Mechanisms of Early Brain Morphogenesis

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Mechanisms of Early Brain Morphogenesis  

by  

Benjamen A. Filas  

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of  

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In structures with obvious mechanical function, like the heart and bone, the relationship of mechanical forces to growth and development has been well studied. In contrast, other than the problem of neurulation (formation of the neural tube), developmental mechanisms in the nervous system have received relatively little attention.

The central aim of this research is to characterize the biophysical mechanisms that shape the early embryonic brain. Experiments were performed primarily in the chicken brain, which is morphologically similar to humans during early stages of development. Proposed mechanisms were tested using computational models to ensure that hypotheses are consistent with physical law.

The brain initially forms as a straight epithelial tube in the embryo (≈ 3 weeks gestation in humans). We first investigated a potential role for mechanical feedback in regulating the development of this structure. We find that the neuroepithelium actively stiffens under decreased loading and softens under increased loading. Nuclear shapes are elongated in stiffer brains and circular in softer brains, consistent with...
changes in cytoskeletal contractility and wall stress. These results suggest a role for stress-based mechanical feedback in regulating epithelial development.

We next investigated the more specific role of cytoskeletal contraction in forming the primary brain vesicles and rhombomeres that subdivide the primitive brain tube. We show that a combination of circumferential contraction in the boundary regions and isotropic contraction between boundaries can generate realistic vesicle morphologies, whereas longitudinal contraction between boundaries likely causes rhombomere formation. Models are used to show how regional variations in contraction may be a function of brain geometry and morphogenetic plasticity.

As an extension of the previous study, we show that enhancing contractility in the embryonic chicken brain induces morphologies reminiscent of more primitive species such as frog and fish. In particular, brain cross sections that are relatively circular transform into diamonds, triangles, and narrow slits, shapes that are present in normal zebrafish and *Xenopus* brains at comparable stages of development. Models show that these shapes are likely produced by locally elevated cytoskeletal contraction, indicating a potential role for differential contractility in early brain development and evolution.

In summary, results from this thesis should improve our understanding of the biophysical mechanisms that establish and regulate phenotype in the developing brain. The research begins to establish the framework necessary to connect early-stage mechanisms to interspecies differences in brain morphogenesis that occur during later development.
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Washington University in Saint Louis
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This dissertation is dedicated to the future scientists and engineers who apply and improve upon this research.
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Chapter 1

Introduction

During development, the brain undergoes a dramatic transformation from a simple tubular structure to (in large mammals) a highly convoluted shape. Most investigators recognize that mechanics plays a major role in this process, but the physical mechanisms of brain morphogenesis are not well understood.

Here, we discuss the current state of the field and some of the challenges to uncovering the biomechanical aspects of early brain morphogenesis. Where appropriate, we emphasize interspecies differences in morphogenetic mechanisms, as understanding these differences can provide insight into the development of individual organisms (Lui et al., 2011). After background in embryology and a discussion of research significance, we summarize the main results from this dissertation.

1.1 Neurulation and brain tube formation

Neurulation is the earliest stage of development specific to the nervous system. This process begins within the first three weeks of conception in humans, as a central region of ectoderm called the neural plate folds to create the neural tube (Fig. 1.1). The wall of the tube is a neuroepithelium composed of a single layer of undifferentiated neural progenitor cells (Lowery and Sive, 2009). The cells are columnar, and the cell nuclei migrate between the apical side (facing the lumen) and basal side (facing the exterior) during the cell cycle, giving the neuroepithelium a pseudostratified, or multi-layered appearance (Sauer, 1935; Miyata, 2008). Eventually, the anterior and posterior regions of the neural tube become the brain and spinal cord, respectively.
Morphogenesis of the neural tube occurs in a specific spatiotemporal pattern along the length of the embryo. In the chicken, mouse, and human embryo, the neural plate elevates, folds, and fuses to form a tube with a hollow lumen (Fig. 1.1A). Depending on the longitudinal position along the tube, this closure is facilitated by the formation of one or three hinge points (Fig. 1.1A, asterisks). Generally, multiple hinge points are present at the anterior end of the tube (prospective brain), while only one hinge point forms posteriorly (prospective spinal cord). The end result is a tube that decreases in cross-sectional area from the brain through the spinal cord. Collectively this folding is known as primary neurulation, which has been shown to require the coordination of forces intrinsic to the neuroepithelium as well as extrinsic forces generated by surrounding tissues (Schoenwolf and Smith, 1990).

In contrast, during later stages of development, an entirely different mechanism sculpts the furthest posterior spinal cord region. Here, undifferentiated mesenchymal (loosely connected, highly migratory) cells condense and cavitate to form an internal lumen in a process known as secondary neurulation (Fig. 1.1B). Hence, the anterior brain and spinal cord form via coordinated bending of the neuroepithelium, whereas the posterior end of the spinal cord forms via the agglomeration, cavitation, and epithelialization of loosely connected cells.

In species such as *Xenopus* (frog) and zebrafish, however, such a difference between neurulation mechanisms is not immediately apparent (Schmitz et al., 1993; Lowery and Sive, 2004; Harrington et al., 2009). Here, neural precursor cells migrate medially to form a neural keel (Fig. 1.1C, arrows), intercalate (exchange neighbors), and remodel to form a slit-like lumen. Interestingly, it remains controversial as to whether the brain forms via a primary or secondary neurulation mode in these species. Dynamic (time lapse) imaging studies suggest that these cells roll into a tube, as occurs during primary neurulation, but in doing so, the cells intercalate and migrate, displaying behaviors more typical of those involved in secondary neurulation (Lowery and Sive, 2004; Harrington et al., 2009). Hence, neurulation in these species may involve a combination of the primary and secondary neurulation mechanisms used by higher vertebrates. Computational models for neural tube closure in amphibians have provided insight into some of these processes (Chen and Brodland, 2008; Brodland et al., 2010).
Figure 1.1: Neurulation mechanisms. (A) Primary neurulation in the chicken. A central region of ectoderm (neural plate) bends to form the neural groove. Multiple (brain) or single hinge points (spinal cord) facilitate subsequent tube closure (asterisks). (B) Secondary neurulation in the chicken. Mesenchymal cells coalesce and cavitate to form the posterior spinal cord. (C) Neurulation in zebrafish. Cells migrate medially (arrows) to form the neural keel and reorganize to form a slit-like lumen. (D) Schematic from Schoenwolf and Smith (1990) showing representative cell morphologies during stages of hinge point formation in the prospective chicken brain. Interrelated processes of cell shape change, contraction at the apical (inner) wall, and nuclear positioning cooperatively shape the bending neuroepithelium.

What does seem to be clear, however, is that hinge points do not form during neural tube formation in *Xenopus* or zebrafish as occurs in chicken, mouse, and human embryos (Fig. 1.1A,C, Harrington et al., 2009). Hinge point formation is characterized by interrelated, intrinsic processes such as cell wedging, possibly caused by apical contraction or the radial positioning of nuclei in the neuroepithelial wall (interkinetic nuclear migration) (Fig. 1.1D, Schoenwolf and Smith, 1990). The nucleus constitutes the bulk of the cell volume (Fig. 1.1D) and its radial position in the neuroepithelial wall depends on the stage of the cell cycle. If, for example, a subset of cells takes longer to undergo DNA synthesis at the outer wall of the neuroepithelium, then the nucleus would force the basal side of these tall, thin cells to expand and potentially generate a hinge point (Smith and Schoenwolf, 1988).
Apical narrowing via contraction may also be involved, however, as proteins that regulate cytoskeletal contraction (rho, phosphorylated myosin light chain, and F-actin) colocalize and accumulate at the inner wall of the neuroepithelium at hinge points (Sadler et al., 1982; Lee and Nagele, 1985; Kinoshita et al., 2008). These distributions hint at a mechanistic role for cytoskeletal contractility in driving neurulation. Consistent with this idea, treatment with contractility inhibitors (blebbistatin: myosin II inhibitor, Y27632: rho kinase inhibitor, and cytochalasin D: F-actin polymerization inhibitor) prevent primary neurulation in these species (Morriss-Kay and Tuckett, 1985; Ybot-Gonzalez and Copp, 1999; Kinoshita et al., 2008). It is currently unclear, however, whether hinge point formation acts as a driving or a stabilizing force during normal neural tube closure (Greene and Copp, 2009). Early finite element models have shown that apical constriction can produce invaginations (Odell et al., 1981) and hinge-like morphologies (Clausi and Brodland, 1993), but this mechanism warrants further study.

1.2 Brain tube morphogenesis

The brain tube of vertebrates subsequently subdivides into three primary vesicles (forebrain, midbrain, and hindbrain) (Fig. 1.2). Depending on the species, the brain vesicles develop from either a hollow tube or a comparatively closed, slit-like tube (Fig. 1.1). This suggests that, as in neurulation, morphogenetic mechanisms driving vesicle formation may vary between species.

1.2.1 Lumen opening in zebrafish brains

To date, mechanistic studies of brain vesicle formation have been conducted primarily in zebrafish embryos. In this species, the internal lumen of the tube differentially opens to generate the primary vesicles. The lumen of the hindbrain opens first, followed closely by the midbrain and the forebrain (Lowery and Sive, 2005). Interestingly, the forebrain, midbrain, and hindbrain lumens open into different cross-sectional shapes (Fig. 1.2A). Specifically, the midbrain lumen is shaped like a diamond, the hindbrain a triangle, while the forebrain opens into a tear-drop shape (see
Differential Lumen Opening Brain Vesicle Formation

**Figure 1.2:** Mechanisms of brain vesicle formation. (A) Differential opening of a slit-like brain tube is concomitant with primary vesicle formation in frog and fish. Shapes vary in transverse cross sections between the forebrain (F), midbrain (M), and hindbrain (H). (B) Primary brain vesicles similarly form in species with comparatively open brain tubes, but vesicle shapes are rounded and relatively homogeneous.

The morphogenetic mechanisms that drive luminal opening in the zebrafish midbrain are beginning to be uncovered. In particular, inhibiting myosin by blebbistatin exposure prevents this process (Nyholm et al., 2009). This result has led to speculation that cytoskeletal contraction at lateral hinge points may facilitate luminal opening in zebrafish (Nyholm et al., 2009), but this potential mechanism has not yet been directly tested.

Once the lumen opens, later expansion of the hindbrain requires relaxation of the cytoskeleton (Gutzman and Sive, 2010). Hence, it seems that the zebrafish brain tube actively contracts to establish a lumen, and later relaxes to facilitate expansion in response to increasing fluid pressure in the lumen (see Section 1.2.2).
1.2.2 Brain vesicle formation

Evidence suggests that brain tube morphology at the mid-hindbrain boundary in zebrafish is not purely a consequence of differential luminal expansion. The decreased radius in this region is associated with wedge-shaped cells produced by a combination of basal constriction and apical expansion (Gutzman et al., 2008). Actin is concentrated on the basal side of these cells, consistent with actomyosin driven basal contraction. (Interestingly, in most other instances of invagination that involve cytoskeletal contraction, the contraction occurs at the cell apex (Davies, 2005).) In embryos that lack laminin, which is a major component of the basement membrane surrounding the outside (basal side) of the brain tube, the mid-hindbrain boundary still forms, but is not as sharp as in wild type embryos (Gutzman et al., 2008). Hence, differential lumen opening may set the initial pattern for the brain vesicles, while ongoing actomyosin activity remodels the tube into its characteristic three-dimensional structure.

Differential contraction is not the only possible mechanism that can drive brain vesicle formation. For example, constrained longitudinal growth could produce a buckling pattern along the tube. Treating zebrafish embryos with aphidicolin to inhibit cell proliferation, however, does not prevent boundary formation, but does reduce vesicle size (Lowery and Sive, 2005). Cell division rates were also found to be significantly greater in the hindbrain relative to the mid-hindbrain boundary during stages of vesicle formation. Taken together these results suggest that differential growth is important for the ongoing shaping and deepening of the brain constrictions, but may not be an essential process driving vesicle formation.

Outside of zebrafish, however, mechanisms of brain vesicle formation have received relatively little attention. One reason for this is because, for example, in the mouse embryo, brain vesicle formation and neurulation occur concurrently (Copp et al., 2003). Since tube closure and boundary formation likely both involve aspects of regulated cytoskeletal contractility (in addition to other conserved morphogenetic mechanisms), it seems reasonable to first parse out the individual components of these processes in alternative experimental systems. However, in the chicken embryo, the brain vesicles form after neural tube closure (Fig. 1.2B), similar to zebrafish (Fig. 1.2A), and are readily amenable to mechanical perturbation studies.
External forces also may play a role in shaping the brain tube. The brain forms on the dorsal side of the embryo surrounded by a loosely packed network of cells and extracellular matrix known as the head mesenchyme. During vesicle formation in chicken and human embryos, the early brain seals at both ends to become a fluid-filled pressure vessel. The brain then begins a period of rapid expansion, and studies have shown that this growth depends on cerebrospinal fluid pressure (Gato and Desmond, 2009) (Fig. 1.3). Specifically, prematurely sealing the brain cavity causes the expansion to begin early (Desmond and Levitan, 2002), whereas relieving the pressure severely retards growth (Desmond and Jacobson, 1977).

Figure 1.3: Brain morphology in the chicken embryo. Optical coherence tomography reconstructions of the lumen of the brain tube. Hamburger and Hamilton Stages 9-21 (HH 9-21; 1.5-3.5 days incubation) are shown (Hamburger and Hamilton, 1951). Vesicles form (≈ HH11) prior to rapid increases in lumen pressure and cavity size (≈ HH14-21). (F, forebrain; M, midbrain; H, hindbrain).

In these embryos, however, the majority of vesicle morphogenesis occurs prior to the brain becoming a sealed, pressurized system. Hence, it is unlikely that vesicle formation depends on the presence of an internal lumen pressure. Consistent with this interpretation, vesicles form in zebrafish pressure inflation mutants (Gutzman et al., 2008). A more significant source of external forces acting on the neuroepithelium during vesicle formation would likely be from surrounding tissues such as the head mesenchyme. To our knowledge, this possibility has not been tested.
1.2.3 Rhombomere formation

As the primary brain vesicles form, a series of smaller, periodic bulges arise in the hindbrain. These rhombomeres (Fig. 1.4A), have received considerable attention since the early 1990s as regions of cell lineage restriction and differential gene expression (reviewed in Kiecker and Lumsden (2005)). With the spotlight on these structures as local signaling centers, interest in the morphogenetic mechanisms of rhombomere formation has receded. Still, some useful mechanistic details can be garnered from the earlier literature.

Figure 1.4: Boundary formation in the brain tube of the chicken embryo. (A) The primary brain vesicles (forebrain, midbrain, and hindbrain) are separated by the permanent fore-midbrain (FM) and mid-hindbrain (MH) boundaries. Rhombomeres are transient, sequential bulges in the early hindbrain. (B) Rhombomere boundaries are characterized by low F-actin content at the apical (inner) wall, reduced cell proliferation rates, and extracellular space. The opposite phenotype is observed in interboundary regions.

In rhombomeres of chicken embryos, cell proliferation rates and apical F-actin concentrations are higher in interboundary regions than in the boundaries (Guthrie et al., 1991) (Fig. 1.4B). In addition, the amount of extracellular space between neighboring cells tends to increase in the boundaries during development (Heyman et al., 1993) (Fig. 1.4B). These results led to early speculation that a bowing or buckling mechanism, due to constrained cell proliferation, drives rhombomere formation.

Interestingly, rhombomeres are transient structures during brain development (as opposed to the primary vesicle boundaries which persist through maturity) (Kiecker
and Lumsden, 2005). Before they disappear, rhombomere boundaries facilitate spatially dependent patterns of axonal migration, cell differentiation, and gene expression. In a recent study in zebrafish, rhombomere boundaries abnormally persisted in hyper-contracted mutants (Gutzman and Sive, 2010), suggesting that rhombomere formation and subsequent dissolution may be a consequence of regulated patterns of cytoskeletal contraction.

Moving forward, the following questions remain:

- Are complementary morphogenetic mechanisms (e.g. regulated contractility) involved in both rhombomere formation and dissolution? How is the segmented phenotype reversed or remodeled?
- Are there interspecies variations in these morphogenetic mechanisms? If so, how do the differences compare to those observed during neurulation and primary vesicle formation?
- How do mechanisms of rhombomere formation differ from vesicle formation?

Answering these types of questions should shed light on methods utilized by embryonic tissues to remodel and confer structural robustness during development. Moreover, this type of analysis could provide insight into evolutionary mechanisms that generate tissue-level shape changes.

1.3 Mechanical feedback in brain development

Early brain development is characterized by rapid changes in the size and shape of the neuroepithelial tube. As discussed above, these processes involve the coordinated action of various morphomechanical processes including cell proliferation, cell growth, and cytoskeletal contractility that are highly variable in time and space. What causes regional variations in these biophysical phenomena? Certainly, differential gene expression can influence tissue patterning, but growing evidence suggests that mechanical stresses may also play a role in regulating tissue shaping and growth. In other
words, the stress state of an embryo may play a fundamental role in directing and regulating morphogenesis.

In particular, computational studies have shown that mechanical feedback may play a role in regulating embryonic growth and remodeling (Ramasubramanian et al., 2008; Taber, 2008). We have recently proposed a general principle for morphogenesis where tissue response to loading depends on the rate of the applied stress (Taber, 2009). These computational models have captured a wide variety of morphogenetic phenomena including growth of pressure vessels (analogous to early brain expansion) and wound healing. The simulations suggest that, in theory, mechanical feedback could amplify relatively small asymmetries in tissue geometry to drive morphogenetic events such as lumen expansion, boundary formation, and neuroepithelial growth.

Initial experimental results seem to support this idea. In one such study, hyper-pressure in the chicken brain was shown to correlate with increased cell proliferation rates after just one hour of treatment (Desmond et al., 2005). Specifically, internal pressure was increased by adding fluid into the brain cavity, which caused a 33% pressure overload compared to physiological values in HH21 embryos (3.5 day incubation) (Hamburger and Hamilton, 1951). Long term (>1 hour) morphogenetic effects from the perturbation were not studied, however. In an alternative experiment, reducing cerebrospinal fluid pressures in the early chick brain decreased neuroepithelial growth rates (Desmond and Jacobson, 1977; Gato et al., 2005).

Together, these computational and experimental results suggest that mechanical stresses may play a role in regulating early brain development. The adaptive morphogenetic mechanisms that regulate this behavior warrant further study.

1.4 Research significance

Many congenital brain defects are rooted in events that occur early in development. Abnormalities in brain size and shape have been implicated in a wide variety of neurological disorders including schizophrenia, autism, and hydrocephalus (Hardan et al., 2001; Kurokawa et al., 2000; Rekate, 1997). In particular, hydrocephalus (excessive intracranial fluid pressure in the skull) occurs frequently and is a leading cause of
brain surgery for children (Fritsch and Mehdorn, 2002; Zhang et al., 2006). Moreover, embryonic-lethal anencephaly results from the lack of an internal lumen pressure in the developing brain (Lowery and Sive, 2009). Aberrant mechanical loading is inherently linked to these malformations, but the sequences of morphomechanical events that cause these disorders are not understood.

While it is well established that abnormal internal pressures in the developing heart cause congenital defects (DeAlmeida et al., 2007; Hove et al., 2003) and lead to adaptive remodeling of the myocardium (Clark et al., 1989; Miller et al., 2003; Nerurkar et al., 2006; Sedmera et al., 1999), similar responses in the brain have not been characterized. Clearly, mechanical forces play an important role, but the biomechanical factors responsible for normal and abnormal development are largely unknown.

Better understanding mechanical feedback in the developing brain could also have important implications for the fields of microsurgery, regenerative medicine, and tissue engineering (Coleman, 2008; Norman et al., 2008). In particular, implementation of neural precursor cells has been suggested as a treatment for neurological disorders, including Parkinson’s disease and spinal cord degeneration (Eggum and Hunter, 2008; Nelson et al., 2002; Saha et al., 2008). Because the mechanical environment plays a critical role in regulating cell differentiation (Engler et al., 2006; Saha et al., 2008) and growth patterns (Nelson et al., 2005), determining how neural progenitors respond to mechanical perturbations in the embryo could provide important predictive information as to how implanted cells may respond to \textit{in vivo} environments.

1.5 Synopsis of dissertation

The overall goal of this dissertation is to elucidate some of the biophysical mechanisms that drive and regulate early brain morphogenesis.

As an extension of our earlier work on the heart (Filas, 2006), we first implemented a technique to quantify morphogenetic deformations in the developing brain tube. Microspheres were injected into the inner lumen of the brain and tracked during morphogenesis. Dynamic optical coherence tomography imaging was used to follow the 3-D movement of the beads in time and space. Reconstructed brain morphologies
provided a physiological relevant coordinate system for computing mathematical measures of deformation. Results from this study indicated that the brain may shorten in a specific, regionally dependent manner to form the brain vesicles that pattern the future nervous system.

Next, we tested the more general role of mechanical feedback in the early brain using controlled levels of compression applied via surface tension. Results suggest that the brain actively adapts to changes in applied loads by altering cytoskeletal contractility (and corresponding nuclear shapes) in ways that tend to restore the original mechanical stress state in the tissue. Similar results were found in the heart, supporting a potential widespread role for stress-based mechanical feedback in epithelial development.

We then investigated a potential role for differential cytoskeletal contraction in brain vesicle formation. Results show that contractility is essential for vesicle formation and that specific contraction patterns vary along the length of the brain tube. Corresponding finite element models indicate that regional contraction mechanisms may depend on brain geometry, and that these mechanisms confer different levels of structural robustness in the embryo.

In a related study, we found that regulated contractility may play a role in early brain evolution. Notably, enhancing contractility in the chicken brain generates morphologies remarkably similar to species such as frog and fish. We suspect that the reasons for these differences are rooted in the initial geometry of the brain tube following neurulation. Consistent with the growing evolutionary developmental biology (evo-devo) synthesis, these results suggest that spatiotemporal differences in contractile protein expression may underly interspecies differences in brain morphology.

Taken together, these studies begin to illuminate some of the biomechanical mechanisms involved in early brain morphogenesis. Future work should focus on connecting these mechanisms to processes that occur during later development, such as cell differentiation, migration, and even brain folding. As an initial step, we present preliminary data in the appendix suggesting that the brain actively responds to perturbations in lumen pressure at later stages of development. Together, these data should be useful in establishing an experimental and computational framework to better characterize morphogenetic adaptation in the embryonic brain.
Chapter 2

Tracking Morphogenetic Deformations in the Chick Embryo

2.1 Summary

Embryonic epithelia undergo complex deformations (e.g. bending, twisting, folding, and stretching) to form the primitive organs of the early embryo. Tracking fiducial markers on the surfaces of these cellular sheets is a well-established method for estimating morphogenetic quantities such as growth, contraction, and shear. Here, we describe a labeling method where polystyrene microspheres are used as contrast agents during non-invasive optical coherence tomography (OCT) imaging to track 3-D tissue deformations. From the coordinates of the markers, various deformation measures are computed as functions of time and space. This technique has been successfully implemented in our lab to study the physical mechanisms of early heart and brain development, and should be adaptable to a wide range of morphogenetic processes.

2.2 Introduction

In the embryo, many morphogenetic processes involve complex three-dimensional (3-D) deformations of cell sheets, or epithelia (Davies, 2005; Gilbert, 2003). In studies of morphomechanics, it is useful to quantify these deformations, and researchers have measured strain distributions in epithelia during gastrulation (Zamir et al., 2005,
2006) and early heart development (Ramasubramanian et al., 2006; Filas et al., 2007). In this chapter, we present the experimental details behind a technique for measuring surface strain that can accommodate virtually any type of deformation during epithelial morphogenesis (Filas et al., 2008). The general approach and overall goal of the strain algorithm is described but mathematical specifics are omitted. For details behind the development, implementation, and validation of the algorithm, readers are directed to Filas (2006) and Knutsen (2008). This method is applicable to a wide range of morphogenetic problems, as well as to functional studies, such as strain measurements in beating hearts.

2.3 Methods

2.3.1 Images, surfaces, markers, and normal vectors

Deformation is measured by following the displacements of a set of fiducial points (markers) attached to a surface (Fig. 2.1A). The surface and the marker locations are typically derived from image volumes acquired, for example, by magnetic resonance imaging (MRI), computed tomography (CT), or optical coherence tomography (OCT). Surfaces are created from segmented image volumes via previously-described algorithms (e.g., CARET (Van Essen et al., 2001)) or standard software (MATLAB, The Mathworks Inc., Natick, MA). The resulting surface created by these algorithms consists of a set of triangular faces, each defined by the (global) coordinates of its three vertices and by a normal unit vector (Fig. 2.1B).

To characterize deformation kinematics, accurate measurements of displacements of points on the surface are needed. In the absence of natural landmarks that can be tracked over time, markers, such as opaque or reflective beads, are attached to the physical surface, so that they move with the material. The location of each marker is tracked over the duration of the imaging study. Typically, the number of markers is much smaller than the number of vertices that represent the surface. Hence, the distance between vertices is usually small compared to the marker spacing.
Figure 2.1: Surface geometry and normal vectors. (A) Schematic of surface geometry. S: reference surface; s: deformed surface; $X_i$, $x_i$: local Cartesian coordinate systems; $e_i$: local Cartesian base vectors; $g_i$: local covariant base vectors at a bead on S; $g_i$: convected base vectors at same bead on s. Note that, in general, $e_1$ and $e_2$ are only approximately tangent to S, and the tangent base vectors ($G_1$, $G_2$ and $g_1$, $g_2$) are not orthogonal. (B) Normals to the surface of a looped heart.

2.3.2 Experimental preparation

White Leghorn chicken eggs were incubated at 38°C for approximately 34 or 42 hours to stage 10 (heart) or stage 11 (brain). The embryo was extracted from the egg using a filter paper carrier method (Voronov and Taber, 2002) and, to gain direct access to the heart, the membrane covering the ventral surface of the heart (the splanchnopleure) was removed using a fine glass needle. Embryos were cultured at 38°C in a heated Delta T culture dish (Bioptechs, Butler, PA) in Dulbeccos Modified Eagles Medium (Sigma). A schematic of the experimental set-up is shown in Figure 2.2.

Surface strains were measured in the developing heart and brain of the chick embryo during stages 10-12 of Hamburger and Hamilton (Hamburger and Hamilton, 1951). During this early phase of development, the relatively straight heart tube bends ventrally and twists rightward into a c-shaped tube (Fig. 2.3) (Manner, 2000). In the ventral view, rotation due to torsion causes markers that are initially located near the right side of the heart tube to move behind the heart (Fig. 2.3A,B). At the same time, the brain forms its major subdivisions (forebrain, midbrain, and hindbrain, see Fig. 2.4).
Cardiac looping

In the heart experiments, morphogenetic deformation was isolated from deformation due to the heartbeat by using verapamil (25 µM) to stop sarcomeric contraction. Previous studies have shown that c-looping is normal in embryos cultured in verapamil (Manasek et al., 1972; Clark et al., 1991). It is important to note that cardiac bending and torsion normally occur simultaneously during c-looping. When the splanchnopleure is removed, however, torsion is delayed until approximately stage 11 but is completed normally by stage 12 (Nerurkar et al., 2006). This behavior is not crucial for the present purpose, but it should be kept in mind when interpreting results. For the heart, carboxylated blue polystyrene microspheres with a mean diameter of 6 µm (Polysciences, Warrington, PA) were sprinkled onto the epicardium at stage 10 with a syringe. Time lapse microscopy studies confirmed that the beads remained firmly attached during development (Filas, 2006).

Brain ventricle morphogenesis

For the brain, beads were injected into the brain tube at stage 11 using a pulled glass micropipette. We found this stage to be ideal for injection because the cranial neuropore had sealed completely (Van Straaten et al., 1996), thus preventing markers
Figure 2.3: Motion of beads attached to the surface of a looping chick heart. (A) Stage 11+; (B) stage 12. Regions outlined in red denote region where a high number of beads initially congregated along the right side of heart (A) and subsequently rotated toward the outer curvature and the backside of the heart (B). Blue asterisks (A, B) show a region of low bead density near the primitive atrium (PA) where strains were not calculated. (C) Surface representation and beads (blue circles) used in deformation analysis generated from images of the stage 12 heart. V: ventricle; CT: conotruncus. Scale bar: 250 µm.

from escaping through this cavity. Additionally, internal pressure in the brain is relatively low at this time (Desmond et al., 2005), allowing the hole made at the bead injection site to heal quickly. Because the brain is surrounded by other tissue, larger polystyrene microspheres were used in these injections (10 µm diameter, Bangs Labs, Fishers, IN) to enhance image contrast. OCT imaging confirmed that beads attached to both the dorsal and the ventral sides of the inner brain lumen and could be reliably tracked during ventricle formation Fig. 2.5.

### 2.3.3 Optical coherence tomography

The relatively new imaging technique of OCT (Huang et al., 1991; Fujimoto, 2003) was used to image the embryo noninvasively approximately once per hour at high resolution (< 10 µm) and in 3-D (image stacks) during the ex-ovo incubation period (∼ 12 hours, stage 10 to 12, for the heart (e.g. Fig. 2.6); ∼ 6 hours, stage 11-12, for the brain). The 3-D coordinates of the microspheres were determined at each time point using thresholding functions in commercially available software (Velocity, Improvision), and deformation measures were calculated relative to the reference configuration using the methods described above.
Figure 2.4: Motion of beads attached to the inner surface of an embryonic chick brain. (A) Stage 11. (B) Stage 12. (C) Surface representation and beads (blue circles) used in deformation analysis generated from images of stage 12 brain. F: forebrain; OV: optic vesicle; M: midbrain; H: hindbrain. Scale bar: 250 µm.

Figure 2.5: (A) Polystyrene microspheres that adhere to the dorsal (black arrow) and ventral (white arrow) sides of the brain tube are discernible from surrounding tissues in transverse cross section. Ventral views of OCT reconstructions show microsphere locations near the mid-hindbrain boundary at (B) HH12 and after (C) 6 hr of incubation (M: midbrain, H: hindbrain). Several groups of beads are outlined to highlight the overall deformation of the tissue. Scale bar: 200 µm.

2.3.4 Image reconstruction

To map computed quantities onto the surface of the heart or brain, cross sections obtained by OCT were manually segmented using the CARET (Computerized Anatomical Reconstruction and Editing Tool Kit) software package (Van Essen et al., 2001).
Figure 2.6: Time lapse OCT imaging and marker tracking in the looping heart. (A-H) 3-D surface projections of the looping heart imaged via OCT. Duration between time points is \( \approx 45 \) minutes. Beads remained attached to the myocardium and are tracked from HH11\(^+\) to HH12 as the heart bends and rotates into a c-shaped tube. Scale bar: 250 \( \mu \)m.

Reconstructed volumes were subsequently transferred into MATLAB and aligned with bead centroids. For the brain, beads were placed on the inner surface, but the reconstructed images show only the outer surface clearly. Thus, the tracked beads were shifted dorsally in Fig. 2.4C to allow their relative positions on the brain surface to be seen. However, the actual bead centroids on the inner surface were used in the strain analysis.

### 2.3.5 Overview

The overall goal of the techniques presented here is to label and culture embryonic tissues for measuring morphogenetic deformations during early development. The methods required to successfully implement this technique for the embryonic chick heart and brain are summarized in Figure 2.7. These approaches should be readily adaptable to alternative experimental systems.
2.4 Results

2.4.1 Cardiac looping

Illustrative data are shown from one heart with 45 beads tracked for about 5 hours from stage 11+ to 12, a period that captures most of the torsion (rotation) that occurs following removal of the splanchnopleure (Fig. 2.3). Note that many of the beads initially near the right side of the heart (Fig. 2.3A, red outline) moved behind the heart during rotation (Fig. 2.3B, red outline). Stretch ratios in the direction of maximum curvature ($\lambda_1$, circumferential) and minimum curvature ($\lambda_2$, longitudinal) and shear ($\gamma$) were computed and mapped onto the fully deformed (c-looped) stage 12 heart surface (Fig. 2.8)). Stretch ratios were predominately positive, while shear was significant near the ends of the heart tube.
Figure 2.8: Myocardial deformation during looping. Circumferential stretch ratio ($\lambda_1$), longitudinal stretch ratio ($\lambda_2$), and shear ($\gamma$) mapped onto a stage 12 (fully c-looped) embryonic chick heart. Quantities were computed relative to the configuration at stage 11+ (approximately five hours earlier). The circumferential and longitudinal directions were defined locally as the directions of maximum and minimum curvature, respectively. Orientations show the ventral, lateral, and dorsal surfaces of the heart. V: ventricle; PA: primitive atrium; CT: conotruncus.

2.4.2 Brain development

In this example, 56 beads were tracked from stage 11 to 12 (6 hour incubation, (Fig. 2.4), and stretch ratios were calculated in the directions of maximum ($\lambda_1$) and minimum ($\lambda_2$) curvature (Fig. 2.9). As indicated by arrows in Fig. 2.9, the direction of maximum curvature approximates the circumferential direction everywhere except the forebrain, where it approximates the longitudinal direction. Stretch ratios in the direction of maximum curvature are positive in the hindbrain and the ‘neck’ of the forebrain (Fig.2.9). In the midbrain and hindbrain, stretch ratios in the direction of minimum curvature (longitudinal direction) were slightly less than one. Shear becomes a significant component of the deformation in the forebrain.
Figure 2.9: Neuroepithelial deformation during ventricle morphogenesis. Stretch ratios in the directions of maximum curvature ($\lambda_1$) and minimum curvature ($\lambda_2$), and shear ($\gamma$) mapped onto a stage 12 embryonic chick brain. Deformation measures were calculated relative to a stage 11 reference state (≈6 hr incubation). Note that, because of the complex geometry, the principal axes of curvature are not uniquely related to anatomical axes. As indicated by arrows, the longitudinal and circumferential directions in the midbrain and hindbrain correspond to the directions of minimum and maximal curvature, respectively. In the forebrain, the situation is reversed. Orientations show the ventral and dorsal surfaces of the brain. F: forebrain; OV: optic vesicle; M: midbrain; H: hindbrain.

2.5 Discussion

Here, we demonstrate the implementation of a new method for computing the deformation of folding surfaces with multiple-valued coordinates. Multi-valued surfaces, which arise in 3-D morphogenetic phenomena such as invagination (Ramasubramanian et al., 2008) and brain folding (Welker et al., 1990; Neal et al., 2007) pose difficulties for methods that rely on a single global fitting function (Hashima et al., 1993; Filas et al., 2007). To address this problem, we analyze locally single-valued patches of the surface.

Our method extends previous work on strain measurements that are based on tracking the motions of tissue labels using non-invasive imaging technologies such as OCT,
MRI, or light microscopy. Several studies have used triangles of markers to measure strains due to the heartbeat in the developing heart (Alford and Taber, 2003; Taber et al., 1994; Tobita and Keller, 2000b,a) and the mature heart (Meier et al., 1980; Villarreal et al., 1988; Omens et al., 1996). The spacing between the markers must be closely controlled to limit the effects of measurement error and to avoid missing large strain fluctuations within the triangle (Alford and Taber, 2003).

Nonhomogeneous strain analyses using arrays of multiple (>3) markers have also been implemented, although not throughout multi-valued surfaces. In one of the earliest of these strain analyses, Hashima et al. (1993) fit polynomial (cubic Hermite) surfaces to 3-D marker coordinates in an end-diastolic reference state and subsequent deformed states during the cardiac cycle in a canine heart. This enabled longitudinal, circumferential, and shear strains to be calculated over the entire domain of their marker array. More recently, a polynomial least squares fitting approach has been used to calculate strains from combined marker arrays (Kindberg et al., 2007). Particle image velocimetry has also proved useful in describing nonhomogeneous, morphogenetic strains in quail embryos, but so far this approach has only been applied in 2-D (Zamir et al., 2005, 2006).

Recently, we have used similar techniques to measure strains in limited regions of the looping chick heart over a limited period of development (Filas et al., 2007). The current method allows us to track deformations for longer periods of time over all regions of the heart containing surface labels. It is important to recognize that, in addition to deformation attributed directly to mechanical stress (e.g., bending), morphogenetic strains can be caused by cell proliferation, cell growth, and cytoskeletal contraction (Soufan et al., 2006, 2007).

During looping, the wall of the heart tube consists of a thin outer layer of myocardium, a relatively thick middle layer of extracellular matrix called cardiac jelly, and a thin endocardium that lines the lumen (Manner, 2000). From the venous to the arterial end, a series of regions form along the tube: the primitive atrium, ventricle, and conotruncus (Fig. 2.3C).

Estimates of longitudinal and circumferential stretch ratios and shear obtained by the current approach are consistent with measurements from past studies (Ramasubramanian et al., 2006; Filas et al., 2007). Generally, the heart tube inflates (due
to expanding cardiac jelly) and lengthens from stage 11+ to 12, giving stretch ratios greater than unity in most regions. The circumferential stretch ratio increases toward the conotruncus, indicating a higher rate of swelling in this region approaching stage 12 (Fig. 2.8). Longitudinal stretch is highest near the poles of the ventricle, showing a higher rate of lengthening in these regions. Shear is relatively small except near the ends of the heart tube, where most of the torsion occurs. (The ends of the heart are still attached to the body of the embryo at this time.) These results are consistent with past observations (Filas et al., 2007).

Because the early brain tube is embedded in surrounding tissue, beads could not be placed on its outer surface. Hence, we measured stains at the inner lumenal surface, which encloses embryonic cerebrospinal fluid. To our knowledge, this marks the first measurement of strain in the developing brain in any type of embryo. Therefore, comparison with other quantitative data is not possible, but some observations are appropriate.

In the direction of maximum curvature in the midbrain and hindbrain (i.e., the circumferential direction), stretch ratios were highest in the hindbrain and in the expanding ‘neck’ region leading into the forebrain (in the longitudinal direction). These regions undergo significant shape change during this 6-hour incubation period (Fig. 2.9). Stretch in the direction of minimum curvature is slightly less than one in the bulges and the forebrain. This reflects slight longitudinal shortening in the bulges and slight shortening in the circumferential direction in the forebrain. This is also consistent with past data showing relatively little growth in the brain in the longitudinal direction between these developmental stages (Van Straaten et al., 1996). Higher longitudinal stretch ratios ($\lambda_1$) on the dorsal side of the forebrain and neck relative to the ventral side reflect the motion of beads towards each other, as the brain undergoes cranial flexure, i.e., it bends downward during this period (Fig. 2.9). Shear was significant in the forebrain (though not in other regions), reflecting non-uniform, possibly anisotropic, growth in this region (Fig. 2.9). These observations represent only an illustrative case study of deformation in the embryonic brain. Clearly, deformation should be characterized in more embryos over longer periods of incubation before firm conclusions can be drawn.
The advantage of the current technique for this particular data set is clear. In the reference configuration, beads are scattered around the lumen, which is a multi-valued surface. The complete strain distribution would have been impossible to estimate by the approach of Filas et al. (2007).

In summary, a new method for calculating surface strains was used to compute deformation measures in the looping embryonic heart and the developing embryonic brain. This method accommodates sparse, randomly scattered marker arrays, with reasonable errors in the location of markers. Application of this method to data from time series of OCT images of developing chick embryos provides maps of longitudinal and circumferential strain that illuminate the mechanics of morphogenesis.
Chapter 3

Mechanical Stress as a Regulator of Cytoskeletal Contractility and Nuclear Shape in Embryonic Epithelia

3.1 Summary

The mechano-sensitive responses of the heart and brain were examined in the chick embryo during Hamburger and Hamilton stages 10-12. During these early stages of development, cells in these structures are organized into epithelia. Isolated hearts and brains were compressed by controlled amounts of surface tension (ST) at the surface of the sample, and microindentation was used to measure tissue stiffness following several hours of culture. The response of both organs was qualitatively similar, as they stiffened under reduced loading. With increased loading, however, the brain softened while heart stiffness was similar to controls. In the brain, changes in nuclear shape and morphology correlated with these responses, as nuclei became more elliptical with decreased loading and rounder with increased loading. Exposure to the myosin inhibitor blebbistatin indicated that these changes in stiffness and nuclear shape are likely caused by altered cytoskeletal contraction. Computational modeling suggests that this behavior tends to return peak tissue stress back toward the levels it has in the intact heart and brain. These results suggest that developing cardiac and neural epithelia respond similarly to changes in applied loads by altering
contractility in ways that tend to restore the original mechanical stress state. Hence, this study supports the view that stress-based mechanical feedback plays a role in regulating epithelial development.

3.2 Introduction

Epithelia (cell sheets) play a central role in embryonic development, as they elongate, shorten, fold, and invaginate to create structures such as the brain, spinal cord, eyes, blood vessels, the heart, lungs, and the gut (Taber, 1995). These morphogenetic processes involve mechanical forces, and, prior to the 1980s, considerable effort was devoted to uncovering the physical mechanisms of development. More recent studies, however, have focused chiefly on genetic and molecular regulation. Although significant advances have been made in these areas, the links between the mechanistic and regulatory aspects of epithelial morphogenesis remain poorly understood.

Recent work has shown that cells are highly sensitive to their local mechanical environment. Substrate stiffness can direct cell differentiation (Engler et al., 2006), motility (Reinhart-King et al., 2008), and growth rate (Saez et al., 2007), while mechanical stress and strain affects cell proliferation (Wozniak and Chen, 2009), cell sorting (Krieg et al., 2008), cytoskeletal remodeling (Galbraith et al., 1998), and patterns of growth (Desmond and Jacobson, 1977; Nelson et al., 2005; Wozniak and Chen, 2009). The specific response depends on cell type, differentiation state, and loading characteristics (Taber, 1995). In mature organisms, mechanical perturbations often elicit an adaptive response that tends to restore a homeostatic or optimal state consistent with evolutionary law (Humphrey, 2008; Kassab and Navia, 2006).

In the embryo, researchers have hypothesized that active responses to mechanical perturbations play a role in driving and regulating morphogenesis (Taber, 2009). Experimental data accumulated by Belousov and co-workers seem to support this view (Belousov and Grabovsky, 2006; Kornikova et al., 2010) and we have found that the looping heart tube stiffens via cytoskeletal contraction when normal loads applied by the overlying splanchnopleuric membrane are removed (Nerurkar et al., 2006; Ramasubramanian et al., 2008). Our data also suggest that this response represents a morphogenetic adaptation that restores normal looping.
Here, we study this contractile response in the heart under more controlled conditions. In addition, because the primitive myocardium is an epithelium, we wondered whether this behavior is a general characteristic of embryonic epithelia. Hence, we also examine the mechano-sensitive response of the neuroepithelium of the early brain tube. Although the present study does not address morphogenesis directly, understanding how embryonic tissues respond to mechanical loads is a crucial step in determining the physical mechanisms of development.

Some features of the brain make it more amenable to analysis than the heart. For example, the brain does not beat and its structure is simpler than that of the heart, as it consists of a single neuroepithelium rather than the three layers (endocardium, cardiac jelly, myocardium) of the heart wall. Also, neural precursor cells in the brain are more highly organized and aligned than myocardial cells in the heart tube. Although experiments were conducted on both the heart and brain, these characteristics foster a more in-depth study of the brain.

We subjected isolated brain and heart tubes from chick embryos to various compressive loads and used microindentation to measure tissue stiffness following several hours in culture. Despite differences in morphology and function, both organs stiffened on decreased loading, while only the brain softened on increased loading. Exposure to the myosin inhibitor blebbistatin indicated that this response is caused by changes in contractility. Further study of the brain revealed that, relative to controls, cell nuclei became more elongated in stiffer brains and more circular in softer brains. Computational modeling indicates that these adaptive changes in nuclear shape are consistent with changes in contractility and wall stress. The results indicate a significant role for tissue stress in regulating epithelial morphology and development.
3.3 Methods

3.3.1 Experimental protocol

Fertilized white Leghorn chicken eggs (Sunrise Farms, Catskill, NY) were incubated for 36 hours to HH10− to 10+ (Hamburger and Hamilton, 1951). Embryos were removed from the egg using a filter paper carrier as previously described (Voronov and Taber, 2002). The brain or heart was then isolated from surrounding tissue using a fine glass needle and microscissors. Isolated tissues were transferred to semisolid 0.3% agar gels containing culture media (89% DMEM, 10% chick serum, 1% penicillin/streptomycin/neomycin) prepared in 24-well culture plates. Surface tension was varied by adding and removing liquid media in each well with a micropipette. Applied loads were tightly controlled for no surface tension (NST) samples (submerged in 0.7 mL liquid media) and high surface tension (HST) samples (excess liquid completely removed from the culture gel). (In alternative experiments, the HST loading condition was implemented by compressing isolated tissues with glass coverslips (see Fig. 3.15).) A third moderate surface tension (MST) loading condition was slightly less controlled as just enough fluid was removed to match heart or brain diameter in the intact embryo. The purpose of this condition was to simulate the natural loading in the embryo as much as possible without accurately knowing the magnitude of these loads. Because of natural variability, applying the same precisely controlled force to each sample would not yield the same deformation.

Isolated brains were cultured dorsal side up for five hours prior to microindentation, whereas hearts were cultured on the left or right side for twelve hours through the process of c-looping. Samples were cultured in an environment maintained at 37°C, 95% humidity, and 5% CO2. Imaging with optical coherence tomography verified that fluid volumes (and surface tension loads) remained relatively consistent during culture in all conditions.

In some experiments, isolated tissues were cultured in 60 µM (-)-blebbistatin (Sigma, St. Louis, MO) in the dark to inhibit myosin-II based contractility. Contraction was enhanced in a group of HST brains using calyculin A (Sigma) mixed into the agar+media culture gel at a final concentration of 20 nM. Additional experiments
were performed at intermediate time points during culture to test the reversibility of imposed loading and chemical perturbations. In a separate study, explant viability was verified for all culture conditions using 0.4% (w/v) Trypan Blue (Sigma).

Bright field images of isolated tissues were captured using a CCD camera (COHU, Model 4915, Coway, CA) attached to a dissecting microscope (Leica MZ8, Wetzlar, Germany). Morphology measurements were performed using commercially available image analysis software (Volocity; Improvision, Waltham, MA).

3.3.2 Optical coherence tomography (OCT)

OCT images were generated by detecting the time delay of backscattered light in a sample path versus that of a fixed reference path (Fujimoto, 2003) as previously described (Filas et al., 2007). Briefly, our system, custom built in the laboratory of Dr. Andrew Rollins at Case Western Reserve University (Jenkins et al., 2006), operates at a wavelength of 1310 nm with a laser output of 15 mW. Four images were acquired every 5 µm in a 2x2 mm scanning window and were later averaged together using a custom MATLAB (Mathworks) program. Alternatively, in isolated culture experiments, a Thorlabs (Newton, NJ) OCT system coupled to a Nikon FN1 microscope was used to more efficiently locate and image samples (isolated hearts were no larger than 1 mm (Itasaki et al., 1989)). Subsequent image analysis for both systems was performed using Volocity software, including image cropping, contrast optimization, and noise filtering. Three-dimensional volumes were automatically generated by cropping surrounding tissue and thresholding the inner cavity of the neural tube (see Fig. 3.6A’).

3.3.3 Micro-indentation and tissue stiffness

Stiffness was measured using a custom-built micro-indentation device (Zamir, 2003). Isolated tissues were supported on one side via suction from a micropipette, and a calibrated indenter (flexible glass cantilever beam) was periodically driven into the opposite side of the sample (0.1 Hz) by a piezoelectric motor. Optical measurements of beam deflection and tissue deformation were used to plot indentation force as a
function of indentation depth (see Fig. 3.8A). Tangent stiffness was computed as the slope of this curve at a particular depth (see Fig. 3.8B). A linear fit was used to approximate the slope of the force-displacement curve at tissue indentation depths \( \leq 5 \mu M \), while a four-parameter exponential function was used for larger indentation depths as previously described (Zamir, 2003).

All tissues were indented at room temperature while fully submerged in phosphate buffered saline. Hence, surface tension had no effect on indentation testing. During this process morphology did not change noticeably. Isolated hearts were indented in 50 \( \mu M \) verapamil (L type calcium channel blocker - Sigma) to inhibit the heart beat. Previous studies have shown that this drug has little effect on diastolic myocardial stiffness (Ramasubramanian et al., 2008). Three indentations were recorded at each sampling location to verify a consistent response. Side studies confirmed measured stiffness was relatively consistent when indenting the same region but in slightly different locations.

### 3.3.4 Nuclear geometry

Embryos were fixed in 3.7% formaldehyde overnight and washed in a series of increasing ethanol concentrations (70, 85, 95, and 100%) to dehydrate the tissues. Samples were next washed in xylene, transferred to paraffin, and 10 \( \mu m \) slices of the brain were cut using a microtome (Bausch and Lomb, Rochester, NY). Slices were rehydrated (reverse ethanol concentrations above), washed in PBS, stained (DAPI, ProLong Gold, Invitrogen, Carlsbad CA), and imaged (Olympus IX70 microscope - 40x magnification). In another test of explant health, we found minimal regions of condensed DNA (indicative of cell death (Jurisicova et al., 1996)) after 5 hours of culture.

Nuclear misalignment (the difference in angle between the major axis of the nucleus and the neuroepithelial wall) and circularity (minor/major axis of the best fit ellipse to the nucleus) were measured using ImageJ (http://rsbweb.nih.gov/ij/) and custom image processing routines implemented in Matlab (The Mathworks, Inc, Natick MA). Misalignment was not calculated in nuclei with a circularity \( \geq 0.9 \). More than 80
nuclei were analyzed for each sample and at least four embryos were analyzed for each loading condition.

### 3.3.5 Actin staining

Fluorescent staining was used to visualize the actin cytoskeleton in the brain. Embryos were removed from the egg using a filter paper carrier and immediately fixed in 3.7% formaldehyde. After overnight fixation, samples were rinsed in PBS, and the head mesenchyme was removed from the brain tube. This step prevented diffusion artifacts and yielded clearer images. Samples were then incubated 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, and the sample was protected from light and gently rocked for an additional hour. Brains were then washed in PBS and imaged using a fluorescent Leica DMLB microscope (5x objective).

### 3.3.6 Finite element modeling

Finite element models were generated to (1) determine the effects of indentation geometry on experimental stiffness measurements and (2) analyze stress distributions in finite element models for compressive loading of the heart and brain. All models were developed using the commercial finite-element software Comsol Multiphysics (v3.5, Burlington, MA).

Growth and contraction are simulated using the theory of Rodriguez et al. (Rodriguez et al., 1994), which assumes the existence of a stress-free configuration for each material element throughout the deformation. 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 ($\mathbf{F} = \mathbf{K} \cdot \mathbf{G}$). In the models, growth is specified in the radial and circumferential directions. Hence, we take $\mathbf{G} = G_r \mathbf{e}_r \mathbf{e}_r + G_\theta \mathbf{e}_\theta \mathbf{e}_\theta$ where the $G_i$ are growth stretch ratios relative to the undeformed cylindrical polar coordinates $(r, \theta)$ and the $\mathbf{e}_i$ are unit vectors. Experiments have shown that the embryonic heart and brain have only moderately nonlinear material properties (Xu
et al., 2010; Zamir and Taber, 2004). Thus, material properties were assumed to be pseudoelastic and nearly incompressible with a modified neo-Hookean strain-energy density function

\[ W = \frac{1}{2} \mu \left( I_1 J^{-\frac{2}{3}} - 3 \right) + p \left( 1 - J - \frac{p}{2\kappa} \right) \tag{3.1} \]

where \( \mu \) and \( \kappa \) are the small-strain shear and bulk modulus, respectively, \( I_1 \) is the first invariant of the right Cauchy-Green deformation tensor \( (K^T \cdot K) \), \( J \) is the elastic volume ratio \( (\det K) \), and \( p \) is a penalty variable. In this theory, stress is only associated with elastic deformation, with the Cauchy stress tensor given by (Taber, 2004)

\[ \sigma = J^{-1}K \cdot \frac{\partial W}{\partial \epsilon} \cdot K^T \tag{3.2} \]

where \( \epsilon = \frac{1}{2}(K^T \cdot K - I) \) is the Lagrangian strain tensor for the elastic part of the deformation and \( I \) is the identity tensor. Full details on the implementation of this protocol in COMSOL software have been published elsewhere (Taber, 2008).

All geometries were estimated from OCT cross sections (Figs. 3.9, 3.1, 3.2). Meshes were made sufficiently dense such that further refinement did not significantly affect the solutions (Figs. 3.1B, 3.2B). In the compressive loading models, a frictionless rigid plate was used to compress the brain and heart using appropriate contact pairs as guided by the Comsol user manual.

**Microindentation model**

Axisymmetric models were used to study the effect of brain geometry on mechanical stiffness (see Fig. 3.9). Reflecting experimental morphology, flattened HST and NST+bleb midbrains are modeled as circular discs (300 \( \mu \)m in diameter and 125 \( \mu \)m thick), while NST and MST midbrains are modeled as hollow spheres (260 \( \mu \)m outer diameter and 130 \( \mu \)m inner diameter). (Spherical geometry takes longitudinal
curvature into account in a first approximation.) Material properties are defined by Eq. 1 with $\mu = 200$ Pa and $\kappa$ three orders of magnitude larger than $\mu^{12}$. The indenter is modeled as a rigid cylinder (26 $\mu$m in diameter) with a prescribed downward displacement and frictionless contact with the brain.

**Heart and brain models: morphological and mechanical background**

Before considering the implementation of finite element models for the embryonic heart and brain, we first describe relevant morphological and mechanical differences between these tissues.

Despite similarities in the tubular geometries of the early heart and brain, fundamental differences exist between their cytoskeletal, nuclear, and cellular organization. The neuroepithelium of the brain consists of radially aligned cells (and nuclei - see Fig. 3.12) that are more than twice as thick as the circumferentially aligned myocardial cells that wrap around the cardiac jelly (CJ) in the heart tube. While cardiomyocyte elongation has been reported at the inner curvature of the looping heart, the cells and nuclei of the heart are generally more randomly oriented than their neuroepithelial counterparts. Indeed, changes in cell size and proliferation are not uniform during looping (Soufan et al., 2006), and to our knowledge, changes in nuclei shape have not been characterized during this process.

In the brain tube, actin microfilaments are radially oriented across the neuroepithelial wall with a localized concentration of circumferential actin at the apical (inner) wall (Fig. 3.5B), similar to younger embryos (van Straaten et al., 2002). In the HH10 heart, myocardial actin is circumferentially aligned in a relatively homogeneous distribution, but this changes during looping (Itasaki et al., 1989; Shiraishi et al., 1992). Swelling of CJ during looping induces a general state of tension in the myocardium (Ramasubramanian et al., 2006; Zamir and Taber, 2004). The stress distribution across the thicker neuroepithelium is less well characterized, but certain details are relevant to our analysis. In particular, prior to ventricle formation, the inner lumen of the brain is not pressurized (Desmond et al., 2005) and a significant residual stress
develops by HH11 (Xu et al., 2010). Taking into account these differences in morphology and stress state, we developed finite element models for the early heart and brain (see Figs. 3.1, 3.2, 3.16, 3.17).

**Isolated heart model**

The heart tube is modeled as a cylinder (2D - plane strain) composed of two layers: an outer layer of myocardium (MY) (10 \(\mu m\) thick) and an inner, softer layer of cardiac jelly (CJ) (115 \(\mu m\) thick) (Figs. 3.1, 3.16). The total diameter of the heart model is 250 \(\mu m\). Due to symmetry conditions, only one quarter of the heart was simulated with appropriate boundary conditions (Fig. 3.1A). Material properties for both layers are approximated by Eq. 1 with \(\mu_{MY} = 26\) Pa and \(\mu_{CJ} = 6\) Pa\(^{13}\). The material contains no interior lumen, because in most cases no lumen was apparent in isolated hearts as determined from OCT cross sections (Fig. 3.4A”-C”).

Cardiac jelly is an acellular passive material, and, during looping, the diastolic myocardium contains little cytoskeletal contractility (Remond et al., 2006). Hence, we took \(G = I\) in the myocardium of the control heart. To simulate swelling of the CJ, we specified \(G_r = G_\theta = 1.3\).

**Isolated brain model**

The brain tube is modeled as a hollow cylinder (2D - plane strain) composed of two layers: an inner, active contractile ring (5\% of the wall thickness) and an outer, passive layer of neuroepithelium (Fig. 3.2A). We note that the neuroepithelium is likely not completely passive in the embryo, but radial contraction and relaxation effects are beyond the scope of this study. Half of the brain tube is modeled with appropriate symmetry conditions (Fig. 3.2A). For the HH10 midbrain, the inner diameter of the tube is 225 \(\mu m\) and the wall thickness is 48 \(\mu m\).

Growth in the midbrain from HH10 to HH11 was prescribed to match experimental changes in luminal diameter (80 \(\mu m\) increase to 305 \(\mu m\)), and wall thickness (10 \(\mu m\) increase to 58 \(\mu m\)), as well as residual stress at HH11 (opening angle \(\approx 80^\circ\)) (Fig. 3.2C,D)\(^{12}\). Uniform radial growth was prescribed to account for changes
Figure 3.1: Finite element modeling scheme for the heart. (A) The heart tube is modeled as a cylinder composed of a relatively thin myocardial layer surrounding a core of cardiac jelly. To simulate the forces due to surface tension in the culture media, a rigid plate with a prescribed downward displacement is used to compress the heart. (B) A sufficiently dense mesh is used to capture local stress variations in the myocardium during compression.

in wall thickness \( (G_r = 1.2) \), while a baseline contraction was specified in the inner actomyosin ring as guided by experimental results for F-actin staining patterns (Fig. 3.5B). Consistent with this idea, recent work has proposed epithelial relaxation (largely on the apical side) as a mechanism for luminal expansion in the zebrafish hindbrain (Gutzman and Sive, 2010).

To generate the measured change in diameter and opening angle, non-uniform circumferential growth was specified across the neuroepithelium in the form
\[ G_\theta = G_a G_i \left[ 1 - \left(1 - \frac{G_o}{G_i} \right) \left( \frac{R - R_i}{R_o - R_i} \right) \right] \]  

(3.3)

where \( G_i = 1.48 \) and \( G_o = 1.22 \) are the circumferential growth stretch ratios at the inner and outer radii respectively, \( G_a \) is the active growth stretch ratio (to simulate contraction), and \( R_i \) and \( R_o \) are the inner and outer radii of the tube at HH10. In the contractile ring, we take \( G_a = 0.9 \), while \( G_a = 1 \) in the rest of the (passive) wall. Outside the inner ring, \( G_\theta \) decreases linearly across the wall (Fig. 3.2C'). This puts the basal (outer) wall in tension \( (\sigma_\theta > 0) \) and the apical (inner) wall in compression \( (\sigma_\theta < 0) \) (Fig. 3.2C), yielding a positive opening angle (Fig. 3.2D). The innermost apical ring \( (G_a = 0.9) \), however, remains in tension.

The radial cut in an opening angle experiment (Fig. 3.2D) was simulated by rerunning the plane-strain model with one of the neuromuscular boundaries switched from a symmetry to a free boundary condition. After cutting, most of the brain becomes stress free, except for the inner contractile ring (Fig. 3.2D). To check for out-of-the-plane artifacts due to the plane-strain assumption, opening angles were verified against separate plane stress models (Alford et al., 2008).

### 3.3.7 Statistics

All data are reported as mean +/- SD. Statistical analyses were performed using the statistics package in SigmaPlot (v11, Systat Software Inc.). Tissue morphology and stiffness as well as nuclear circularity and misalignment were compared across multiple sample groups using one-way ANOVA, with post hoc pairwise comparison made using the Bonferroni - Dunn Test \( (p < 0.05 \) for statistical significance). To compare samples indented before and after changes in loading or blebbistatin treatment, a two tailed, paired t-test was used.
3.4 Results

3.4.1 Isolated hearts stiffen under reduced loads

First, we consider the morphology, culturing, and mechanical testing of the isolated heart. The embryonic chick heart forms as a relatively simple tube at Hamburger-Hamilton stage 10 (HH10, approximately 33 hours) (Hamburger and Hamilton, 1951). At this stage of development, an inner endocardium (one cell, approx. 10 µm thick) is separated from an outer myocardium (two cells, approx. 20 µm thick) by a viscous extracellular matrix dubbed the cardiac jelly (CJ) (Fig. 3.3A). Isolated hearts were
studied over a time period of about 12 hours (HH10–HH12). These stages of development encompass the morphogenetic process of cardiac c-looping, as the initially straight heart deforms into a c-shaped tube (Manner, 2000).

Isolated HH10 hearts were cultured in fluid whose depth was adjusted to provide three types of loading (Fig. 3.4): no surface tension (NST), moderate surface tension (MST), and high surface tension (HST). The NST condition (fully submerged heart) effectively eliminated external loads from surrounding tissues (Voronov and Taber, 2002) (Fig. 3.4A). The MST case was used to approximate loads in the intact embryo, as liquid media was removed until the horizontal diameter of the isolated heart was
similar to that of the intact heart (Fig. 3.4B). Finally, to culture explants under high surface tension (HST), liquid media was wicked away from the surface of the culture gel, greatly compressing the heart (Fig. 3.4C).

Cross sections obtained by optical coherence tomography (OCT) reveal that, as compressive loading increased, samples were flattened and less cardiac jelly separated the myocardium and the endocardium (Fig. 3.4A”-C”). In general, the internal lumen was closed after culture (Fig. 3.4A”-C”), but the isolated heart looped into a c-shaped tube in all culture conditions, with the dorsal mesocardium located at the inner curvature (Fig. 3.4A’-C’), further confirming the robust nature of this process in extraembryonic conditions (Butler, 1952; Remond et al., 2006).

Microindentation tests were used to measure tissue stiffness, as hearts were indented on the outer curvature of the loop (OC, see Fig. 3.4B’). Past studies have found the outer layer of myocardium to be an order of magnitude stiffer than the inner layer of cardiac jelly (Zamir, 2003), suggesting that measured stiffness is primarily that of the myocardium. During these tests, verapamil (calcium channel blocker - 50 µM) was used to inhibit sarcomeric contraction, arresting the heart without changing the diastolic mechanical properties of the myocardium (Nerurkar et al., 2006; Ramasubramanian et al., 2008). Note that verapamil affects sarcomeric contraction, but not non-sarcomeric (cytoskeletal) contraction.

Because force-displacement curves were nonlinear, the stiffness (slope) varied with indentation depth (Remond et al., 2006). Consistent with our previous results for unloaded hearts in ovo (Nerurkar et al., 2006), hearts cultured under NST for 12 hours were significantly stiffer than hearts cultured under MST or HST at indentation depths ≥ 15 µm (Fig. 3.4D) . Hearts cultured under MST were similar in stiffness to HST hearts (Fig. 3.4D). In summary, the stiffness of the heart increased significantly when compressive loads were removed, but the stiffness changed relatively little under loads greater than control. Previously, we showed that the increased stiffness in response to decreased loading is caused by cytoskeletal contraction (Remond et al., 2006).
Figure 3.4: Isolated heart morphology and stiffness under altered loading conditions. (A-C) Embryonic hearts were isolated at HH10^-10^+ and cultured under NST (no surface tension, n = 6), MST (moderate surface tension, n = 6), or HST (high surface tension, n = 5). (A'-C') After 12 hours, the hearts were looped. (A''-C'') Less cardiac jelly (CJ) separated the endocardium (EN) and the myocardium (MY) as loads increased. Dashed lines in A'-C' indicate OCT cross section locations in A''-C''. Heart tubes are outlined for clarity in bright field images; OC = outer curvature, IC = inner curvature. Scale bar: 250 µm. (D) Hearts were indented on the outer curvature after culture. Stiffness was similar at a 5 µm indentation depth, but diverged thereafter. NST hearts were significantly stiffer (*, p < 0.05) than MST and HST hearts at indentation depths ≥15 µm. MST hearts were similar in stiffness to HST hearts at all indentation depths.
3.4.2 Isolated brains shorten, curl, and stiffen under reduced loads

The above experiments were repeated for the brain. Brains were removed from the embryo at HH10. At this stage, the brain is a tubular, pseudostratified neuroepithelium (one cell, approx. 40-50 µm thick) surrounded by the vitelline membrane, surface ectoderm, head mesenchyme, and notochord (Fig. 3.3B). Actin microfilaments are concentrated along the apical (inner) side of the tube (red outline in Fig. 3.3B, Fig. 3.5B). In addition, the optic vesicles (OVs) have evaginated laterally from the cranial end of the brain tube, although the brain ventricles cannot yet be easily identified (Fig. 3.6A, A'). In the intact embryo, the midbrain is compressed by the vitelline membrane and the head mesenchyme, yielding a slightly elliptic cross section (Fig. 3.6A’).

Under NST conditions, the lumen of the isolated brain opens further (Fig. 3.6B). As in the heart experiments, the MST state was obtained by removing liquid media until the diameter of the isolated brain was similar to that of the intact brain (Fig. 3.6C). Finally, removing most of the fluid gave HST, which compressed the brain until the lumen was no longer apparent (Fig. 3.6D). Isolated brains were cultured for 5 hours in each loading condition and resulting tissue lengths, stiffnesses, and nuclear shapes were measured.

In the NST condition, the length of isolated brains decreased by approximately 25% during culture, and the brain tube curled with the relatively stiff notochord located at the inner curvature (similar to the dorsal mesocardium in the heart) (Fig. 3.7A, A’, E). MST and HST brains decreased in length but significantly less than the NST samples (MST, p < 0.05; HST, p < 0.001), and did not curl (in contrast to the heart) as these explants were pressed into the semi-solid media by the surface tension (Fig. 3.7B, B’, C, C’, E). While a significant difference in length change was not found between MST and HST conditions, in general, it appeared that shortening was inversely proportional to mechanical loading (Fig. 3.7E).

Microindentation was used to measure tissue stiffness in the dorsal midbrain for each loading condition. In general, due in part to differences in global structure, isolated brains were stiffer than isolated hearts (Figs. 3.4D and 3.8B), a result consistent with
Figure 3.5: Actin localization in the early brain. (A) At HH10 the brain is a relatively uniform cylindrical structure with evaginated optic vesicles (OV) at the cranial end. (B) Phalloidin staining shows that F-actin is concentrated at the apical (Ap) wall of the neuroepithelium. This localized region comprises only a small percentage of the total wall thickness of the brain. (Ba: basal side of the wall). White outline in (A) corresponds to the fluorescent region in (B). Scale bars: 250 \( \mu \text{m} \).

past studies (Remond et al., 2006; Xu et al., 2010). Explant stiffness increased as external loading decreased (Fig. 3.8B), a difference that was statistically significant for NST samples relative to HST brains for indentation depths \( \geq 10 \mu \text{m} \) (Fig. 3.8B).

To explore the reversibility of these perturbations, we performed additional experiments in which loading was changed at an intermediate time point during culture. Brains were cultured for half the time (2.5 hours) with increased (HST) or decreased (NST) loading, indented, cultured the remaining 2.5 hours under the opposite loading and then indented again (Fig. 3.8C, D). At the halfway point, stiffness values were similar to those of brains cultured for the full five hours in each respective loading condition. Following transfer to the opposite loading condition (HSTt, NSTt), the
Figure 3.6: Morphology and loading of the brain tube. (A) At HH10 the brain is a relatively cylindrical tube with evaginated optic vesicles (OVs) at the cranial end. (A’) 3-D reconstruction of the inner lumen (white outline in A) from optical coherence tomography (OCT) imaging. Transverse OCT cross sections show the prospective midbrain (dashed black line in A) in normal (A”) and altered loading conditions (B-D). (A”-D) For clarity, the basal (solid line) and apical (dashed line) sides of the brain tube have been delineated. After removing surrounding tissues (except the notochord, N), the brain is cultured under (B) no surface tension (NST), (C) moderate surface tension (MST), or (D) high surface tension (HST), depending on the amount of liquid media present during culture. HM = head mesenchyme; SE = surface ectoderm; VM = vitelline membrane. Scale bar: 250 µm.

Stiffnesses of HSTt and NSTt brains decreased and increased, respectively. These results show that the responses to altered loading conditions are reversible.

Structural stiffness depends on both material properties and geometry. Because brain geometry varied greatly between sample groups (Fig. 3.6B-D), it was important to determine how geometry affected our results. To estimate the effects of geometry on apparent tissue stiffness, finite-element models for brains undergoing micro-indentation were created using Comsol Multiphysics software (Fig. 3.9). For the same material properties, the models showed that apparent stiffness was approximately 1.6 times higher in collapsed brains relative to those with an internal lumen (Fig. 3.9C). Hence,
Figure 3.7: Isolated brain morphology under altered loading conditions. Isolated brains were cultured for 5 hours under no surface tension (NST, n = 38), moderate surface tension (MST, n = 18), or high surface tension (HST, n = 20). Additional brains were cultured under NST in blebbistatin (60 µM, NST+bleb, n = 13) to inhibit cytoskeletal contraction. (A-D) HH10 brains at the beginning of the experiment. (A’-D’) Same brains after 5 hours of culture. (E) Ratio of brain tube length after culture to length before culture. Brains became shorter, with NST and MST brains shortening more than HST brains. Treating NST brains with bleb prevented the length decrease and curling observed in NST samples. (E’) All brains were measured along the midline. Statistics: * = p < 0.05 and ** = p < 0.001 relative to the NST loading condition, and + = p < 0.05 relative to the MST loading condition. Scale bar: 500 µm.
Figure 3.8: Stiffness of isolated brains cultured under different loading conditions. Midbrain stiffness was measured on the dorsal side using a micro-indentation device. (A) Representative force-displacement curves for each of the sample groups are shown. Wall stiffness is the slope of this curve at a particular indentation depth. (B) Stiffness versus indentation depth. Brains cultured under no surface tension (NST, n = 13) for 5 hours were significantly stiffer than those cultured under high surface tension (HST, n = 7) or no surface tension + blebbistatin (NST + bleb, n = 5) at indentation depths ≥ 10 µm (*, p < 0.05). The stiffness of moderate surface tension (MST, n = 5) brains fell between NST and HST samples but differences were not statistically significant. (C,D) Effect of reversed loading conditions on brain stiffness. (C) Brains were isolated and cultured under NST for 2.5 hours, indented, transferred to HST, cultured for an additional 2.5 hours (HSTt), and then indented again. Stiffness significantly decreased at all indentation depths following this change in loading. (D) The opposite experiment (HST culture followed by NST culture) led to an opposite significant stiffening effect. Stiffness values were similar to those measured after 5 hours of culture in each respective culture condition (n ≥ 3 for each load reversal experimental set).

the flattening effect of high surface tension would increase the measured tissue stiffness, and it follows that for the same geometry, stiffness differences between HST versus NST and MST samples may in fact be even larger than our data indicate.
Figure 3.9: Micro-indentation model to determine the effect of brain geometry on measured tissue stiffness. Generally, HST and NST + bleb brains were flattened and lacked an internal lumen as opposed to MST and NST brains. (A, B) To compare these groups, representative midbrain geometries were estimated from OCT cross sections of HST and NST brains. (A’, B’) In these axisymmetric models, the HST sample is a flattened disc of tissue, while the NST sample is a hollow sphere. (C) Simulating micro-indentation in these models with a rigid cylindrical indenter reveals that the apparent stiffness (slope of the force-displacement curve) of the HST sample is approximately 1.6 times higher than the NST sample for the same material properties. Scale bar: 100 µm.

3.4.3 Changes in brain morphology and stiffness correlate with changes in cytoskeletal contractility

To test whether these changes in morphology and stiffness were caused by cytoskeletal contraction, we inhibited myosin II by culturing specimens in 60 µM blebbistatin
(bleb) under NST conditions. The length of explants exposed to bleb decreased only slightly (<5%) (Fig. 3.7D, D’, E), and these explants curled less than those cultured under NST alone (Fig. 3.7A’, D’). Bleb-treated brains shortened significantly less than the MST (p<0.05) and NST (p<0.001) brains without bleb (Fig. 3.7E). Taken together, these data suggest that the embryonic brain actively shortens via cytoskeletal contraction when external loads are reduced.

In additional experiments, NST brains were cultured in bleb for 2.5 hours and then transferred to normal media. After bleb washout, curling resumed (Fig. 3.10A”). Also, when bleb was added to brains undergoing curling for 2.5 hours, curling ceased and the overall tissue size increased (Fig. 3.10B”). These results show that inhibiting contraction stops brain curling after it begins and that this effect is reversible.

![Figure 3.10: Effect of blebbistatin washout on brain curling. (A, B) Isolated brains were cultured for 2.5 hours under NST with (A, n = 4) and without (B, n = 3) bleb-treatment (60 µM). (A’, B’) After 2.5 hours, bleb-treated brains curled much less than NST brains in normal media. (A”) Following bleb washout and culture for an additional 2.5 hours, substantial tissue curling resumed. (B”) Treating with bleb following NST culture inhibited further curling, and brain size increased. Scale bar: 500 µm.](image)

Brain stiffness was measured to confirm the effects of bleb on contractility. Stiffness of HST brains was similar to the NST+bleb brains at all indentation depths, suggesting
reduced contraction in HST explants (Fig. 3.8B). To further examine the effects of bleb exposure time, a subset of the NST explants (NSTs) were reincubated for an additional hour in 60 μM blebbistatin and reindented (Fig. 3.11). A statistically significant decrease in tissue stiffness was found at indentation depths ≥ 10 μm. Stiffness decreased to approximately the same value as tissues cultured for the full five hours in blebbistatin (dashed line in Fig. 3.11).

![Indentation Depth (µm)]

<table>
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<th>10.0</th>
<th>12.5</th>
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<tr>
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<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
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<td>NST + Bleb (5 hr)</td>
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Figure 3.11: Effect of blebbistatin exposure time on NST (stiffened) brains. A subset of NST explants (NSTs, n = 5) were reincubated for an additional hour in 60 μM bleb and reindented. Tissue stiffness significantly decreased at indentation depths ≥ 10 μm (*, p<0.05). The stiffness of brains treated for one hour with bleb were similar to those cultured for the full five hours in the drug (dashed line).

In summary, brain stiffness increased as externally applied loads decreased (Fig. 3.8B). This response was myosin-II dependent, as stiffness and length changes did not occur in blebbistatin-treated samples cultured under NST conditions (Figs. 3.7, 3.8, 3.10, 3.11). The brain, therefore, contracts and stiffens when compressive loads are decreased, but relaxes and softens when loads are increased.

3.4.4 Brain nuclei change shape under changes in load

Lastly, we speculated that changes in contractility alter wall stresses, which in turn affect the shape of cell nuclei. Studying nuclear shape in the brain is easier than in the developing heart, because brain nuclei have more consistent patterns than those in the heart. In the normal brain tube, nuclei are generally elongated and aligned in
the radial direction from HH10 to HH12 (Fig. 3.12). Hence, we analyzed changes in nuclear shape and orientation in the midbrain for each loading condition (Fig. 3.13). Nuclear misalignment was defined as the difference in angle between the major axis of the elliptically shaped nucleus and the normal to the neuroepithelial wall. Nuclear circularity (minor/major axis of the best-fit ellipse, 1 = perfect circle, 0 = straight line) was also calculated.

As in normal HH11 brains (Fig. 3.12B'), nuclei were radially elongated and relatively aligned in control brains cultured from HH10 to HH11 (Fig. 3.13A). This morphology was consistent across the neuroepithelial wall and did not vary in different regions of the midbrain (Fig. 3.14). In MST brains, nuclear morphology was similar to control (HH10-11) brains (Fig. 3.13C, G, H), while NST nuclei were more aligned and elliptical than controls, although differences were not statistically significant (Fig. 3.13B, G, H). Exposure to bleb resulted in rounder (p < 0.001), more randomly oriented (p < 0.05) nuclei (Fig. 3.13E, G, H), which were similar to nuclei of HST brains (Fig. 3.13D, G, H). Brains compressed with a glass coverslip (as opposed to surface tension) also had a similar rounded nuclear phenotype (Fig. 3.15).

As a corollary to the bleb experiments, we enhanced myosin contractility in HST samples by adding calyculin (cy - phosphatase inhibitor) to the culture gels at a final concentration of 20 nM. After the same 5 hour culture period, the nuclei of HST+cy brains were more elliptical and radially aligned than HST brains, and had a phenotype similar to the MST samples and the HH10-11 controls (Fig. 3.13F, G, H).

Taken together, our data suggest that as contractility increases, neuroepithelial nuclei become more elliptical in shape and more radially aligned. As contractility decreases, nuclei become rounder and more randomly oriented.

3.5 Discussion

The main finding of this study is that epithelia of the embryonic brain and heart respond similarly to altered mechanical loads. Our results indicate that these organs decrease in stiffness as compressive loads increase, and vice versa. Exposure to the myosin II inhibitor blebbistatin shows that these changes in stiffness are associated
Figure 3.12: Nuclear morphology in normal brains. Representative DAPI-stained midbrain sections in embryos fixed immediately after removal from the egg. Dashed regions in A-C correspond to enlarged regions in A'-C'. From HH10 to 12 (36 to 48 hours of incubation), midbrain nuclei are elliptical in shape and oriented in the radial direction. In general, nuclear density increases and circularity decreases with embryonic stage. Example nuclei have been outlined in A'-C' for clarity. Scale bar: 50 µm.

This response is also accompanied by changes in nuclear shape (heart nuclei were not studied). As discussed below, all of these effects are consistent with an adaptive response that tends to restore the original mechanical state.
Figure 3.13: Nuclear shape and orientation in the brain under different loading conditions. (A) Nuclei are elliptically shaped and radially aligned relative to the inner lumen in control embryos cultured from HH10 to 11. Ap: apical side of the neuroepithelial wall; Ba: basal side of the wall. (B, C) Culturing brains under reduced loading (NST) led to highly organized, more elliptical nuclei, while the morphology of MST (approximating normal loading conditions) samples mimicked HH10-11 controls. (D, E) Nuclei of highly loaded (HST) brains were more circular and randomly oriented than controls, and resembled the phenotype of blebbistatin-treated brains. (F) Stimulating contraction in HST brains with 20 nM calyculin (cy) prevented increases in nuclear circularity and misalignment. (G, H) Nuclear circularity and misalignment relative to the radial direction were quantified and compared between sample groups. As loading decreased, nuclei generally became more elliptical in shape and more aligned. The nuclei of loaded brains (MST and HST) were more circular and misaligned. Statistics: * = p < 0.05 and ** = p < 0.001 relative to the NST+bleb loading condition; + = p < 0.05 and ++ = p < 0.001 relative to the HST loading condition.

Even though different types of cells can have widely disparate functions, studies have shown a remarkable similarity in how they respond to certain types of mechanical stimuli. For example, fibroblasts, smooth muscle cells, and endothelial cells all turn away from the direction of oscillatory stretch on a 2-D substrate (Chien, 2007), and, for many cell types, the cytoskeleton rapidly fluidizes and then re-solidifies in response to transient stretch (Trepat et al., 2007). Of particular relevance here is the study of Mizutani et al. (2004), who found that fibroblasts become softer or stiffer in response to stretch or shortening, respectively. Like our results, these responses for single cells correlate with changes in actomyosin contractility. At the tissue level, the
Figure 3.14: Nuclear circularity distribution in HH11 brain. (A) Nuclei were grouped into four regions of DAPI-stained midbrain sections in embryos cultured from HH10 - 11; D = dorsal, V = ventral, R = right, L = left. Nuclei in the notochord (N) were not included in these analyses. (B, C) Circularity is plotted as a function of wall thickness in regions with similar wall stress distributions (B - dorsal and ventral, C - left and right). No significant differences in circularity were observed between these regions. (Linear regressions show little variation between sampling groups). (D) Pooling the data shows that nuclear circularity is relatively homogeneous across the neuroepithelial wall in control embryos.

Present results for the embryonic heart are consistent with previous findings, whereby removing a membrane (splanchnopleure) that normally compresses the looping heart elicits a contractile response that stiffens the myocardium (Nerurkar et al., 2006; Ramasubramanian et al., 2008).

During early embryonic development, the heart and brain share some common features. Both are tubes composed primarily of epithelia. In the heart, the rate of growth depends on blood pressure (Clark et al., 1989); in the brain, growth rate depends on cerebrospinal fluid pressure (Desmond and Jacobson, 1977; Desmond et al., 2005). In addition, mechanically driven changes in tissue shape are crucial to both heart and brain morphogenesis (Filas et al., 2008; Gutzman et al., 2008; Taber, 1995). Hence, it
Figure 3.15: Phenotype of isolated brains compressed by glass coverslips. As an alternative method, small pieces of coverslips were used to compress isolated brains. (A) Brain before and after (B) compression with a coverslip (load $\approx 1$-4 dynes). (C) Transverse OCT cross section through the midbrain (dotted line in B). The internal lumen is nearly closed. (D) Following five hours of culture, nuclei are relatively round, similar to the HST phenotype. Scale bar: 250 $\mu$m.

is no surprise that both types of epithelia (myocardium and neuroepithelium) exhibit similar mechano-sensitive behaviors.

In our experiments, both the isolated heart and brain contracted and stiffened when loads from surrounding tissues were removed (Figs. 3.4D and 3.8). Increased compressive loading induced cytoskeletal relaxation and softening of the brain (Fig. 3.8), but, in the heart, the stiffness of highly compressed (HST) samples was similar to that of control (MST) samples. The lack of softening in the heart is consistent with the finding that the myocardium of the heart tube contains relatively little myosin II-based cytoskeletal contractility (as opposed to sarcomeric contractility) under normal conditions (Remond et al., 2006). In other words, the diastolic cytoskeleton is already in a relatively relaxed state. In addition, isolated brains were stiffer than isolated hearts in all culture conditions. While individual brain cells generally are softer than heart cells (Engler et al., 2006), in the early embryo, the neuroepithelium (ectoderm) is much thicker than the myocardium (mesoderm), explaining in part the higher neuroepithelial indentation stiffness. Moreover, Krieg et al. (2008) found that
early ectodermal cells are significantly stiffer (due to higher cortical tension) than mesodermal and endodermal cells.

In the brain, these changes in contractility and stiffness are also associated with global changes in brain size and shape, as well as changes in the shape of cell nuclei. Longitudinal contraction causes the brain to shorten and curl (Figs. 3.7, 3.10). The curling, with the notochord along the inner curvature, is consistent with flexure that occurs during normal development (Goodrum and Jacobson, 1981). (Similarly, like hearts in ovo, isolated hearts loop with the dorsal mesocardium at the inner curvature.) The enhanced magnitude of the bending in NST brains may be caused by contraction of the actin-rich notochord (Munro and Odell, 2002).

During early development in ovo, neuroepithelial nuclei become increasingly elliptical and radially aligned (Fig. 3.12). In our experiments, nuclei became even more elliptical and aligned with the increased contraction induced by decreased loading (Fig. 3.13B). Decreasing contractility, whether through compressive loading (HST) or drug treatment (NST+bleb), produced circular nuclei with a less organized orientation (Fig. 3.13D, E, G, H). Since contraction affects wall stress, these results are consistent with studies showing that mechanical forces can alter nuclear shape (Guilak, 1995; Maniotis et al., 1997).

Forces deform the nucleus through cytoskeletal connections, causing conformational changes in DNA or chromatin structure, which can alter gene expression (Dahl et al., 2008; Wang et al., 2009). Progenitor cells are potentially more sensitive to changes in mechanical loads, as their nuclei are softer and more deformable than the nuclei of terminally differentiated cells (Pajerowski et al., 2007). Therefore, we speculate that changes in nuclear shape correlate with changes in wall stress. To illustrate the feasibility of this mechanism, we developed finite element models simulating compressive loading of the tubular heart and brain (Figs. 3.16, 3.17). The results and interpretation are summarized in Figs. 3.18 and 3.19.

According to the model for the heart, reducing the normal compressive load decreases the myocardial tension caused by swelling and squeezing of cardiac jelly (Figs. 3.16, 3.18 MST to NST). In response to this perturbation, actomyosin contraction restores myocardial tension (Figs. 3.16, 3.18, NSTc). In a previous study, we
Figure 3.16: Computed stress distributions in the embryonic heart. Loads due to surface tension are simulated via various amounts of compression by a rigid plate (see Fig. 3.1). Colors indicate circumferential stress ($\sigma_\theta$) in directions convected with the deformation, and the graph shows the distribution along the outer surface of the myocardium (MY). **MST:** moderate surface tension (control). The cardiac jelly (CJ) has baseline uniform swelling (growth, $G_r = G_\theta = 1.3$) that stretches the passive MY. With moderate compression, the entire MY is in a state of circumferential tension. The jump in stress in the graph occurs at the boundary of contact between the plate and the heart. **NST:** no surface tension (unloaded). Tension in MY is reduced. **NSTc:** Active contractile response in MY ($G_\theta = 0.9$) increases tension. The contractile response (NSTc) reverses the direction of the original stress perturbation.

found that a similar response to decreased loading can play a role in morphogenesis of the looping heart (Nerurkar et al., 2006; Ramasubramanian et al., 2008).

The situation in the brain is a little more complicated. The main reason is that, since the neuroepithelial wall is relatively thick, compressing the brain causes bending stresses that are strongly inhomogeneous (Fig. 3.17). We assume that (1) contractile stress is generated predominately by the actomyosin-rich network concentrated at the apical (inner) side of the neuroepithelium (Fig. 3.5B); (2) apical actin is a key mechano-sensor in the brain tube; and (3) peak actin stress modulates the response of the entire actin network. The last assumption is consistent with recent work.
Figure 3.17: Computed stress distributions in embryonic brain. Surface tension loads on the embryonic midbrain are simulated via compression with a rigid plate (see Fig. 3.2A). Circumferential stress ($\sigma_\theta$) in directions convected with the deformation is plotted in the neuroepithelium. Larger stresses in the apical actomyosin ring were outside surface plotting bounds. Hence, the contractile ring graph shows the circumferential stress along the apical (inner) wall for each condition. (Labels 1a, 1b, etc. in the graph correspond to labels between models.) \textbf{MST:} moderate surface tension (control). The apical ring has a baseline contraction ($G_a=0.9$, 10% contracted) and the brain is moderately compressed. The ring is under maximum tension in the middle. \textbf{NST:} ring stress is uniform; maximum ring tension is reduced (unloaded). \textbf{NSTc:} Active contractile response in the inner ring ($G_\theta=0.8$) increases maximum ring tension. \textbf{NST to NSTc:} This contraction increases compressive stress in the circumferential direction across the remainder of the neuroepithelial wall (favoring elliptical nuclei). \textbf{HST:} high surface tension. Relative to MST state, maximum tension increases in the ring. \textbf{HSTr:} apical ring relaxes ($G_a=1.0$, 0% contracted), which decreases maximum tension in the ring. \textbf{HST to HSTr:} Following relaxation, circumferential compression decreases in the remainder of the neuroepithelium (favoring circular nuclei). Note that active responses in the inner actomyosin ring reverse the direction of the original stress perturbations. Transmural wall stresses (NST to NSTc and HST to HSTr graphs) are from the dorsal wall of the brain (e.g. dashed line in the NSTc plot) and plotted from the apical (Ap) to the basal (Ba) side.

suggesting that local changes in tension can induce large-scale changes in contractility in neighboring cells (Chowdhury et al., 2010; Fernandez-Gonzalez and Zallen, 2009; Pouille et al., 2009).
Figure 3.18: Response of heart to altered mechanical loads. Schematic for active myocardial response to changes in compressive loading. **MST:** moderate surface tension (control). The myocardium (MY) is in a state of circumferential tension (shown by arrow in the MY). Tension is caused by loads applied by the cardiac jelly (CJ) as it swells. *In ovo* compression by the overlying splanchnopleure (SPL) membrane is simulated by the surface tension. The lumen surrounded by the endocardium (EN) is closed in isolated hearts (see Fig. 3.4). **NST:** no surface tension. Removing external loads decreases myocardial tension. **NSTc:** Active contraction increases tension in the MY, counteracting the initial loss in tension following compressive load removal.

In the brain tube, increasing compressive loading (HST) increases the maximum tension in the apical actomyosin ring, while decreasing compression (NST) has the opposite effect (Fig. 3.17). In response, the inner ring contracts when loads are removed (NSTc) and relaxes when loads are increased (HSTr) (Figs. 3.17, 3.19). These responses tend to reverse the direction of the original perturbation in peak actin stress (Fig. 3.17).
Figure 3.19: Response of brain to altered mechanical loads. Schematic for active response of the neuroepithelium to changes in loading. Line thickness of the apical actomyosin ring (red) reflects relative contractility causing these adaptive responses. **MST:** Moderate surface tension (control). Radial compressive loads are imposed by surrounding tissues in the intact embryo (see Fig. 3.3B). Nuclei are elliptical in shape and radially oriented (black circle). Actin ring is in a state of circumferential tension due to contraction. **NST:** Removing surrounding tissues decreases peak tension in actin ring. **NSTc:** Elevated contraction in the actomyosin ring increases to counter this decrease in tension. Circumferential compression increases in the remainder of the neuroepithelium, favoring radially elongated nuclei. **HST:** High compressive loading increases peak tension in ring. **HSTr:** The actomyosin ring relaxes to offset this increase in tension. This reduces circumferential tension across the remainder of the wall, favoring circular nuclei.

Furthermore, apical contraction causes circumferential compression throughout the remainder of the wall (Fig. 3.17, NST to NSTc). This change in stress favors elliptical, radially aligned nuclei as seen experimentally (Fig. 3.19, NSTc case; Fig. 3.13B). Conversely, apical relaxation has the opposite effect (Fig. 3.17, HST to HSTr), favoring the relatively circular nuclei observed after HST culture (Fig. 3.19, HSTr case; 3.13D).

The non-uniform stresses in the brain under all loading conditions (Figs. 3.2, 3.17), however, does not seem consistent with the relatively uniform patterns in nuclear
shape observed in the neuroepithelial wall (Fig. 3.14). One explanation for this discrepancy may be that our model is too simple, as it neglects, for example, radial contraction and the effects of 3-D geometry. Another possibility is that the nuclei intrinsically maintain a preferred shape that is modulated by a uniformly distributed molecular signal triggered by peak actin stress. Clearly more work is warranted to decipher how epithelial cell (and nuclear) shape is optimized with respect to changes in mechanical stress during development.

Together, our data suggest that embryonic epithelia respond to stress perturbations in ways that tend to restore the original (possibly peak) stress in the tissue, and that this adaptive response is brought about by alterations in cytoskeletal contractility. However, if our mechanically perturbed specimens were simply adapting ‘toward’ an original stress state, the nuclear shapes should begin to resemble those in control samples. In contrast, our results indicate that NST nuclei eventually become more elliptical than controls (MST), while HST nuclei become rounder than controls (Fig. 3.13). These data suggest that the response causes overshoots of the original nuclear shape and peak stress, in agreement with the hyper-restoration hypothesis proposed by Belousov and co-workers (Belousov, 2008; Belousov and Grabovsky, 2006). The significance of the overshoot has been demonstrated by computational models, which show that if tissue stress returns to its original homeostatic value as in mature tissues, then morphogenesis ceases when homeostatic conditions are restored (Taber, 2008). On the other hand, an overshoot in stress can induce further perturbations. In this way, a single perturbation can drive a relatively complete morphogenetic process (Belousov, 1998).

In conclusion, we have shown that changes in mechanical loading lead to changes in cytoskeletal contractility, which cause alterations in epithelial stiffness and nuclear shape in the developing embryo. We speculate that these changes are mediated by changes in tissue stress. Future studies are needed to determine the role of mechanical feedback in morphogenesis. Better understanding of how precursor cells adapt to changes in mechanical stress also could provide insight into the mechanisms of stress regulation and disease pathology in mature tissues (Humphrey, 2008; Jaalouk and Lammerding, 2009).
Chapter 4

Actomyosin Contraction Drives Formation of the Primary Vesicles in the Embryonic Chick Brain

4.1 Summary

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 boundaries 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.

4.2 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 (spatially and temporally) boundaries between 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 neurepithelium 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.

4.3 Materials and Methods

4.3.1 Embryo preparation, microdissection, 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. 4.2).

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.

4.3.2 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 Bioptechs 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 datasets were captured automatically. 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).
4.3.3 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 - 60 µM). 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. 4.4C).

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.

4.3.4 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. 4.6E) 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 a 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 µg/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).

### 4.3.5 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. 4.9A). 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. 4.9A,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. 4.9B’). 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. 4.9B”). 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 $R_0$ (to a first approximation). For an upper bound, the curvature of a compressed circular beam changes from $1/R_0$ to zero. Then, the wall strain at the inner surface is approximately

$$\epsilon = \frac{H}{2R_0}$$  \hspace{1cm} (4.1)

for full compression, where $H$ is the wall thickness. Inserting the mean values $H = 45 \, \mu m$ and $R_0 = 150 \, \mu m$ yields $\epsilon = 0.15$. Hence, the effect of compression on measured aspect ratio is relatively small ($\approx 15\%$) 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.
4.3.6 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; Belousov, 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 remains closed indicates little stress or compression in intact tissue. 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.

4.3.7 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. 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 at the apical (inner) wall of the brain using the theory of Rodriguez et al. (1993). In this theory, the deformation gradient tensor $F$ is decomposed into a growth tensor $G$ and an elastic deformation gradient tensor $K$ relative to the local zero-stress state ($F = K \cdot G$). 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):

$$W = \frac{1}{2}\mu \left( I_1 J^{-\frac{2}{3}} - 3 \right) + p \left( 1 - J - \frac{p}{2\kappa} \right).$$  \hspace{1cm} (4.2)
Here, $\mu$ is the small-strain shear modulus, $\kappa$ is the bulk modulus, $J$ is the elastic volume ratio ($\det K$), $I_1$ is the first invariant of the right Cauchy-Green deformation tensor ($K^T \cdot K$), and $p$ is a penalty variable introduced for nearly incompressible materials. In terms of $W$, the Cauchy stress tensor is given by (Taber, 2004)

$$\sigma = J^{-1}K \cdot \frac{\partial W}{\partial \epsilon} \cdot K^T$$

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

In the simulations, $G$ 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 ($G_\theta$ 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).

To account for the concomitant tissue stiffening that occurs during contraction, we assume that the shear modulus varies as

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

where $\mu_p$ is the passive modulus ($\approx 200$ 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. 4.12A.

As a first approximation, we assume that the values of $G_\theta$ and $G_z$ within each apical region are reflected in the aspect ratio and orientation angle of the neuroepithelial cells. This assumption is consistent with recent data showing that contractile force correlates with increased cell elongation in the direction of contraction (Alford et al., 2011). As shown later, three regions showed a statistically significant correlation between cell elongation and orientation indicating contraction of aligned cells: mid-hindbrain boundary ($\theta$), rhombomere boundary ($\theta$), and rhombomere ($z$) (see Fig. 4.9E-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 $\approx 0.6$. Thus, we take (see Fig. 4.10)

\[
\text{Primary vesicles: } \frac{G_\theta}{G_z} = 1 \\
\text{Boundaries: } \frac{G_\theta}{G_z} = \overline{AR} = 0.6 \\
\text{Rhombomeres: } \frac{G_z}{G_\theta} = \overline{AR} = 0.6.
\] (4.5)

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. 4.6). From these data, we assume that the magnitude of contraction ($C$) at the apical wall is given by the first approximations

\[
\text{Rhombomere boundaries: } C = G_\theta G_z = 0.75^2 \\
\text{Elsewhere: } C = G_\theta G_z = 0.5^2.
\] (4.6)

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 (Gorfinkiel et al., 2009) in Drosophila. The effects of varying the value of $C$ are explored in Fig. 4.12C.

Solving equations (4.5) and (4.6) gives values for $G_\theta$ and $G_z$ in each region, which are specified to change linearly with time.

### 4.3.8 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

\[
y_i = b_0 + b_1 \cos(a_i) + b_2 \sin(a_i)
\] (4.7)

where for every cell ($i = 1\ldots n$), $y_i$ 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. (4.7) 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 +/- SD with $p < 0.05$ for statistical significance.

### 4.4 Results

#### 4.4.1 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. 4.1A, 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. 4.1B, B’). The tube continues to expand through HH11+ (8 hours) as primary cranial flexure (ventral bending of the forebrain) occurs (Fig. 4.1C, C’).

Next, the cranial neuropore and the spinal neurocoel close at the top and bottom of the brain tube, respectively. Cerebrospinal fluid (CSF) 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. 4.1D, D’). Note that cross sections of the rhombomeres (R) and rhombomere boundaries (Rb) in the hindbrain remain relatively round.

These images show that all sections of the brain tube remain relatively round and open 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.

4.4.2 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. 4.2A, A’) nor periodically dissecting away tissue that normally occludes the spinal neurocoel from HH10 inhibited boundary formation (n = 3, Fig. 4.2B, 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. 4.3, arrows). These data suggest that boundary formation in the chick brain is intrinsic to the neuroepithelium.

Figure 4.2: Vesicle boundaries form independently of lumen pressure. Dotted lines indicate lumen. (A, A’) Boundaries form in brains intubated with a glass micropipette (black arrows). This perturbation maintains an open lumen during culture (FM = fore-midbrain boundary, MH = mid-hindbrain boundary, white arrows) (n = 8). (B, B’) In an alternative experiment, anterior somites are removed and the spinal neurocoel (SN) is dissected open (black arrows). (Embryos were checked hourly and re-dissected as necessary to ensure the tube remained open during culture.) Vesicle boundaries formed (n = 3). Scale bar: 200 µm.
Figure 4.3: Isolated brain culture. Dotted lines indicate lumen. Brains were isolated at HH10 and cultured for five hours to HH11 under moderate compressive loads (applied via surface tension) that mimic those in the intact embryo. The mid-hindbrain (MH) boundary forms (arrows) (M, midbrain). Arrowheads indicate the fluid-gas interface in OCT cross sections. Scale bars: 100 µm.

4.4.3 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 and 30 µM) and cultured for six hours. Boundaries formed in control embryos (n = 18 / 18, Fig. 4.4A, A’), but not in blebbistatin (bleb)-treated embryos (n = 1 / 19, Fig. 4.4B, B’, C). Boundary formation was rescued, however, following blebbistatin washout and 10 hours of subsequent culture (n = 7 / 8, Fig. 4.4B”, C).
Figure 4.4: 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 µM), 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: 200µm.

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. 4.4C). In control brains incubated for 6hr, MH boundary diameter was approximately 30-40% less than the vesicles, while MH and vesicle diameters were similar in bleb-treated brains. After washout of bleb and overnight culture (14hr), MH diameter became significantly less than the vesicles ($\approx$
Hence, bleb-treatment inhibited boundary formation, an effect that was reversible by washout of the drug. Together, these results indicate that myosin-II based contractility is required for boundary formation.

### 4.4.4 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). Because of the permeabilization step, however, long-term (\(\gtrsim 1\) 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. 4.5A, B). In contrast, similar culture with calyculin A (20nM) caused the mid-hindbrain boundary to close nearly completely (Fig. 4.5B’, arrows; Fig. 4.5G). Culture of embryos in media enriched with ATP (5mM, 0.05% Triton-X 100) induced a similar response in only 25 minutes (Fig. 4.5C’). 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. 4.5C). Note that the FM boundary (arrowheads in Fig. 4.5B’, 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. 4.5D-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. 4.5G-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.

4.4.5 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. 4.6A). 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. 4.6B, quantified in Fig. 4.7). 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. 4.6B'). Phosphorylated myosin light chain expression patterns were similar to that of F-actin (Fig. 4.6C). At HH12, boundary formation was largely complete, and F-actin was predominately absent at rhombomere boundaries but persisted at the larger vesicle boundaries (Fig. 4.6D). During vesicle formation, F-actin filaments were circumferentially aligned at the apical wall of the MH boundary (shown at HH11+ in Fig. 4.6E, 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
Figure 4.5: 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 ($A/A_o$, 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 ($L/L_o$, 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.
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).
4.4.6 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. 4.8, 4.9). (Note that cell shapes are shown for all regions and at higher magnification in Fig. 4.8). Cell boundaries were outlined and converted to binary, and contours were extracted for further analysis (Fig. 4.9A-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. 4.9C, 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. 4.9D). No predominant orientation was found in the brain vesicles (midbrain, hindbrain), however. In the aligned regions, cellular aspect ratio correlated with orientation angle (Fig. 4.9E-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
Figure 4.8: Cell shapes at the apical neuroepithelium. Anterior is to the left. (A) Representative image of cell shapes at rhombomeres (R) and rhombomere boundaries (Rb, red shading) (H = hindbrain). Cells are aligned in the circumferential direction at boundaries and in the longitudinal direction between boundaries. Arrows indicate cell orientation. (A’) High magnification image of boxed region in (A). The transition from longitudinal to circumferential alignment near a boundary is shown. (B, C) Cells were traced at the midbrain, mid-hindbrain boundary, hindbrain, rhombomeres, and rhombomere boundaries as described in the Materials and Methods. Cells were aligned circumferentially in all boundaries, longitudinally in rhombomeres, and showed no preferred orientation in the primary vesicles. Scale bars: 50 µm.
Figure 4.9: 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 MHB 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 µm.
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. 4.10.

Figure 4.10: 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. 4.11 and 4.13.
4.4.7 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. 4.11A, A'; 4.13A, A'), with relative contractile protein density and cell shape used to estimate contraction magnitude and directionality (Figs. 4.11B, 4.13B; see Materials and Methods). The effects of varying these parameters, as well as the dependence of tissue stiffness on contractility, are explored in Fig. 4.12.

First, we consider the model for primary vesicle formation (Fig. 4.11). Consistent with our data (Fig. 4.10), 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. 4.3). 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. 4.11C). 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. 4.11C). The model reasonably reproduced these trends, as well as overall brain morphology (Fig. 4.11B, 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. 4.13). Here, based on our experimental data (Fig. 4.10), longitudinal contraction is simulated between boundaries, with weaker circumferential contraction at the boundaries (Fig. 4.13A'). The model generates bulges similar to normal rhombomere morphology (Fig. 4.13B, B'). This mechanism of contraction shortens the tube, similar to the model prediction (Fig. 4.13C). However, whereas the model also predicts small decreases in lumen diameter, measurements gave a small increase. This discrepancy may be caused by
Figure 4.11: 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 $G_{\theta}$ 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. 4.3) ($A_V$ = 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.
Figure 4.12: Model parameter study. (A) Dependence of shear modulus on contractility (see Eq. 4.4). Increasing $a$ amplifies the contractile response. Axial length and diameter of the tube decrease. Note that the mid-hindbrain boundary deepens. (B) Ratio of circumferential to longitudinal contraction at the mid-hindbrain boundary ($C_\theta = G_z/G_\theta$). Increasing $C_\theta$ deepens the mid-hindbrain boundary with little effect on tube length. (C) Baseline contraction at the apical wall ($C = G_\theta G_z$). Increasing baseline contractility at the apical wall induces effects similar to (A).

tissue growth, which is not included in the model. These data suggest a potential role for longitudinal contraction during rhombomere development.
Figure 4.13: 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 $G_\theta$ 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.

4.4.8 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. 4.11C and 4.13C). 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. 4.14). 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. 4.14B). 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. 4.14C). Lastly, we tested the effect of isotropic contraction in the boundary alone (Fig. 4.14D). 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. 4.15). 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. 4.15B). 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. 4.15C).

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
Figure 4.14: 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.

brain culture (Fig. 4.5H, 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. 4.12C). 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. 4.16A, A').
Figure 4.15: 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 $\mu$m.

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. 4.16B, 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. 4.16C).

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.

### 4.4.9 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. 4.17A),
but circumferential contraction at rhombomere boundaries with isotropic contrac-
tion elsewhere (‘vesicle mechanism’) yielded relatively little deformation (Fig. 4.17B).
These results show why the rhombomere mechanism may be needed to create the rel-
atively 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. 4.1) (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. 4.17C) in
the two models for primary vesicle formation (Figs. 4.11B and 4.17A). For the vesicle
mechanism, the ratio of mid-hindbrain boundary to midbrain lumen diameter remains
relatively constant as pressure increases (Fig. 4.17C), but the ratio steadily increases
for the rhombomere mechanism as the boundary eventually disappears (Fig. 4.17D).
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 main-
tain 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.
Figure 4.17: A hypothesis for regional variations in boundary formation mechanisms. (A, B) Contraction mechanisms are switched in the vesicle (Fig. 4.11B) and rhombomere (Fig. 4.13B) 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 ($\approx 7.1$ mm H$_2$O) increase in lumen pressure (consistent with experimental measurements from Jelinek and Pexieder (1968)). Tube diameter increases in both models, but the boundary is lost 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’. 

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4.5 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 primitive vesicles in the brain of the early 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 (Figs. 4.3, 4.5E) and depressurized brains (Fig. 4.2).

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. 4.1). 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. 4.6B’). 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 note that morphological boundaries form between HH10 and HH11 in the chicken embryo, a range encompassing only 5-6 hours of development (Hamburger and Hamilton, 1951). Since the cell cycle for the neuroepithelium is 8-12 hours long (Goodrum and Jacobson, 1981), cell proliferation likely only plays a relatively minor role in vesicle formation. Still, underlying circumferential growth may be one potential reason why rhombomere diameters slightly increased in these experiments (Fig. 4.13C).

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. 4.6). 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. 4.15B). 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. 4.6) raise the possibility that differential tension
also could play a role in preventing cell intermixing in the rhombomeres. Interestingly, transmural 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 transmural 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. 4.17B), 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. 4.17C). 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.

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.
Chapter 5

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

5.1 Summary

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 corresponding sections normally found in species such as zebrafish and *Xenopus laevis* (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 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.

5.2 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 primitive 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.

5.3 Materials and Methods

5.3.1 Embryo culture and optical coherence tomography

Fertilized white Leghorn chicken eggs were incubated (37°C, 90% humidity) for 45 - 48 hours to reach Hamburger-Hamilton stages 12− to 12 (HH12− to 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., 2011a) 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.

### 5.3.2 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, extracted 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. 5.3C). 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).

5.3.3 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 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. 5.5A”-D”, 5.6D, and 5.1B (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 5.1.

### 5.3.4 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, Burlington, MA). The effects of local apical contraction were simulated in tubes of circular (250 µm inner diameter) or slit-like cross-section (major axis: 250 µm inner diameter, minor axis: 20 µm inner diameter) (see Figs. 5.5A’-D’, 5.6C). Total wall thickness is 65 µm 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. 5.5, 5.6), 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
Figure 5.1: Method to quantify fluorescence intensity in a representative brain section. (A) F-Actin distribution (green) in a calyculin-treated mid-hindbrain boundary section in the chicken embryo. (B, C) Fluorescence intensity is measured across the wall at tissue vertices (blue, red, purple) and at mid-vertices (yellow, green, grey) and normalized to background. (B’, C’) Distributions are fit with a polynomial function to smooth the data as outlined in the Materials and Methods. (D) Vertex and mid-vertex fluorescence intensities are averaged and plotted as mean +/- standard deviation. In hyper-contracted brain sections actin density is highest at the apical side of the brain tube at tissue vertices relative to non-vertices.

stiffening. Briefly, the deformation gradient tensor $F$ is decomposed into a growth tensor $G$ and an elastic deformation gradient tensor $K$ relative to the current zero-stress state ($F = K \cdot G$). During active contraction, $G$ changes the stress-free configuration of the material element, while $K$ generates stress and ensures geometric compatibility.
Here, $G$ 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. Details on the implementation of growth in Comsol software have been published elsewhere (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., 2011a):

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

Here, $\mu$ and $\kappa$ are the small-strain shear and bulk modulus, respectively, $I_1$ is the first invariant of the right Cauchy-Green deformation tensor ($K_T \cdot K$), $J$ is the elastic volume ratio ($\det K$), 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)

$$\sigma = J^{-1}K \cdot \frac{\partial W}{\partial \epsilon} \cdot K^T$$

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

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

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5.3.5 Statistics

All data are reported as mean +/- SD. Statistical analyses were done using Sigma-Stat (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).

5.4 Results

5.4.1 Interspecies differences in early brain morphology

The primitive 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. 5.2A). 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. 5.2B). Longitudinal sections and 3-D reconstructions of the lumen confirm that control embryos maintain a predominately open anterior brain tube (Figs. 5.2B, E, H).
Figure 5.2: 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-contrasted 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-contrasted chick brains strongly resemble control zebrafish brains. Scale bars: 50 µm.
5.4.2 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). More generally, calyculin is a serine/threonine phosphatase inhibitor that enhances contractility in the embryonic chick brain (Filas et al., 2011a), induces cortical contraction in sea urchin eggs (Asano and Mabuchi, 2001), and speeds up chromosome movement during anaphase (Fabian et al., 2007). Proper regulation of myosin phosphatase is essential for liver organogenesis (Huang et al., 2008) and hindbrain expansion in zebrafish embryos (Gutzman and Sive, 2010).

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. 5.2C). This hyper-contracted morphology strongly resembled the morphology of normal zebrafish brains at 22 hpf (hours post fertilization) in longitudinal section (Figs. 5.2C, D, F, G) (18). Three-dimensional reconstructions of the luminal space (Filas et al., 2011a) 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. 5.2I, 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 \((n \geq 7)\) confirmed that calyculin-treated chicken brains strongly resembled control zebrafish brains, possibly even more so than control chicken brains (Fig. 5.3A). To test this possibility, these data were pooled to generate a
cumulative shape model (Fig. 5.3B). 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. 5.3C). 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. 5.2E-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. 5.4). (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 (see section 5.6, Fig. 5.11).

Cross-sectional morphology of the brain tube was analyzed via OCT imaging. Representative morphologies (Fig. 5.4, left) and average shapes (Fig. 5.4, right, $n \geq 5$ 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. 5.4, 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
Figure 5.3: 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 5.4: 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.

(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. 5.4, 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.

5.4.3 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-contrasted 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) 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. 5.5A, A”; green). In calyculin-treated embryos, however, greater accumulations of F-actin were observed in regions with sharp vertices (Figs. 5.5B-D, B”-D”; 5.1). 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. 5.5A’-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. 5.5A’). 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. 5.5B’-D’). These results indicate that the characteristic cross-sectional morphologies observed in hyper-contrasted 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.
Figure 5.5: 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-phallodin - 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 ($g_\theta < 1$) 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.
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. 5.6B, asterisks; Fig. 5.6D). 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. 5.6B, 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. 5.7). These actin staining patterns are similar to those found in hyper-contracted chicken brains, presumably supporting the differential contraction idea.

### 5.4.4 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. 5.6A), 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. 5.6C). When local apical contraction is simulated at two lateral hinge points, the lumen opens into a diamond shape, as observed experimentally (Fig. 5.6C). 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.
Figure 5.6: 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 µM, 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 5.7: F-actin distributions in normal Xenopus midbrain. (A) F-actin (green) and nuclei (red) were stained in Xenopus midbrains (n = 3) as described in the Materials and Methods. (B) F-actin was concentrated at the apical side of the mediolateral hinge points (blue) relative to surrounding regions, a result similar to zebrafish embryos. Note that the disparity between F-actin content of the vertices (blue) and surrounding regions (red) spans more than half of the wall thickness from the apical side. An upslope in fluorescence intensity toward the outer wall indicates a notable amount of F-actin localization on the basal side of the neuroepithelium in these samples. F-actin staining intensity at mediolateral tissue vertices (asterisks) is approximately 1.1 times greater than dorsoventral (arrowheads) vertices (this value was greater than 1.3 in zebrafish embryos). This disparity may be due to slight differences in stage comparisons as zebrafish midbrains were in the process of opening, while Xenopus midbrains were fully opened. Scale bar: 50 µm.

5.4.5 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. 5.5). 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. 5.8A). 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.

Figure 5.8: 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.

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. 5.8B) and compared with the complementary simulation. Taking $G_\theta = 1, 0.7, 0.4, \text{and} 0.1$ provided reasonable agreement for steadily increasing contraction (Fig. 5.8C). These results suggest that the drug-induced contraction increases approximately linearly with time during the incubation period.

### 5.4.6 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 $\mu$M; a concentration previously shown to significantly alter cytoskeletal stiffness and nuclear shape in the early chick brain (Filas et al., 2011a)) (Fig. 5.9A-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. 5.9C, C’), a morphology that remained hypercontracted in appearance relative to controls (Fig. 5.9D, 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. 5.9B’, 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. 5.9E’), but gross morphology did not seem to be affected (Fig. 5.9E). Hence, it seems that morphogenetic shape changes caused by enhanced cytoskeletal contraction are largely unidirectional.

### 5.5 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...
Figure 5.9: 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 µm.

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. 5.2-5.4). 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. 5.5). 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. 5.2A; 5.4, top row; 5.5A) (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. 5.6A), 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. 5.6B, 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. 5.6B, D). However, we note that this difference in F-actin intensity between vertices is less in *Xenopus* brains (Fig. 5.7), 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. 5.5B).

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. 5.4, 5.5, 5.8). Differences in contractility between disparate species may, therefore, be a function of the initial geometry of the brain tube when it first forms (Fig. 5.10).

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.
Figure 5.10: 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.

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 (see section 5.6). 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. 5.9B-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 ($G_\theta \approx 0.1$), 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. 5.5A”-D’; 5.6D). We also did not attempt to model the dramatic changes that occur longitudinally in hypercontracted brain tubes (Fig. 5.2C). 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).

5.6 Addendum

5.6.1 Neurulation and vesicle shape in the mouse embryo

The appropriate developmental stage at which to compare the embryonic mouse brain to the chick, zebrafish, and *Xenopus* morphologies reported in this study is approximately E8.5 (Davis et al., 2008) (E = embryonic day). However, at E8.5/9, neurulation (closure of the neural folds) is ongoing, making interspecies morphological comparisons more complicated. The reason behind this is that neurulation likely involves the spatiotemporal regulation of cytoskeletal contractility (Greene and Copp, 2009; Kinoshita et al., 2008; Ybot-Gonzalez and Copp, 1999). Since brain vesicle formation and neurulation appear to occur concurrently in the mouse, it is difficult to separate one morphogenetic process from the other. However, some useful information can still be extracted from optical coherence tomography images (Fig. 5.11). First, the lumen of the mouse brain is largely open as the neural folds fuse, as is the case in the chick brain. The transverse brain sections also tend to round out from E8.5 to E9.5 as the neural folds fuse and luminal pressure begins to increase, which is again similar to the chick. Notably, however, a vertex is observed on the ventral
side of the mouse brain in all sections, likely where the tissue is anchored to the notochord. A similar vertex is not observed in chick brain sections, and the reasons for these differences are currently unknown.

Figure 5.11: Transverse cross sections of the embryonic mouse brain. At E8.5/9 (top row) the brain is not sealed and remains open on the dorsal side of the tube (except near the fore-midbrain boundary, FM). Note that the size of the brain already varies considerably between regions (M = midbrain, MH = mid-hindbrain boundary, H = hindbrain). By E9.5 (second row), however, neurulation (closure of the neural folds) is complete and the mouse brain is generally round except where the brain attaches to the notochord on the ventral side of the tissue. Scale bar: 100 µm.

5.6.2 Neurulation patterns in the chick embryo do not predict induced contractile behavior

Contractile patterns reported in this study do not tend to correspond to those observed during neurulation. In the normal chick embryo, specific neuroepithelial geometries encountered during closure of the neural folds seem to depend on neural tube diameter, with a gradient of shapes observed from the future forebrain through the future spinal cord (Schoenwolf and Smith, 1990). In contrast, specific geometries in hyper-contracted chick brains vary significantly between adjacent regions. For example, the contracted mid-hindbrain boundary is triangular in shape with the base of the triangle located on the ventral side of the brain tube (Fig. 5.4), an orientation opposite to the somewhat triangular shape observed in this region during neurulation (Schoenwolf and Smith, 1990). Moreover, in hyper-contracted chick brains,
this region is flanked by the disparate morphology of the diamond-shaped midbrain (Fig. 5.4), which requires four hinge points to form (Fig. 5.5B, B’), whereas only one or three hinge points are typically reported during neurulation (Schoenwolf and Smith, 1990; Kinoshita et al., 2008). Hence, results from this study suggest that after vesicle formation, chicken brains have the capability to contract in a manner that is independent of the morphogenetic mechanisms present during neurulation.
Chapter 6

Conclusions

In this thesis, a combination of experimental and computational techniques were used to investigate the mechanics of early embryonic brain morphogenesis. In this chapter we summarize our findings and outline paths for future study.

6.1 Summary of results and discussion

Primary advancements from this research include:

1. We developed and implemented a technique to characterize multi-valued tissue deformations that is applicable to a wide range of morphogenetic problems and functional studies (e.g., strain measurements in beating hearts).

2. Epithelia of the embryonic brain and heart respond similarly to altered mechanical loads. Both organs decrease in stiffness as compressive loads increase, and vice versa. These results support a role for stress-based mechanical feedback in regulating epithelial development.

3. Differential cytoskeletal contraction plays a significant role in establishing the morphological boundaries that subdivide the embryonic brain. We hypothesize that observed contraction patterns depend on brain geometry and confer different levels of structural robustness to the brain tube.
4. Spatiotemporal differences in cytoskeletal contractility can explain differences in early brain morphology between disparate species. We speculate that these patterns play a role in early brain evolution.

These results were obtained using a unique combination of experimental (ex ovo culture, dynamic optical coherence tomography imaging) and mathematical (finite element modeling, quantitative shape analysis) techniques. This type of approach should be widely applicable to other experimental systems, and in the following section, we outline future directions for such studies.

Lastly, we stress that, although the early brain may not beat like the heart, it is still a highly mechanically active structure. With a significant number of congenital brain defects linked to abnormal mechanical events (e.g. aberrant internal pressures and folding patterns), and their etiology not being well understood, it is essential that more attention be placed on understanding the fundamental mechanical behavior of the embryonic brain. Linking early mechanisms of brain morphogenesis to those that occur later in development should illuminate the biomechanical events that cause anomalies associated with pathological conditions.

6.2 Future directions

6.2.1 Deformation analyses

In chapter 2, we developed techniques to dynamically culture the chicken embryo and track tissue motions during morphogenesis. Using a piecewise approach, we showed how measures of deformation can be computed and mapped to multi-dimensional surfaces to study complex 3-D tissue movements. This is a powerful technique that should find numerous applications in developmental biology and other nonlinear studies of deformation. Subsequent studies should consider:

1. Exploring and exploiting natural tissue contrast for tracking motion.

2. Developing injectable contrast agents for studies of transmural wall deformations.
3. Optimizing microsphere coatings for superior tissue adhesion.

4. Automating 4-D bead tracking techniques.

5. Automatically or semi-automatically generating 3-D reconstructed surfaces.

The first three items are directed at adapting and improving the current labeling technique. In particular, exploring the local injection of smaller OCT contrast agents, such as gold or silver nanoparticles, could be one future research strategy (Lee et al., 2003).

The latter two items focus on improving method throughput. The current technique requires tissue labeling, embryo culture, time lapse imaging, marker tracking, generating 3-D reconstructions, and mapping calculated deformation measures to reconstructed surfaces. Since the full implementation of the method is relatively time-consuming, focusing on automating certain parts of the analysis should not only improve throughput, but may also facilitate a more widespread adoption of the technique.

6.2.2 Mechanical feedback in the embryo

In chapter 3, we studied how isolated epithelia adapt to mechanical perturbations by systematically adjusting surface tension applied at the surface of samples during culture. This is a relatively straightforward approach to test the mechano-sensitivity of embryonic tissues. To improve this method, future studies should consider integrating imaging and mechanical testing platforms.

For example, one approach would be to use a vertical (as opposed to a horizontal) indenter to dynamically assess tissue stiffness during culture. Such a modification would minimize experimental complexity and duration. Moreover, interfacing this setup with an OCT imaging system would allow tissue morphology and 3-D microindentation profiles to be captured in real-time. As an alternative, the setup could be integrated with a confocal microscope. In this case, appropriate fluorescent markers could be used to dynamically monitor changes in cell and nuclear shapes. Notably,
fluorescent probes have been used to track nuclear deformations in single cells (Lombardi et al., 2011), but toxicity could be a concern in long term experiments.

As a variation of the surface tension method, we also applied compressive loads to isolated brains using glass coverslips. The advantage of using a coverslip (or other similar small object) for compression is that the effects of locally applied loads can be studied. Care must be taken, however, to ensure that these objects remain stationary throughout the experiment. Time lapse culturing and imaging techniques described in chapter 2 could be helpful in this case. Alternatively, local loads could be applied using magnetic forces, similar to techniques previously implemented to study single cells (Na et al., 2008; Chowdhury et al., 2010). Both approaches warrant further exploration.

To study mechanical loading at later stages of brain development, we have begun to analyze the effects of lumen pressure perturbations in the brain tube. Preliminary results from these experiments are discussed in the appendix.

Lastly, we note that testing for mechanical feedback in artificial tissue constructs composed of embryonic precursor cells could be an altogether different approach for future research efforts (Legant et al., 2009; Lam and Wakatsuki, 2011). Inherent limitations in using embryonic tissues for mechanical testing (size, developmental variability, throughput) could be minimized in this case. However, the power of demonstrating feedback during normal morphogenesis would be lost.

6.2.3 Structural plasticity and optimization in the developing brain

From model results presented in chapter 4 we speculated that contraction patterns affect the structural robustness and morphogenetic plasticity of the brain tube. Specifically, we postulated that regional variation in contraction patterns may explain why primary vesicle boundaries (mid-hindbrain and fore-midbrain) are permanent structures, whereas rhombomeres are only transiently observed during early brain development. Future studies should consider investigating this hypothesis more directly by
studying actomyosin dynamics in the rhombomeres from boundary formation through dissolution.

Although speculative, it is interesting to think of the developing brain as a structurally optimized tissue. In particular, the mechanism of primary vesicle formation suggested by this research seems to be ideal. The brain contracts circumferentially at the boundary (which confers structural robustness to growing lumen pressures), while surrounding vesicles contract isotropically and with similar strength. Contraction in the vesicles could be important to prevent collapse of the internal lumen (see appendix) before a significant pressure builds in the brain tube. This possibility warrants further study.

6.2.4 Evolution and development

In chapter 5, we proposed a role for differential cytoskeletal contraction in the evolution and development of the brain. This result is consistent with growing evidence from the field of evolutionary developmental biology (evo-devo) suggesting that morphological differences between species result largely from changes in the spatiotemporal regulation of gene and protein expression during development.

This study required integrating information across the fields of evolutionary and developmental biology. A review of the literature reveals fundamental differences in how information is reported between these disciplines. In particular, morphological differences between species are stressed in an evolutionary context, while similarities tend to be emphasized in developmental studies.

Whatever the underlying reason for this difference, we propose that interspecies differences in developmental mechanisms and morphology should be suitably addressed (when relevant), rather than downplayed. As demonstrated in chapter 5, better understanding these differences can provide insight into the development of individual organisms and can help explain seeming discrepancies in the literature (e.g. why basal contraction occurs at the mid-hindbrain boundary in zebrafish but not at comparable stages in chickens).
6.2.5 Quantitative shape analysis

Extension to three dimensions

In chapter 5, we incorporated a principal components analysis (PCA) procedure to analyze interspecies differences in brain morphology. This robust method ensures that meaningful variance in the data is not overlooked (Pincus and Theriot, 2007). However, for our purposes, the main limitation of this technique was that it could not be applied to 3-D data (e.g. surfaces or volumes) in its current form.

Evolutionary studies have used sets of points known to correspond to morphological landmarks to extend the PCA technique to three dimensions (Bastir et al., 2011; Mitteroecker and Gunz, 2009). However, we note that global alignment procedures (e.g. registration algorithms; (Knutsen et al., 2010)) applied to surface outlines could eliminate this added complexity of manually selecting landmarks. Future studies should consider the development and implementation of this technique.

Application: finite element model validation

Beyond a purely morphological analysis, mathematical models ensure that proposed hypotheses for morphogenesis are consistent with physical law. These models can be quantitatively tested by comparing stress and strain distributions to those found in experiments (Varner et al., 2010).

To quantitatively ensure that deformed model geometries match shapes observed experimentally, future studies could employ PCA-based techniques to compare these data. This approach could be especially valuable for evaluating predictive model performance (i.e. perturbation studies) when an additional comprehensive stress and strain analysis is beyond the scope of the study. In the appendix, we highlight an instance where such an approach could be used.
Appendix A

Morphogenetic Adaptation of the Pressurized Brain to Altered Mechanical Loads

Following stages of vesicle formation, the embryonic brain begins a phase of rapid expansion, and studies have shown that this growth depends on an internal lumen pressure. In this section, we present preliminary results that suggest a role for mechanical feedback in regulating the growth and development of the pressurized brain. Results from these studies should be useful in further characterizing how the brain actively adapts and responds to changes in mechanical stress.

A.1 Mechanical perturbations

A.1.1 Brain tube collapse

The embryonic chicken brain begins a period of rapid, pressure-driven inflation after the spinal neurocoel and anterior neuropore close at Hamburger and Hamilton stage 11 (HH11, ≈ 42 hour incubation) (Hamburger and Hamilton, 1951; Desmond and Levitan, 2002; Desmond and Jacobson, 1977; Gato and Desmond, 2009). Previous studies have shown that the internal lumen of the pressurized brain collapses following wounding at HH16-24 (2.5 - 4 days incubation) (Desmond and Jacobson, 1977; Lawson
and England, 1996), but a similar response has not been reported in earlier-stage brains.

To explore this possibility, we wounded the HH11+ brain by piercing the forebrain with a small glass micropipette (≈ 50 µm diameter). Subsequent changes in brain morphology were imaged using optical coherence tomography (OCT). After wounding, the elliptical lumen of the midbrain gradually collapsed during 4.5 hours of culture (Fig. A.1, left). Collapse patterns were similar to theoretical predictions for thin-walled tubes (Shapiro, 1977). Interestingly, collapse occurs far more rapidly (on the order of minutes) after wounding at later stages of development (Desmond and Jacobson, 1977). These results suggest that the early-stage brain is prone to collapse when mechanical loads are perturbed, but this collapse may occur more gradually than in older embryos.

Next, we investigated the loads required to collapse the brain tube using finite element models (Comsol Multiphysics, v3.5). (Since the modeling strategy is similar to that described in chapters 3-5, it is only briefly restated here.) We developed a plane strain model for the brain tube (of elliptical cross section) with initial geometry estimated from the HH11+ midbrain (Fig. A.2A). The shape of the brain was varied by adjusting the minor radius of the tube in the simulations (parameter (b) in Fig. A.2A, B). We take the tube to be uniformly passive (shear modulus = µ = 200 Pa, estimated from Xu et al. (2010)) and pressure-free (p_i = 0, to simulate wounding). External (follower) pressures (p_e) were gradually increased until the inner walls of the tube came into contact (Fig. A.2B). We call this critical value the ‘contact pressure’ (Fig. A.2C).

For all geometries tested, the ‘contact pressure’ is small (≈ 1 mm H_2O), and decreases even further as tubes became more elliptical (Fig. A.2B, C). Interestingly, experimentally measured lumen pressures in normal HH12-13 brains are only slightly larger than contact pressures reported here (≈ 2-3 mm H_2O) (Jelinek and Pexieder, 1968). From these data, we speculate that early lumen pressures are slightly larger than loads exerted by surrounding tissues. This would ensure that internal pressures are sufficient to prevent collapse, but still relatively small so that excess tension does not develop in the neuroepithelium. Related to the latter idea, over-inflation of the pressurized brain tube has been linked to abnormally high cell proliferation rates (Desmond et al., 2005).
Figure A.1: Midbrain collapse patterns. (Left) HH11+ brain is wounded and changes in morphology are imaged for 4.5 hours. The initially elliptical lumen of the midbrain (dotted line, upper left) collapsed into a figure eight shape at $t = 3$hr, and then flattened. (Right) Theoretically predicted collapse patterns for a thin-walled tube of elliptical cross section are similar to those observed in the midbrain. Tubes collapse as internal pressures ($p_i$) decrease relative to external pressures ($p_e$). Schematic adopted from Shapiro (1977).

These results suggest that the brain may be susceptible to collapse before internal pressures build in the tube. Moreover, the brain may be especially vulnerable in regions with elliptical cross-sectional shapes, such as the primary vesicles. This may be a key reason why the primary vesicles remain contracted during boundary formation (see chapter 4): to buffer against collapse. This possibility warrants further testing in more realistic models including the effects of cytoskeletal contraction.
Figure A.2: Modeling collapse. (A) External pressure ($p_e$) at the outer wall of an elliptical tube is steadily increased. Model geometries estimated from the embryonic midbrain. (Wall thickness = 50 µm; major radius (a) = 150 µm; minor radius (b) = 100, 75, or 50 µm.) The tube is homogeneous and passive (shear modulus = $\mu$ = 200 Pa). (B) The external pressure $p_e$ required to bring the inner walls of the tube into contact (generating a figure eight shape) is determined. (C) This ‘contact pressure’ is relatively small ($\approx 1$ mm H$_2$O) for all tested geometries, and decreases even further with increasing tube eccentricity ($b / a$ decreases).

A.1.2 Estimating loads exerted by neighboring tissues

Accumulating cerebrospinal fluid generates internal pressures in the brain that can be directly measured using either passive (Jelinek and Pexieder, 1968) or active techniques (Desmond et al., 2005; Chabert and Taber, 2002). Loads exerted by surrounding tissues on the brain tube, on the other hand, can be estimated indirectly using a combination of imaging and mathematical modeling. Systematically removing surrounding tissues and tracking changes in brain morphology can be used to gauge applied stresses. This type of approach is demonstrated in Figure A.3.

Removing the vitelline membrane and head mesenchyme caused the lumen of the midbrain to differentially expand (Fig. A.3A-A”). These results suggest that both tissues constrain the developing brain, but potentially in different directions. This
Figure A.3: Estimating loads from surrounding tissues. Top row: bright field images. Lower row: Midbrain shapes imaged via OCT. Location of each transverse section denoted by dashed line in corresponding bright field image. (A) Intact HH12 brain. (VM = vitelline membrane, HM = head mesenchyme.) (A’) Vitelline membrane and then the (A”) head mesenchyme are removed. Arrows indicate direction of lumen expansion following each dissection. (B) The isolated brain is wounded (asterisk) and imaged after 15 minutes. (A”, B) Lumen size slightly decreases. (C) Intact HH12 brain is wounded (asterisk) and imaged after 15 minutes. The midbrain collapses more dramatically than in isolated samples.

Compression is further evidenced by differences in midbrain morphology between isolated and intact brains following wounding (Fig. A.3B, C). In intact brains, the lumen of the midbrain collapsed within 15 minutes of wounding, but only slightly deflated in isolated brains. This experiment shows that loads exerted by surrounding tissues cause the depressurized brain to collapse.

Together, these results indicate that surrounding tissues exert compressive loads on the developing brain tube. The magnitude of these loads can be estimated by comparing deformed brain geometries to corresponding finite-element models. As discussed at the end of chapter 6, a principal components shape analysis could be useful in this case to quantitatively compare experimental and model shapes. (Mathematical comparisons of elliptical and figure eight-like shapes are not obvious using standard methods.)
A.1.3 Collapse and (rapid) re-inflation

As outlined in the previous section, the brain may be especially susceptible to collapse just before lumen inflation begins. If closure of the anterior neuropore or spinal neurocoel is delayed and surrounding tissues continue to grow, the tube could collapse. We speculated that the brain may maintain an innate ability to counteract collapse if this were to occur. Consistent with this idea, we found that if the HH11+ brain collapses (as occurs in Fig. A.1), it often re-inflates. Surprisingly, this response is rapid and dramatically increases brain size.

To visualize this dynamic process, we reconstructed the inner lumen of the brain tube during collapse and re-inflation. (For clarity, this technique is first demonstrated in Fig. A.4.) After wounding, we imaged the HH11+ brain as it collapsed and subsequently re-inflated using OCT (Fig. A.5). Lumen volume (computed from OCT reconstructions) steadily decreased as brains collapsed during 5-6 hours of culture. Collapse was followed by a rapid (1-2 hour) re-inflation, which caused a four-fold increase in lumen volume (Fig. A.5).

These results suggest that, although collapse occurs gradually at HH11+, re-inflation is rapid and may restore a normal, unperturbed phenotype. We suspect that the brain maintains this latent ability to re-inflate to buffer against congenital malformations. This possibility warrants further study. Interestingly, previous studies have noted a similar rapid cavity re-inflation following wounding, but the phenomenon was not discussed in further detail (Lawson and England, 1996).

A.2 In ovo culture for long term studies of morphogenesis

To facilitate long term studies of mechanical feedback in the pressurized brain, we have begun to explore the use of in ovo culture techniques. We have found that embryos develop normally in shell-less culture inside standard heated and humidified incubators (Fig. A.6). However, embryo morphology is difficult to visualize in the yolk. In particular, prior to blood vessel formation, the embryo is virtually invisible
$t = 0$, Wound $t = 2.5$hr

Figure A.4: Lumen reconstructions during collapse. (Left) The inner lumen of the brain tube is reconstructed from OCT cross sections after wounding at HH11+. Locations of transverse sections are indicated (F = forebrain, M = midbrain, H = hindbrain). (Right) After 2.5 hours of culture, the lumen has begun to collapse. Note the figure eight shape of the midbrain lumen and its corresponding representation in the reconstruction.

using standard bright field imaging (Fig. A.7A). Injecting concentrated Fast Green (food coloring) into the side of the yolk (Fig. A.7B, asterisk) greatly improves visibility. Notably, the brain can be easily located for subsequent mechanical manipulations or micro-injections. Importantly, dye injection does not seem to adversely affect embryo health during culture (Fig. A.8). This method should prove useful for long term studies of normal and perturbed brain development.
Figure A.5: Lumen collapse and re-inflation. Two HH11 brains (dark and open circles) were wounded and then imaged via OCT for 8 hours. Lumen reconstructions are shown in longitudinal and sagittal views at various times during culture. Lumen volume is calculated directly from reconstructions. Following wounding (t = 0), the inner lumen of the brain gradually collapses (5-6 hours) and then rapidly re-inflates (1-2 hours). In both samples, lumen volume increased by an approximate factor of four during re-inflation.
Figure A.6: *In ovo* shell-less culture. (A) Egg shells are removed at HH16 (2.5 days incubation) and the yolk (containing the embryo) is deposited in a small petri dish. (B) Embryo development appears normal after an additional 2.5 days inside a heated and humidified incubator.

Figure A.7: Dye improves *in ovo* embryo visibility. (A) Bright field image of *in ovo* HH11 embryo. Embryo location in the yolk is unclear. (B) Same field of view as in (A). Concentrated Fast Green is injected into side of the yolk (asterisk); embryo location becomes visible.
Figure A.8: Shell-less culture with dye. (A) Bright field image of in ovo HH14 embryo. Dye injected into yolk for contrast. (B) Same embryo after 1.5 days of culture. Increases in embryo size, flexure, and vasculature suggest normal development.
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