Calcium signals drive cell shape changes during zebrafish midbrain–hindbrain boundary formation

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ABSTRACT One of the first morphogenetic events in the vertebrate brain is the formation of the highly conserved midbrain–hindbrain boundary (MHB). Specific cell shape changes occur at the point of deepest constriction of the MHB, the midbrain–hindbrain boundary constriction (MHBC), and are critical for proper MHB formation. These cell shape changes are controlled by nonmuscle myosin II (NMII) motor proteins, which are tightly regulated via the phosphorylation of their associated myosin regulatory light chains (MRLCs). However, the upstream signaling pathways that initiate the regulation of NMII to mediate cell shape changes during MHB morphogenesis are not known. We show that intracellular calcium signals are critical for the regulation of cell shortening during initial MHB formation. We demonstrate that the MHB region is poised to respond to calcium transients that occur in the MHB at the onset of MHB morphogenesis and that calcium mediates phosphorylation of MRLC specifically in MHB tissue. Our results indicate that calmodulin 1a (calm1a), expressed specifically in the MHB, and myosin light chain kinase together mediate MHBC cell length. Our data suggest that modulation of NMII activity by calcium is critical for proper regulation of cell length to determine embryonic brain shape during development.

INTRODUCTION Cell shape changes are central to the formation of tissue structure. One of the earliest and most highly conserved vertebrate brain structures that forms during development is the tissue fold at the midbrain–hindbrain boundary (MHB; Rhinn and Brand, 2001; Lowery and Sive, 2009; Hirth, 2010). Using the zebrafish MHB as a model to understand brain morphogenesis, we characterized the basic cell shape changes that are required to fold the neuroepithelium in this region (Gutzman et al., 2008, 2015). Zebrafish MHB formation is initiated at the 18-somite stage (ss) after the neural tube has formed. Cells at the point of deepest constriction at the MHB—the MHB constriction (MHBC)—shorten and narrow by 24 ss (Gutzman et al., 2015). By primordium-6 stage, cells at the MHBC constrict basally and expand apically, leading to an acute tissue angle and clear delineation of the midbrain from the hindbrain (Gutzman et al., 2008). The initial cell shape changes are differentially regulated by myosin motor proteins. Apical-basal cell length is specifically regulated by nonmuscle myosin IIA (NMIIA), and anterior-posterior cell width is specifically regulated by nonmuscle myosin IIB (NMIIIB; Gutzman et al., 2015). However, the molecular pathways that activate NMII proteins to differentially mediate these cell shape changes remain unknown.
NMII activity can be regulated by several different kinase cascades via phosphorylation of myosin regulatory light chain (MRLC). Calcium (Ca$^{2+}$) signaling plays a critical role in mediating phosphorylation of MRLC via myosin light chain kinase (MLCK; Berridge et al., 2000; Somlyo and Somlyo, 2003; Vicente-Manzanares et al., 2009) and is critical for many embryonic developmental processes (Webb and Miller, 2003; Slusarski and Pelegri, 2007). However, the specific role for Ca$^{2+}$ signaling in brain morphogenesis is unknown. We hypothesized that Ca$^{2+}$ functions as a morphogenetic signal to mediate cell shape changes at the MHB. To investigate further the role of Ca$^{2+}$ signals in regulating zebrafish MHB morphogenesis, we used pharmacological inhibitors to manipulate intracellular Ca$^{2+}$ levels. We used 2-aminoethoxydiphenyl-borate (2-APB) to decrease intracellular Ca$^{2+}$ levels (Kreiling et al., 2002; Ashworth et al., 2007) and thapsigargin (Thaps) to increase intracellular Ca$^{2+}$ levels (Kreiling et al., 2008; Zhang et al., 2011). Wild-type embryos were treated at 18 ss with dimethyl sulfoxide (DMSO), 2-APB, or Thaps. We analyzed cell shapes at 24 ss, after the initial morphogenetic events had occurred (Figure 2, A–C). We found that embryos with decreased intracellular Ca$^{2+}$ (2-APB treated) had significantly longer cells at the MHBC, whereas embryos with increased intracellular Ca$^{2+}$ (Thaps treated) had significantly shorter cells at the MHB (Figure 2D). Efficacy of 2-APB and Thaps was determined using the genetically encoded calcium indicator GCaMP6s-GFP (Supplemental Figure S2, A–D). Apoptosis and cell adhesion within the neuroepithelium, which are known to depend on intracellular Ca$^{2+}$ homeostasis (Orrenius et al., 1992; Lagunowich et al., 1994), were not affected by these treatments at the doses used (Supplemental Figure S2, E–K).

Quantification of Ca$^{2+}$ effects on tissue shape showed that the MHB angle was abnormal, as expected with changes in MHB cell length, whereas MHB cell width was only slightly affected with Thaps treatment (Supplemental Figure S3, A and B). However, cells 40 μm posterior to the MHB were not significantly different from controls after either treatment (Figure 2E), indicating a region-specific effect on cell shape with Ca$^{2+}$ manipulation. Ca$^{2+}$ has been previously...
Calcium regulates cell length at the MHBC. (A–C) Confocal images of embryos injected with mGFP and treated at 18 ss with (A, A') DMSO, (B, B') 100 μM 2-APB, or (C, C') 2 μM Thaps. Embryos were washed, incubated, and live imaged at 24 ss. (A–C′) Magnifications of A–C. Arrowheads indicate the MHBC. Asterisks in A–C indicate cell outlined in A′–C′. (D, E) Cell length quantification at the MHBC and 40 μm outside the MHBC. For statistical analysis, one-way ANOVA with Tukey’s HSD post hoc test was done. ***p < 0.001 compared with DMSO, mean ± SEM. For each measurement, DMSO (n = 29; 58 cells), 2-APB (n = 16; 32 cells), and Thaps (n = 14; 28 cells). Scale bars, 25 μm.

reported to regulate cell shape in other cell types. Thaps treatment causes cell retraction, gap formation, and actin rearrangement in cultured endothelial cells (Moore et al., 1998), and in cardiac myocytes, Ca²⁺ regulates cellular contraction, causing shortening of cells in vitro (Bramlage et al., 2001).

Calcium signals to nonmuscle myosin II to mediate cell shape at the MHB

On the basis of our previous work demonstrating that NMII proteins are critical mediators of cell shape at the MHB (Gutman et al., 2015) and the known role for Ca²⁺ in regulation of NMII activity (Somlyo and Somlyo, 2003), we hypothesized that Ca²⁺ may mediate its effects on MHB cell shape via downstream activation of NMII. To test this hypothesis, we first determined whether increasing Ca²⁺ in the MHB would modulate phosphorylation of MRLC (pMRLC), an indicator of NMII activity (Vicente-Manzanares et al., 2009). We treated wild-type embryos with DMSO or Thaps and microdissected MHB tissue for Western analysis of pMRLC. MHB dissections were confirmed to be specific using reverse transcription-PCR (RT-PCR) with tissue-specific markers (Supplemental Figure S4, A–D). Western analysis of MHB protein showed that pMRLC levels increased approximately twofold after Thaps treatment (Figure 3, A and B), suggesting that increased intracellular Ca²⁺ leads to increased NMII activity. We were unable to detect a change in pMRLC with 2-APB treatment. This is consistent with the observed difference in the magnitude of effects on Ca²⁺ between 2-APB and Thaps. Specifically, 2-APB decreases Ca²⁺ by half, whereas Thaps increases Ca²⁺ by fivefold (Supplemental Figure S2, A–D).

Next we tested our hypothesis that Ca²⁺ mediates cell shape via NMII protein function, using two complementary rescue experiments that coupled modulation of intracellular Ca²⁺ levels with manipulation of NMII function. We used 2-APB or Thaps treatment to manipulate Ca²⁺ levels, as described in Figure 2. NMII activity was manipulated using the pharmacological reagent blebbistatin (Bleb) or with mypt1 gene knockdown to inhibit or activate NMII, respectively. Bleb is a well-established myosin II inhibitor (Kovacs et al., 2004), and mypt1 encodes the regulatory subunit of myosin phosphatase and is required to inactivate pMRLC. Therefore mypt1 knockdown leads to an increase in pMRLC, resulting in NMII overactivation (Ito et al., 2004; Gutman and Sieve, 2010).

In our first rescue experiments, we increased Ca²⁺ levels and rescued the cell-length phenotype with inhibition of NMII. We hypothesized that we could rescue Thaps-induced short cells by inhibiting NMII with Bleb (Figure 3C). As predicted, we found that Thaps decreased cell length and Bleb increased cell length at the MHBC, and treatment of embryos with Thaps followed by Bleb rescued the abnormal-cell-length phenotypes specifically at the MHBC (Figure 3, D–I). These data suggest that the Ca²⁺ signal affects cell length via modulation of NMII function. Quantification of tissue shape showed that the MHBC angle was abnormal, as expected with changes in MHBC cell length, but was also partially rescued with Thaps and Bleb treatment together. Bleb had a slight effect on MHB cell width, which is expected because Bleb inhibits both NMIIA and NMIIB, and we know that NMIIB is essential in mediating cell width (Supplemental Figure S3, C and D; Gutman et al., 2015). Cell length outside of the MHBC was not significantly different from controls for any treatment (Figure 3I), suggesting that MHBC cells are poised to respond to slight changes in intracellular Ca²⁺ levels.

In a complementary set of experiments, we overactivated NMII and rescued the cell-length phenotype by decreasing Ca²⁺ levels. We hypothesized that decreasing Ca²⁺ with 2-APB would specifically rescue short cells induced by NMII overactivation (Figure 4A). The mypt1 morphants revealed cells that were both shorter and wider than controls, as we previously reported (Figure 4B;
Calmodulin and MLCK regulate cell shape at the MHB

Because Ca$^{2+}$ is a ubiquitous ion and additional manipulations of Ca$^{2+}$ levels are not region specific, the question remains as to how Ca$^{2+}$ transients might lead to modulation of cell shape specifically at the MHB. Ca$^{2+}$ interacts with calcium-binding proteins to initiate downstream signaling cascades (Berridge et al., 2000). Therefore one possible mechanism for a region-specific Ca$^{2+}$ response to transient activity would be through tissue specific expression of a calcium-binding protein required to mediate cell shape changes. We hypothesized that the calcium-binding partner calmodulin, a highly conserved multifunctional Ca$^{2+}$ sensor and signal transducer (Crivici and Ikura, 1995; Tidow and Nissen, 2013), might play a role in allowing MHBC cells to respond specifically to Ca$^{2+}$.

Mammalian vertebrates express three calmodulin genes, whereas zebrafish have six calmodulin genes due to genome duplication that all encode the same calmodulin protein (Friedberg and Taliaferro, 2005). In zebrafish, calmodulin 3b is ubiquitously expressed in the brain during development, whereas other calmodulin genes have region-specific expression (Thisse et al., 2001; Thisse and Thisse, 2004), and the protein exhibits cell type–specific subcellular localization (Caceres et al., 1983; Barreda and Avila, 2011). Of particular interest is calmodulin 1a (calm1a), which was shown to have potential MHB-specific expression during development (Thisse et al., 2001). Differential expression patterns of calmodulin have been observed in the eye and nervous system in other vertebrates (Friedberg and Rhoads, 2001; Thut et al., 2001; Kobayashi et al., 2015); however, comparative expression patterns during neural tube formation and brain morphogenesis at the time points examined here have not been reported. We used in situ hybridization to confirm the MHB localized expression of calm1a during brain morphogenesis. We found that calm1a is expressed specifically at the MHB before and during the initiation of MHB morphogenesis, with other specific expression detectable in the trigeminal ganglia and otic vesicles (Figure 5, A–C).

Gutzman and Sive, 2010; Gutzman et al., 2015). As predicted, cell length in mypt1 morphants treated with 2-APB was rescued compared with mypt1 morphant controls (Figure 4, B–D). No other significant differences were observed (Figure 4E and Supplemental Figure S3, E and F). We did not observe a rescue of the MHB tissue angle in these experiments, which is expected because we know that cell width also contributes to normal MHB tissue angle (Supplemental Figure S3, E and F).

Taking the results together, we showed in vivo that MHBC cell length is regulated by Ca$^{2+}$ and NMII activity. These complementary rescue experiments further suggest an important correlation between regulation of cell length and NMII. In our previous work, we determined that NMIIA specifically regulates cell length at the MHB (Gutzman et al., 2015). Here 2-APB rescues only the cell-length phenotype in mypt1-knockdown embryos, without the rescue of cell width. These results lead us to speculate that Ca$^{2+}$ might signal through intermediate molecules specifically to NMII to differentially mediate cell length at the MHB. Additional experiments are needed to specifically address this possibility.

FIGURE 3: Calcium signals to NMII at the MHB to modulate MHBC cell length. (A) Representative Western blot for pMRLC in MHB-specific tissue dissected after DMSO or 2 μM Thaps treatment. (B) pMRLC Western quantification using α-tubulin as a control (n = 4). (C) Hypothesized role of Ca$^{2+}$ and NMII interactions. IF, increase in Ca$^{2+}$ leads to increased pMRLC to activate NMII and causes shorter cells at the MHBC; AND, Bleb inhibits NMII function; THEN, Thaps treatment to increase Ca$^{2+}$ leading to shorter cells can be rescued with NMII inhibition by Bleb. (D–G′) Confocal images of mGFP-injected embryos treated with (D, D′) DMSO/DMSO, (E, E′) Thaps/DMSO, (F, F′) DMSO/Bleb, or (G, G′) Thaps/Bleb. (D′–G′) Magnifications of D–G. Arrowheads indicate the MHBC. Asterisks in D–G indicate cell outlined in D′–G′. (H, I) Cell length quantification at the MHBC and 40 μm outside the MHBC. For statistical analysis, one-way ANOVA with Tukey’s HSD post hoc test was done. ***p < 0.001 compared with DMSO/DMSO; mean ± SEM. For each measurement, DMSO/DMSO (n = 7; 14 cells), Thaps/DMSO (n = 9; 18 cells), DMSO/Bleb (n = 7; 14 cells), and Thaps/Bleb (n = 8; 16 cells). Scale bars, 25 μm.
Therefore, we hypothesized that the spatial localization of calm1a expression may provide an MHBC region-specific response to the Ca\(^{2+}\) transients. To test this, we knocked down calm1a using morpholino antisense oligonucleotides. We chose a knockdown method rather than a mutant approach because we hypothesize that the multiple calmodulin genes, all encoding the same protein, would lead to gene compensation, masking any specific role for calm1a in MHB tissue (Rossi et al., 2015). The calm1a morpholino (MO) effectiveness and specificity were confirmed using RT-PCR, MHB tissue-specific Western analysis, and calm1a mRNA rescue (Supplemental Figure S4). The morpholino resulted in a deletion of calm1a mRNA expression (Supplemental Figure S4, E–G). Together our results suggest a model in which Ca\(^{2+}\)/calmodulin specifically activates MLCK at the MHBC to mediate cell length via activation of NMII (Figure 5L). This is the first time a role for Ca\(^{2+}\)-mediated regulation of cell length during brain morphogenesis has been demonstrated. We hypothesize that the specific expression of calm1a in this region is what allows the MHBC neuroepithelial cells to respond to Ca\(^{2+}\) transients that occur in the MHB region at the onset of morphogenesis.

**MATERIALS AND METHODS**

**Zebrafish maintenance and husbandry**

Zebrafish maintenance, husbandry procedures, and staging were followed as per Kimmel et al. (1995) and Westerfield (2000). Wild-type (AB and EK) zebrafish embryos were used for all experiments. Somites were counted to establish specific and consistent staging and eliminate concerns regarding potential developmental delay for all experiments. This study was approved by the University of Wisconsin–Milwaukee Institutional Animal Care and Use Committee.

**Live imaging of calcium transients and analysis**

For imaging of Ca\(^{2+}\) transients, wild-type single-cell embryos were injected with 100 ng of GCaMP6s-GFP mRNA encoding the Ca\(^{2+}\) indicator, a gift from Philipp Keller and Yinan Wan (Howard Hughes Institute).
Live time-lapse imaging was conducted beginning at 18, 20, or 24 ss using a Nikon CS2 scanning confocal microscope. Time-lapse data sets were acquired as single slices or 5-μm z-stacks and taken ~15–20 μm into the neural tube tissue from the dorsal surface at a frame rate of 10–30 s per image. Overall capture time was between 10 and 120 min, with the average total time being 35 min per embryo. Ca²⁺ transients observed were quantified spatially within the MHB region (~10 μm on either side of the MHB, ~20 μm total) and the outside MHB region (~30 μm on either side of the MHB region, ~60 μm total). Therefore the MHB region accounted for 25% of the total area analyzed, and the outside MHB region accounted for 75% of the total area analyzed. To determine Ca²⁺ transient frequency, we normalized the number of Ca²⁺ transients observed during each time-lapse experiment to the number of transients per hour at an acquisition rate of one image per 10 s. Each region was normalized to represent an equal area of the neural tube tissue for comparison. To examine change in cell shape after Ca²⁺ transients, we measured the apical-basal length of single cells using the mCherry cell outline images. Single cells were measured during the Ca²⁺ transient, and the same cell was measured again immediately after the transient. Only cells in the MHBC region demonstrated decreases in cell length after Ca²⁺ transients. All confocal images were analyzed using the Nikon Imaging Systems (NIS) Elements software.

Drug treatments

Wild-type embryos were injected at the one-cell stage with 200 ng/μl membrane GFP (mGFP) mRNA (CAAX-eGFP). At 18 ss, embryos were dechorionated and treated for 10 min with 100 μM 2-APB (D9754-1G; Sigma-Aldrich), an inositol triphosphate receptor (IP₃R) antagonist that blocks Ca²⁺ release from the endoplasmic reticulum, to decrease intracellular Ca²⁺ levels (Bootman et al., 2002; Ashworth et al., 2007). Alternatively, embryos were treated for 15 min with 2 μM Thaps (T9033-1MG; Sigma-Aldrich), a sarcoplasmic and endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor that depletes Ca²⁺ stores, to increase intracellular Ca²⁺ levels (Kreiling et al., 2008; Zhang et al., 2011). After the treatment times, embryos were washed and allowed to mature to 24 ss in 1% agarose-lined Petri dishes at 28°C and imaged using live confocal microscopy.

FIGURE 5: Calmodulin 1a and MLCK mediate cell length at the MHBC. (A–C) calm1a gene expression by in situ hybridization. Arrowheads indicate MHBC, and arrows indicate trigeminal ganglia; asterisks indicate otic vesicle. F, forebrain; M, midbrain; H, hindbrain. (D–G’) Confocal images of embryos injected with mGFP and (D, D’) control MO, (E, E’) calm1a MO, (F, F’) MLCK mRNA, or (G, G’) calm1a MO and MLCK mRNA. (D’–G’) Magnification of D–G. Arrowheads indicate the MHBC. Asterisks in D–G indicate cell outlined in D’–G’. (H, I) Cell length quantification at the MHBC and 40 μm outside the MHBC. (J) Representative Western blots for pMRLC in MHB-specific tissue dissected after control MO or calm1a MO injection. (K) pMRLC Western quantification using α-tubulin as a control (n = 5). (L) Proposed signaling pathway for Ca²⁺ regulation of cell length at the MHBC. For statistical analysis, one-way ANOVA with Tukey’s HSD post hoc test was done. **p < 0.01 and *p < 0.05 compared with control, mean ± SEM. Control MO (n = 17; 34 cells), calm1a MO (n = 19; 38 cells), MLCK mRNA (n = 13; 26 cells), and calm1a MO plus MLCK (n = 9; 18 cells). Scale bars, 25 μm.
DMSO control treatments were conducted using the same volume percentage as other treatment groups in each experiment. For Thaps/Bleb combined drug treatment rescue experiments, wild-type embryos were injected at the one-cell stage with mGFP mRNA. At 18 ss, embryos were separated into two treatment groups: DMSO or 2 μM Thaps. After treatment, embryos were washed and incubated at 28°C until 20–21 ss. At 20–21 ss, embryos from each treatment group were separated into two additional treatment groups: DMSO or 50 μM Bleb (BoS560-1MG; Sigma-Aldrich). Finally, each group was incubated at 28°C until 24 ss and imaged using live confocal microscopy.

Live confocal imaging and cell shape analysis
All mGFP live confocal imaging was conducted as previously described (Graeden and Sive, 2009; Gutzman et al., 2015) using a Nikon CS2 scanning confocal microscope. Live confocal images presented in each figure are single slices taken from a z-series of images ~15–20 μm into the tissue from the dorsal surface. All confocal images were processed using Nikon Imaging Systems (NIS) Elements software and Photoshop (Adobe). Cell shape analysis was performed as described in Gutzman et al. (2015). Briefly, cell length was determined using the NIS-Elements software measurement tool by measuring a single cell spanning the neuroepithelium from apical to basal in the region described, either directly at the MHBC or 40 μm posterior to the MHBC (outside the MHBC) on the hindbrain side.

Antisense MO oligonucleotide and mRNA injections
All knockdown experiments were performed using splice site-blocking MO antisense oligonucleotides. MO details are as follows: mypt1 MO (5′-ATTTTTTGTACTTACTCAGGGATG-3′; Gutzman et al., 2008, 2015), calmodulin 1a (ENSDART00000034580), and calm1a MO (5′-CCACAAAAAGACTGGCTTACCTGCA-3′). Zebar® fish p53 MO (5′-GCCGCATGCGTTTTCGAAGATG-3′) was used in conjunction with the calm1a MO at equal concentration. Standard control MO (5′-CCCTTACCTCAGGTTACATTATA-3′). All MOs are from Gene Tools. Morpholinos were injected into one-cell-stage embryos either alone or in conjunction with mGFP mRNA as indicated. MO concentrations were 5 ng mypt1 MO, 2 ng calm1a MO, and 2 ng p53 MO. Control MO concentration was equal to the highest concentration of any MO used in that experiment. For MLCK mRNA expression experiments, 50 ng of MLCK mRNA (zeMLCK fused to the globin 3’ untranslated region, kindly provided by Erez Raz, University of Munster, Munster, Germany), was injected with 200 ng/μl mGFP mRNA separately or combined with calm1a MO or control MO. All mRNA was in vitro transcribed for injections using the mMachine Transcription Kit (Ambion). Embryos were incubated at 28°C to 24 ss and imaged using live confocal microscopy.

In situ hybridization
In situ hybridization was conducted according to standard procedures. For the EST/cDNA clone, calm1a, cd617, from ZFIN was used to produce the calmodulin 1a in situ probe. Bright-field imaging was conducted using an Olympus SZX12 stereomicroscope with an Olympus DP72 camera.

Western blot analysis with MHB-specific tissue
Embryos were dechorionated and treated as described with either DMSO or Thaps at 18 ss. Alternatively, embryos were injected with calm1a MO or control MO. MHB tissue was dissected from 18 to 24 ss for protein isolation and analysis (see Supplemental Figure S4 for details). Primary antibodies were pMLC2 (pMLC; 3671; Cell Signaling Technology) at 1:500 (Gutzman and Sive, 2010) and α-tubulin (T6199; Sigma-Aldrich) at 1:1000. Secondary antibodies were anti-mouse horseradish peroxidase (HRP; 70765; Cell Signaling Technology) and anti-rabbit HRP (7074S; Cell Signaling Technology) at 1:2000. Blots were imaged on a UVP Biospectrum Imaging System.

Statistical analyses
Statistical analysis between two groups was carried out using the Mann–Whitney U test; p values denoting significance are reported in each figure legend. Statistical analysis for comparisons between more than two treatment groups was carried out by one-way analysis of variance (ANOVA). For ANOVA p < 0.05, Tukey’s honest significant difference (HSD) post hoc tests were performed to determine significance between control treatment and experimental treatment groups. The p values for post hoc comparisons are presented in each figure legend. All ANOVA and Tukey’s HSD post hoc analyses were carried out using R-3.1.2. At least three independent experiments were conducted for all data presented; n indicates the total number of embryos analyzed, and numbers of single cells analyzed are indicated where necessary.

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