The Spemann organizer meets the anterior-most neuroectoderm at the equator of early gastrulae in amphibian species

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The dorsal blastopore lip (known as the Spemann organizer) is important for making the body plan in amphibian gastrulation. The organizer is believed to involute inward and migrate animaly to make physical contact with the prospective head neuroectoderm at the blastocoel roof of mid- to late-gastrula. However, we found that this physical contact was already established at the equatorial region of very early gastrula in a wide variety of amphibian species. Here we propose a unified model of amphibian gastrulation movement. In the model, the organizer is present at the blastocoel roof of blastulae, moves vegetally to locate at the region that lies from the blastocoel floor to the dorsal lip at the onset of gastrulation. The organizer located at the blastocoel floor contributes to the anterior axial mesoderm including the prechordal plate, and the organizer at the dorsal lip ends up as the posterior axial mesoderm. During the early step of gastrulation, the anterior organizer moves to establish the physical contact with the prospective neuroectoderm through the “subduction and zippering” movements. Subduction makes a trench between the anterior organizer and the prospective neuroectoderm, and the tissues face each other via the trench. Zippering movement, with forming Brachet’s cleft, gradually closes the gap to establish the contact between them. The contact is completed at the equator of early gastrulae and it continues throughout the gastrulation. After the contact is established, the dorsal axis is formed posteriorly, but not anteriorly. The model also implies the possibility of constructing a common model of gastrulation among chordate species.

Key words: amphibian, chordate, gastrulation, movement, Spemann organizer.

Introduction

Spemann and Mangold found that the dorsal blastopore lip of an amphibian gastrula can induce a secondary axis when transplanted into another embryo’s ventral side (Spemann & Mangold 1924). The dorsal blastopore lip was named as “the organizer” because it induces the differentiation of cells that attach to it into well-organized axial structures (such as head, tail, and neural tissue).

Gastrulation is a critical stage in the formation of the central nervous system. Neurulation of the dorsal ectoderm is regulated by the axial mesoderm, known as the organizer which includes prechordal plate and the notochord. In the amphibian gastrulation process, the axial mesoderm, which is derived from the dorsal marginal zone, invaginates, involutes into the body, and then migrates toward the animal pole on the inner surface of the blastocoel roof. The inner surface of the blastocoel roof is ready for the directional migration of the leading edge along the surface of fibronectin substrate (Nakatsuji et al. 1982; Boucaut et al. 1984; Winklbauer & Nagel 1991; Johnson et al. 1993), and the migration was observed in vivo and in vitro (Hara et al. 2013; Moosmann et al. 2013).
Anterior terminal neural tissue is determined when the anterior tip of the axial mesoderm reaches the anterior-most portion of the prospective neuroectoderm at the blastocoel roof. In this work, the establishment of this physical contact is called "anterior contact establishment" (ACE) (cf. Fig. 10A–6). The establishment of physical contact between the organizer and the prospective neuroectoderm is one of the important steps for axis formation. As the prospective head neuroectoderm is known to locate around the animal pole in fate maps of early gastrula (Vogt 1929; Nakamura 1942; Keller 1975), ACE should occur around the animal pole area of mid- to late-gastrula embryo.

However, we found that gastrulation movement in X. laevis is distinct from this conventional model of amphibian gastrulation (cf. Fig. 10A) (Koide et al. 2002). In that report, we suggested that ACE occurs at the equator of early gastrula, and the physical contact of the anterior organizer with the prospective head neuroectoderm continues throughout the gastrulation process. The leading edge tissue migrates animaly beyond the region of anterior contact after ACE. Our model was supported by live imaging of a single embryo using microscopic magnetic resonance imaging (MRI; Papan 2007). It is still unknown whether the X. laevis gastrulation movement is applicable to the movement of other amphibians because it is believed that the features of gastrulation vary among species (Shook et al. 2002; Moya et al. 2007). Here, we show that the X. laevis model is fundamentally applicable to a wide variety of amphibian species, and propose a unified model of amphibian gastrulation movement (cf. Fig. 10B). In the model, the anterior organizer is present at the blastocoel floor at the onset of gastrulation, and moves to the equator to make physical contact with the prospective head neuroectoderm through the "subduction and zippering" (S&Z) movement (cf. Fig. 9) during the early step of gastrulation. The blastocoel roof becomes not neural but epidermal tissue after the contact. This physical contact continues to the end of gastrulation movement, indicating that the head is fixed at the dorsal equator of early gastrula so that the A-P axis is formed toward the posterior as the posterior organizer involutes inward (cf. Fig. 10B–6,7). This model of amphibian gastrulation would enable us to make a direct comparison with the gastrulation movements of other chordate species.

Materials and methods

Adults

Xenopus laevis purchased from Watanabe Zoushoku (Hyogo, Japan) and Cynops pyrrhogaster collected in Hyogo, Japan were maintained in JT Biohistory Research Hall (Osaka, Japan). Rana japonica was collected in Kagoshima and Hiroshima, Japan. Odorana supranarina was collected in Okinawa, Japan. Silurana tropicalis was maintained by the Institute for Amphibian Biology (Hiroshima University) through the National Bio-Resource Project of MEXT, Japan. The other amphibians were kept by the strain maintenance team of the Institute for Amphibian Biology.

Embryos

Embryos of R. japonica, Rana porosa brevipoda, X. laevis, and S. tropicalis were obtained by in vitro fertilization. Embryos of the other species were obtained by natural mating. The jelly coat was removed chemically with 1.5% thioglycolic acid (pH 9.5) and/or removed mechanically. The embryos were kept in 0.1× Steinberg’s solution (58 mmol/l NaCl, 0.67 mmol/l KCl, 0.44 mmol/l Ca(NO₃)₂, 1.3 mmol/l MgSO₄, 4.6 mmol/l Tris, pH 7.8) until they reached the desired stage at 13°C for X. laevis; at 19°C for R. porosa brevipoda; at 20°C for Ambystoma mexicanum, Bombina orientalis, and Cynops ensicauda; at 22°C for S. tropicalis; at 23°C for C. pyrrhogaster; and at 18°C for the other amphibia. Staging of gastrulae was performed according to the time length after the time of blastopore appearance. Note that we raised X. laevis embryos at low temperature (13°C) to determine the embryonic stages exactly. In this condition, gastrulation completes in 20 h. The time of blastopore appearance was defined by the appearance of a pigment line formed by the accumulation of bottle cells. As the need arises, the embryos were fixed with 10% formalin in PBS (175 mmol/l NaCl, 7.4 mmol/l Na₂HPO₄, 1.9 mmol/l NaH₂PO₄, pH 7.8) at the indicated stages, and were sagittally dissected with a microtome blade.

Staining of inner surface of blastocoel cavity

For internal labeling, 20–250 nl (depending on egg size) of 1% neutral red solution in saline was injected into the blastocoel cavity at the indicated stages in 3% Ficoll/0.5× Steinberg’s solution. Then, the embryos were transferred to 0.5× Steinberg’s solution without Ficoll where they developed into neurulae.

Labeling of external surface

Vital dye staining of the equatorial region of the embryos was performed as described previously (Kaneda et al. 2009) with minor modifications. To precisely stain tissue lined by the leading edge tissue, we
measured the distance between the leading edge tissue and the dorsal lip of blastopore at 7 h after blastopore appearance (ACE) in the sagittal sections of *C. pyrrhogaster* embryos. The distance was 1.3 mm on average. Then, superficial vital dye staining of *C. pyrrhogaster* embryos was performed at the distance of 1.3 mm from the dorsal lip using an ocular micrometer in 0.1× Steinberg’s solution.

**Blastocoel roof-less embryos**

The blastocoel roof was removed at the level of the blastocoel floor with fine forceps and eyebrow knives at the indicated stages. The blastocoel roof-less embryos were developed in 0.5× Steinberg’s solution containing 100 µg/ml kanamycin.

**Graft assay**

The dorsal blastopore lip at blastopore appearance or the blastocoel floor at ACE was detached from the embryos with eyebrow knives, and grafted to the prospective ventral side of the blastopore-appearance-stage recipient embryos by the Einsteck method (Slack & Isaacs 1994). The recipient *X. laevis* embryos were allowed to develop in 1× Barth’s solution containing 100 µg/ml kanamycin at 18°C. The recipient *C. pyrrhogaster* embryos were allowed to develop in 1× Steinberg’s solution containing 25 units/ml penicillin G and 25 µg/ml streptomycin and 100 µg/ml kanamycin at 20°C.

**Preparation of probes**

*Xenopus laevis* and *C. pyrrhogaster* chordin probes (Yokota *et al.* 1998) were synthesized from cDNA independently cloned from each gastrula cDNA library in pCS2AT+ linearized with EcoRI, and transcribed from the T7 promoter.

In situ hybridization

Whole-mount in situ hybridization was performed as reported (Hemmati-Brivanlou *et al.* 1990). Section in situ hybridization was performed essentially as described (Butler *et al.* 2001) with the following modifications. The embryos were fixed for 2 h in MEMFA for *X. laevis* and 4% paraformaldehyde in PBS for *C. pyrrhogaster*. The embryos were sectioned (10 µm thickness) and expanded in the water bath (Section Transfer System; Thermo, MA, USA). The sections were arranged in layers on MAS-coated glass slides, and left to dry overnight on a hot plate set at 40°C. As the prehybridization treatment, the slides were rinsed with 1× PBST. The RNase treatment subsequent to the hybridization was omitted, and probe washes were carried out at 60°C with 5× standard saline citrate (SSC) and 2× SSC three times for 10 min each, respectively, and then at room temperature with 1× TBST three times for 5 min each.

**Time-lapse microscopy**

For time-lapse microscopy, the embryos were embedded in 10% gelatin/0.1× Steinberg’s solution, cut out as cuboids with gelatin, and transferred to a new dish containing 0.1× Steinberg’s solution with their positions fixed. Photomicrographs were taken every 10 min using a digital optical microscope (VHX-200; Keyence, Osaka, Japan) in a temperature-controlled room at 18–22°C.

**Results**

Prospective head neuroectoderm makes physical contact with leading edge tissue at early gastrula stage

As the first step toward understanding the mechanism of amphibian gastrulation, we examined when the physical contact between the organizer and the prospective head neuroectoderm is established in various amphibian species. We analyzed anuran amphibian embryos of six species belonging to three families: fire-bellied toad *B. orientalis*, Japanese brown frog *R. japonica*, Nagoya daruma pond frog *R. porosa brevipoda*, wrinkled frog *Rana rugosa*, *X. laevis*, and Western clawed frog *Silurana tropicalis*. We also observed the gastrulation of urodelan amphibian embryos of four species belonging to three families: Mexican salamander *A. mexicanum*, sword-tail newt *C. ensicauda*, Japanese fire-bellied newt *C. pyrrhogaster*, and Japanese clouded salamander *Hynobius nebulosus*. We labeled the inner surface of blastocoel cavities of the embryos at various gastrula stages with neutral red (Fig. 1A–C). This labeling allows us to determine the extent of the physical contact between the leading edge tissue and the prospective head neuroectoderm, by observing the embryos at the neurula stage. We categorized the labeled neurula embryos into three types. In type-A embryo, the neural plate is entirely stained with vital dye, which means that the entire prospective neuroectoderm is still in the blastocoel roof when labeled (Fig. 1A). In type-B embryo, the anterior portion of the neural plate is stained, which means that the leading edge tissue is on the way to the anterior end of the prospective neural region when labeled (Fig. 1B). In type-C embryo, no neural plate...
staining is observed, which means that the inner tissue lines the entire prospective neuroectoderm when labeled (Fig. 1C). Therefore, type-C embryo is characterized by the establishment of physical contact at the neurula stage (Table 1).

To know when the leading edge tissue reaches the anterior-most portion of neural tissue, we examined when the vital dye should be injected to make the type-C embryo. The results are summarized in Figure 1D (detailed results are shown in Fig. S1). To accommodate the difference in developmental speed among species, the horizontal axis in Figure 1D indicates the ratio of the length of time of blastopore appearance ("0") to that of circular blastopore pigment line formation ("1"). At the time length ratios of 0.4–0.6, the rate of type-C embryo emergence exceeded 70% in all the species examined. The formation of the circular blastopore is considered to be the onset of the mid-gastrula stage (Keibfl 1925; Okada & Ichikawa 1947; Nieuwkoop & Faber 1956). In fact, it takes a very short time from the blastopore appearance to ACE (ACE occurs in the first 3 h in the whole 20-h process of gastrulation in X. laevis, and 7 h in 24-h in C. pyrrogaster). Taken together, those results indicate that the establishment of physical contact between the leading edge tissue and the anterior neuroectoderm occurs at the early gastrula stage. In other words, blastocoel roof at this stage does not contribute to future neural tissue.

Equatorial region at ACE ends up to anterior-most neural plate

To observe the internal morphology of the embryos after physical contact was established, we fixed the embryos at ACE and cut them along the mid-sagittal plane (Fig. 2A–F,I–K). Figure 2 shows that the leading edge is located at the equatorial region in all the embryos. This suggests that the prospective head neuroectoderm is located at the equatorial region at ACE. If this were so, the ectoderm associated with the leading edge around the dorsal equatorial region at ACE would end up as the anterior-most neural tissue, such as the forebrain. To confirm this possibility, we labeled the dorsal equatorial region (tissue precisely lined by the leading edge) of C. pyrrogaster at ACE.
and examined whether the labeled tissue contributes to the anterior portion of the neural plate. As a result, the labeled cells (Fig. 3A) were found in the anterior-most neural plate (Fig. 3B). In combination with our previous result (Koide et al. 2002), it seems that the ectodermal layer on the equator at ACE corresponds to the prospective head neuroectderm both in anuran and urodele species.

The blastocoel roof at ACE does not contribute to neural tissue but contribute to epidermis

From these observations, we hypothesized that the blastocoel roof at ACE is dispensable for the formation of dorsal structures. To check this hypothesis, we removed blastocoel roofs at ACE in R. rugosa, X. laevis, B. orientalis, C. pyrrhogaster, and A. mexicanum embryos, and monitored how the embryos would develop. In all the blastocoel roof-less embryos, dorsal structures, including complete head structures, showed normal development, but the ventral epidermis were absent (Fig. 4A–E 3/3 in B. orientalis B. orientalis, 4/5 in R. rugosa, 17/20 in X. laevis, 5/6 in A. mexicanum, and 5/5 in C. pyrrhogaster). In addition, we recognized the cement gland in the blastocoel roof-less anuran embryos (Fig. 4A–C, black arrows), reinforcing our conclusion that the anterior-most neural structure was completely formed in the embryo. Interestingly, when the blastocoel roof was removed before ACE, at stages corresponding to type-A or -B, the dorsal structure was absent or formed only posteriorly, according to the timing of blastocoel roof removal (Fig. 5). From these results, we concluded that the blastocoel roof at ACE contributes not to neural tissue but to ventral epidermis.

The physical contact between prospective head neuroectderm and the leading edge tissue at ACE continues throughout the gastrulation process

In X. laevis, it was shown that the anterior organizer tissue remained attached to the future head neuroectoder during gastrulation (Koide et al. 2002). This was supported by the results of C. pyrrhogaster experiment (Fig. 6). The outer surface of the dorsal-equatorial region of C. pyrrhogaster embryo at ACE, a region that corresponds to the prospective head neuroectoderm, was labeled with Nile blue. An agarose bead was placed on the leading edge tissue correctly lining the labeled ectoderm (Fig. 6A), and the embryo was allowed to develop to observe the relative positions of the labeled tissue and the bead. At the end of gastrulation, the Nile blue labeled tissue and the agarose bead were in contact with each other (Fig. 6B),
strongly indicating that the leading edge tissue of *C. pyrrhogaster* is continuously associated with the prospective head neuroectoderm from ACE throughout gastrulation, as was observed in *X. laevis* gastrulation.

Brachet’s cleft is closely involved in the physical contact

In the previous paper, we showed that the *X. laevis* embryo at ACE had a just-formed Brachet’s cleft (Koide et al. 2002). Observation of the internal morphology of other species’ embryos at ACE revealed that all the embryos had Brachet’s cleft (Fig. 2A–K). It is also shown that type A and B embryos in Figure 5 had very poor Brachet’s cleft, if any, when the blastocoel roof was removed (data not shown), but the type C embryo definitely had the well-formed Brachet’s cleft.

Those observations underscored the importance of the formation of Brachet’s cleft for ACE. If Brachet’s cleft formation indicates the completion of ACE, the blastocoel roof should be dispensable for the neural formation when Brachet’s cleft is just formed. In East-
Fig. 3. Vital dye staining of equatorial outer surface in Cynops pyrrhogaster. *C. pyrrhogaster* neurula with Nile blue staining of the equatorial surface at 7 h after blastopore appearance. The labeled region is indicated by a red arrowhead. (A) Immediately after labeling. (B) Labeled cells are localized in the anterior-most neural plate (15/25). Black arrowhead in panel A indicates blastopore.

Fig. 4. Blastocoel roof after anterior contact establishment (ACE) is dispensable for neural formation. Tailbud embryos whose blastocoel roof was removed at the gastrula stage. Lateral view. Head is left. (A) Bombina orientalis, (B) Rana rugosa, (C) Xenopus laevis, (D) Ambystoma mexicanum, (E) Cynops pyrrhogaster, (F) Bufo japonicus formosus, and (G) Bufo gargarizans miyakonis. (A–E) The blastocoel roof was removed at ACE. Black arrows in A–C indicate cement gland. Broken line in (E) indicates the border of epidermis. (F, G) The blastocoel roof was removed 4 h after blastopore appearance when Brachet’s cleft was just formed.

Fig. 5. Formation of neural tissue is influenced by blastocoel roof removal time in Xenopus laevis. The blastocoel roof was removed 1, 2, and 3 h after blastopore appearance. The resultant embryos showed some differences during development to the tailbud stage. (A) The blastocoel roof was removed 1 h after blastopore appearance. The embryo has only a posterior dorsal axis. (B) The blastocoel roof was removed 2 h after blastopore appearance. The embryo has an anterior axis but no head. (C) The blastocoel roof was removed 3 h after blastopore appearance (ACE). The embryo has whole dorsal axis including cement gland.
amphibian embryos (5/5 in B. gargarizans miyakonis and 2/2 in B. gargarizans japonicus, the embryos are too dark to enable visualization of vital dye, and so the labeling experiments shown in Figure 1 could not be performed. Brachet’s cleft was observed 4 h after the time of blastopore appearance in both embryos (Fig. 2G,H). To check whether anterior contact was established in the embryos 4 h after blastopore appearance, we removed the blastocoel roof from the embryos and allowed the embryos to develop to the tailbud stage (Fig. 4F,G). We found that the resultant embryos had complete head structures with reduced ventral epidermis as observed in other amphibian embryos (5/5 in B. japonicus formosus and 2/2 in B. gargarizans japonicus). The results suggest that Brachet’s cleft formation may have a significance in relation to the physical contact in amphibian species.

Anterior organizer is located at the dorsal blastocoel floor of the embryo at the onset of gastrulation

It may be important to know how the Brachet’s cleft is formed during gastrulation processes. Since it was suggested that Brachet’s cleft is formed not by delamination but by the physical contact of blastocoel roof with blastocoel floor (Winklbauer & Schürfeld 1999), the tissue in contact with the prospective head neuroectoderm at ACE should be located at the dorsal blastocoel floor prior to ACE. To confirm this possibility, we placed an agarose bead on the blastocoel floor (Fig. 7A). When we placed the bead close to the dorsal end of the blastocoel floor at the time of blastopore appearance, it ended up around the tip of Brachet’s cleft at ACE (Fig. 7B,C). The bead was also found in the middle of Brachet’s cleft when it was placed on the blastocoel floor with a small distance from the dorsal end (Fig. 7D,E) in both X. laevis and C. pyrrhogaster. The results confirm that the contact surface of Brachet’s cleft is constructed between the blastocoel floor and the blastocoel roof in both amphibian species at the time of blastopore appearance. Taken together, the leading edge tissue is localized at the dorsal blastocoel floor at the time of blastopore appearance, and moves to establish physical contact with the prospective head neuroectoderm at ACE through the movement for Brachet’s cleft formation.

If this inner tissue at the dorsal blastocoel floor is the anterior organizer, it should have the ability to induce a secondary axis when grafted ventrally. As expected, when we grafted the inner tissue (Fig. 7F), 56% of X. laevis embryos (n = 16) and 27% of C. pyrrhogaster embryos (n = 15) had the secondary axes (Fig. 7G,H). When the dorsal blastopore lip was transplanted, 51% of X. laevis embryos (n = 37) and 22% of C. pyrrhogaster embryos (n = 9) had the secondary axes (Fig. 7I, J). Together, the results indicate that the dorsal blastocoel floor can act as a functional organizer in normal development.

The physical contact is established by “subduction and zippering” (S&Z) movement

As the organizer equivalent tissue can be identified from the expression of its marker genes, we checked the expression pattern of chordin in C. pyrrhogaster and X. laevis. Careful observation of the shift of chordin expression pattern may help us to understand how the anterior organizer moves to establish the anterior contact.

Chordin is initially expressed in the dorsal blastocoel roof at the blastula stage in both species (Fig. 8A,F; Kuroda et al. 2004) The chordin-negative ectoderm animal located relative to the organizer (chordin-positive) tissue should correspond to the prospective neuroectoderm, meaning that the prospective neuroectoderm and the organizer are aligned in tandem in the blastocoel roof of late blastula (Fig. 9A), as indicated by the fate map of amphibian late blastula/early gastrula embryos (Vogt 1929; Nakamura 1942; Keller 1975).

The chordin-positive tissue moves vegetally and expands medially on the blastocoel floor (Fig. 8B,G) by the time of blastopore appearance. At this stage,
the **chordin**-positive tissue no longer exists in the blastocoel roof, and it turns out that the prospective neuroectoderm becomes oriented at an angle of approximately 90 degrees from the anterior organizer located in the blastocoel floor (Fig. 9B). It may be important to remark that the boundary between the **chordin**-positive and -negative regions forms a pivot for the following movements. As the downward movement of the prospective neuroectoderm (**chordin**-negative ectoderm) progresses, the **chordin**-positive blastocoel floor is forcibly dragged down, forming a trench (Fig. 8C,H). This trench-making movement resembles “subduction” (Fig. 9B–D), which means one of the movements of plate tectonics. It may be noteworthy to say that the bottom of the trench corresponds to the boundary between **chordin**-positive and -negative tissues, and will contribute to the vegetal tip of future Brachet’s cleft. Then, the **chordin**-positive side of the trench approaches the other side to make physical contact (Fig. 8D,E,I,J). As a result, Brachet’s cleft is formed from the posterior (vegetal) toward the anterior (equatorial), just like “zippering” (Fig. 9D–F). The S&Z movement model shows the significance of Brachet’s cleft for the proper function of the organizer.

**Discussion**

In this study, we showed that the *X. laevis* gastrulation movement proposed previously (Koide et al. 2002) is applicable to a wide variety of amphibian species. Here we propose a unified model of amphibian gastrulation (Fig. 10B).

In this model, the prospective organizer tissue is initially located in the blastocoel roof at the late blastula stage (Fig. 8A, see also Kuroda et al. 2004). Since the
prospective neuroectoderm is shown to locate in the animal hemisphere at the onset of gastrulation in the fate maps of various amphibian species (Vogt 1929; Nakamura 1942; Keller 1975), it follows that the prospective neuroectoderm and the organizer are aligned in tandem at the animal hemisphere prior to gastrulation (Fig. 10B-1). Subsequently, the prospective neuroectoderm moves downward to expand the area of the organizer medially on the blastocoel floor by the time of blastopore appearance. This downward movement is also partially supported by the observation of MRI (Papan et al. 2007). Then, the prospective neuroectoderm and the anterior organizer area make a 90-degree angle at the blastocoel corner between the dorsal blastocoel floor and the blastocoel roof (Fig. 10B-2). The “subduction” movement creates a trench (red arrowheads) formed between chordin-positive blastocoel floor and chordin-negative blastocoel roof. (D, I) Expression in X. laevis 2 h after blastopore appearance, and in C. pyrrogaster 4 h after blastopore appearance. (E, J) Expression in ACE embryos (in X. laevis 3 h after blastopore appearance, and in C. pyrrogaster 7 h after blastopore appearance). Insets are schematic drawings of the expression pattern. The developmental stages are shown at the bottom of each panel. Black arrowheads indicate blastopore. The outline of the embryo is shown by broken lines in panel (A). Note that the boundary between chordin-positive and -negative tissues corresponds to the bottom of the trench, and contributes to the tip of Brachet’s cleft (red arrows).

Kuroda et al. (2004) reported that the BCNE (Blastula Chordin- and Noggin-Expressing) center, which is defined by the region that expresses chordin and noggin at the blastula stage, contains both the prospective neuroectoderm and the Spemann organizer. In the report they claimed that the prospective neuroectoderm in the BCNE center expresses chordin when it was located at the blastocoel roof, and the expression disappears when it reaches the equatorial region. However, we found that the prospective neuroectoderm does not express chordin when it is located in the blastocoel roof at the time of blastopore appearance (Figs 1, 5, 7 and 8). And the prospective neuroectoderm is found at the outer layer of the dorsal

Fig. 8. Expression patterns of chordin during early gastrulation in Xenopus laevis and Cynops pyrrogaster. (A–E) Expression patterns of X. laevis chordin from late blastula to anterior contact establishment (ACE) (3 h after blastopore appearance). (F–J) Expression patterns of C. pyrrogaster chordin from late blastula to ACE (7 h after blastopore appearance). (A, F) Expression in late blastula embryos. Chordin expression is first detected in the dorsal blastocoel roof. (B, G) Expression at the onset of gastrulation. Chordin is expressed not only in the surface layer (dorsal lip) but also internally in the blastocoel floor. (C, H) Expression in X. laevis 1 h after blastopore appearance, and in C. pyrrogaster 2 h after blastopore appearance. A trench (red arrowheads) is formed between chordin-positive blastocoel floor and chordin-negative blastocoel roof. (D, I) Expression in X. laevis 2 h after blastopore appearance, and in C. pyrrogaster 4 h after blastopore appearance. (E, J) Expression in ACE embryos (in X. laevis 3 h after blastopore appearance, and in C. pyrrogaster 7 h after blastopore appearance). Insets are schematic drawings of the expression pattern. The developmental stages are shown at the bottom of each panel. Black arrowheads indicate blastopore. The outline of the embryo is shown by broken lines in panel (A). Note that the boundary between chordin-positive and -negative tissues corresponds to the bottom of the trench, and contributes to the tip of Brachet’s cleft (red arrows).
marginal zone at ACE. These results make us think that the BCNE center contains only the future organizer tissue and that the prospective neuroectoderm has never expressed chordin, and move downward from blastocoel roof to the equator to meet the anterior organizer at ACE.

The blastocoel floor derived tissue makes physical contact with the prospective neuroectoderm at the equator at the early gastrula stage (ACE), and the contact continues throughout gastrulation at the equatorial region (Fig. 6, Koide et al. 2002). The earliest neural marker gene, such as Xotx2, is expressed at an earlier stage than the mid-gastrula stage (Blitz & Cho 1995). This blastocoel floor derived tissue could be regarded as a direct inducer of the anterior neuroectoderm, although it is well known that various signals emanating from a variety of tissues are necessary for the maintenance and patterning of the anterior neural tissues during development.

It has long been believed that the dorsal blastopore lip plays an important role in the organizer function (Spemann & Mangold 1924). The tissue located originally at the dorsal lip at the time of blastopore appearance was found in a region slightly farther from the prospective head neuroectoderm at ACE in both X. laevis and C. pyrrhogaster (Fig. S2B,C). This means that the dorsal lip derived tissue may never obtain an opportunity to directly contact the prospective neural tissue, and indicates that the dorsal blastopore lip may not function to induce anterior neural tissue in normal development, although the dorsal lip derived tissue actually has the ability to do so. As both the dorsal lip and the tissue at the dorsal blastocoel floor show the same expression patterns of various genes for organizer function, such as chordin, goosecoid, and noggin (Cho et al. 1991; Smith & Harland 1992; Sasai et al. 1994), it is not unusual that the dorsal lip is able to mimic the organizer function, even though it is not the functional organizer in normal development. The prospective head neuroectoderm and the anterior organizer are tissues derived from the blastocoel roof and the blastocoel floor, respectively, meaning that the contact surface has never been exposed to the external surface of the embryo at any time. Vogt’s drawing supports this prediction (Fig. S3). Vogt labeled the surface of late blastula embryos with vital dye (Fig. S3A), grew the embryos up to the tailbud stage to see where the labeled tissues were localized, and found that the anterior portion of chordamesoderm remained unlabeled (indicated by the green bracket in Fig. S3B).
although most of the stained tissue contributed to the internal axial tissue, including the pharyngeal endoderm and the posterior notochord (Vogt 1929). If our explanation is correct, the unlabeled tissue shown in Vogt's drawing should be labeled when the inner surface of the blastocoel cavity at the time of blastopore appearance is labeled (the green bracket in Fig. S3B). The result was what we expected (data not shown). This indicates that the organizer tissue can be divided into two regions: one is originally located at the blastocoel floor in early gastrulae and ends up as the anterior axial mesoderm including the prechordal plate, and the other is located at the surface of early gastrula and ends up as the posterior axial mesoderm. This may correspond to the upper blastopore lip because it is known that the dorsal lip involutes into the embryo at the early gastrula stage and contributes to the axial mesoderm (Shih & Keller 1992; Winklbauer & Schürfeld 1999), though the bottle cells are known to come to lie in the anterior archenteron (Kaneda & Motoki 2012).

In this report, we show that the anterior-most axial mesoderm and the anterior neuroectoderm are in contact with each other from the time of ACE through the end of gastrulation (Fig. 10B-6). But the anterior migration of the leading edge after ACE is definitely observed, as many researchers have reported (Nakatsui et al. 1982; Boucaut et al. 1984; Winklbauer & Nagel 1991; Johnson et al. 1993). In our model, we propose that the leading edge tissue consists of the predicted organizer (chordin-expressing cells) from the time of blastopore appearance through ACE, and after ACE, the prospective endodermal tissue precedes the anterior terminus of the axial mesoderm. This means that different cells contribute to the leading edge before and after ACE (Fig. 10B-6). It was also reported that the dorsal blastocoel floor of early gastrula embryo ends up as the pharyngeal endoderm (Winklbauer & Schürfeld 1999). This may suggest that only the dorsal-most portion of the blastocoel floor prior to ACE functions as the organizer, and another part of the blastocoel floor ends up as the pharyngeal endoderm, or the dorsal-most portion of blastocoel floor after ACE contributes to the pharyngeal endoderm. Indeed, it is known that a tissue of the anterior archenteron roof including the leading edge tissue of the late gastrula does not express marker genes of the axial mesoderm (such as chordin and goosecoid, Yamaguti et al. 2005; Kaneda & Motoki 2012).

We showed that the leading edge tissue can induce the secondary axis as well as the dorsal lip (Fig. 7F-J). However, Bouwmeester et al. (1996) showed that the leading edge tissue is a rather weak inducer of the secondary axis. This contradiction might be caused by the difference in embryonic stages. Since it takes very short time from the time of blastopore appearance to ACE, it is difficult to know exactly the embryonic stage from the external morphology. Moreover, it is said that
the external morphology does not necessarily reflect the internal morphology. Therefore, the leading edge tissue used in their experiments may be slightly different from what we used. The important thing is that “the anterior contact” of the anterior axial mesoderm with the head neuroectoderm is preserved until the end of gastrulation, and they never slide from each other. Therefore the leading edge tissue may be the mesoderm or the endoderm depending on the developmental stage.

Although limited anterior migration of the head mesoderm was reported recently for a few amphibian species (Poznanski & Keller 1997; Kaneda & Motoki 2012), it is still widely believed that the axial mesoderm migrates anteriorly during amphibian gastrulation. This confusion might make it difficult to construct a general model of amphibian gastrulation. We propose herein a unified model of amphibian gastrulation, which may allow us to directly compare the gastrulation movements of the model of amphibian gastrulation, which may allow us to construct a common model of chordata gastrulation.

We thank Drs A. Kashiwagi and K. Kashiwagi for the gift of C. pyrrhogaster chordin probe. We also thank Ms H. Satsuki for technical assistance.

Acknowledgments

We thank Drs A. Kashiwagi and K. Kashiwagi for providing the research environment, and Dr M. Asashima for the gift of C. pyrrhogaster chordin probe. We also thank Ms H. Satsuki for technical assistance.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Rate of each type in the experiment of neutral red injection into blastocoel roof.

Fig. S2. Not dorsal lip but dorsal blastocoel floor acts as anterior organizer in normal development.

Fig. S3. Anterior axial tissue is prevented from labeling surface cells at blastopore appearance.

Fig. S4. Proposed model of amphibian gastrulation is comparable to protochordate gastrulation.

Movie S1. Time-lapse movie of vegetal view of Rana nigromaculata embryo.

Movie S2. Time-lapse movie of vegetal view of Xenopus laevis embryo.