Published in final edited form as:

Phys Biol. 2012 December ; 9(6): 066007. doi:10.1088/1478-3975/9/6/066007.

Regional Differences in Actomyosin Contraction Shape the Primary Vesicles in the Embryonic Chicken Brain

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Abstract

In the early embryo, the brain initially forms as a relatively straight, cylindrical epithelial tube composed of neural stem cells. The brain tube then divides into three primary vesicles (forebrain, midbrain, hindbrain), as well as a series of bulges (rhombomeres) in the hindbrain. The boundaries between these subdivisions have been well studied as regions of differential gene expression, but the morphogenetic mechanisms that generate these constrictions are not well understood. Here, we show that regional variations in actomyosin-based contractility play a major role in vesicle formation in the embryonic chicken brain. In particular, boundaries did not form in brains exposed to the nonmuscle myosin II inhibitor blebbistatin, whereas increasing contractile force using calyculin or ATP deepened boundaries considerably. Tissue staining showed that contraction likely occurs at the inner part of the wall, as F-actin and phosphorylated myosin are concentrated at the apical side. However, relatively little actin and myosin was found in rhombomere boundaries. To determine the specific physical mechanisms that drive vesicle formation, we developed a finite-element model for the brain tube. Regional apical contraction was simulated in the model, with contractile anisotropy and strength estimated from contractile protein distributions and measurements of cell shapes. The model shows that a combination of circumferential contraction in the boundary regions and relatively isotropic contraction between boundaries can generate realistic morphologies for the primary vesicles. In contrast, rhombomere formation likely involves longitudinal contraction between boundaries. Further simulations suggest that these different mechanisms are dictated by regional differences in initial morphology and the need to withstand cerebrospinal fluid pressure. This study provides a new understanding of early brain morphogenesis.

1 Introduction

In the vertebrate embryo, the embryonic brain is initially a relatively straight neuroepithelial tube devoid of constrictions. This cylindrical structure then grows, differentiates, and undergoes dramatic topological transformations as the brain inflates, bends, and locally constricts (Goodrum and Jacobson, 1981; Gato and Desmond, 2009). Boundaries develop to partition the brain tube into the three primary vesicles (forebrain, midbrain, and hindbrain),

and a series of temporary bulges (rhombomeres) form in the hindbrain. These regions vary in size and shape and establish the basic pattern for growth and development of the early brain (Lowery and Sive, 2009). Studies have shown that the boundaries also divide the brain into regions of localized gene expression and play an ongoing morphogenetic role as sites for cell signaling and differentiation (Kiecker and Lumsden, 2005). However, the physical mechanisms that drive brain vesicle formation are not well understood.

The first morphological boundaries to form in the embryonic brain are the prominent mid-hindbrain boundary and the periodic boundaries between the rhombomeres. Studies in zebrafish have begun to address the role of cytoskeletal contraction in boundary morphogenesis. In particular, contraction at the basal (outer) side of the neuroepithelial wall increases the sharpness of the mid-hindbrain boundary (Gutzman et al., 2008), while rhombomere boundaries persist abnormally in brains with overactive myosin activity (Gutzman and Sive, 2010). These studies demonstrate that regulated cytoskeletal contractility is necessary for normal brain morphogenesis, but the specific function of contraction in boundary formation has not been previously explored.

The purpose of this study was to determine the role of actomyosin contraction in vesicle formation in the embryonic chicken brain, which resembles human brain morphology during early development (Gato and Desmond, 2009). Using experiments and computational modeling, we focus on some of the first morphological boundaries that partition the tube, with a special emphasis on the mid-hindbrain boundary.

Our results suggest that differential contractility at the apical side of the neuroepithelium is essential for boundary morphogenesis. To determine the specific contractile mechanisms, we used actomyosin staining patterns and cell shape distributions to define realistic parameters in finite element models for vesicle and rhombomere formation. The models demonstrate that a combination of circumferential contraction at vesicle boundaries and isotropic contraction between boundaries captures realistic vesicle geometries, while longitudinal contraction between boundaries likely generates the rhombomeres. Together, these results shed light on the spatiotemporal cytoskeletal dynamics involved in patterning the early embryonic brain.

2 Materials and Methods

Embryo preparation, dissection, and culture

Fertilized white leghorn chicken eggs were incubated (37 C, 90% humidity) for 36 – 42 hours to reach Hamburger and Hamilton stage 10 to 11 (HH10 – HH11) (Hamburger and Hamilton, 1951). Embryos were removed from the egg using a filter paper carrier (demonstrated in Filas et al. (2011b)) and rinsed in PBS. For subsequent culture, embryos were submerged in Dulbeccos Modified Eagles Medium (Sigma) supplemented with 10% chick serum (Sigma) and 1% penicillin/ streptomycin/neomycin from 5000 U/mL stock (Invitrogen), and superfused with a 95% oxygen, 5% carbon dioxide mixture (Voronov and Taber, 2002).

For isolated brain experiments, the vitelline membrane and surrounding head mesenchyme were dissected from the embryo using pulled glass micropipettes and microscissors under a Leica MZ8 microscope. (Pulled glass micropipettes were also used to intubate the brain in experiments designed to test the potential role of internal pressure in boundary formation; see Fig. S1).

Two different types of isolated brain culture were used in this study. In the first, isolated brains were left attached to posterior tissues to act as an anchor during culture. This

technique enabled changes in morphology to be more readily observed by limiting some of the tissue bending and curling that occurs during standard, fully isolated culture (Filas et al., 2011a). In the second method, fully isolated brains were cultured dorsal side up on semisolid 0.3% agar gels containing culture media prepared in 24-well culture plates. As previously described (Filas et al., 2011a), surface tension at the media-gas interface was used to apply loads to the isolated brains. To simulate normal loading exerted by surrounding tissues, just enough liquid culture medium was added to compress the brain to match morphology in the intact embryo.

Time lapse imaging and optical coherence tomography

Time lapse imaging was performed using both bright field microscopy and optical coherence tomography (OCT). For both systems, embryos were cultured as described above at 37°C using a Biopetechs Delta T4 Culture Dish Controller with heated and humidified 95% oxygen, 5% carbon dioxide gas supplied to the embryo via a Mini-Pump Variable Flow (Fisher Scientific) device.

Bright field images were captured on a Retiga 1300 camera mounted on a Leica DMLB microscope over a user-specified time interval using commercial software (Openlab, PerkinElmer Inc., Waltham, MA). OCT datasets were acquired using a Thorlabs (Newton, NJ) OCT system coupled to a Nikon FN1 microscope. The total number of image stacks to be captured and the delay between each volume were input into the Thorlabs imaging software, and 4-D time lapse datasets were captured. Image analysis, reconstructions and measurements were performed using Volocity software (PerkinElmer Inc.) or ImageJ. Three-dimensional volumes of the brain were automatically generated by cropping surrounding tissues and thresholding the inner cavity of the tube (Filas et al., 2011a).

Biochemical perturbations

Chemical inhibitors were used to assess the role of cytoskeletal contraction in boundary formation. Prior to compartment formation (HH10, 36 hours of incubation), contraction was inhibited using the non-muscle myosin II inhibitor (-)-blebbistatin (Sigma) (15 – 30 μ M). Blebbistatin-treated embryos were cultured in the dark for six hours to HH11 and compared to controls. To ensure that observed results were caused by altered myosin-II activity instead of general toxicity effects, blebbistatin was washed out in a subset of embryos after the initial six hours of treatment. Samples were washed three times in PBS, cultured in normal medium for an additional 10 hours, and compared to embryos cultured for the full 16 hours in blebbistatin (with the treated media refreshed after the first six hours of culture).

To quantitatively assess vesicle formation, the diameter of the mid-hindbrain boundary was compared to the average diameters at the center of the midbrain and hindbrain, as measured from bright field images. If the difference in boundary and vesicle diameter was less than 10%, then boundary formation was said to be inhibited. Note that, in normal HH11 brains, boundary diameter is typically 30–40% less than the vesicles (see Fig. 2C).

Contractility was enhanced using calyculin A (20 nM), a compound with strong inhibitory effects on myosin phosphatase (myosin-II is kept in a hyper-activated, phosphorylated state (Fabian et al., 2007)). These experiments were performed just as the vesicles were beginning to form at HH11– HH11 (39–41 hours of incubation). To show the effect of enhancing contractility while eliminating possible effects due to growth, we treated embryos in a contraction medium of 5mM ATP (Sigma) and 0.05% Triton-X 100 (for cell permeabilization) in DMEM (Hilfer et al., 1977; Maloney and Wakely, 1982). These experiments, also performed at HH11– 11, induced a contractile response in less than 30 minutes.

Fluorescence imaging

Fluorescent staining was used to visualize the distribution of contractile proteins in the brain. Embryos were removed from the egg and immediately fixed in 3.7% formaldehyde. After overnight fixation, samples were rinsed in PBS, and the head mesenchyme was dissected from the brain tube.

For F-actin staining, samples were blocked for two hours in 0.1% Triton X-100 (Sigma) and 1% bovine serum albumin (Sigma). Phalloidin-TRITC (Sigma) was added to this mixture at a final concentration of 0.4 μM for one hour under gentle rotation. Samples were washed in PBS and imaged using a fluorescence Leica DMLB microscope (5X, 10X, 20X objectives). F-actin alignment at the apical wall of the brain tube (see Fig. 4E) was quantified using the intensity gradient technique, as described in Karlon et al. (1998).

For phosphorylated myosin light chain staining, the forebrain and tissue posterior to the hindbrain were removed to ensure antibody could diffuse into the brain. Tissues were blocked and permeabilized in 5% Normal Goat Serum (NGS) and 0.1% Triton X-100 for two hours. Explants were rinsed (3X) in PBS supplemented with 0.1% Tween-20 under gentle rotation. Activated myosin light chain (p-MLC) was located with a rabbit pAb (1:100; Cell Signaling Technology) in 1.5% NGS (overnight) and visualized with a goat anti-rabbit IgG (AlexFluor 488, Invitrogen, overnight application prior to rinsing) as previously described (Zhou et al., 2009).

Cell shapes were visualized at the apical side of the brain tube in HH11⁺ brains using the fixable analog of an FM 1–43 membrane probe (Invitrogen) (Shi et al., 2009). Fixed brains were washed in PBS, the head mesenchyme was dissected from the brain, and the tube was cut in half axially along the dorsal and ventral sides using microscissors. The isolated left and right halves of the brain tube were gently rocked in a fresh solution of FM 1–43 FX in PBS (5 $\mu\text{g}/\text{mL}$) for 10 minutes, washed in PBS, lightly compressed with a small coverslip fragment (weight ≈ 5 –10 mg, similar to flat-mounting methods previously described in Guthrie et al. (1991)), and imaged using a Leica DMLB microscope (20X, 40X objectives).

Quantitative cell shape analysis

Cells were manually outlined and saved using the ROI manager in ImageJ. All images were manually oriented such that the x and y axes in each image corresponded to the longitudinal (z) and circumferential (θ) directions in the brain, respectively (see Fig. 5A). In each region (midbrain, mid-hindbrain boundary, hindbrain, rhombomere, rhombomere boundary), cell shapes were analyzed across the neuroepithelial wall in arrays approximately 3–4 cells wide (see Fig. 5A,B). Tracings were filled, converted to binary, and a watershed algorithm was applied to separate cell borders. Binary images were verified against original tracings to ensure accurate image processing (see Fig. 5B'). From this point forward, we used the open source software *Celltool* developed by Z. Pincus (Pincus and Theriot, 2007) to quantitatively analyze cell shapes.

Briefly, contours were extracted from binary images with each cell represented as a natural cubic spline fit through 100 evenly spaced points (see Fig. 5B''). Contours were aligned and mean cell shapes were calculated for individual regions. Aspect ratio (minor/major axis of the best-fit ellipse) and alignment angle (orientation of the best-fit ellipse relative to the longitudinal direction in the brain tube) were calculated directly from the extracted contours using *Celltool*.

To estimate the effect of coverslip-induced compression on measured cell morphology, we assumed that the cross section bends like a thin circular beam of undeformed radius R_o (to a

first approximation). For an upper bound, the curvature of a compressed circular beam changes from $1/R_o$ to zero. Then, the wall strain at the inner surface is approximately

$$\varepsilon = \frac{H}{2R_o} \quad (1)$$

for full compression, where H is the wall thickness. Inserting the mean values $H = 45 \mu\text{m}$ and $R_o = 150 \mu\text{m}$ yields $\varepsilon = 0.15$. Hence, the effect of compression on measured aspect ratio is relatively small ($\cong 15\%$) and close to the experimental error of the technique. Moreover, this analysis would predict variations in cell aspect ratio between regions of different diameter (e.g. midbrain v. rhombomeres). However, observed cell aspect ratios (but not orientation) were relatively homogeneous in all measured locations, supporting our analysis that flat-mounting does not substantially alter cell morphology.

Wall stress

Local dissection was used to assess the regional stress state in the brain, similar to other studies (Zamir et al., 2003; Varner et al., 2010; Beloussov, 1998). Lateral cuts were made into the neuroepithelium at HH11 in the midbrain, mid-hindbrain boundary, and hindbrain using microscissors. A wound that opens indicates tension normal to the cut direction, whereas a wound that pushes closed indicates compression. Experiments were repeated ($n = 3$) in all regions in both intact and isolated samples. To avoid a wound-healing response, images were taken within 10–15 seconds of making the cut.

Finite-element modeling

Axisymmetric models (circular cylinders) for the brain tube were created using the finite-element software COMSOL Multiphysics (v3.5 COMSOL AB, Burlington, MA). In the undeformed configuration, each model is a tube with a uniform cross section. Presumptive vesicular and boundary lengths were estimated from experimental measurements of brain morphology. Two boundaries are included in the rhombomere model to emphasize periodicity, whereas only the mid-hindbrain boundary is included in the vesicle model because the fore-midbrain boundary does not form until later in development. For boundary conditions, unless noted otherwise, zero axial displacement is specified at one end of the tube, while the other end is free to move axially without rotation (symmetry condition).

Contraction is simulated by specifying negative growth within a thin layer (10% of the total wall thickness: $5 \mu\text{m}$) at the apical (inner) wall of the brain using the theory of Rodriguez et al. (1993). In this theory, the deformation gradient tensor $\mathbf{F}$ is decomposed into a growth tensor $\mathbf{G}$ and an elastic deformation gradient tensor $\mathbf{K}$ relative to the local zero-stress state ($\mathbf{F} = \mathbf{K} \cdot \mathbf{G}$). Methods for implementing this theory in COMSOL software are described in Taber (2008).

A modified neo-Hookean strain-energy density function is used to characterize the pseudoelastic, nearly incompressible neuroepithelium as previously described (Xu et al., 2010; Filas et al., 2011a):

$$W = \frac{1}{2}\mu \left(I_1 J^{-\frac{2}{3}} - 3 \right) + p \left(1 - J - \frac{p}{2\kappa} \right). \quad (2)$$

Here, μ is the small-strain shear modulus, κ is the bulk modulus, J is the elastic volume ratio ($\det \mathbf{K}$), I_1 is the first invariant of the right Cauchy-Green deformation tensor ($\mathbf{K}^T \cdot \mathbf{K}$), and p is a penalty variable introduced for nearly incompressible materials. Since even slight

compressibility can have a significant effect on aspects of the mechanical behavior, we ran the model for various values of the bulk modulus, κ (see Fig. S6). (For an incompressible material, $\kappa \rightarrow \infty$.) Significant differences are only observed when the bulk modulus is similar to, or less than the shear modulus (for comparison, this ratio is $\approx 6-7$ in cartilage (Hayes and Mockros, 1971)). Here, we take $\kappa/\mu = 10^3$.

In terms of W , the Cauchy stress tensor is given by (Taber, 2004)

$$\boldsymbol{\sigma} = J^{-1} \mathbf{K} \cdot \frac{\partial W}{\partial \boldsymbol{\varepsilon}} \cdot \mathbf{K}^T \quad (3)$$

where $\boldsymbol{\varepsilon} = \frac{1}{2}(\mathbf{K}^T \cdot \mathbf{K} - \mathbf{I})$ is the Lagrangian strain tensor for the elastic part of the deformation and $\mathbf{I}$ is the identity tensor.

In the simulations, $\mathbf{G}$ is taken as a diagonal tensor with components G_i . For passive material, $G_i = 1$; for active contraction in direction i , $G_i < 1$. Relative to local cylindrical polar coordinates, contraction components in the circumferential and longitudinal directions (G_θ and G_z) were estimated from experimental data (see below). In the radial direction, we take $G_r = 1$, as changes in wall thickness are small during the stages of vesicle formation (Xu et al., 2010).

Note that the growth tensor $\mathbf{G}$ describes the active change in stress-free dimensions of a tissue element, which does not always entail a change in volume. Here, we prescribe G_θ and G_z to decrease as the tissue contracts, but we set $G_r = 1$. Hence, $\det \mathbf{G} < 1$, which implies that the volume of the inner layer decreases as it contracts. This can be interpreted as fluid being extruded from the cell apex into the cytoplasm. However, since the thickness of the contractile layer is small relative to the total wall thickness ($5 \mu\text{m}$, 10%), this shift in fluid would have a relatively small mechanical effect. Hence, taking the neuroepithelium to be a nearly incompressible material should still be a reasonable approximation.

To account for the concomitant tissue stiffening that occurs during contraction (Ramasubramanian et al., 2008; Zhou et al., 2009; Filas et al., 2011a), we assume that the shear modulus varies as

$$\mu = \frac{\mu_p}{(G_\theta G_z)^a} \quad (4)$$

where μ_p is the passive modulus ($\approx 200 \text{ Pa}$ (Xu et al., 2010)) and a is positive constant. Here, we take $a = 1.5$; the effects of varying this parameter are explored in Fig. S5A.

As a first approximation, we assume that the values of G_θ and G_z within each apical region are reflected in the aspect ratio and orientation angle of the neuroepithelial cells. Our reasoning is based on the following argument. Consider a cell at the inner wall that is round in its zero-stress state. If the cell contracts circumferentially, we would expect it to become shorter in the circumferential direction and longer in the longitudinal direction, with stresses imposed by neighboring cells reducing this deformation somewhat. As shown later, we found the opposite to be true, with cells being elongated in the presumed direction of contraction. Although this may seem contradictory, experimental studies have suggested that cells elongated in a particular direction preferentially contract in the same direction (Gorfinkiel et al., 2009). Presumably, this change in shape facilitates *a priori* alignment of

actomyosin fibers so they can generate more contractile force (Alford et al., 2011). Our estimates for the contraction parameters (G_i) are based on this idea.

We found that three regions showed a statistically significant correlation between cell elongation and orientation indicating contraction of aligned cells: mid-hindbrain boundary (θ), rhombomere boundary (θ), and rhombomere (z) (see Fig. 5E-G). No correlation between these parameters was observed in the primary vesicles (midbrain, hindbrain), suggesting isotropic contraction. In all regions, the average aspect ratio $\overline{AR}$ was consistently ≈ 0.6 . Thus, we take (see Fig. 6)

$$\begin{aligned} \text{Primary vesicles: } \frac{G_\theta}{G_z} &= 1 \\ \text{Boundaries: } \frac{G_\theta}{G_z} &= \overline{AR} = 0.6 \\ \text{Rhombomeres: } \frac{G_z}{G_\theta} &= \overline{AR} = 0.6. \end{aligned} \quad (5)$$

We also assume that the intensities of contractile proteins correlate with contractile strength, defined here by $C = G_\theta G_z$. As shown below, F-actin and phosphorylated myosin light chain are concentrated at the apical wall, but are expressed less intensely at rhombomere boundaries (Fig. 4). From these data, we assume that the magnitude of contraction (C) at the apical wall is given by the first approximations

$$\begin{aligned} \text{Rhombomere boundaries: } C &= G_\theta G_z = 0.75^2 \\ \text{Elsewhere: } C &= G_\theta G_z = 0.5^2. \end{aligned} \quad (6)$$

Taking $C = 0.5^2$ corresponds to 50% shortening in the longitudinal and circumferential directions of a stress-free element. Similar decreases in cell area (3 to 4-fold) have been observed during other contraction-mediated morphogenetic processes, such as ventral furrow formation (Martin et al., 2009) and dorsal closure (Gorfinkel et al., 2009) in *Drosophila*. The effects of varying the value of C are explored in Fig. S5C. Solving equations (5) and (6) gives values for G_θ and G_z in each region, which are specified to change linearly with time.

Statistics

The correlation between angular (cell orientation) and linear (aspect ratio) data was tested as described by Zar (2009). For statistically significant correlations, a linear-circular regression was computed using the equation

$$y_i = b_0 + b_1 \cos(a_i) + b_2 \sin(a_i) \quad (7)$$

where for every cell ($i = 1 \dots n$), y_i is the linear dependent variable (aspect ratio), and a_i is the circular independent variable (alignment angle) (Zar, 2009). To determine the constants b_0 , b_1 , and b_2 , Eq. (7) was fit to the data in a least squares sense using the Matlab routine *lsqcurvefit*.

One-way ANOVA, with post hoc pairwise comparison made using the Bonferroni–Dunn test was used to compare control and blebbistatin-treated (15 and 30 μM) morphologies. A two-tailed, paired t-test was used to compare blebbistatin washout and hyper-contracted morphologies, as well as fluorescence intensity distributions. All data are reported as $\pm$ SD with $p < 0.05$ for statistical significance.

3 Results

Brain morphology during primary vesicle formation

Following neurulation (closure of the neural folds), the embryonic chicken brain is a relatively straight cylindrical tube. Transverse cross sections are round or elliptical except near the forebrain where the optic vesicles evaginate from the cranial end of the brain tube (Fig. 1A, A'). By HH11⁻ (approximately 4 hours culture from HH10), the basic constrictions (mid-hindbrain boundary, MH; fore-midbrain boundary, FM) that separate the primary brain vesicles (forebrain, F; midbrain, M; hindbrain, H) are apparent, but the tube remains relatively round and uniform in transverse cross section (Fig. 1B, B'). The tube continues to expand through HH11⁺ (8 hours) as primary cranial flexure (ventral bending of the forebrain) occurs (Fig. 1C, C').

Next, the cranial neuropore and the spinal neurocoel close at the cranial and caudal ends of the brain tube, respectively. Cerebrospinal fluid (CSF) pressure then builds in the lumen, and the brain undergoes a period of rapid expansion (Desmond and Levitan, 2002). At HH12 (12 hr), boundary lumens are elongated in the dorsoventral direction relative to the laterally elongated vesicles (Fig. 1D, D'). Note that cross sections of the rhombomeres (R) and rhombomere boundaries (Rb) in the hindbrain remain circular.

These images show that all transverse sections of the brain tube are generally round as morphological boundaries form from HH10–11. As intraluminal pressure builds toward HH12, increases in brain size become more significant and differences in cross-sectional (elliptical) orientation become apparent between regions.

Boundary formation is intrinsic to the neuroepithelium

First, we explored whether external loads exerted by CSF pressure and surrounding tissue play a role in boundary formation. Neither intubating the brain with a small glass micropipette to relieve pressure ($n = 8$, Fig. S1A, A') nor periodically dissecting away tissue that normally occludes the spinal neurocoel from HH10 inhibited boundary formation ($n = 3$, Fig. S1B, B'). We also cultured isolated HH10 brains for six hours to eliminate loads due to surrounding tissues. Boundaries formed in isolated explants, similar to intact controls (Fig. S2, arrows). These data suggest that boundary formation in the chick brain is intrinsic to the neuroepithelium.

Reducing cytoskeletal contractility inhibits boundary formation

Because contractility is involved in numerous morphogenetic processes (Ettensohn, 1985; Martin, 2010), we investigated the role of cytoskeletal contraction in vesicle formation. Whole stage HH10⁻ to HH10 embryos without FM or MH boundary constrictions were submerged in culture media containing the nonmuscle myosin-II inhibitor blebbistatin (15 or 30 μ M) and cultured for six hours. Boundaries formed in control embryos ($n = 18 / 18$, Fig. 2A, A'), but not in blebbistatin (bleb)-treated embryos ($n = 1 / 19$, Fig. 2B, B', C). Boundary formation was rescued, however, following blebbistatin washout and 10 hours of subsequent culture ($n = 7 / 8$, Fig. 2B'', C).

To quantify the effects of blebbistatin (bleb) treatment on boundary formation, we compared mid-hindbrain (MH) boundary diameter to average primary vesicle (midbrain and hindbrain) diameter (Fig. 2C). In control brains incubated for 6hr, MH boundary diameter was approximately 30–40% less than the vesicles. In bleb-treated brains there was little difference between MH and vesicle diameters, but after washout of bleb and overnight culture (14hr), MH diameter became significantly less than the vesicles ($\approx 30\%$). Hence, bleb-treatment inhibited boundary formation, an effect that was reversible by washout of the

drug. Together, these results suggest that myosin-II based contractility is required for boundary formation.

Enhancing cytoskeletal contractility deepens vesicle boundaries

As a corollary to the myosin-inhibitor experiments, we artificially enhanced contractility by exposing embryos to calyculin A or ATP. We used two different compounds because calyculin and (permeabilized) ATP treatment operate on different time scales. Effects of calyculin are gradual and occur over several hours of culture, potentially allowing growth or other morphogenetic processes to affect the results. In contrast, the effects of ATP are relatively rapid (order of minutes) and demonstrate the capacity of the tissue to contract at a given time during development (Hilfer et al., 1977; Maloney and Wakely, 1982). Experiments using ATP eliminate most effects due to growth as the cell cycle length is between 8 and 12 hours during early brain development (Goodrum and Jacobson, 1981). Because of the permeabilization step, however, long-term (≥ 1 hr) ATP experiments are not feasible, and hence, studying the effects of enhanced cytoskeletal contraction during morphogenesis are better suited to calyculin treatment.

Cytoskeletal contractility was enhanced in brains with partially formed boundaries at HH11. Control brains cultured for 4 hours expanded, but vesicle boundaries did not deepen (Fig. 3A, B). In contrast, similar culture with calyculin A (20nM) caused the mid-hindbrain boundary to close nearly completely (Fig. 3B', arrows; Fig. 3G). Culture of embryos in media enriched with ATP (5mM, 0.05% Triton-X 100) induced a similar response in only 25 minutes (Fig. 3C'). Corresponding controls in detergent alone did not contract to a similar degree, indicating that this response is not purely due to the calcium influx that occurs during tissue permeabilization (Fig. 3C). Note that the FM boundary (arrowheads in Fig. 3B', C') deepens in calyculin, but not in ATP-treated brains. Since the FM boundary forms later during development than the MH boundary, the different effects on the FM boundary may be due to the duration of the experiments (4 hours versus 25 minutes).

To confirm that observed morphological responses were intrinsic to the brain tube, these experiments were repeated on isolated brains (Fig. 3D-F). Morphologies were similar to intact cultures, except shorter culture times were required to induce similar effects.

Geometric changes were quantified by measuring the change in cross-sectional area of the MH boundary (via OCT) and the axial length relative to controls (Fig. 3G-J). In most cases, the cross-sectional area and tube length decreased significantly in treated brains (both intact and isolated). We note that isolated brains tended to contract more than intact samples, consistent with our recent finding that the overall contractility of the neuroepithelium increases as external loads decrease (Filas et al., 2011a). Taken together, these results show that elevated contraction causes the MH boundary to deepen and the brain tube to shorten.

Contractile protein content varies regionally in the brain tube

Cytoskeletal protein distributions provide important clues into contractile mechanisms. At HH10, F-actin was localized primarily to the apical (inner) side of the neuroepithelium and was uniformly distributed along the length of the brain tube (Fig. 4A). As boundaries began to form, however, F-actin intensity became relatively lower in rhombomere boundary regions, while the intensity of F-actin at the MH boundary remained similar to the surrounding regions (Fig. 4B, quantified in Fig. S3). Some basal F-actin staining was observed at higher magnifications at the MH boundary, but the intensity was far weaker than that observed on the apical side (Fig. 4B'). Phosphorylated myosin light chain expression patterns were similar to that of F-actin (Fig. 4C). At HH12, boundary formation was largely complete, and F-actin was predominately absent at rhombomere boundaries but persisted at

the larger vesicle boundaries (Fig. 4D). During vesicle formation, F-actin filaments were circumferentially aligned at the apical wall of the MH boundary (shown at HH11⁺ in Fig. 4E, E').

The relatively uniform staining intensities at the midbrain, MH boundary, and the hindbrain suggest that the overall magnitude of contraction between these regions is similar during normal development. Reduction in F-actin and p-MLC content at rhombomere boundaries, however, indicates reduced contractile strength in these regions during morphogenesis. Alignment of F-actin filaments in the MH boundary suggests that anisotropic contraction also plays a role in vesicle formation. The degree of anisotropy was estimated from measurements of cell shape and orientation (see below).

Cell shape and orientation vary regionally in the brain tube

Cell shapes on the inner surface of the wall were analyzed from membrane staining in five regions: midbrain, mid-hindbrain boundary, hindbrain, rhombomere boundary, and rhombomere (Figs. 5, S4). (Note that cell shapes are shown for all regions and at higher magnification in Fig. S4). Cell boundaries were outlined and converted to binary, and contours were extracted for further analysis (Fig. 5A-B''). Cell shapes were relatively homogeneous, as mean shapes were elliptical (aspect ratio: 0.61 ± 0.03) with small variations in shape in all regions analyzed (Fig. 5C, $n = 5$ embryos and $n > 300$ cells in each region).

Analysis of orientation showed that cells were aligned primarily in the circumferential direction in the MH and Rh boundaries and in the longitudinal direction in the rhombomeres (Fig. 5D). No predominant orientation was found in the brain vesicles (midbrain, hindbrain), however. In the aligned regions, cellular aspect ratio correlated with orientation angle (Fig. 5E-G).

These data show that cells at the apical side of the neuroepithelium are relatively homogeneous in shape but differ in orientation. Cells are aligned circumferentially at the MH and Rh boundaries, and longitudinally in the rhombomeres. Although the aspect ratio was similar, no predominant orientation was found elsewhere. To interpret these results mechanistically, we note that Alford et al. (2011) found that contractile direction and force correlate with the direction and magnitude of cell elongation, respectively. Therefore, taken together with our actin and myosin staining data, our results suggest that contraction is primarily circumferential in the MH boundary, isotropic in primary vesicles, longitudinal in rhombomeres, and weakly circumferential in rhombomere boundaries. These findings are summarized in Fig. 6.

Differential contraction as a mechanism for vesicle formation

To determine whether the contraction patterns inferred from our data are consistent with the observed morphogenesis, we created computational models (see Materials and Methods). For simplicity, each model consists of a circular pseudoelastic tube with either two presumptive vesicles or three rhombomeres separated by boundary regions. The tube is initially straight, and axisymmetric contraction is prescribed in thin regions adjacent to the lumen (Figs. 7A, A'; 8A, A'), with relative contractile protein density and cell shape used to estimate contraction magnitude and directionality (Figs. 7B, 8B; see Materials and Methods). The effects of varying these parameters, as well as the dependence of tissue stiffness on contractility and material compressibility, are explored in Figs. S5 and S6.

First, we consider the model for primary vesicle formation (Fig. 7). Consistent with our data (Fig. 6), the magnitude of the simulated contraction is homogeneous at the apical wall, but occurs circumferentially in the boundary and isotropically in the vesicles. Since external

forces are excluded in this model, results are compared to experimental data from isolated brain culture (see Fig. S2). To quantify changes in geometry, relative changes in brain length and lumen cross-sectional areas were measured during culture at 0, 2.5, and 5hr from HH10 (Fig. 7C). Axial length and lumen area decreased during vesicle formation in isolated culture, with the cross-sectional area of the MH boundary decreasing more than the vesicles (Fig. 7C). The model reasonably reproduced these trends, as well as overall brain morphology (Fig. 7B, B', C). These results suggest that the contractile mechanisms inferred from cell shape and contractile protein distributions produce changes in morphology consistent with those occurring during formation of the MH boundary.

Next, we consider the model for rhombomere formation (Fig. 8). Here, based on our experimental data (Fig. 6), longitudinal contraction is simulated between boundaries, with weaker circumferential contraction at the boundaries (Fig. 8A'). The model generates bulges similar to normal rhombomere morphology (Fig. 8B, B'). This mechanism of contraction shortens the tube, similar to the model prediction (Fig. 8C). However, whereas the model also predicts small decreases in lumen diameter, measurements gave a small increase. This discrepancy may be caused by tissue growth, which is not included in the model. These data suggest a potential role for longitudinal contraction during rhombomere development.

Additional model tests

When models are used to help understand morphogenesis, it is important to recognize their limitations. For example, a model that predicts correct morphology under normal conditions may not necessarily be realistic, as other combinations of mechanisms may yield similar shapes. Hence, it is important to test a model using other data (Ramasubramanian et al., 2006; Varner et al., 2010).

Toward this end, the cross-sectional area and length change measurements provide some additional data (Figs. 7C and 8C). To understand the significance of the general agreement with these results, we investigated the effect of each contraction component on the deformation of the vesicle formation model (Fig. 9). If circumferential contraction is simulated in the boundary region, but the vesicles remain entirely passive, a boundary forms but the tube does not shorten axially as observed in experiments (Fig. 9B). On the other hand, isotropic contraction in the vesicles with a passive boundary region shortens the tube and forms a boundary. However, the boundary is considerably shallower and longer than experimentally-observed shapes (Figs. 9C). Lastly, we tested the effect of isotropic contraction in the boundary alone (Fig. 9D). Here, the longitudinal component of contraction caused the boundary to bow outward. These results suggest that a combination of circumferential contraction at the boundary and isotropic contraction in the surrounding vesicles is necessary to fully capture the changes in tube diameter and length observed during primary vesicle formation.

To test the model further, we examined the axial stress state in the brain tube by making lateral cuts into the neuroepithelial wall (Fig. 10). Longitudinal cuts (to probe circumferential stresses) were also made but were difficult to visualize using standard bright field microscopy. In both intact and isolated brains, and in all regions analyzed (midbrain, MH boundary, and hindbrain), the wall of the brain tube did not open following the incision (dotted lines, Fig. 10B). Rather, the wall pushed together (sometimes even producing local bulges), indicating that most of the wall of the brain is in a state of axial compression. In contrast, wounds in the head mesenchyme (HM) opened in intact embryos, indicating axial tension in this tissue (to equilibrate the compression in the brain). Similarly, the model predicts a relatively uniform distribution of compressive axial stress ($\sigma_z < 0$) across the neuroepithelial wall following boundary formation except in the apical regions, where contraction generates tension (Fig. 10C).

Next, we explored the mechanism of contraction in the vesicles in greater detail using a combination of experimental and model perturbations. As observed in isolated brain culture (Fig. 3H, J), artificially enhancing contraction shortens the tube axially and deepens the mid-hindbrain boundary. Notably, similar effects are observed in the vesicle model when the overall magnitude of contraction is increased ($G_{\theta}G_z$ decreases; see Fig. S5C). In addition, an increase in contractility should reduce cell size. Consistent with this idea, cell areas in calyculin-treated brains were smaller than controls ($\approx 50\%$) after two hours of culture (Fig. 11A, A').

As another predictive test for the model, we cut off the posterior end of the hindbrain, and the free walls of the isolated brain curled inward (Fig. 11B, B'). To simulate this condition, the lower boundary was released in the vesicle formation model. Tension at the inner wall causes free end of the tube to bend inward, similar to the mechanically-perturbed morphology (Fig. 11C).

Together, these data show that the model reasonably reproduces axial stress distributions and mechanically perturbed shapes in the brain tube. In addition, we observed smaller cells in hyper-contracted brains relative to controls. This observation supports our underlying modeling assumption that contractile force correlates with changes in cell size. These results provide further support that our models accurately capture the mechanical behavior of the brain during vesicle formation.

Why do different contraction patterns create vesicle and rhombomere boundaries?

Lastly, we investigated why the embryo may use different contractile mechanisms to create the primary vesicles and rhombomeres. We began by switching the proposed mechanisms between the vesicle and rhombomere models. Interboundary longitudinal contraction with relatively weak circumferential contraction in the boundary region ('rhombomere mechanism') produced a boundary in the vesicle model (Fig. 12A), but circumferential contraction at rhombomere boundaries with isotropic contraction elsewhere ('vesicle mechanism') yielded relatively little deformation (Fig. 12B). These results show why the rhombomere mechanism may be needed to create the relatively short rhombomeres, but the same mechanism seems to work as well for the vesicles. So, why do the vesicles use a different mechanism? As discussed next, the answer to this question may lie in events that occur later in development.

The primary vesicle boundaries (mid-hindbrain and fore-midbrain) are permanent structures that eventually separate the cerebral aqueduct from the third and fourth ventricles in the adult (Lowery and Sive, 2009). However, rhombomere boundaries are transient structures that facilitate spatially dependent patterns of axonal migration, cell differentiation, and gene expression, before disappearing later in development (Kiecker and Lumsden, 2005). Following vesicle formation, CSF pressure in the brain lumen rapidly increases and accelerates brain expansion (Fig. 1) (Desmond and Jacobson, 1977; Desmond et al., 2005; Jelinek and Pexieder, 1968). This expansion is caused by a combination of viscoelastic inflation and stress-induced growth. We speculated that the structural robustness of the vesicles as lumen pressure increases depends on the specific morphogenetic mechanism.

To test this possibility, we specified an increasing lumen pressure (Fig. 12C) in the two models for primary vesicle formation (Figs. 7B and 12A). For the vesicle mechanism, the ratio of mid-hindbrain boundary to midbrain lumen diameter remains relatively constant as pressure increases (Fig. 12C), but the ratio steadily increases for the rhombomere mechanism as the boundary eventually disappears (Fig. 12D). The reason for these differences is that the stiffening that accompanies contraction allows the boundaries to resist deformation when subjected to pressure loads. Hence, circumferential contraction within the

MH boundary region may be needed to maintain the boundary geometry in the pressurized brain tube. Together, these results suggest that spatially varying patterns of contraction depend on both early and later development of the brain tube.

4 Discussion

The results from this study suggest that regional differences in contractile strength and anisotropy along the apical side of the wall drive formation of the early vesicles in the brain of the chicken embryo. In particular, we postulate that the boundary between the midbrain and hindbrain is created by a combination of circumferential contraction in the boundary regions and isotropic contraction (circumferential and longitudinal) in the vesicles. In contrast, longitudinal contraction between boundaries, with only weak circumferential contraction within the boundaries, likely generates the rhombomeres. Our results also indicate that these mechanisms are intrinsic to the neuroepithelium, as vesicle boundaries form in both isolated (Fig. 3E, S2) and depressurized brains (Fig. S1).

Both laboratory experiments and computational modeling have provided supporting evidence. The importance of cytoskeletal contraction was shown by experiments where inhibiting nonmuscle myosin II with blebbistatin prevented vesicle formation, while enhancing contractility using calyculin A or ATP deepened vesicle boundaries. Cell shape and contractile protein distributions appear to support the postulated regional variations in contractile strength and anisotropy, and a finite element model has shown that these variations are consistent with observed changes in morphology. Experiments used to test model predictions offer additional data supporting our hypothesis.

To our knowledge, the morphogenetic role of cytoskeletal contractility has not been previously explored during the stages of vesicle formation in the chicken embryo. This is surprising considering that early brain development in chicken is similar to that in human embryos, and abnormal vesicle structure has been linked to various neurological disorders including schizophrenia, autism, and mental retardation (Lowery and Sive, 2009). In contrast, the problem of neurulation has received considerable attention for many decades (Schoenwolf and Smith, 1990). Recently, Kinoshita et al. (2008) have shown that the contractility inhibitors blebbistatin and Y27632 prevent neurulation in the chicken embryo. Moreover, spatiotemporal patterns of rho, F-actin, and phosphorylated myosin light chain expression suggest a mechanistic role for contractility in driving neural tube closure. These results indicate that regulated patterns of contraction are necessary for formation of the brain tube, which derives from the anterior end of the neural tube. Likewise, the present study shows that cytoskeletal contractility is also involved in subsequent vesicle development.

Studies in zebrafish embryos have begun to address the brain vesicle formation problem. Gutzman et al. (2008) have shown that basal contraction (on the outside of the wall) increases the sharpness of the mid-hindbrain boundary, which normally has a kink-like appearance in fish, as opposed to the comparatively smooth (no kinks) MH boundary in the chick (Fig. 1). This observation is consistent with the less intense F-actin staining on the basal side relative to the apical side in the normal chicken brain (Fig. 4B'). There also are some important differences in brain tube morphology between chicken and zebrafish. In the chicken embryo, for example, the brain vesicles develop from an initially open tube, whereas in zebrafish, the vesicles arise from a comparatively closed tube (Harrington et al., 2009). With these differences in initial structure, it is not surprising that there may be differences in the mechanisms that pattern the early brain across species (Filas et al., 2012).

Other mechanisms also can contribute to this process. For example, the vesicles may grow faster than the boundary regions in the circumferential direction, creating similar morphology. Supporting this possibility, Lowery and Sive (2005) found higher rates of cell

proliferation in the hindbrain relative to the mid-hindbrain boundary during vesicle formation in zebrafish. However, treatment with the cell proliferation inhibitor aphidicolin did not inhibit vesicle formation. Guthrie et al. (1991) also found higher rates of cell proliferation in rhombomere interboundaries across a wide range of developmental stages in the chicken brain. Here, we observed that the rhombomeres shorten in the longitudinal direction during culture from HH10 to HH11 (Fig. 8C), a result more consistent with a contraction-based mechanism. Underlying circumferential growth, however, may be one potential reason why rhombomere diameters slightly increased in these experiments (Fig. 8C).

Classic studies using ATP have shown that elevated contraction induces precocious invagination of the lens of the eye and the thyroid placode (Hilfer et al., 1977; Maloney and Wakely, 1982). Treating tissues in this manner demonstrates what actomyosin contraction can accomplish at a particular time point during development, rather than what occurs during normal morphogenesis. Using a similar approach, we found significant circumferential constriction of the mid-hindbrain boundary and axial shortening of the brain tube within 30 minutes at HH11. This result demonstrates the importance of contraction in boundary formation, as effects due to growth are eliminated. Morphologically similar responses found using calyculin A further confirmed a role for cytoskeletal contractility in sculpting the brain tube.

Except in rhombomere boundaries, our data suggest that the apical side of the wall contracts with relatively uniform magnitude ($G_\theta G_z = \text{constant}$), as indicated by F-actin and phosphorylated myosin light chain staining (Fig. 4). We speculate that the brain tube is normally maintained in a partially contracted state to provide structural stability, as it is compressed by surrounding tissues such as the notochord, foregut, and the head mesenchyme (Fig. 10B). A baseline level of contractility could help prevent collapse under increasing loads before a significant lumen pressure builds later in development (Jelinek and Pexieder, 1968; Gato and Desmond, 2009). Moreover, a uniform contractile strength between the midbrain, mid-hindbrain boundary, and hindbrain is consistent with past results demonstrating that brain stiffness is relatively uniform in these regions at HH11 (Xu et al., 2010). In addition, contraction patterns prescribed in the model appear to produce deformations consistent with previously measured strains on the inner wall of the brain during early development (Filas et al., 2008). Specifically, more longitudinal shortening was found in the vesicles relative to the mid-hindbrain boundary.

Studies have shown that actomyosin-based contraction regulates the spatial organization of cells in the embryo (Martin and Wieschaus, 2010) and is essential for progenitor cell sorting (Krieg et al., 2008). Preventing cell mixing across morphological boundaries is critical for cell fate determination as populations begin to differentiate. Recent work in *Drosophila* suggests that tension resulting from active contraction can prevent cell mixing and establish morphological compartments (Landsberg et al., 2009; Monier et al., 2010).

Importantly, cells do not mix across boundaries in the rhombomeres of the early chick brain (Fraser et al., 1990), a result that has been traditionally attributed to differential cell adhesion. Regionally varying patterns of F-actin and phosphorylated myosin light chain expression reported here (Fig. 4) raise the possibility that differential tension also could play a role in preventing cell intermixing in the rhombomeres. Interestingly, transmurals actomyosin cables form at rhombomere boundaries (similar to the lineage restricting actomyosin ‘fences’ reported in *Drosophila* (Monier et al., 2010)) at later stages (HH17) of development (Guthrie et al., 1991). Hence, initial lineage restriction could be a consequence of differential contractility, which is later reinforced by the formation of transmurals actomyosin cables. This possibility warrants further study.

Not addressed here are the genetic and molecular mechanisms responsible for the specific location of the boundaries. Recent studies have shown that the organizer gene *Fgf8* is expressed in a narrow band at the mid-hindbrain boundary and plays a role in lineage restriction (Sunmonu et al., 2011; Tossell et al., 2011; Dworkin et al., 2012). It would be interesting to examine whether inducing local, abnormal constrictions in the brain tube via banding or clipping (procedures that have been applied to the embryonic chicken heart (DeAlmeida et al., 2007; Rugonyi et al., 2008)), could lead to altered *Fgf8* expression patterns. Such an approach could provide important information about the interplay between mechanical forces and gene expression during early brain development.

Lastly, we propose that producing similar but somewhat different geometries sometimes requires different morphogenetic mechanisms, perhaps explaining the need for regional variations in contractility in the brain. For example, the circumferential contraction mechanism that creates the mid-hindbrain boundary is unable to generate significant constriction between rhombomeres (Fig. 12B), because the rhombomeres are too short for this mechanism to be effective. However, circumferential contraction in the mid-hindbrain boundary makes the primary vesicles more structurally robust, as increasing lumen pressure has relatively little effect on boundary shape as the brain grows (Fig. 12C). Consistent with this idea, boundaries that separate the primary vesicles are permanent, whereas rhombomere boundaries are transient and do not need to withstand the increasing lumen pressure during development.

A limitation to the current study is the inability to perform live imaging of the cell shape changes that occur at the apical wall during boundary formation. Such an approach would provide useful insight into the dynamics of cell shape distributions reported here and could also improve model accuracy. For example, using techniques developed by Graner et al. (2008) and Blanchard et al. (2009), live imaging data could be seamlessly integrated within a continuum modeling framework, taking into account both the elastic and fluid-like material behavior in the tissue. The current model assumes a purely elastic material and does not take into account processes such as cell intercalation, which may involve a more viscous-like response. Moreover, dynamic images of cell shapes could be used to calibrate the temporal changes in the growth tensor. Future studies should consider developing live-imaging techniques for comprehensively tracking epithelial cells in the chicken embryo, with a focus on visualizing these dynamics in complex 3-D structures such as the early brain.

In summary, results from our study show that circumferential contraction plays a crucial role in brain vesicle formation. Contraction mechanisms vary regionally at the apical neuroepithelium to pattern the brain tube, and these differences may be a function of brain geometry and morphogenetic plasticity. Future work is needed to define the genetic and molecular mechanisms that regulate these contractile patterns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants R01 GM075200 and R01 NS070918 (LAT), as well as a fellowship for BAF from NIH T90 DA022871 and the Mallinckrodt Institute of Radiology, Washington University. We gratefully acknowledge Zachary Pincus (Yale University) and Yanrong Shi (Washington University, laboratory of Steven Bassnett) for helpful advice in analyzing and visualizing cell shapes, respectively. We also thank Ruth Okamoto (Washington University) for developing the code used to calculate F-actin fiber orientation.

References

- Alford PW, Nesmith AP, Seywerd JN, Grosberg A, Parker KK. Vascular smooth muscle contractility depends on cell shape. *Integrative biology : quantitative biosciences from nano to macro*. 2011; 3(11):1063–70. [PubMed: 21993765]
- Beloussov, LV. *The Dynamic Architecture of a Developing Organism: An Interdisciplinary Approach to the Development of Organisms*. Kluwer; Dordrecht, the Netherlands: 1998.
- Blanchard GB, Kabla AJ, Schultz NL, Butler LC, Sanson B, Gorfinkiel N, Mahadevan L, Adams RJ. Tissue tectonics: morphogenetic strain rates, cell shape change and intercalation. *Nat Methods*. 2009; 6(6):458–464. [PubMed: 19412170]
- DeAlmeida A, McQuinn T, Sedmera D. Increased ventricular preload is compensated by myocyte proliferation in normal and hypoplastic fetal chick left ventricle. *Circ Res*. 2007; 100(9):1363–1370. [PubMed: 17413043]
- Desmond ME, Jacobson AG. Embryonic brain enlargement requires cerebrospinal fluid pressure. *Developmental Biology*. 1977; 57(1):188–198. [PubMed: 863106]
- Desmond ME, Levitan ML. Brain expansion in the chick embryo initiated by experimentally produced occlusion of the spinal neurocoel. *Anatomical Record*. 2002; 268(2):147–159. [PubMed: 12221721]
- Desmond ME, Levitan ML, Haas AR. Internal luminal pressure during early chick embryonic brain growth: descriptive and empirical observations. *Anat Rec A Discov Mol Cell Evol Biol*. 2005; 285(2):737–747. [PubMed: 15977221]
- Dworkin S, Darido C, Georgy SR, Wilanowski T, Srivastava S, Ellett F, Pase L, Han Y, Meng A, Heath JK, Lieschke GJ, Jane SM. Midbrainhindbrain boundary patterning and morphogenesis are regulated by diverse grainy head-like 2-dependent pathways. *Development (Cambridge, England)*. 2012; 139(3):525–36.
- Ettensohn CA. Mechanisms of epithelial invagination. *Quarterly Review of Biology*. 1985; 60:289–307. [PubMed: 3901078]
- Fabian L, Troszczanek J, Forer A. Calyculin A, an enhancer of myosin, speeds up anaphase chromosome movement. *Cell Chromosome*. 2007; 6:1. [PubMed: 17381845]
- Filas BA, Bayly PV, Taber LA. Mechanical stress as a regulator of cytoskeletal contractility and nuclear shape in embryonic epithelia. *Ann Biomed Eng*. 2011a; 39(1):443–454. [PubMed: 20878237]
- Filas BA, Knutsen AK, Bayly PV, Taber LA. A new method for measuring deformation of folding surfaces during morphogenesis. *J Biomech Eng*. 2008; 130:61010.
- Filas BA, Oltean A, Beebe DC, Okamoto RJ, Bayly PV, Taber LA. A potential role for differential contractility in early brain development and evolution. *Biomechanics and modeling in mechanobiology*. 2012
- Filas BA, Varner VD, Voronov DA, Taber LA. Tracking morphogenetic tissue deformations in the early chick embryo. *Journal of visualized experiments : JoVE*. 2011b; (56):e3129.
- Fraser S, Keynes R, Lumsden A. Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature*. 1990; 344(6265):431–435. [PubMed: 2320110]
- Gato A, Desmond ME. Why the embryo still matters: CSF and the neuroepithelium as interdependent regulators of embryonic brain growth, morphogenesis and histiogenesis. *Dev Biol*. 2009; 327(2):263–272. [PubMed: 19154733]
- Goodrum GR, Jacobson AG. Cephalic flexure formation in the chick embryo. *J Exp Zool*. 1981; 216(3):399–408. [PubMed: 7276892]
- Gorfinkiel N, Blanchard GB, Adams RJ, Martinez Arias A. Mechanical control of global cell behaviour during dorsal closure in *Drosophila*. *Development (Cambridge, England)*. 2009; 136(11):1889–98.
- Graner F, Dollet B, Raufaste C, Marmottant P. Discrete rearranging disordered patterns, part I: robust statistical tools in two or three dimensions. *The European physical journal E, Soft matter*. 2008; 25(4):349–69.
- Guthrie S, Butcher M, Lumsden A. Patterns of cell division and interkinetic nuclear migration in the chick embryo hindbrain. *J Neurobiol*. 1991; 22(7):742–754. [PubMed: 1722508]

- Gutzman JH, Graeden EG, Lowery LA, Holley HS, Sive H. Formation of the zebrafish midbrain-hindbrain boundary constriction requires laminin-dependent basal constriction. *Mech Dev.* 2008; 125(11–12):974–983. [PubMed: 18682291]
- Gutzman JH, Sive H. Epithelial relaxation mediated by the myosin phosphatase regulator Mypt1 is required for brain ventricle lumen expansion and hindbrain morphogenesis. *Development.* 2010; 137(5):795–804. [PubMed: 20147380]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *Journal of Morphology.* 1951; 88:49–92.
- Harrington MJ, Hong E, Brewster R. Comparative analysis of neurulation: first impressions do not count. *Molecular reproduction and development.* 2009; 76(10):954–65. [PubMed: 19653285]
- Hayes WC, Mockros LF. Viscoelastic properties of human articular cartilage. *Journal of applied physiology.* 1971; 31(4):562–8. [PubMed: 5111002]
- Hilfer SR, Palmatier BY, Fithian EM. Precocious evagination of the embryonic chick thyroid in ATP-containing medium. *J Embryol Exp Morphol.* 1977; 42(1):163–175.
- Jelinek R, Pexieder T. The pressure of encephalic fluid in chick embryos between the 2nd and 6th day of incubation. *Physiol Bohemoslov.* 1968; 17:297–305. [PubMed: 4234872]
- Karlon WJ, Covell JW, McCulloch AD, Hunter JJ, Omens JH. Automated measurement of myofiber disarray in transgenic mice with ventricular expression of ras. *The Anatomical record.* 1998; 252(4):612–25. [PubMed: 9845212]
- Kiecker C, Lumsden A. Compartments and their boundaries in vertebrate brain development. *Nat Rev Neurosci.* 2005; 6(7):553–564. [PubMed: 15959467]
- Kinoshita N, Sasai N, Misaki K, Yonemura S. Apical accumulation of Rho in the neural plate is important for neural plate cell shape change and neural tube formation. *Mol Biol Cell.* 2008; 19(5):2289–2299. [PubMed: 18337466]
- Krieg M, Arboleda-Estudillo Y, Puech PH, Kafer J, Graner F, Muller DJ, Heisenberg CP. Tensile forces govern germ-layer organization in zebrafish. *Nat Cell Biol.* 2008; 10(4):429–436. [PubMed: 18364700]
- Landsberg KP, Farhadifar R, Ranft J, Umetsu D, Widmann TJ, Bittig T, Said A, Jülicher F, Dahmann C. Increased cell bond tension governs cell sorting at the *Drosophila* anteroposterior compartment boundary. *Current biology : CB.* 2009; 19(22):1950–5. [PubMed: 19879142]
- Lowery LA, Sive H. Initial formation of zebrafish brain ventricles occurs independently of circulation and requires the *nanog* and *atp1a1a.1* gene products. *Development.* 2005; 132(9):2057–2067. [PubMed: 15788456]
- Lowery LA, Sive H. Totally tubular: the mystery behind function and origin of the brain ventricular system. *BioEssays : news and reviews in molecular, cellular and developmental biology.* 2009; 31(4):446–58.
- Maloney C, Wakely J. Microfilament patterns in the developing chick eye: their role in invaginations. *Experimental eye research.* 1982; 34(6):877–86. [PubMed: 7200910]
- Martin AC. Pulsation and stabilization: contractile forces that underlie morphogenesis. *Dev Biol.* 2010; 341(1):114–125. [PubMed: 19874815]
- Martin AC, Kaschube M, Wieschaus EF. Pulsed contractions of an actinmyosin network drive apical constriction. *Nature.* 2009; 457(7228):495–499. [PubMed: 19029882]
- Martin AC, Wieschaus EF. Tensions divide. *Nature cell biology.* 2010; 12(1):5–7.
- Monier B, Péliissier-Monier A, Brand AH, Sanson B. An actomyosin-based barrier inhibits cell mixing at compartmental boundaries in *Drosophila* embryos. *Nature cell biology.* 2010; 12(1):60–5. sup pp 1–9.
- Pincus Z, Theriot JA. Comparison of quantitative methods for cell-shape analysis. *Journal of microscopy.* 2007; 227(Pt 2):140–56. [PubMed: 17845709]
- Ramasubramanian A, Latacha KS, Benjamin JM, Voronov DA, Ravi A, Taber LA. Computational model for early cardiac looping. *Ann Biomed Eng.* 2006; 34(8):1355–1369.
- Ramasubramanian A, Nerurkar NL, Achtien KH, Filas BA, Voronov DA, Taber LA. On Modeling Morphogenesis of the Looping Heart Following Mechanical Perturbations. *Journal of Biomechanical Engineering.* 2008; 130:61018.

- Rodriguez EK, Omens JH, Waldman LK, McCulloch AD. Effect of Residual Stress on Transmural Sarcomere Length Distributions in Rat Left Ventricle. *American Journal of Physiology*. 1993; 264:H1048–H1056. [PubMed: 8476082]
- Rugonyi S, Shaut C, Liu A, Thornburg K, Wang RK. Changes in wall motion and blood flow in the outflow tract of chick embryonic hearts observed with optical coherence tomography after outflow tract banding and vitelline-vein ligation. *Physics in medicine and biology*. 2008; 53(18):5077–91. [PubMed: 18723935]
- Schoenwolf GC, Smith JL. Mechanisms of Neurulation: Traditional Viewpoint and Recent Advances. *Development*. 1990; 109:243–270. [PubMed: 2205465]
- Shi Y, Barton K, De Maria A, Petrash JM, Shiels A, Bassnett S. The stratified syncytium of the vertebrate lens. *Journal of cell science*. 2009; 122(Pt 10):1607–15. [PubMed: 19401333]
- Sunmonu NA, Li K, Guo Q, Li JYH. Gbx2 and Fgf8 are sequentially required for formation of the midbrain-hindbrain compartment boundary. *Development (Cambridge, England)*. 2011; 138(4): 725–34.
- Taber, LA. *Nonlinear Theory of Elasticity: Applications in Biomechanics*. World Scientific; New Jersey: 2004.
- Taber LA. Theoretical study of Belousov's hyper-restoration hypothesis for mechanical regulation of morphogenesis. *Biomech Model Mechanobiol*. 2008; 7:427–441. [PubMed: 17909868]
- Tossell K, Kiecker C, Wizenmann A, Lang E, Irving C. Notch signalling stabilises boundary formation at the midbrain-hindbrain organiser. *Development (Cambridge, England)*. 2011; 138(17):3745–57.
- Varner VD, Voronov DA, Taber LA. Mechanics of head fold formation: investigating tissue-level forces during early development. *Development (Cambridge, England)*. 2010; 137(22):3801–11.
- Voronov DA, Taber LA. Cardiac looping in experimental conditions: the effects of extraembryonic forces. *Developmental Dynamics*. 2002; 224:413–421. [PubMed: 12203733]
- Xu G, Kemp PS, Hwu JA, Beagley AM, Bayly PV, Taber LA. Opening angles and material properties of the early embryonic chick brain. *Journal of biomechanical engineering*. 2010; 132(1):011005. [PubMed: 20524743]
- Zamir EA, Srinivasan V, Perucchio R, Taber LA. Mechanical asymmetry in the embryonic chick heart during looping. *Ann Biomed Eng*. 2003; 31(11):1327–1336. [PubMed: 14758923]
- Zar, JH. *Biostatistical Analysis*. 5. Prentice Hall/Pearson; Upper Saddle River, NJ: 2009.
- Zhou J, Kim HY, Davidson LA. Actomyosin stiffens the vertebrate embryo during crucial stages of elongation and neural tube closure. *Development*. 2009; 136(4):677–688. [PubMed: 19168681]

Figure 1. Morphology of the early embryonic chick brain

Optical coherence tomography reconstructions of the lumen of the brain tube. (A–D) Longitudinal view, transverse cross sections, and (A'–D') sagittal view during stages of vesicle formation (HH10–12; OV, optic vesicle; F, forebrain; FM, fore-midbrain boundary; M, midbrain; MH, mid-hindbrain boundary; H, hindbrain; Rb, rhombomere boundary; R, rhombomere). Supplementary Movies 1–4 allow the reader to view these reconstructions in any orientation. Scale bars: 250 μm.

Figure 2. Inhibiting cytoskeletal contraction prevents vesicle boundary formation

Dotted lines indicate lumen. (A, A') In a control HH10 embryo, boundaries (arrows) formed during six hour culture to HH11 (F, forebrain; M, midbrain; H, hindbrain). (B, B') Blebbistatin (bleb)-treated embryo: vesicle formation was inhibited. Note that at these low concentrations, development of other organs such as the optic vesicles (OV) and the heart tube (HT) appear to be normal. (B'') Washout of blebbistatin and overnight culture in control medium rescued vesicle formation (arrows: boundaries). (C) In control brains, mid-hindbrain diameter (D_{MH}) was approximately 30% less than that of the surrounding vesicles (D_V) following culture to HH11. These diameters were similar in bleb-treated brains (30 or 15 μ M), indicating that a boundary did not form (*** = $p < 0.001$ relative to controls). Bleb-washout and overnight culture restored the boundary (* = $p < 0.05$ relative to bleb-treated brains). Scale bar: 200 μ m.

Figure 3. Hyper-contraction deepens vesicle boundaries

(A) HH11 embryo after boundaries first form. Dark arrows denote the mid-hindbrain (MH) boundary. Dotted lines indicate lumen. (B) The brain grows but vesicle boundaries do not deepen in control embryos cultured for 4 hours. (B') In contrast, the lumen of the MH boundary closes in calyculin-treated (20 nM) brains. (C, C') Alternative hyper-contraction experiments were performed via permeabilized ATP (5mM) treatment. Control brains cultured in detergent alone contracted less than ATP-treated samples; the lumen of the MH boundary closes in ATP-treated samples, similar to calyculin-treated embryos. The fore-midbrain boundary does not deepen, as occurs in calyculin-treated brains (arrowheads in B', C'), a result likely due to different culture times (see Results section). (D-F') Corresponding experiments were performed on isolated brains. Morphologically similar contractile responses occurred in isolated brains with shorter culture times. (G, H) The MH boundary lumen significantly decreases in cross-sectional area (A/A_0 , measured from transverse OCT sections) following calyculin or permeabilized ATP treatment in both intact and isolated samples (treated = spotted bars). (I, J) Change in axial length (L/L_0 , shown in panel A) between the FM boundary and the hindbrain-rhombomere boundary. Statistically significant decreases in length were observed in most conditions following calyculin or ATP exposure (** = $p < 0.01$, * = $p < 0.05$, number of experiments indicated under each bar). Scale bar: 200 μm .

Figure 4. Contractile protein localization in the brain

(A) At HH10, F-actin was concentrated in a relatively uniform distribution at the apical side of the brain tube (Ap = apical; Ba = basal). (B) At HH11, F-actin staining intensity was relatively lower between rhombomere boundaries (asterisks), but staining near the mid-hindbrain boundary (MH) remained relatively homogeneous (M = midbrain; H = hindbrain). (B') Although the majority of F-actin localized to the apical side of the neuroepithelium, some local regions of F-actin were present near kinks in the tissue on the basal side (arrowheads). (C) At HH11, phosphorylated myosin light chain (p-MLC) was similarly located at the apical side with a reduction in staining intensity at rhombomere boundaries (asterisks). (D) By HH12⁺, relatively little F-actin was observed at rhombomere boundaries (asterisks), but F-actin content remained similar at the fore-midbrain (FM) and mid-hindbrain (MH) boundaries relative to surrounding vesicles. (E) On the apical side of HH11⁺ brains, F-actin filaments were oriented predominantly in the circumferential (θ) direction at the MH boundary. (E') Relative frequency circular histogram shows this alignment for the boxed region (MH) in E, as calculated using an intensity gradient algorithm (Karlson et al., 1998). Scale bars: 50 μ m.

Figure 5. Cell shape analysis

Cells borders (plasma membranes) were (A) stained on the apical side of isolated brain explants, (B) manually outlined, and (B') converted to binary. (B'') From these data, contours were extracted and resampled such that each cell was represented by a smooth curve. (C) Mean cell shapes were computed from extracted contours at the midbrain, mid-hindbrain boundary, hindbrain, rhombomere boundary, and rhombomere ($n = 5$ embryos and $n > 300$ cells in each location). Mean cell shapes were relatively elliptical in all regions analyzed. (D) Relative frequency circular histograms show circumferential cell alignment at the MH and rhombomere boundaries, and longitudinal alignment in rhombomeres. Cells are more randomly oriented in the vesicles (midbrain, hindbrain). (E-G) Aspect ratio correlated with cell orientation in regions with aligned cells. (E, F) Cells at the mid-hindbrain and rhombomere boundaries were aligned and elongated in the in the circumferential (θ) direction. (G) A similar trend was found in the longitudinal (z) direction between rhombomere boundaries. Correlations were statistically significant ($p < 0.05$ in each case); the corresponding linear-circular regression (see Eq. (7)) is shown for each region. Similar correlations were not found in the midbrain and hindbrain. Scale bar: $50 \mu\text{m}$.

Figure 6. Differential contraction schematic

Contraction patterns at the inner wall of the brain tube as suggested by experimental data. Magnitude of contraction is similar in all regions (red) but is relatively reduced in rhombomere boundaries (yellow). Circumferential contraction occurs at the mid-hindbrain (MH) boundary with isotropic contraction in the vesicles. Longitudinal contraction between weakly contractile rhombomere boundaries (Rb) creates periodic bulges. Proposed mechanisms are simulated in Figs. 7 and 8.

Figure 7. Model for vesicle formation

(A) 3-D representation of finite-element model for the brain tube. Outlined region corresponds to axisymmetric model geometry shown in (A'). Model consists of the posterior half of the midbrain (M) and the anterior half of the hindbrain (H), separated by the mid-hindbrain (MH) boundary. (B) Contraction occurs only within a thin layer next to the lumen (blue and green regions). Circumferential contraction in the boundary region and isotropic contraction in the vesicles causes the boundary to form. (Values for G_θ and G_z were estimated from distributions of contractile proteins; see Materials and Methods.) (B') Representative isolated brain cultured through HH11. Model shape is similar to brain morphology. (C) Model (solid lines) reasonably reproduces geometric changes in isolated brains (symbols, $n = 6$). Average lumen cross-sectional areas were computed from OCT cross sections (e.g., Fig. S2) (A_V = average vesicle area computed from midbrain and

hindbrain; A_{MH} = mid-hindbrain area). Axial length (L) is distance between midbrain and hindbrain (measured at maximum diameter of the vesicles). Scale bar: 50 μm .

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Figure 8. Model for rhombomere formation

(A) 3-D representation of model for the rhombomeres (R). Outlined region corresponds to model geometry in (A'). Two prospective boundaries subdivide the tube. Contraction is specified in a thin layer of the inner wall (blue and green regions). (B) Longitudinal contraction between boundary regions and weak circumferential contraction at boundaries causes bulges to form. (Values for G_θ and G_z were estimated from distributions of contractile proteins; see Materials and Methods.) (B') Deformed model geometry resembles normal rhombomere morphology. (C) Consistent with the model (solid lines), axial length (L) of the first rhombomere decreases and average tube diameter (D_R = diameter of rhombomere; D_{Rb} = diameter of rhombomere boundary) does not change significantly during culture from HH10–11 (symbols, $n = 7$). Scale bar: 50 μm .

Figure 9. Contraction mechanisms and model shape

Type of contraction in each apical region is indicated. (A) Nominal model for vesicle formation. Circumferential contraction between isotropically contracting vesicles shortens the tube and generates a boundary. (B) Circumferential contraction only in the boundary region. A constricted boundary forms but the tube does not shorten and the vesicles do not decrease in diameter. (C) Isotropic contraction only between the boundaries. The diameter and axial length of the tube decrease, but only a shallow boundary forms. (D) Isotropic contraction only in the boundary region. The tube bows outward slightly at the boundary.

Figure 10. Axial stress in brain tube

(A, A', B, B') Lateral (transverse) cuts were made in the neuroepithelium at various craniocaudal locations in intact and isolated brains at HH11 (M = midbrain; MH = mid-hindbrain boundary; H = hindbrain; HM = head mesenchyme). Arrowheads show cut location. (A, B) Intact embryos (A', B') Isolated brains. In all regions analyzed (midbrain, mid-hindbrain boundary, hindbrain) in both intact and isolated samples, the wound in the brain did not open. The neuroepithelial walls pushed together (dotted lines), suggesting the brain is in a state of axial compression. The head mesenchyme (HM) opens in intact embryos, however, indicating axial tension in this tissue. (C) Computational model predicts a relatively homogeneous distribution of compressive axial stress ($\sigma_z < 0$) outside the inner contractile ring, consistent with experimental results. Scale bars: 100 μm .

Figure 11. Perturbation studies

(A, A') Cells are smaller ($\approx 50\%$) and aligned in the circumferential (θ) direction at the mid-hindbrain boundary in hyper-contracted brains. Cells at the apical wall of control and calyculin-treated brains are shown after two hours of culture from HH11. (B) The posterior half of an isolated hindbrain was removed (dashed line). (B') The neuroepithelium (dotted lines) curled inward. (C) Releasing the lower boundary in the model qualitatively reproduces this result. Scale bars: $50\ \mu\text{m}$.

Figure 12. A hypothesis for regional variations in boundary formation mechanisms
 (A, B) Contraction mechanisms are switched in the vesicle (Fig. 7B) and rhombomere (Fig. 8B) models. The 'rhombomere mechanism' forms a boundary in the vesicle model, but the 'vesicle mechanism' produces relatively little deformation in the rhombomere model. (C, D) The effects of increasing lumen pressure (p) were explored in the vesicle models. Vesicle formation models before (purple) and after (orange) a 70 Pa (≈ 7.1 mm H₂O) increase in lumen pressure (consistent with experimental measurements from Jelinek and Pexieder (1968)). Tube diameter increases in both models, but the diameter becomes uniform in the model utilizing the 'rhombomere mechanism'. These results indicate that the 'vesicle mechanism' is more structurally robust to increasing internal pressure than the 'rhombomere mechanism'.