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A Potential Role for Differential Contractility in Early Brain Development and Evolution

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Abstract

Differences in brain structure between species have long fascinated evolutionary biologists. Understanding how these differences arise requires knowing how they are generated in the embryo. Growing evidence in the field of evolutionary developmental biology (evo-devo) suggests that morphological differences between species result largely from changes in the spatiotemporal regulation of gene expression during development. Corresponding changes in functional cellular behaviors (morphogenetic mechanisms) are only beginning to be explored, however. Here we show that spatiotemporal patterns of tissue contractility are sufficient to explain differences in morphology of the early embryonic brain between disparate species. We found that enhancing cytoskeletal contraction in the embryonic chick brain with calyculin A alters the distribution of contractile proteins on the apical side of the neuroepithelium and changes relatively round cross sections of the tubular brain into shapes resembling triangles, diamonds, and narrow slits. These perturbed shapes, as well as overall brain morphology, are remarkably similar to those of corresponding sections normally found in species such as zebrafish and frog. Tissue staining revealed relatively strong concentration of F-actin at vertices of hyper-contracted cross sections, and a finite element model shows that local contraction in these regions can convert circular sections into the observed shapes. Another model suggests that these variations in contractility depend on the initial geometry of the brain tube, as localized contraction may be needed to open the initially closed lumen in normal zebrafish and *Xenopus* (frog) brains, whereas this contractile machinery is not necessary in chick brains, which are already open when first created. We conclude that interspecies differences in cytoskeletal contraction may play a larger role in generating differences in morphology, and at much earlier developmental stages, in the brain than previously appreciated. This study is a step toward uncovering the underlying morphomechanical mechanisms that regulate how neural phenotypic differences arise between species.

1 Introduction

Recent studies have shown that much of the morphological diversity between species is likely caused by differences in the regulation of gene expression during embryonic development (Carroll, 2008). Consequently, some researchers have speculated that temporal

changes in gene regulatory networks form the basis of morphological evolution (Carroll, 2008; Olson, 2006). The association of these changes with corresponding changes in morphogenetic mechanisms is not well understood, however (Breuker et al., 2006; Northcutt, 2001; Salazar-Ciudad et al., 2003). For each change in form that occurs during evolution, identifying the underlying biophysical mechanism should help explain how a particular feature changes from one phenotype to another. This idea is a major tenet of the relatively new field of evolutionary developmental biology (evo-devo).

The present study focuses on the brain. During evolution, the vertebrate brain has undergone dramatic changes in morphology, with interspecies variations in brain structure being most apparent in mature organisms. For example, the cerebral cortex of most larger mammals is highly convoluted, while brains of smaller mammals are relatively smooth (Welker et al., 1990). While it is known that size and shape of the brain correlate with cognition and other functions (Hofman, 1989; Barton and Harvey, 2000), how these different morphologies have evolved remains relatively unknown.

Most proposed mechanisms of evolutionary change in the developing brain have focused on late-stage differences in embryonic cell proliferation and differentiation between ventricular compartments (Northcutt, 2001; Sylvester et al., 2010). Differences in patterning during early brain development have received considerably less attention (Sylvester et al., 2010; McGowan et al., 2011). Recent work suggests, however, that variations in morphology and spatiotemporal patterns of gene expression during the earliest stages of brain development may help explain differences in form between adult species (Hofmann, 2010; McGowan et al., 2011).

Here, we show that spatiotemporal variations in cytoskeletal contraction are sufficient to explain some of the morphological differences observed in the embryonic brain tube of various species. Interestingly, we found that enhancing contractility in chick embryos induces their brains to acquire shapes that strongly resemble the characteristic brain shapes found in zebrafish and frog (*Xenopus laevis*) embryos. Computational modeling confirms that these various shapes are consistent with locally elevated cytoskeletal contraction in regions that exhibit relatively strong concentrations of F-actin. These results suggest a link between regulated tissue contractility and brain evolution.

2 Materials and Methods

Embryo culture and optical coherence tomography (OCT)

Fertilized white Leghorn chicken eggs were incubated (37° C, 90% humidity) for 45 – 48 hours to reach Hamburger-Hamilton stages 12⁻ to 12 (HH12⁻ - HH12) (Hamburger and Hamilton, 1951). Embryos were removed from the egg using a filter paper carrier and cultured while submerged in growth medium as previously described (Voronov and Taber, 2002). To enhance contractility, calyculin A (Sigma, St. Louis, MO) was mixed into the medium at a final concentration of 20 nM prior to culture. In other experiments, tissues were cultured in 60 μM (–)-blebbistatin (Sigma, St. Louis, MO) in the dark to inhibit myosin-II based contractility. Bright field images were captured during each experiment using a CCD camera (COHU, Model 4915, Coway, CA) attached to a dissecting microscope (Leica MZ8, Wetzlar, Germany).

Zebrafish embryos were fixed in 3.7% p-formaldehyde at 22hpf (hours post-fertilization) and imaged. Live *Xenopus laevis* embryos were obtained at stage 19 as described by Nieuwkoop and Faber (1967) and cultured in 0.2X Marc's modified Ringer's solution. These embryos were fixed and imaged at various time points through stage 36.

OCT datasets were acquired using a Thorlabs (Newton, NJ) OCT system coupled to a Nikon FN1 microscope. Image analysis and 3-D luminal reconstructions (Filas et al., 2011) were performed using Volocity software (PerkinElmer Inc., Waltham, MA). In addition to standard image cropping, contrast optimization, and noise filtering techniques, datasets were aligned into desired orientations using image stack reslicing tools. This method was especially valuable when analyzing OCT datasets of zebrafish brains. The early zebrafish embryo is attached to a large, non-planar yolk sac that can make a priori sample alignment problematic. Hence, post-hoc volume realignment tools were useful to display transverse optical cross sections in the desired orientation.

Quantitative analysis of brain morphology

Observed differences in brain morphology between species and culture conditions are complex, and hence, measuring standard quantities such as changes in the aspect ratio or surface area of the brain lumen would be of limited value. Therefore, to quantify differences in brain morphology, we performed a principal components analysis of brain shapes using the open source software *Celltool*, developed by Z. Pincus. Employing a systematic analysis of shape ensures that meaningful variance in the data is not overlooked. Further details behind the development and implementation of this robust analytical technique are described in Pincus and Theriot (2007).

Bright field microscopy images (dorsal view) were used to obtain longitudinal shapes. The apical (inner) wall of the brain lumen was manually outlined from the hindbrain through the forebrain (excluding the optic vesicles). For zebrafish embryos, the yolk sac was dissected away prior to imaging so brain structure could be more clearly visualized. *Xenopus* embryos were omitted in the bright field imaging shape analysis because brain shape could not be clearly visualized due to embryo pigmentation. In all cases, OCT images were used for analysis of transverse cross sections. Usually, OCT contours could be automatically extracted after smoothing and thresholding the neuroepithelial wall from the internal lumen, similar to the method used to reconstruct luminal morphologies in 3-D.

All images were subsequently size-normalized and converted to binary in ImageJ. Brain shapes were extracted from binary images using a contour-extraction method and analyzed using *Celltool*. Briefly, these contours were resampled to have 100 evenly spaced points and fit with a natural cubic spline to yield a parametric plane curve. Principal components analysis was performed on the aligned and processed contours to generate shape modes describing the maximum variance in the data. For the mathematical details behind the implementation of this technique, readers are again directed to Pincus and Theriot (2007).

Pairs of coordinates corresponding to the relative contributions of the first and second principal components were plotted for each sample in the shape space (see Fig. 2C). Although these values have no simple physical interpretation, the plotted positions accurately reflect the relative contribution of each of the different axes (as opposed to plotting in terms of standard deviations from the mean, which would be physically interpretable, but would obscure the relative contribution of each component). Because the results from these relatively small datasets passed tests for both normality and equal variance, standard parametric, multi-sample statistical comparisons were performed along individual shape components as described below. It should be emphasized, however, that this is not a general statistical technique to be applied to all principal component shape-space data, as distributions can be decidedly non-Gaussian. In such instances, more involved non-parametric analyses are warranted (Dye et al., 2005).

Quantitative fluorescence imaging

Following culture, chicken embryos were fixed in p-formaldehyde for at least one hour. Samples were rinsed in PBS, and tissues surrounding the anterior brain tube were removed to prevent diffusion artifacts and to yield clearer images. Isolated brains were blocked for two hours in 0.1% Triton X-100 (Sigma) and 5% normal goat serum (Sigma). Tissues were stained in this mixture with Alexa-546 phalloidin (final concentration: 0.2 U/mL, Invitrogen, Carlsbad, CA) and DRAQ-5 (final concentration: 0.5 M, Cell Signaling Technology, Danvers, MA), protected from light, and gently rocked for 90 minutes. Brains were washed in PBS, embedded in 3.5% agarose, sectioned (50 μ m thick) with a vibratome (Model OTS 400, Electron Microscopy Sciences, Fort Washington, PA), and imaged with a Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Zebrafish and *Xenopus* brains were stained as above in the chick, except, in lieu of dissecting away tissues surrounding the brain, individual sections were stained following slicing to prevent diffusion artifacts.

Pixel intensities were measured in ImageJ as specified in Figs. 4A''–D'', 5D, and S3B (line width: 10 pixels). Resulting transmural fluorescence distributions were fit with a polynomial function (to smooth the data), normalized to the background staining intensity and total wall thickness, and averaged together as shown for each geometry. Average pixel intensities and standard deviations are plotted as function of wall thickness for regional comparisons (minimum of three embryos per distribution). The method is demonstrated with an illustrative brain section in Figure S2.

Finite element modeling

Two-dimensional plane strain models of transverse cross sections of the embryonic brain were developed using the commercial finite-element software COMSOL Multiphysics (v3.5 COMSOL AB, Burlington, MA). The effects of local apical contraction were simulated in tubes of circular (250 μ m inner diameter) or slit-like cross-section (major axis: 250 μ m inner diameter, minor axis: 20 μ m inner diameter) (see Figs. 4A'–D', 5C). Total wall thickness is 65 μ m in both models including an inner contractile layer (10% of the wall thickness) and an outer passive layer (90% of the wall thickness). Some models were further divided into subsections (Figs. 4, 5), enabling contraction to be specified in local regions of the inner wall as observed experimentally. Meshes were made sufficiently dense such that further refinement did not significantly affect model solutions.

Apical contraction is specified in the circumferential direction using the growth theory of Rodriguez et al. (1994), which assumes the existence of local stress-free configurations. Using this theory, we model contraction as negative growth with material stiffening. Briefly, 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 current zero-stress state ($\mathbf{F} = \mathbf{K} \cdot \mathbf{G}$). During active contraction, $\mathbf{G}$ changes the stress-free configuration of the material element, while $\mathbf{K}$ generates stress and ensures geometric compatibility. Here, $\mathbf{G}$ is taken as a diagonal tensor with components G_i . For passive material, $G_i = 1$; for active contraction in direction i , $G_i < 1$. For circumferential contraction in the model, we specify $G_\theta < 1$ and $G_r = G_z = 1$ relative to local cylindrical polar coordinates. For details on the implementation of this theory in COMSOL software, readers are directed to Taber (2008).

Since material properties of the early brain are only moderately non-linear (Xu et al., 2010), 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., 2011):

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

Here, μ and κ are the small-strain shear and bulk modulus, respectively, I_1 is the first invariant of the right Cauchy-Green deformation tensor ($\mathbf{K}_T \cdot \mathbf{K}$), J is the elastic volume ratio ($\det \mathbf{K}$), and p is a penalty variable. In this theory, stress is associated only with elastic deformation, with the Cauchy stress tensor given by (Taber, 2004)

$$\boldsymbol{\sigma} = J^{-1} \mathbf{K} \cdot \frac{\partial W}{\partial \boldsymbol{\varepsilon}} \cdot \mathbf{K}^T \quad (2)$$

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.

Material properties are defined by Eq. (1) with $\mu = 200$ Pa for the passive brain (Xu et al., 2010) and κ taken three orders of magnitude larger than μ . Because tissue stiffness correlates with cytoskeletal contractility, as a first approximation μ is specified to increase linearly with contraction magnitude. A maximum value of $\mu = 1000$ Pa is reached when $G_0 = 0.1$.

Statistics

All data are reported as mean $\pm$ SD. Statistical analyses were done using SigmaStat (SPSS Science). Interregion changes in cross-sectional area and shape-space distributions were compared using one-way analysis of variance with post hoc pairwise comparison made using the Bonferroni / Dunn Test ($p < 0.05$ for statistical significance).

3 Results

Normal interspecies differences in early brain morphology

The early embryonic brain forms at the anterior end of the neural tube, which is created by the morphogenetic process of neurulation (Schoenwolf and Smith, 1990). Although the vertebrate brain begins as a simple tube composed of a one-cell-thick neuroepithelium, some substantial interspecies differences in morphology exist in the early embryo. For example, the lumens of some brain tubes are open initially (e.g., human, mouse, and chicken), while others are comparatively closed and slit-like (e.g., frog and fish) (Lowery and Sive, 2004; Harrington et al., 2009). For illustration, consider the brain of the chicken embryo at stage 12⁻ of Hamburger and Hamilton (HH12⁻, approximately 46 hours incubation, comparable to three weeks of development in the human embryo) (Hamburger and Hamilton, 1951). The lumen is open throughout the brain tube, although a series of constrictions have formed to delineate the three primary brain vesicles (forebrain, F; midbrain, M; hindbrain, H; Fig. 1A). During a subsequent five hours of culture, the brain grows in response to increasing cerebrospinal fluid pressure in the lumen (Desmond and Jacobson, 1977), but vesicle boundaries do not deepen (Fig. 1B). Longitudinal sections and 3-D reconstructions of the lumen confirm that control embryos maintain a predominately open anterior brain tube (Figs. 1B,E,H).

Hyper-contracted chick brain morphology resembles that of zebrafish and *Xenopus*

Recent work has suggested an important role for cytoskeletal contractility in early zebrafish brain morphogenesis (Gutzman et al., 2008; Gutzman and Sive, 2010). To test whether contractility is important in species with comparatively open brain tubes following closure

of the neural folds, we cultured chicken embryos in media containing calyculin A (20 nM) to enhance actomyosin contraction of the cytoskeleton. Calyculin is a phosphatase inhibitor with a high specificity for myosin phosphatase (Fabian et al., 2007). Inhibiting myosin phosphatase prevents the dephosphorylation of myosin light chain, which leaves myosin in a hyper-activated state (Gutzman and Sive, 2010) (Supplementary Information - SI). In treated embryos, the boundaries between the primary vesicles deepened dramatically, to the point where the fore-midbrain (FM) and mid-hindbrain (MH) boundaries closed nearly completely (Fig. 1C). This hyper-contracted morphology strongly resembled the morphology of normal zebrafish brains at 22 hpf (hours post fertilization) in longitudinal section (Figs. 1C,D,F,G) (18). Three-dimensional reconstructions of the luminal space (Filas et al., 2011) of hyper-contracted chick brains and normal zebrafish brains reveal that the mid-hindbrain (MH) boundary remained open only near the ventral floor of the brain tube (Fig. 1I,J). These data suggest that increasing contractile force in the early chick brain produces morphology similar to that of normal zebrafish brains.

To quantitatively assess variations in shape between these groups, we performed a principal components analysis of the bright-field brain morphology data (see Materials and Methods). Mean shapes ($n = 7$) confirmed that calyculin-treated chicken brains strongly resembled control zebrafish brains, possibly even more so than control chicken brains (Fig. 2A). To test this possibility, these data were pooled to generate a cumulative shape model (Fig. 2B). Across the first shape mode, the depth of the vesicle boundaries and the curvature of the vesicles vary, while the second shape mode captures more subtle differences in shape in the longitudinal direction, especially in the boundary regions. According to this analysis, the first and second shape modes (describing approximately 66 and 13% of the variance in the data, respectively) effectively separated the three sample groups in the shape-space (Fig. 2C). Along the first component, which represented the largest source of variance in the data, shapes were significantly different between control and calyculin-treated chicken brains but not between calyculin-treated chicken brains and control zebrafish brains ($p < 0.001$). However, in the second principal component, which describes a smaller fraction of the data variance, calyculin-treated chicken brains were significantly different from zebrafish ($p < 0.01$).

These results suggest that the shapes of calyculin-treated chicken brains are more similar to normal brains from a different species than normal brains from the same species. However, although these brains share many morphological similarities, some reproducible, albeit more subtle, differences in morphology do exist between calyculin-treated chicken brains and control zebrafish brains. Notably, the longitudinal length of the fore-midbrain boundary appears to be greater in zebrafish brains relative to the calyculin-treated chicken brains.

As shown in Fig. 1E–J, embryonic brains are distinctly 3-D structures. Hence, we next explored whether morphological similarities also were present in transverse cross section. Moreover, we examined the generality of our results by including *Xenopus laevis* (stage 33/34) embryos in our analysis (Fig. 3). (We were not able to visualize the brain structure of this embryo using traditional bright field microscopy.) Mouse brains were also considered, but comparisons were complicated by the incomplete closure of the neural tube (SI, Fig. S1).

Cross-sectional morphology of the brain tube was analyzed via OCT imaging. Representative morphologies (Fig. 3, left) and average shapes (Fig. 3, right, $n = 5$ embryos) are reported for each sample group. In all regions shown (FM, M, MH, H), transverse cross sections of untreated chick brains were rounded and free of sharp edges (Fig. 3, top row of sections). In the FM and MH boundary regions, the lumens were elongated in the dorsoventral direction, while the lumens of the ventricles (M, H) were either circular or transversely elongated. In contrast, cross sections in hyper-contracted chicken brains

contained sharp vertices and had shapes that were remarkably similar to corresponding sections in normal zebrafish and *Xenopus* brains (Fig. 3, lower three rows of sections). Distinct shapes characterized the midbrain (diamond), hindbrain (triangle), and fore-midbrain boundary (dorsoventral aligned slit). Mid-hindbrain boundary morphology was slightly more heterogeneous, but in all sections the luminal space of this region was small relative to the neighboring ventricles.

Taken together, these results show that elevated cytoskeletal contraction transforms the early brain tube of the chick embryo into a shape that strongly resembles that of fish and frog brains at comparable stages of development. These morphological similarities are apparent in both longitudinal and transverse sections.

Differential contractility may underlie interspecies differences in phenotype

To reconcile the striking similarities in morphology between calyculin-treated chick brains and normal fish and frog brains, we examined the transformation of the rounded chicken brain to the sharply constricted, hyper-contracted phenotype. We speculated that these morphological changes were caused by regional differences in contractility. Since cytoskeletal proteins that regulate contractile activity (e.g., rho and phosphorylated myosin light chain, p-MLC) colocalize with F-actin on the apical side of the neuroepithelium (Kinoshita et al., 2008), we used F-actin intensity as a marker for contractile strength. In control brains, F-actin was distributed relatively uniformly around the apical (inner) side of the wall (Fig. 4A,A'; green). In calyculin-treated embryos, however, greater accumulations of F-actin were observed in regions with sharp vertices (Figs. 4B–D, B'–D'; S2). These observations suggest a relationship between locally elevated contraction (in regions of high actin density) and cross-sectional morphology.

To test this idea, we developed computational (finite element) models for brain cross sections (Fig. 4A'–D'). In all cases, the model is initially a circular tube. As expected, simulating uniform circumferential contraction within a relatively thin ring adjacent to the lumen (apical side) decreases the radius of the tube while maintaining a circular shape (Fig. 4A'). In contrast, simulating localized contraction only in regions of the highest actin density produces vertices and cross-sectional shapes similar to those found experimentally (Fig. 4B'–D'). These results indicate that the characteristic cross-sectional morphologies observed in hyper-contracted brain tubes may be caused by differential cytoskeletal contraction. Moreover, the morphological similarity to normal fish and frog brains suggest that differential contraction determines the shape of the early brain across species.

To explore this possibility further, we stained normal zebrafish brains for F-actin. In zebrafish midbrains beginning to open into a diamond shape, we found local accumulations of F-actin at the mediolateral hinge points (Fig. 5B, asterisks; Fig. 5D). Further analysis revealed F-actin density to be 1.33 ± 0.05 times higher ($n = 4$) at the mediolateral (asterisks) relative to the dorsoventral (arrowheads) hinge points in these samples (Fig. 5B,D). (This ratio was 1.08 ± 0.03 ($n = 3$) in *Xenopus* embryos, which we suspect is due to slight differences in embryo staging as described in Fig. S3). These actin staining patterns are similar to those found in hyper-contracted chicken brains, presumably supporting the differential contraction idea.

Interspecies contraction patterns depend on initial brain shape

Next, we explored the reasons for these differences in contractile and morphological patterning between species. We speculated that these differences are dictated by the shape of the brain immediately following neurulation. Notably, the brain tube is initially open in human, mouse, and chicken embryos, but is comparatively closed and slit-like in zebrafish

and *Xenopus laevis* embryos (Copp et al., 2003; Lowery and Sive, 2004; Harrington et al., 2009). Moreover, previous studies have shown that inhibiting cytoskeletal contractility in zebrafish using the nonmuscle myosin-II inhibitor blebbistatin prevents opening of the midbrain lumen (Nyholm et al., 2009) (Fig. 5A), suggesting that contraction plays a role in opening the tube.

To integrate these observations, we created a model for an initially slit-like brain section (Fig. 5C). When local apical contraction is simulated at two lateral hinge points, the lumen opens into a diamond shape, as observed experimentally (Fig. 5C). This result is consistent with the idea that opening the zebrafish brain requires cytoskeletal contraction, which also gives the cross section its characteristic diamond-like shape. In contrast, this mechanism is not needed in the chick brain, which is already open following neurulation.

Induced changes in brain morphology are caused by steadily increasing contraction

Thus far, our computational models have shown that simulating circumferential contraction in regions consistent with experimental distributions of F-actin is sufficient to reproduce various cross-sectional shapes observed in the brain tube (Fig. 4). Not considered in this analysis, however, are the temporal dynamics of the induced contractile response. Following the time course of calyculin exposure indicates that changes in tissue morphology are not immediate, but occur gradually over the five-hour duration of these experiments (Fig. 6A). Morphological changes are first observed in the fore-midbrain (FM) and mid-hindbrain (MH) boundaries, as rounded cross sections become asymmetric within three hours of culture. As contraction continues, the midbrain and hindbrain acquire characteristic diamond and triangle shapes, respectively, in transverse cross section.

For the mid-hindbrain boundary, which contracted the greatest amount, we investigated whether the model reasonably captured the changes in geometry that occur in this region following calyculin exposure. In this analysis, the boundary was imaged in six embryos and luminal shapes were extracted at equal time intervals, following 0, 1.5, 3, and 4.5 hours of calyculin exposure. Mean shapes were calculated (Fig. 6B) and compared with the complementary simulation. Taking $G_0 = 1, 0.7, 0.4$, and 0.1 provided reasonable agreement for steadily increasing contraction (Fig. 6C). These results suggest that the drug-induced contraction increases approximately linearly with time during the incubation period.

Induced changes in brain morphology are irreversible

Lastly, we investigated the reversibility of the response after the contractile phenotype (local vertices in transverse section) had been established at the mid-hindbrain boundary. We replaced the culture media of calyculin-treated embryos with media containing the myosin-II inhibitor blebbistatin ($60 \mu\text{M}$; a concentration previously shown to significantly alter cytoskeletal stiffness and nuclear shape in the early chick brain (Filas et al., 2011) (Fig. 7A–C, A'–C'). Washout of calyculin and addition of blebbistatin led to only a slight reversal of the original, contracted phenotype with an overall increase in tissue size (Fig. 7C, C'), a morphology that remained hyper-contracted in appearance relative to controls (Fig. 7D, D'). Notably, the lumen of the mid-hindbrain boundary did not return to a circular shape following washout, as the induced triangular shape persisted but opened slightly (Fig. 7B', C'). As a separate control, in embryos treated with blebbistatin alone, the lumen of the mid-hindbrain boundary expanded in the dorsal-ventral direction as it relaxed (Fig. 7E'), but gross morphology did not seem to be affected (Fig. 7E). Hence, it seems that morphogenetic shape changes caused by enhanced cytoskeletal contraction are largely unidirectional.

4 Discussion

The results of the present study suggest a link between the spatiotemporal regulation of cytoskeletal contraction and interspecies brain patterning in the early vertebrate embryo. Enhancing contractility in the chick embryo causes dramatic changes in the shape of the brain tube, as it takes a form that strongly resembles those of normal zebrafish and *Xenopus laevis* brains at comparable stages of development (Figs. 1–3). Our data indicate that these changes in shape are caused by a transition from uniform to localized contraction along the apical side of the neuroepithelial wall (Fig. 4). From these findings, we speculate that interspecies variations in early brain morphology are caused in part by regional differences in cytoskeletal contraction in the neuroepithelium.

We postulate that the reasons for these early differences in contractility are rooted in the initial cross-sectional geometry of the brain tube. Notably, the lumens of fish and frog brains are initially closed, whereas those of human, mouse, and chick are already open when the neural folds fuse (Figs. 1A; 3, top row; 4A) (Copp et al., 2003; Lowery and Sive, 2004; Harrington et al., 2009). Recently, Nyholm et al. (2009) have shown that inhibiting contraction prevents luminal opening in the zebrafish brain (Fig. 5A), while Gutzman and Sive (2010) have shown that later expansion of the hindbrain lumen requires relaxation of the neuroepithelial cytoskeleton. These results indicate that the zebrafish brain requires contraction to open the lumen, but further expansion requires a return to a more relaxed state.

To help understand how contraction can cause an initially closed lumen to open into the proper shape, we used a computational model. When local apical contraction is simulated at mediolateral hinge points, the slit-like lumen opens into a diamond-like shape, similar to the normal zebrafish brain (Fig. 5B, C). This result is consistent with our finding that F-actin content is greater at the mediolateral vertices (asterisks) relative to the dorsoventral vertices (arrowheads) in opening zebrafish midbrains (Fig. 5B, D). However, we note that this difference in F-actin intensity between vertices is less in *Xenopus* brains (Fig. S3), a result that may be due to differences in embryo staging. Interestingly, this phenotype in *Xenopus* (four hinge points) is similar to that of the hyper-contracted chicken midbrain (Fig. 4B).

We speculate that the initial contractile step is not needed in the chick brain, because it already has an open lumen when it forms. Accordingly, the early chick brain remains comparatively relaxed, although contraction may be involved later in other aspects of brain development. Significant cross-sectional asymmetries develop in the chick brain only when cytoskeletal contraction is artificially enhanced (Figs. 3, 4, 6). Differences in contractility between disparate species may, therefore, be a function of the initial geometry of the brain tube when it first forms (Fig. 8).

Apical contraction has been implicated in the formation of a wide variety of structures in the embryo, including tubes, folds, pits, and invaginations (Ettensohn, 1985; Martin, 2010). Thus, it should not be surprising that biophysical processes may play a role in generating the morphological differences observed between species. Recently, computer models linking mechanical forces to molecular signaling events have illustrated how morphogenetic processes, such as differential growth, can be a factor in mammalian tooth evolution (Salazar-Ciudad and Jernvall, 2010). Similarly, our models and experiments suggest a potential role for the spatiotemporal regulation of cytoskeletal contraction in evolution of the early stages of brain development.

Previous studies have shown that concentrations of contractile proteins at the ventral and mediolateral hinge points in the neural tube may play a role in the cell wedging observed at these locations during neurulation (Kinoshita et al., 2008; Lee and Nagele, 1985). However,

hinge points created during neurulation are not necessarily precursors to the vertices in the brain tube induced in the present study (SI). Apparently, the early chick brain maintains a latent ability to contract asymmetrically in a manner analogous to zebrafish and *Xenopus* brains that is independent of the morphogenetic processes involved in creating the neural tube. This feature also may explain why calyculin exposure appears to have greater effects in certain locations (hinge points) in our experiments. In the chick, the brain tube may maintain this ability to contract asymmetrically to buffer against morphogenetic errors during development (Nerurkar et al., 2006; Koshiba-Takeuchi et al., 2009). For example, if the brain tube were to collapse due to a loss of cerebrospinal fluid pressure, differential contraction could be a potential mechanism for re-opening the lumen. The capacity to continuously adapt to perturbations would help minimize congenital malformations.

Once a hyper-contracted phenotype is established, relaxing the cytoskeleton does not fully restore normal morphology (Fig. 7B–D, B'–D'). Recent work has shown that apical contraction in epithelial cells occurs via a ratchet-like mechanism, whereby actomyosin networks undergo large changes in geometry via alternating steps of contraction and remodeling (Martin et al., 2009; Solon et al., 2009). Besides allowing very large shortening in contracting regions ($G_0 \approx 0.1$), remodeling stabilizes the cytoskeleton after each contractile step, causing it to lose its memory of past configurations and making reversal difficult. Hence, the reverse experiment (e.g., inducing a chicken-like morphology from a zebrafish brain) may not be possible. Consistent with this idea, Gutzman and Sive (2010) found that treating comparably staged zebrafish embryos with blebbistatin affects brain size, but not shape.

We speculate that the lumens of zebrafish and *Xenopus* embryos begin to open relatively homogeneously, generating diamond shapes in transverse cross section, that subsequently remodel into the various shapes observed in the present study. The extent to which similar mechanisms may facilitate the opening and expansion of epithelial tubes both in vivo and in vitro (Raghavan et al., 2010) remains to be determined. Moreover, the significance behind the specific shapes observed in each region of the developing brain warrant further study.

Morphological differences created by specific patterns of contraction in the early embryo may set a blueprint for later-stage tissue growth and neurogenesis (Sylvester et al., 2010; Hofmann, 2010). The combined effects of geometry, contractile force, cavity pressure, and external loads exerted by surrounding mesenchyme generate wall stresses that can affect cellular processes in the brain such as proliferation, migration, differentiation, contraction, relaxation, and apoptosis (Desmond et al., 2005; Nelson et al., 2005; Wozniak and Chen, 2009). At the tissue level, morphogenetic processes such as flexure and luminal expansion, which occur later during brain development in human, mouse, and chicken embryos (but are less apparent in zebrafish and frog embryos), are likely to be influenced by neuroepithelial morphology and cytoskeletal stiffness (Manner et al., 1993; Gato and Desmond, 2009).

The purpose of our models is to help interpret our experimental results. They should be considered qualitative, as they do not include, for example, the complete transmural distribution of contractility suggested by our data (Figs. 4A'–D"; 5D). We also did not attempt to model the dramatic changes that occur longitudinally in hyper-contracted brain tubes (Fig. 1C). A realistic model should include 3-D geometry and the effects of the fluid filling the lumen. This is beyond the scope of the present study.

In summary, our results suggest that interspecies differences in early brain morphology can be generated by spatiotemporal variations in cytoskeletal contraction during development. These effects are likely caused by spatiotemporal differences in the expression of contractile

proteins. Future work is necessary to uncover the implications of these early-stage morphological differences on late-stage brain development (Lui et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Images of early chicken and zebrafish brains

(A) At HH12⁻ the early chicken brain is a cylindrical tube with a hollow lumen. Local constrictions separate the primary brain vesicles (F = forebrain, M = midbrain, H = hindbrain). (B) After 5 hours of culture, the tube expands as lumen pressure increases. (C) For similar incubation time, enhancing cytoskeletal contractility with calyculin significantly alters brain morphology, as vesicle boundaries (asterisks) close off nearly completely. The lumen of the brain tube is highlighted in red (excluding the telencephalon of the forebrain, which includes the optic vesicles) to highlight differences in morphology. (D) Normal zebrafish brain at 22 hpf. Note similarity to hyper-contracted chick brain in (C). (E–G) Sagittal views of panels B–D. Compare the outlined region in (F) with the dyed (red) region

in (G). (Zebrafish brains were injected with a fluorescent dye for contrast; panels D and G from Lowery and Sive (2005).) d = diencephalon of forebrain, t = telencephalon of forebrain. (H–J) Lateral views of reconstructions of the inner lumen of the brain near the mid-hindbrain (MH) boundary. Hyper-contracted chick brains strongly resemble control zebrafish brains. Scale bars: 50 μ m.

Figure 2. Quantitative analysis of brain morphologies

(A) Mean brain shapes constructed from bright field images. Anterior is to the left. Shapes are compared following a five hour culture from HH12⁻ in control (blue, $n = 9$) and calyculin-treated (orange, $n = 10$) chicken embryos. Zebrafish morphology is analyzed at 22hpf (green, $n = 7$). (B) Samples are grouped and a mean shape is computed (center, $n = 26$). The first and second shape modes describe nearly 80% of the total variance in the data: 65.7% in the first mode and 13.3% in the second mode. Shapes one standard deviation away from the mean are shown. (C) Shape-space distribution of brain morphologies (A.U. = arbitrary units). The first mode (horizontal axis) significantly separates control chicken brains from zebrafish and calyculin-treated chicken brains ($p < 0.001$). The second mode (vertical axis) significantly separates calyculin-treated chicken embryos from control zebrafish embryos ($p < 0.01$). Calyculin-treated chicken brains, therefore, share more morphological characteristics with control brains from an entirely different species. Some

more subtle, but reproducible differences, however, do exist between zebrafish and calyculin-treated chicken brains (e.g. longitudinal length of the fore-midbrain boundary).

Figure 3. Comparison of transverse cross sections of brains from three species

(Left) Sections acquired via optical coherence tomography are from the regions indicated in the schematics. (Right) Corresponding average cross sectional shapes ($n = 5$ embryos in each case). Normal chicken brains (top row of images) are rounded and free of vertices. Enhancing contraction in the chick brain (second row of images) generates morphologies that closely resemble normal zebrafish and *Xenopus* brain sections (third and fourth rows), which are characterized by diamond, triangular, and slit-like shapes (FM: fore-midbrain, M = midbrain, MH = mid-hindbrain, H = hindbrain). Stages for comparison: chick (HH12), zebrafish (22hpf), *Xenopus* (stage 33/34). Scale bar: 100 μm .

Figure 4. Local apical contraction as a mechanism for brain morphogenesis

(A–D) Experimental cross sections show the shape of the chick brain (nuclei, DRAQ-5 - red) and distributions of F-actin (rhodamine-phalloidin - green) at various craniocaudal locations (indicated by the schematic). Normal and calyculin-treated (+ contract) chick brains are shown (M = midbrain, MH = mid-hindbrain boundary, N = neuroepithelium caudal to the hindbrain). Normal midbrains (A) are relatively round in transverse section with a relatively uniform distribution of F-actin, whereas sharp corners on the apical side (B–D) in calyculin-treated brains are characterized by high F-actin density (asterisks). (A'–D') Computational models of corresponding brain sections simulate apical circumferential contraction ($G_0 < 1$) as suggested by F-actin distributions (blue and green regions; see

magnified views of boxed regions below). (Undeformed model and mesh are shown on the left.) Shapes closely resemble experimental geometries. (A''–D'') Transmural distributions of F-actin stain intensity indicate elevated actin density near the apical (inner) side of the wall. Apical intensity is relatively uniform around the circumference of control brains (A''), but is stronger near vertices (blue) of calyculin-treated brains (B''–D''). In all samples, F-actin distributions are relatively similar at the basal side regardless of sampling region. Scale bar: 50 μm .

Figure 5. Local apical contraction as a mechanism for opening the lumen of the brain tube (A) The zebrafish midbrain is initially slit-like (as opposed to the circular chick brain) and remains closed when cytoskeletal contraction is inhibited with blebbistatin (100 μ M, hematoxylin-stained section from Nyholm et al. (2009)). (B) In normal zebrafish brains, the lumen opens into a diamond shape, as F-actin accumulates at mediolateral vertices (asterisks). A similar accumulation is not observed at the dorsoventral vertices (arrowheads). (C) Simulating a similar distribution of circumferential apical contraction (asterisks) in a model of a slit-like brain opens the tube and generates a diamond-like morphology as observed experimentally. (D) In normal zebrafish midbrains, F-actin is preferentially localized at the apical side of the mediolateral hinge points (blue) relative to surrounding

regions (red). F-actin staining intensity at mediolateral tissue vertices (asterisks) is approximately 1.3 times greater than at dorsoventral vertices (arrowheads). Scale bar: 50 μm .

Figure 6. Tracking dynamic changes in chicken brain morphology

(A) Transverse sections of control chick brains (HH12⁻, t = 0) are rounded, but become constricted with sharp vertices after 4.5 hours of calyculin exposure (FM = fore-midbrain, M = midbrain, MH = mid-hindbrain, H = hindbrain). Intermediate time points (1.5hr, 3hr) show that this transition from rounded to constricted shapes is not immediate, but gradual throughout the duration of the experiment. (B) Mean lumen shapes at the mid-hindbrain boundary calculated from six embryos at the indicated time points. Shapes change gradually from circular to triangular. (C) In the complementary simulation, taking $G_{\theta} = 1, 0.7, 0.4$, and 0.1 (in the indicated regions of the left panel) reasonably reproduces this change in shape.

These results suggest that calyculin-induced contraction increases approximately linearly with time in the mid-hindbrain boundary during culture. Scale bar: 100 μm .

Figure 7. Reversibility of contraction effects

(A, B) HH12⁻ brains cultured for 3.5 hours in calyculin contract. Mid-hindbrain diameter decreases (arrowheads), axial length of the embryo decreases (white arrows), and the optic vesicles change shape (dotted lines). (A', B') In transverse section, the round mid-hindbrain region becomes triangular in shape. (C, C') Following calyculin washout and blebbistatin exposure, axial length and mid-hindbrain diameter increase and the original shape of the optic vesicles is partly restored. However, the hyper-contracted triangular shape of the mid-hindbrain persists in treated embryos (C'), in contrast to the comparably rounded shape of this region in controls (D, D'). (E) As a separate control, embryos treated with blebbistatin for 7 hours from HH12⁻ were similar to controls, but the mid-hindbrain boundary elongated in the dorsoventral direction (E'). Scale bars: 100 μ m.

Figure 8. Summary schematic for interspecies differences in cytoskeletal contractility during early brain development

Local apical contractility drives luminal opening in species with slit-like brain tubes such as zebrafish and *Xenopus*. Chick (as well as mouse and human) brains are comparatively open following neurulation, and hence, local apical contraction is not required for subsequent morphogenesis. Artificially enhancing contractility in the chick brain, however, recapitulates locally-contracted morphologies observed in normal zebrafish and *Xenopus* brains. Due to tissue remodeling that occurs during this process, this change in phenotype is not fully reversible following drug-induced relaxation.