are mostly two to three cell diameters from the interface with the half expressing Nodal, and they are reduced in density (Fig. 5I; 100% of 45 embryos). Chimeras with Antivin or BMP RNA co-expressed in one half of the embryo have serotonergic neurons in both halves, as in controls expressing Δ-cadherin only (Fig. 5G,H; Antivin, 98% of 59 embryos; BMP, 85% of 41 embryos). Thus, when the animal plate is adjacent to cells expressing Nodal, there is a suppression of serotonergic neuron formation in cells of the plate that are close to the interface.

The effect of suppressing neuron formation in adjacent cells could result directly from secretion of Nodal protein or from an indirect effect of half of the embryo expressing Nodal and becoming oral ectoderm. SpGsc lies downstream of Nodal and expression appears to induce an oral ectoderm phenotype (Angerer et al., 2001; Duboc et al., 2004). In embryos that are expressing Δ-cadherin to block vegetal signaling and SpGsc to specify oral ectoderm, permanent blastulae form that are similar to those formed when SpGsc is expressed alone. The embryos lack a thickened animal plate, express SpNK2.1 uniformly, yet they do not express neural markers, and SpHnf6 can not be detected with in situ hybridization probes (Fig.

5M-O; 100% of 150 embryos have this phenotype). Thus, without vegetal signaling, SpGsc induces the SpNk2.1 expressing region of oral ectoderm. We prepared chimeras, in which vegetal signaling is blocked throughout the entire embryo, and half of the embryo is converted to SpNk2.1-expressing oral ectoderm by co-expression of SpGsc. In the half of the embryo co-expressing SpGsc and Δ-cadherin, no serotonergic neurons form. In the half expressing Δ-cadherin alone, there are abundant serotonergic neurons (Fig. 5P; 77% of 22 embryos have this phenotype). The serotonergic neurons form in cells directly at the interface with cells co-expressing SpGsc and Δ-cadherin, indicating that there is no suppression of serotonergic neuron in adjacent cells as a consequence of SpGsc specification of SpNK2.1-expressing oral ectoderm. This supports a model in which Nodal, or a signal produced as a consequence of Nodal, but not part of the Gsc pathway, acts to suppress neuron formation.

To further assess this, we used SB431542 to block Nodal receptors. Embryos co-injected with Δ-cadherin and Nodal have an average of two serotonergic neurons (Fig. 5F, Fig. 6). When we treat these Nodal/Δ-cadherin co-injected embryos with SB431542, there is an average of 17 serotonergic neurons scattered throughout the thickened animal plate ectoderm (Fig. 5R, Fig. 6). Thus, blocking the Nodal receptor is able to partially rescue the suppression of serotonergic neuron formation by Nodal. To control for SB431542 having an independent enhancing effect on serotonergic neuron formation in the absence of Nodal, we treated embryos that have vegetal signaling blocked by injection of Δ-cadherin. An average of 40 serotonergic neurons form in an unpatterned apical organ that is similar to that of embryos expressing Δ-cadherin alone (Fig. 5Q, Fig. 6).

DISCUSSION

Vegetal signaling acts indirectly to restrict neurogenesis

Animal half embryos or embryos in which vegetal signaling is blocked form permanent blastulae with one hemisphere comprising columnar epithelium with long cilia (Wikramanayake et al., 1998; Emily-Fenouil et al., 1998; Logan et al., 1999). The thickness of the ectoderm and the long cilia suggest that, in these embryos, the animal plate is expanded. In normal embryos, the animal plate ectoderm is unique in being the only region where there is an overlap in the expression of SpNK2.1 and SpHnf6. These transcription factors are expressed throughout the thickened animal plate ectoderm in permanent blastulae, providing specific molecular markers that indicate this is an expansion of the animal plate. The neural markers indicate that the thickened cap of ectoderm differentiates as a field of neurons. The region lacks the characteristic bilateral symmetry and a well-organized neuropil, but the form and types of neurons suggest this is an expanded, but poorly patterned, apical organ. Expression of stabilized β-catenin and dnGsk enhances nuclearization of β-catenin and converts animal blastomeres to endomesodermal fates (Wikramanayake et al., 1998; Emily-Fenouil et al., 1998). We are not able to identify neurons in these vegetalized embryos. Thus, treatments that block vegetal signaling result in an expansion of the animal plate and treatments that enhance vegetal signaling seem to repress it.

Our results with chimeras expressing Δ-cadherin in animal blastomeres and octomere injections of Δ-cadherin indicate that repression of neurogenesis is not directly dependent on Wnt/β-catenin signaling. It appears that the embryo is patterned by signals that specify successive tiers of cells, beginning at the

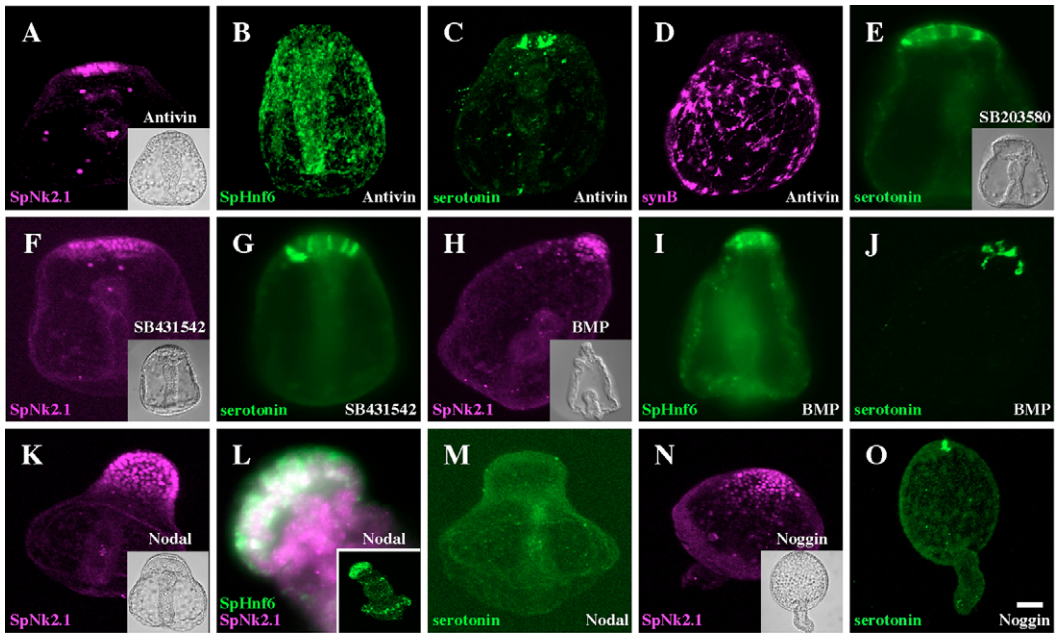

Fig. 4. Effects of treatments that alter ectoderm specification on formation and patterning of neurons in the animal plate. (**A-D**) Antivin-injected embryos (72 hours). (A) SpNk2.1 proteins in the animal plate of injected embryos. Inset is transmitted light image. (B) Ciliary band marker, SpHnf6, is expressed throughout the thickened ectoderm. (C) Serotonergic neurons are detected at the animal plate, but they are not normally patterned. (D) Ciliary band neurons (SpSynB) are scattered throughout lateral ectoderm. (**E**) Embryos treated with kinase inhibitor SB203580 have supernumerary, unpatterned serotonergic neurons. (**F**) Embryos treated with the TGFβ receptor inhibitor, SB431542, express SpNk2.1 in the animal plate. Inset shows transmitted light image. (**G**) SB431542-treated embryos have extra serotonergic neurons lacking normal symmetry. (**H-J**) BMP-injected embryos (72 hours). (H) SpNk2.1 is restricted to the thickened ectoderm at the animal pole. (I) SpHnf6 is also restricted to the animal pole. (J) Serotonergic neurons in the animal plate lack normal patterning. (**K-M**) Nodal-injected embryos. (K) Distinctive bell-shaped embryos express SpNk2.1 throughout the dome. Inset shows transmitted light image. (L) SpHnf6 is expressed in the central part of the SpNk2.1 domain; thus, the animal plate is restricted to the thickened ectoderm at the pole. Inset shows entire SpHnf6 expression, including the postoral transverse ciliary band. (M) Serotonergic neurons are rarely detected in Nodal-injected embryos. (**N**) SpNk2.1 expression in Noggin-injected embryo. Inset shows transmitted light image. (**O**) In Noggin-injected embryos, few serotonergic neurons form in the animal plate. Scale bar: 20 μm.

vegetal pole and ultimately specifying oral and aboral ectoderm (Davidson et al., 1998). The endomesoderm specification pathway is unlikely to be involved in restricting the size of the animal plate, as suppression of Notch/Delta signaling does not affect development of the apical organ (Sherwood and McClay, 1999; Sweet et al., 2002) (S.Y., J.Y. and R.D.B., unpublished). We do not know how vegetal signaling initiates expression of p 38 or Nodal, but it has been demonstrated that oral ectoderm specification depends on vegetal signaling (Duboc et al., 2004; Bradham and McClay, 2006).

The animal plate is restricted in size by signals that specify ectoderm

Blocking vegetal signaling produces embryos that have an expanded animal plate, suggesting that there is a process in the embryo that normally restricts neurogenesis to a small region. Restriction of the size of the animal plate ectoderm does not appear to be a consequence of Nodal or BMP signaling, because with vegetal signaling blocked, expression of these signaling molecules results in embryos with expanded animal plates. The thin ectoderm that is formed expresses markers that indicates it is either oral, when Nodal is expressed, or aboral, when BMP is expressed (Duboc et al., 2004; Angerer et al., 2000). However, in embryos in which the vegetal pathway is not altered, but Nodal expression is blocked with a morpholino, or counteracted by expression of Antivin or treatment with SB431542, the resulting embryos have a small animal plate. In these embryos, the small animal plate is surrounded by ectoderm that expresses markers for ciliary band; Plα2 tubulin (Duboc et al., 2004), SpHnf6 and ciliary band neurons. Similarly, blocking BMP signaling with a morpholino, or counteracting it with Noggin, in embryos in which vegetal signaling is intact also produces embryos with a restricted animal plate surrounded by ectoderm expressing

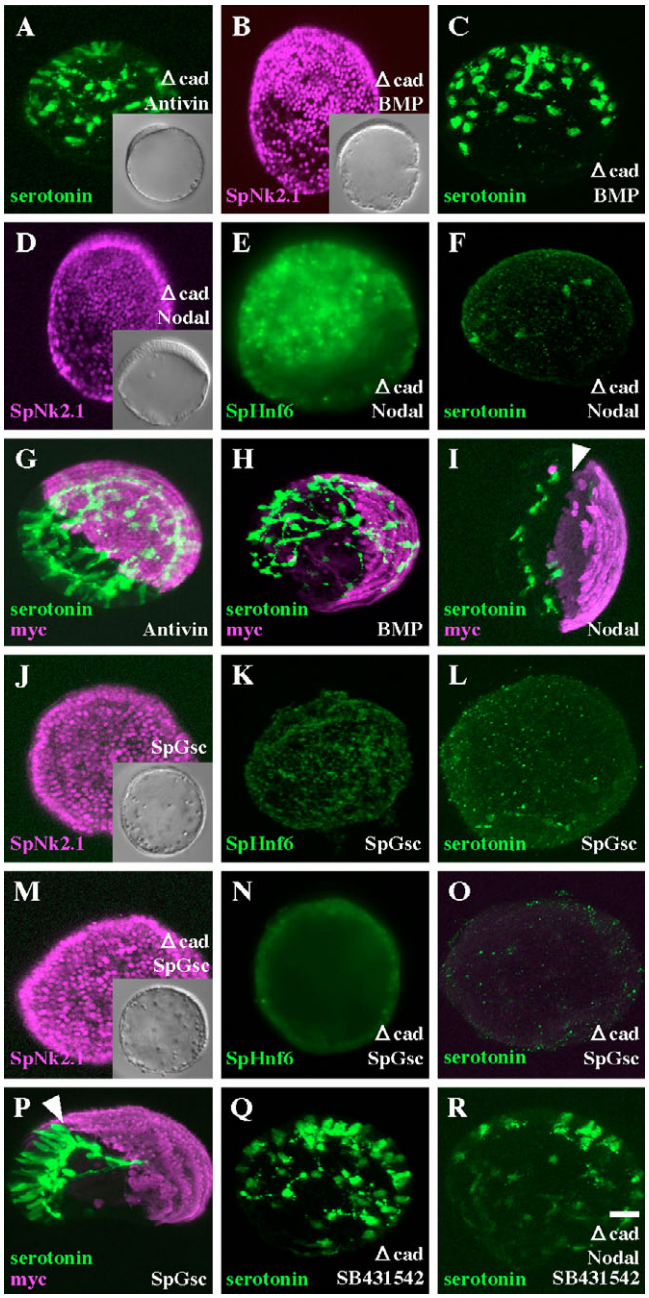

Fig. 5. Alterations of ectoderm specification in embryos and chimeras in which vegetal signaling is blocked. (**A**) Co-injection of Δ-cadherin and Antivin results in permanent blastulae with numerous unpatterned serotonergic neurons in an enlarged animal plate. Inset shows transmitted light image. (**B**,**C**) Embryos co-injected with Δ-cadherin and BMP. (B) SpNk2.1 is expressed throughout. Inset shows transmitted light image. (C) Numerous serotonergic neurons are scattered in thickened epithelium. (**D-F**) Embryos co-injected with Δ-cadherin and Nodal. SpNk2.1 (D) and SpHnf6 (E) expression is identical to Δ-cadherin alone. However, fewer serotonergic neurons form (F). Inset in S shows transmitted light image. (**G**,**H**) Chimeras in which the entire embryo expresses Δ-cadherin and half co-expresses Antivin (G) and myc as a lineage tracer or BMP (H) and myc. Serotonergic neurons form in similar numbers as embryos injected with Δ-cadherin. (**I**) Chimeras expressing Δ-cadherin throughout and Nodal in one half have fewer serotonergic neurons. The serotonergic neurons that form in the half expressing Δ-cadherin alone are several cell diameters from the interface with Nodal-expressing cells (arrowhead). (**J-L**) SpGsc-injected embryos. (J) SpNk2.1 protein is in all cells of embryos expressing SpGsc. Inset shows transmitted light image. Neither SpHnf6 (K) nor serotonin (L) are detected. (**M-O**) Embryos co-injected with Δ-cadherin and SpGsc. (M) SpNk2.1 is expressed uniformly throughout the embryo. Inset shows transmitted light image. Neither SpHnf6 (N) nor serotonergic neurons (O) are detected. (**P**) In chimeras expressing Δ-cadherin throughout, one half co-expresses SpGsc (myc-positive). Serotonergic neurons differentiate in the half of the embryo expressing only Δ-cadherin. The serotonergic neurons form at the interface with cells expressing SpGsc (arrowhead). (**Q**) SB431542-treated Δ-cadherin-injected embryos have a similar number of unpatterned serotonergic neurons as untreated embryos expressing Δ-cadherin. (**R**) Embryos co-expressing Δ-cadherin and Nodal, then treated with SB431542 form serotonergic neurons. Blocking Nodal receptors rescues the formation of serotonergic neurons. Scale bar: 20 μm.

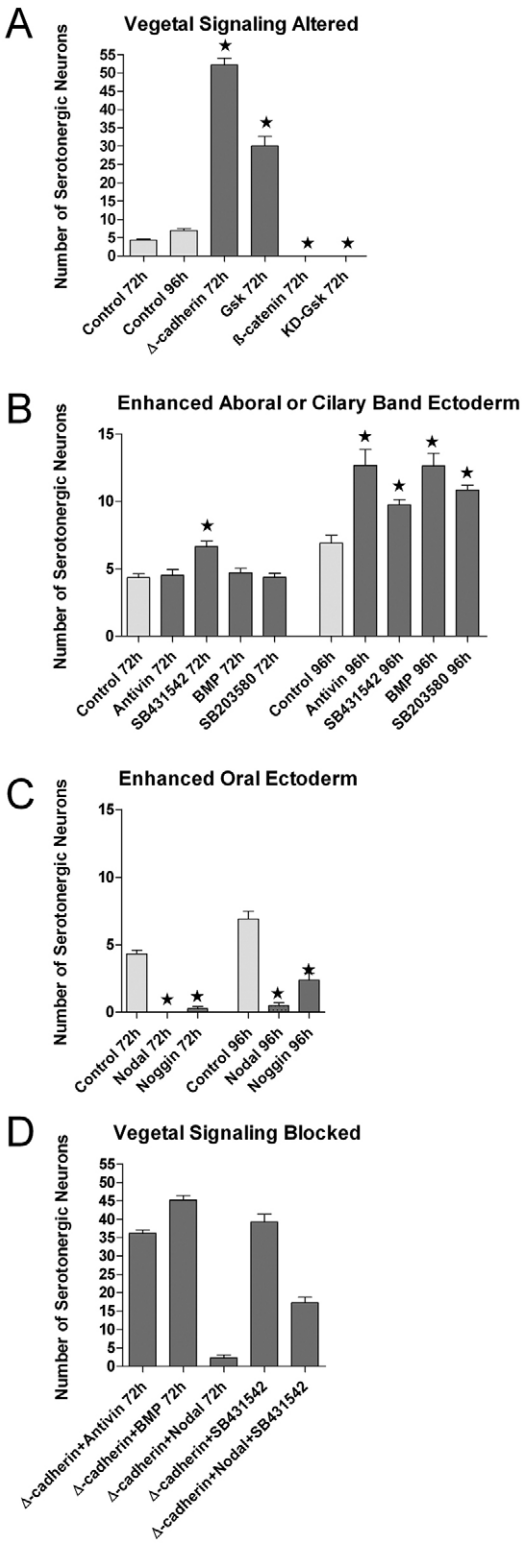

Fig. 6. Graphs showing the mean and standard error of the number of serotonergic cells in the animal plate of treated embryos. (**A**) Controls and treatments that block or enhance vegetal signaling. (**B**) Treatments that enhance the specification of ciliary band or aboral ectoderm. (**C**) Treatments that enhance the specification of oral ectoderm (**D**) Treatments of embryos in which vegetal signaling is blocked to eliminate endogenous specification of ectoderm. The control bars in A-C are the same data. Stars indicate treatments that produce changes that are significantly different from controls ($P<0.01$).

oral ectoderm markers (Angerer et al., 2000; Duboc et al., 2004). Thus, expression of BMP and Nodal when vegetal signaling is blocked cannot rescue restriction in the size of the animal plate. However, blocking specification of oral and aboral ectoderm by inhibiting Nodal and, consequently, BMP results in embryos in which there is a restricted animal plate surrounded by ectoderm with a ciliary band phenotype. These observations suggest that there is a signal, or signals, that are β-catenin dependent that restrict the size of the neurogenic animal plate and produce ectoderm with a ciliary band phenotype. Duboc et al. (Duboc et al., 2004) propose that the initial specification of ectoderm is to a ciliary band phenotype and that oral and aboral domains arise later. Bradham and McClay (Bradham and McClay, 2006) propose that p38MAPK acts upstream of Nodal and is necessary for oral specification. Embryos in which p38MAPK is inhibited or a kinase inactive form is overexpressed have a small animal plate, indicating p38MAPK is not necessary for restricting the size of the animal plate. Although we do not know the molecular basis of this signal, it appears specification of ectoderm that precedes Nodal and BMP specification of oral and aboral ectoderm restricts neurogenesis to a small domain near the animal pole (Fig. 7).

Specification of oral ectoderm patterns neurogenesis in the animal plate

To determine how ectoderm specification influences neurogenesis in the animal plate, we examined how altering specification of oral and aboral ectoderm affected the apical organ. We consistently find that the form of the apical organ and the number of serotonergic neurons is a consequence of the type of ectoderm that is specified. When the ectoderm surrounding the animal plate is specified as oral, fewer serotonergic neurons form in the apical organ. Conversely, more serotonergic neurons form when the ectoderm surrounding the animal plate is specified as ciliary band or aboral, although this is not apparent until 96 hours and may be an indirect effect. In all of these embryos, the bilateral patterning of the apical organ is lost. We propose that the patterning of serotonergic neuron specification is a consequence of a suppressive interaction between oral ectoderm and the animal plate ectoderm.

To determine how oral suppresses serotonergic neuron formation, we prepared embryos in which vegetal signaling is blocked by injection of Δ-cadherin. We believe this to be a model in which we can examine the effects of ectoderm specification on the expanded animal plate without the influence of endogenous vegetal signaling. When Nodal is co-expressed in these embryos, there is a marked reduction in serotonergic neurons, suggesting Nodal acts to suppress this phenotype. The experiments with chimeras indicate that Nodal affect cells adjacent to the cells expressing it. By contrast, cells expressing SpGsc, although apparently oral, have no effect on the animal plate cells adjacent to them. In vertebrates, the inhibitor SB431542 blocks the activity of Alk4, Alk7 (nodal/activin type I receptor) and Alk5 (TGFβ type I receptor) (Inman et al., 2002), and has been demonstrated to block Nodal function in sea urchin embryos (Duboc et al., 2005). When embryos are treated at 19 hours with SB431542, the result is a phenotype similar to the Nodal morpholino or expression of antivin (Duboc et al., 2004). These embryos have extra serotonergic neurons in the apical organ and bilateral symmetry is lost. When we use SB431542 to treat embryos with vegetal signaling blocked and Nodal expression suppressing serotonergic neuron formation, there is a significant rescue of serotonergic neurons. This suggests that blocking perception of Nodal in animal plate cells is sufficient for specification of serotonergic phenotype to proceed. Nodal clearly has roles in specification of oral ectoderm and

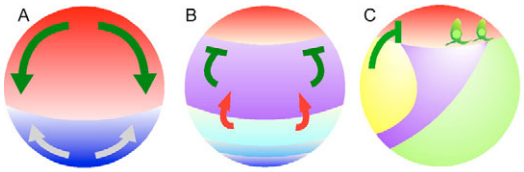

Fig. 7. Model of restriction of size and patterning of animal plate. (**A**) The egg has a neuralizing potential in the animal half. The size and patterning of the neural tissue depends on the β-catenin-mediated specification of endomesoderm. (**B**) During cleavage, specification of endomesoderm in vegetal blastomeres also specifies ectoderm as ciliary band, which restricts neurogenic ectoderm to a small animal plate. (**C**) As the ectodermal domain is specified as ciliary band, oral and aboral ectoderm, neuron formation is patterned in the animal plate. Nodal acts as a paracrine repressor of serotonergic neuron formation where oral ectoderm is adjacent to the animal plate. Serotonergic neurons form where the animal plate is adjacent to ciliary band and aboral ectoderm. Red, animal plate; blue, endomesoderm; purple, ciliary band; yellow, oral ectoderm; light green, aboral ectoderm; green cells, serotonergic neurons.

establishing the position of the adult rudiment (Duboc et al., 2004; Duboc et al., 2005). However, in ascidians and vertebrates, Nodal has also been demonstrated to function in patterning neural tissues (Thisse et al., 2000; Hudson and Yasuo, 2005). Our experiments indicate that, in urchins, Nodal secreted by oral ectoderm acts as a paracrine factor that represses the differentiation of serotonergic neurons at the interface with the animal plate.

The hypothesis that Nodal is able to block serotonergic neuron formation explains the normal patterning of the apical organ. The apical organ is derived from the elliptical patch of animal plate cells that appears to be uniformly neurogenic. As a consequence of inhibition from oral ectoderm, serotonergic neurons form only at the interface of the animal plate with ciliary band and aboral ectoderm (Fig. 7). In prisms, there is usually a row of four neurons along the aboral edge of the animal plate. In embryos lacking oral ectoderm, the animal plate is surrounded by aboral or ciliary band ectoderm and neurons form around the entire perimeter of the animal plate.

The animal plate is a neurogenic ectoderm

The animal plate is resistant to re-specification, and expands in the absence of vegetal signaling (Angerer and Angerer, 2003). The observation that the expanded animal plate differentiates as neural tissue indicates that the animal plate is a region of neurogenic ectoderm. The robust phenotype of the cells that resist respecification to other cell types is neural, and the animalizing influence, which researchers have attributed to this region of the embryos for over 100 years, is a neuralizing influence. The vegetal influences, which we now interpret as the effects of nuclearization of β-catenin, appear to be specification of cell types that restrict the expansion of this neurogenic region of ectoderm.

In vertebrates, ectoderm has a tendency to differentiate into neural tissue, and epidermis results only under the influence of BMP. As BMP4 signaling is widespread, the neural plate forms only where this signaling is directly inhibited, by molecules such as noggin, follistatin and chordin (Hammati-Brivanlou et al., 1994; Hammati-Brivanlou and Melton, 1997). This hypothesis, termed the default model, is currently being revised to accommodate observations that indicate there may be an early requirement for fibroblast growth factor signaling (Delaune et al., 2005; Stern, 2005). In *Drosophila* embryos, dorsal is established initially during oogenesis but is maintained by dorsal cells secreting a BMP4 ortholog, decapentaplegia; ventral neural ectoderm is protected from its dorsalizing effect by secretion of Short gastrulation, a molecule similar to Chordin (Biehs et al., 1996). The suggestion has been made that anti-neural inhibition is a mechanism of neural specification that will be found throughout the metazoans (Bier, 1997). In echinoids, unspecified ectoderm appears to become neurogenic animal plate ectoderm. Thus, a default neuralizing influence appears to be a shared metazoan mechanism of ectoderm specification that is retained in a deuterostome with a planktotrophic larval form.

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