A ryanodine receptor-dependent Ca\textsuperscript{i}\textsuperscript{2+} asymmetry at Hensen’s node mediates avian lateral identity

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In mouse, the establishment of left-right (LR) asymmetry requires intracellular calcium (Ca\textsuperscript{i}\textsuperscript{2+}) enrichment on the left of the node. The use of Ca\textsuperscript{i}\textsuperscript{2+} asymmetry by other vertebrates, and its origins and relationship to other laterality effectors are largely unknown. Additionally, the architecture of Hensen’s node raises doubts as to whether Ca\textsuperscript{i}\textsuperscript{2+} asymmetry is a broadly conserved mechanism to achieve laterality. We report here that the avian embryo uses a left-side enriched Ca\textsuperscript{i}\textsuperscript{2+} asymmetry across Hensen’s node to govern its lateral identity. Elevated Ca\textsuperscript{i}\textsuperscript{2+} was first detected along the anterior node at early HH4, and its emergence and left-side enrichment by HH5 required both ryanodine receptor (RyR) activity and extracellular calcium, implicating calcium-induced calcium release (CICR) as the novel source of the Ca\textsuperscript{i}\textsuperscript{2+}. Targeted manipulation of node Ca\textsuperscript{i}\textsuperscript{2+} randomized heart laterality and affected expression. Bifurcation of the Ca\textsuperscript{i}\textsuperscript{2+} field by the emerging prechordal plate may permit the independent regulation of LR Ca\textsuperscript{i}\textsuperscript{2+} levels. To the left of the node, RyR/CICR and H\textsuperscript{+}V-ATPase activity sustained elevated Ca\textsuperscript{i}\textsuperscript{2+}. On the right, Ca\textsuperscript{i}\textsuperscript{2+} levels were actively repressed through the activities of H\textsuperscript{+}K\textsuperscript{−} ATPase and serotonin-dependent signaling, thus identifying a novel mechanism for the known effects of serotonin on laterality. Vitamin A-deficient quail have a high incidence of situs inversus hearts and had a reversed calcium asymmetry. Thus, Ca\textsuperscript{i}\textsuperscript{2+} asymmetry across the node represents a more broadly conserved mechanism for laterality among amniotes than had been previously believed.

KEY WORDS: Chick embryo, Hensen’s node, Intracellular calcium, Left-right asymmetry, Proton ATPases, Ratiometric calcium imaging, Retinoic acid, Ryanodine receptors, Serotonin

INTRODUCTION

Establishment of the vertebrate left-right (LR) body plan initiates proper positioning of the internal organs. Early differences in small molecules and signals across the midline organizer are translated into an asymmetric genetic cascade that enforces LR identity. Effectors of these early events include cilia motility (Essner et al., 2005; McGrath et al., 2003; Tabin and Vogan, 2003), cell coupling (Levin and Mercola, 1999), ion pumps (Levin et al., 2002; Adams et al., 2006; Shu et al., 2007), serotonin (Fukumoto et al., 2005a; Fukumoto et al., 2005b) and retinoic acid (Chauzad et al., 1999; Tsukui et al., 1999; Zile et al., 2000). Although genetic effectors, including sonic hedgehog (Shh), Nodal, lefty and Pitx2, are largely conserved across vertebrates, there may be significant differences as to which upstream participants are used. Some differences might reflect evolutionary divergence and structural changes in the embryo. The precise relationships among these participants are also unclear.

One poorly understood early asymmetry signal is calcium. In mouse and zebrafish, intracellular calcium (Ca\textsuperscript{i}\textsuperscript{2+}) is enriched along the left margin of the node and Kupffer’s vesicle, respectively (McGrath et al., 2003; Sarmah et al., 2005). In mouse, a loss-of-function mutation in left-right dynein (iv/iv) or polycystin 2 collapses calcium asymmetry at the node and randomizes LR identity (McGrath et al., 2003), prompting suggestions that it is cilia-generated flow across the ventral node that generates the calcium asymmetry and subsequent left-sided gene expression. A role for calcium asymmetry in zebrafish is less clear, as Ca\textsuperscript{i}\textsuperscript{2+} fluxes are also linked to KV formation and subsequent laterality establishment (Sarmah et al., 2005; Schneider et al., 2008).

Whether and how Ca\textsuperscript{i}\textsuperscript{2+} asymmetry across the node might contribute to lateral identity in other vertebrates is unknown. Although the node architecture of the avian embryo might preclude nodal flow (Maner et al., 2001), its node monolalia are positioned toward the ventral endoderm (Essner et al., 2002) and it expresses several ciliary-related genes, including left-right dynein, kinesin 3B and polycystin 2 (Qiu et al., 2005). Thus, at least some elements of this laterality mechanism might be conserved in chick. Ca\textsuperscript{i}\textsuperscript{2+} levels have not been examined in chick, although, at neurulation, a left-sided external calcium pool affects laterality through the activation of notch signaling (Raya et al., 2004).

We report here that the avian embryo uses an asymmetric enrichment of Ca\textsuperscript{i}\textsuperscript{2+} at the node to mediate laterality. This Ca\textsuperscript{i}\textsuperscript{2+} enrichment emerges at early gastrulation and originates from the activity of ryanodine receptors and calcium-induced calcium release (CICR). Several previously identified laterality mediators, specifically serotonin, H\textsuperscript{+}V-ATPase and H\textsuperscript{+}K\textsuperscript{−} ATPase, regulate LR identity by directly affecting node Ca\textsuperscript{i}\textsuperscript{2+} concentrations. Our data significantly advance the understanding of how the avian embryo achieves its LR identity, and show that, despite differences in spatial architecture, Ca\textsuperscript{i}\textsuperscript{2+} asymmetry across the node might be a conserved mechanism of laterality in amniotes.

MATERIALS AND METHODS

Embryos

Chick eggs (Hyline W98) were obtained from the UW Poultry Research Laboratory, Madison, WI. Normal and retinoid-deficient Japanese quail eggs were generated as described previously (Zile et al., 2000). Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951).

Fura-2 ratiometric imaging

HH3+ to HH6 embryos were incubated (60 minutes, 37°C) in a slide chamber with Tyrode’s buffer (TWC) containing 25 μM Fura-2-AM and 0.1% Pluronic F-127 (Molecular Probes), rinsed and then incubated...
(20 minutes) with one of the following pharmacological agents: Bapta-AM (1 mM), EGTA (1.0-2.5 mM), xestospongin C (1 μM), U73122 (10 μM), 9,21-didehydroxyandrostenone (2 μM), ryanodine isomers (10 μM), dantrolene (2 μM), fluoxetine (10 μM), 2-methyl-5HT (25 μM), ondansetron (25 μM), ML10302 (25 μM), GR125487 (25 μM), concanamycin (100 μM) or lansoprazole (7 μM). Concentrations were determined experimentally. Embryos were rinsed and immediately imaged. Studies of CICR (ryanodine versus right regions, we used the equation Cai2+ = Kd × [(R–Rmin)/(Rmax–R)] × (Fmax380/Fmin380) (Grynkiewicz et al., 1985), where the constant terms Kd and Fmax380/Fmin380 were cancelled because a ratio was calculated. Rmin is the emission ratio after MnCl2 treatment, Rmax is the emission ratio after ionomycin treatment, and R represents the emission ratio of experimental interest. Rmin and Rmax were calculated individually for each region. Results were expressed as the mean left-right ratio for five to nine embryos per treatment.

Determination of cardiac laterality
A microbead soaked in the agent of interest was implanted to the left or right of Hensen's node of in ovo HH3++/HH4 embryos: Bapta-AM (30 mM), ionomycin (1 mM), U73122 (10 mM), mixed ryanodine isomers (2.5 mM), EGTA (5 mM), calmidizolium (5 mM), fluoxetine (100 μM), 2-CH3-5HT (5 mM), ondansetron (5 μM), ML10302 (5 mM), GR125487 (5 mM), concanamycin (100 μM), lansoprazole (7 μM) or DMSO only. Ryanodine and EGTA were applied as small agarose plugs. Beads were removed 4 hours later. At HH10/HH11, embryos with significant cranial or midline defects were discarded. The remainder were scored for cardiac laterality as left or right loop, by two treatment-blinded individuals. We analyzed eight to 25 embryos per treatment.

In situ hybridization and immunostaining
cDNA encoding chick Nodal (cNR-J) (Levin et al., 1995) was provided by C. Tabin. HH4 embryos were implanted to the left or right of Hensen's node with DMSO, Bapta-AM, ionomycin, ryanodine-EGTA or calmidizolium-soaked microbeads. At HH8, embryos were processed for whole-mount in situ hybridization as described previously (Smith et al., 1997). For whole embryo immunostaining, antibody directed against the carboxy-terminus of all three RyR isoforms (C-18, 1/500, Santa Cruz) was reacted with HH3+/HH6 embryos as described (Smith et al., 1997). Signal was visualized using alkaline phosphatase-conjugated secondary antibody (Southern Biotech) and BM-Purple (Roche). Embryos were processed batch-wise to ensure consistent treatment. We analyzed eight to 16 embryos per treatment.

Statistical analysis
Binary data (e.g. heart laterality) were analyzed using χ2 analysis (SAS 9.1, SAS Institute, Cary, NC). Normally distributed data were subjected to an unpaired, two-tailed t-test employing the appropriate variance parameter using SigmaStat v.2.0 (Systat Software, Point Richmond, CA). Data not normally distributed were examined using the Mann-Whitney U-test. P<0.05 was set as the level of significance.

RESULTS
Ca2+ is enriched to the left of Hensen's node
We used Fura-2-based ratiometric imaging to evaluate Ca2+ concentrations at the chick Hensen's node. At HH3+/HH4, a modest and bilaterally symmetric Ca2+ elevation was first detected along the anterior margin of the node (Fig. 1A,D). A modest left-side enrichment was occasionally observed (Fig. 1D). At HH4+/HH5–, the calcium signal extended posteriorly and the emerging prechordal plate split this signal into distinct left and right Ca2+ fields; the left Ca2+ is more posterior and is enriched relative to the right. The blue spot in F is an artifact. *, Hensen’s node; pc, prechordal plate; ps, primitive streak. Scale bar in A,D: 100 μm.

Fig. 1. High magnification imaging of Ca2+ in early embryos.
Ratiometric images of Fura-2-loaded HH4 or HH5 embryos. (A-C) Bright-field images at HH4 (A), HH4+ (B) and HH5– (C). (D-F) Fura-2 ratiometric imaging of embryos depicted in A-C. (Bottom row) Line-scan quantitation of the Fura-2 signal in embryos depicted in D-F plotted against right-left axis, at the position indicated by the white line in D-F. A-F are venral views with anterior at the top; right (R) and left (L) are as indicated in A. (A,D) At HH4, a modest and symmetric Ca2+ enrichment appears at the anterior margin of the node. (B,E) At HH4+, Ca2+ enrichment expands posteriorly. (C,F) At HH5–, the prechordal plate splits the Fura-2 signal into distinct left and right Ca2+ fields; the left Ca2+ is more posterior and is enriched relative to the right. The blue spot in F is an artifact. *, Hensen’s node; pc, prechordal plate; ps, primitive streak. Scale bar in A,D: 100 μm.

Hydroxyapatite (HAP) microbeads coated with a drug of interest (Bapta-AM, concanamycin, Bapta-AM (30 mM), ionomycin (1 mM), EGTA (1.0-2.5 mM), 2-methyl-5HT (25 μM), ondansetron (25 μM), mixed ryanodine isomers (2.5 mM), calmidizolium (5 mM), fluoxetine (100 μM), 2-CH3-5HT (5 mM), ondansetron (5 μM), ML10302 (5 mM), GR125487 (5 mM), concanamycin (100 μM), lansoprazole (7 μM) or DMSO only. Ryanodine and EGTA were applied as small agarose plugs. Beads were removed 4 hours later. At HH10/HH11, embryos with significant cranial or midline defects were discarded. The remainder were scored for cardiac laterality as left or right loop, by two treatment-blinded individuals. We analyzed eight to 25 embryos per treatment.
LR Ca\textsuperscript{2+} asymmetry contributes to lateral identity

We manipulated Ca\textsuperscript{2+} levels to the left or right side of Hensen’s node and analyzed the effects on asymmetry. An early asymmetry indicator in chick is the right loop of the heart tube at HH10. DMSO treatment had little effect and most embryos had normal, right-looped hearts (Fig. 3, Fig. 4A). A small percentage had midline-positioned heart tubes that failed to loop or fully fuse; their incidence was not treatment dependent and they were excluded from the analysis. Left but not right Bapta-AM treatment at HH4 significantly increased the incidence of left-looped hearts (40% situs inversus; Fig. 3, Fig. 4B). To test whether Ca\textsuperscript{2+} enrichment alone was sufficient to influence laterality, we applied the potent Ca\textsuperscript{2+} ionophore ionomycin. Right but not left ionomycin treatment increased the frequency of left-looped hearts (31%; Fig. 3, Fig. 4C).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Fura-2 signal from line-scan analysis</th>
<th>Ratio of left/right Ca\textsuperscript{2+} enrichment</th>
</tr>
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<tbody>
<tr>
<td>HH5, untreated</td>
<td>149.5±15.5 (6)*</td>
<td>1.06±0.26 (9)</td>
</tr>
<tr>
<td>HH6, untreated</td>
<td>167.1±4.8 (7)</td>
<td>2.85±0.44 (8)</td>
</tr>
<tr>
<td>Bapta-AM, 1 mM</td>
<td>127.3±16.3 (10)**</td>
<td>0.99±0.05 (9)**</td>
</tr>
<tr>
<td>EGTA, 1 mM</td>
<td>142.0±6.8 (6)**</td>
<td>0.86±0.21 (6)**</td>
</tr>
<tr>
<td>Xestospongin C, 1 μM</td>
<td>172.3±15.1 (11)</td>
<td>3.20±1.22 (5)</td>
</tr>
<tr>
<td>U73122, 10 μM</td>
<td>173.3±16.6 (9)</td>
<td>4.60±1.96 (5)</td>
</tr>
<tr>
<td>Dehydroxyanodine, 2 μM</td>
<td>139.5±13.0 (8)**</td>
<td>1.05±0.28 (8)**</td>
</tr>
<tr>
<td>Ryantodine mix, 10 μM</td>
<td>150.8±13.6 (13)**</td>
<td>0.95±0.32 (12)**</td>
</tr>
<tr>
<td>Dantrolene, 2 μM</td>
<td>141.4±17.5 (8)*</td>
<td>1.10±0.20 (7)**</td>
</tr>
<tr>
<td>Ryanodine (10 μM) + EGTA (2.5 mM)</td>
<td>128.7±13.8 (11)**</td>
<td>0.96±0.15 (7)**</td>
</tr>
<tr>
<td>Calmidizolium, 100 mM</td>
<td>117.8±16.1 (13)**</td>
<td>0.81±0.27 (15)**</td>
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Left and right mean Fura-2 signal, relative Ca\textsuperscript{2+} levels and the LR Ca\textsuperscript{2+} ratio were calculated as described in the Materials and methods. The number of embryos analyzed is indicated in parentheses and embryos were HH6 unless indicated. Asterisks indicate that values differ from HH6 control signal on that side at *P<0.05 and **P<0.005 by unpaired t-test.
**Nodal** is an early laterality determination gene that is restricted to the left-side of Hensen’s node and the lateral plate mesoderm during early somitogenesis (Levin et al., 1995). Although DMSO did not affect Nodal expression (left treatment, 10/10 normal; right treatment, 8/8 normal; Fig. 5A,B), left-sided Bapta-AM treatment at HH4 caused bilateral Nodal expression in the lateral plate mesoderm in 60% of embryos (n=10; Fig. 5C) and normal expression in the remainder. Right-sided Bapta-AM did not affect Nodal (8/8 normal; Fig. 5D). Conversely, right-sided ionomycin reduced Nodal expression (4/6 embryos, 67%; Fig. 5E), but left-sided treatment did not (1/7 lacked Nodal). Both calcium effectors also affected cranial morphology consistent with the known effects of calcium on neural fold elevation. Although we cannot rule out that such cranial changes may have disrupted midline formation and thus affected LR identity (Schneider et al., 2008), this seems unlikely because, while left and right treatment equally affected cranial morphology, only left Bapta and right ionomycin significantly affected Nodal, suggesting that the effects on Nodal and the neural folds were separable events. This suggested that Ca\(_{2+}\) acted upstream of Nodal to affect laterality.

**Ca\(_{2+}\) originates from CICR/RyR activity**

Both extracellular (Raya et al., 2004) and intracellular calcium (McGrath et al., 2003; Sarmah et al., 2005; Shu et al., 2007) affect LR laterality and could originate the Ca\(_{2+}\) enrichment. The left Ca\(_{2+}\) enrichment overlaps at later stages (HH6) with a left-side enriched extracellular calcium pool that has previously been demonstrated to govern chick laterality, heart looping and Nodal expression (Raya et al., 2004). EGTA treatment ablated the Ca\(_{2+}\) asymmetry and attenuated LR Ca\(_{2+}\) levels (Fig. 2D, Table 1), suggesting that extracellular calcium contributed to the Ca\(_{2+}\) enrichment. EGTA also reversed heart looping when applied to the left side (25%, P<0.006) but not the right (0%, Fig. 3).

Intracellular calcium could originate from either phosphoinositide or RyR activity. Phosphoinositides are implicated in zebrafish laterality through roles in Kupffer’s vesicle formation (Schneider et al., 2008) and ciliary beating (Sarmah et al., 2005; Sarmah et al., 2007). However, neither xestospongin C, which inhibits IP\(_3\)-mediated Ca\(_{2+}\) release, nor the phosphoinositidyl-phospholipase C antagonist U73122, affected absolute Ca\(_{2+}\) levels (Fig. 2E,F) or the LR Ca\(_{2+}\) ratio (Table 1), nor did U73122 affect asymmetry (Fig. 3). Thus the Ca\(_{2+}\) did not originate from IP\(_3\)-mediated sources.

RyRs mediate calcium mobilization from sarcoplasmic stores following stimulation by smaller calcium quantities that often originate from extracellular sources, a process known as calcium-induced calcium release (CICR) (Zucchi and Roncha-Testoni, 1997). RyRs have not been previously implicated in laterality. Treatment with the RyR antagonist 9,21-dehydroxystrophanthin ablated the LR Ca\(_{2+}\) asymmetry and significantly lowered Ca\(_{2+}\) levels (Fig. 2G, Table 1), as did a ryanodine isomer mixture and a distinct RyR antagonist, dantrolene (Table 1). Ca\(_{2+}\) levels were not as low as those obtained with Bapta-AM, probably because the CICR component of RyR activity was still present. Accordingly, the ryanodine-EGTA combination was more potent than was each separately in reducing Ca\(_{2+}\) (Table 1), implicating CICR as the RyR stimulus. Ryanodine/EGTA also abolished the Ca\(_{2+}\) enrichment in the HH3++/4 node (Table 2), suggesting that CICR/RyR originated this signal. Similarly, left-side ryanodine treatment had little effect on heart looping (12.5%), whereas the ryanodine/EGTA combination significantly reversed heart looping (36%; Fig. 3, Fig. 4).
Calcium asymmetry in the avian node

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Fig. 5. *Nodal* expression after treatment with antagonists of Ca\(^{2+}\) asymmetry. Embryos received the indicated agent on the left or the right at HH3+++/HH4 and *Nodal* expression was evaluated at HH8 or HH9 by using in situ hybridization. Images are dorsal views with anterior to the top, left (L) and right (R) are as indicated in A; arrows indicate expression. (A, B) Normal, left-sided *Nodal* expression in a DMSO-treated embryo having 3 somites (A) or 5 somites (B). (C) Bilateral *Nodal* expression in a 3-somite embryo that received left-sided Bapta-AM. (D) Normal *Nodal* expression in 4-somite embryo receiving right-sided Bapta-AM. (E) Loss of left-sided *Nodal* expression in a 3-somite embryo receiving right-sided ionomycin (arrow); a faint signal is seen at the left node (arrowhead). (F) Six-somite embryo receiving left-sided ryanodine plus EGTA; *Nodal* expression is absent apart from a small amount to the left of the tailbud (arrowhead); compare with the 5-somite control embryo in B. (G) Loss of *Nodal* expression in a 4-somite embryo that received left-sided calmidizolium; note *Nodal* expression in the tailbud region (arrowhead).

4E). Left-side ryanodine/EGTA treatment at HH3+++/4 repressed *Nodal* expression in five out of nine embryos (Fig. 5F).

An important Ca\(^{2+}\) effector is the Ca\(^{2+}\) sensor protein calmodulin, which interacts with and activates numerous signaling proteins, including RyRs (Berridge et al., 2000; Zalk et al., 2007). Treatment with the calmodulin inhibitor calmidizolium significantly reduced Ca\(^{2+}\) levels at both HH3+/4 (Table 2) and HH6 (Table 1), actions that are consistent with the known ability of calmodulin to directly regulate RyR activity (Zalk et al., 2007). Left but not right calmidizolium treatment also caused a significant incidence of cardiac situs inversus (36%; Fig. 3, Fig. 4D). Left calmidizolium treatment abolished *Nodal* expression in 59% of embryos (10/17, Fig. 5G).

Avians, like mammals, have three RyR isoforms with differing expression and regulatory control. Their contributions to early development are unknown. Immunostaining against all RyR isoforms at HH4 revealed low but discernable levels along the anterior node margin (Fig. 6B,D), overlapping the Ca\(^{2+}\) Fura-2 signal. Expression expanded along the primitive streak at HH5 (Fig. 6E) and the neural plate at HH6 (Fig. 6F). Its expression was symmetric, indicating that its asymmetric activity was regulated post-translationally, a mechanism that commonly governs RyR activity (Zucchi and Ronca-Testoni, 1997). Thus, RyRs are present at the right time and location to mediate the Ca\(^{2+}\) enrichment.

**Serotonin signaling represses right-sided Ca\(^{2+}\)**

The relationship between node Ca\(^{2+}\) asymmetry and other laterality effectors is unknown. The initial symmetry of the Ca\(^{2+}\) signal, followed by its left-side enrichment, suggested the existence of asymmetric regulators. The laterality effector serotonin affects both *Nodal* expression and heart looping in chick (Fukumoto et al., 2005a; Fukumoto et al., 2005b). Treatment at HH3+++/4 with the serotonin re-uptake inhibitor fluoxetine, which prolongs serotonin

**Table 2. Fura-2 levels at the anterior node of HH3+++/HH4 embryos treated with Ca\(^{2+}\) effectors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Fura-2 signal from line-scan analysis</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>136.6±3.7 (5)</td>
</tr>
<tr>
<td>Ryanodine (10 μM) + EGTA (2.5 mM)</td>
<td>104.6±10.1* (7)</td>
</tr>
<tr>
<td>Calmidizolium (100 nM)</td>
<td>112.7±12.9* (5)</td>
</tr>
<tr>
<td>Fluoxetine (10 μM)</td>
<td>113.6±20.9* (5)</td>
</tr>
<tr>
<td>Concanamycin (100 nM)</td>
<td>115.6±10.5* (6)</td>
</tr>
<tr>
<td>Lansoprazole (7 μM)</td>
<td>112.8±16.5* (5)</td>
</tr>
</tbody>
</table>

Mean Fura-2 signal at the anterior node was determined via linescan at the position indicated in Fig. 1D in HH3+++/HH4 embryos treated as indicated. The number of embryos imaged is indicated in parentheses.

*Value differs from controls at P<0.05 using an unpaired t-test.

**Fig. 6. RyR expression in early chick.** (A-F) Antibodies directed against all three RyR isoforms. Symmetric signal is detected around Hensen’s node at HH4 (arrows, B,D). RyR signal expands into the primitive streak at HH5 (left arrow, E) and neural plate by HH6 (arrows, F). Signal was absent from embryos reacted with irrelevant antibody (A,C). Images are dorsal views with anterior at the top.
signaling, repressed \( \text{Ca}^{2+} \) at the H3\( ^{++}/4 \) anterior node (Table 2). At HH6, fluoxetine ablated the LR \( \text{Ca}^{2+} \) differential (1.38±0.13; Table 3) by reducing left-side \( \text{Ca}^{2+} \) levels (Fig. 7B; Table 3), suggesting that serotonin might normally suppress right-side \( \text{Ca}^{2+} \). Consistent with this, left but not right fluoxetine treatment significantly randomized heart looping (36%; Fig. 8).

Serotonin affects laterality through its 5-HT\(_3\) and 5-HT\(_4\) receptors (Fukumoto et al., 2005b). 5-HT\(_3\) (2-methyl-5-HT) and 5-HT\(_4\) (ML10302) agonists rapidly reduced left-side \( \text{Ca}^{2+} \) levels and ablated \( \text{Ca}^{2+} \) asymmetry (Fig. 7C,E; Table 3), whereas their respective antagonists (ondansetron, GR125487) elevated right \( \text{Ca}^{2+} \) and sustained left \( \text{Ca}^{2+} \) levels (Fig. 7D,F; Table 3), indicating that serotonin acted to keep right \( \text{Ca}^{2+} \) low. Similarly, right but not left application of 5-HT\(_3\) and 5-HT\(_4\) antagonists significantly randomized heart-looping direction (ondansetron, 32%; GR125487, 30%), whereas their agonists reversed heart-loop direction only when applied to the left (2-methyl-5HT, 31%; ML10302, 32%; Fig. 8). These findings endorse the model of Fukumoto et al. (Fukumoto et al., 2005a; Fukumoto et al., 2005b) that serotonin operates on the right side of the chick to affect LR identity. The repression of right-sided \( \text{Ca}^{2+} \) by serotonin is a novel mechanism for this laterality effector.

Also implicated in avian laterality are the H\(^+\)-V-ATPase (Adams et al., 2006) and H\(^+\)K\(^+\)-ATPase proton pumps (Levin et al., 2002; Raya et al., 2004); their inhibition randomizes heart laterality and alters asymmetric gene expression. The H\(^+\)-V-ATPase antagonist concanamycin prevented the node \( \text{Ca}^{2+} \) enrichment at HH3\( ^{++}/4 \) (Table 2) and at HH6 (Table 3; Fig. 9B). Left but not right concanamycin treatment also increased the incidence of cardiac situs inversus (58%; Fig. 9D). Thus, H\(^+\)V-ATPase may affect laterality through its ability to initiate and sustain node \( \text{Ca}^{2+} \) asymmetry.

H\(^+\)K\(^+\)-ATPase affects avian laterality by preventing a transient depolarization to the right of Hensen’s node (Levin et al., 2002); its inhibition flattened the LR asymmetry of extracellular calcium (Raya et al., 2004). It affected \( \text{Ca}^{2+} \) in a complex manner. At HH4, the H\(^+\)K\(^+\)-ATPase antagonist lansoprazole significantly reduced the node \( \text{Ca}^{2+} \) enrichment (Table 2). However, at HH6, its action opposed H\(^+\)V-ATPase, and it abolished \( \text{Ca}^{2+} \) asymmetry (1.03±0.19; Table 3) by elevating right \( \text{Ca}^{2+} \) (Fig. 9C), an action consistent with suggestions that it acts upon the right-side of the embryo (Levin et al., 2002). Interestingly, right-side inhibition of H\(^+\)K\(^+\)-ATPase did not affect heart looping (Fig. 9D), a finding that is at odds with its ability to elevate right \( \text{Ca}^{2+} \). Previous laterality studies of H\(^+\)K\(^+\)-ATPase used bilateral inhibitor treatment (Levin et al., 2002).

### Table 3. Left-right \( \text{Ca}^{2+} \) levels in chick embryos treated with serotonin agonists and antagonists, or proton ATPase antagonists

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Fura-2 signal from line-scan analysis</th>
<th>Ratio of left/right ( \text{Ca}^{2+} ) enrichment</th>
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<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Untreated</td>
<td>171.1±9.4 (3)</td>
<td>186.6±12.2**</td>
</tr>
<tr>
<td>Fluoxetine, 10 ( \mu ) M</td>
<td>143.5±23.0 (11)*</td>
<td>158.3±17.7**</td>
</tr>
<tr>
<td>2-Methyl-5HT, 25 ( \mu ) M</td>
<td>135.0±21.8 (11)**</td>
<td>153.6±17.6**</td>
</tr>
<tr>
<td>Ondansetron, 25 ( \mu ) M</td>
<td>188.4±19.2 (10)*</td>
<td>200.5±8.7*</td>
</tr>
<tr>
<td>ML 10302, 25 ( \mu ) M</td>
<td>119.8±18.8 (9)**</td>
<td>130.2±25.5**</td>
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<tr>
<td>Concansesame, 100 nM</td>
<td>151.2±13.7 (13)**</td>
<td>150.8±13.6**</td>
</tr>
<tr>
<td>Lansoprazole, 7 ( \mu ) M</td>
<td>186.1±13.7 (14)**</td>
<td>183.8±18.1**</td>
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Left and right mean Fura-2 signal, relative \( \text{Ca}^{2+} \) levels and the LR \( \text{Ca}^{2+} \) ratio were calculated as described in the Materials and methods. The number of embryos analyzed is indicated in parentheses. Asterisks indicate that values differ from HH6 controls on that side at *\( P<0.05 \) and **\( P<0.005 \) using an unpaired \( t \)-test.
Calcium asymmetry in the avian node

Retinoic acid, laterality and Ca\textsuperscript{2+}

Vitamin A-deficient (VAD) quail embryos have a marked tendency (72%) to form left-sided hearts (Zile et al., 2000); the expression of laterality genes, such as Nodal and Pitx2, is also altered (Zile et al., 2000). Vitamin A-sufficient (VAS) HH6 quail had a modest LR Ca\textsuperscript{2+} enrichment compared with Gallus gallus chicks of equivalent stage (LR ratio 1.44±0.40, Fig. 10A), although the mean Fura-2 signal of the quails did not differ appreciably from that of the chick (right, 168.4±13.7; left, 187.0±14.9; L versus R, P=0.014). Interestingly, HH6 VAD quail had an inverted Ca\textsuperscript{2+} asymmetry, and, in a majority (5/7), Ca\textsuperscript{2+} levels were significantly higher on the right of the node versus the left (Fig. 10B); the mean LR Ca\textsuperscript{2+} ratio for those five embryos was 0.60±0.21. This was not due to increased right Ca\textsuperscript{2+}, but to significantly reduced left Ca\textsuperscript{2+} levels (right, 164.1±9.9; left, 161.6±17.0; n=7; left Fura-2 of VAD versus VAS, P=0.016). Although unexpected, this inverted LR Ca\textsuperscript{2+} ratio in the majority of VAD quail embryos is consistent with their high incidence of reversed cardiac laterality (Zile et al., 2000).

**DISCUSSION**

Here, we show that, like mouse, the chick embryo uses node Ca\textsuperscript{2+} asymmetry to mediate laterality. Ca\textsuperscript{2+} is first enriched along the anterior margin of the node at HH3++, and overlaps with RyR expression. Its induction and left-side enrichment requires RyR and extracellular calcium, implicating CICR as a novel origin for this signal. H’K\textsuperscript{-}ATPase and H’V-ATPase activity are also contributory, although by HH6 their activities are distinct and opposing. 5-HT\textsubscript{3} and 5-HT\textsubscript{4} keep right-side Ca\textsuperscript{2+} levels low, representing a novel mechanism for the laterality effects of serotonin. Although the avian node structure may preclude nodal flow, its use of Ca\textsuperscript{2+} asymmetry supports the hypothesis that aspects of this laterality mechanism are conserved among amniotes.

Figure 11 summarizes our findings and details underlying this model are discussed below. At HH2 and HH3 (not shown), the elongating primitive streak already expresses much of the machinery that governs CICR/RyR activity, including serotonin and its receptors and transporters (Fukumoto et al., 2005a; Fukumoto et al., 2005b), H’V-ATPase (Adams et al., 2006) and H’K\textsuperscript{-}ATPase (Levin et al., 2002); the surrounding blastoderm expresses Cx43 (Levin and Mercola, 1999). At HH3+, the first asymmetric gene, cAct-RIIa, appears (Levin et al., 1995). At this same time, Ca\textsuperscript{2+} enrichment emerges along the anterior margin of Hensen’s node commensurate with RyR expression; its ablation by EGTA/ryanodine and calmidizolium suggests that it originates from CIRC/RyR. Ca\textsuperscript{2+} expression coincides with a transient depolarization of cells along the left side of the node (Levin et al., 2002), and could enhance CICR/RyR activity. Left-sided depolarization, the instigator of which is unknown, may thus be a crucial early step to create asymmetry. By HH4+/HH5, Ca\textsuperscript{2+} levels are asymmetric and enriched along the left side of the node. The previously symmetric Shh expression also becomes restricted to the left side of the node (Levin et al., 1995), and, importantly, the sodium-calcium transporter NCX1 is enriched along the right node (Linask et al., 2001). This right-side restricted NCX1, which uses Na\textsuperscript{+} influx to drive Ca\textsuperscript{2+} export, may steepen further the LR Ca\textsuperscript{2+} asymmetry. By neurulation (HH6), the Ca\textsuperscript{2+} asymmetry has increased and extends posteriorly. H’V-ATPase sustains the left-side Ca\textsuperscript{2+} elevation, whereas H’K\textsuperscript{-}ATPase keeps right Ca\textsuperscript{2+} levels low. Although the mechanism underlying Ca\textsuperscript{2+} repression by serotonin is unclear, the physiological properties of the right side of the node suggest that SHT\textsubscript{3} may function as a Na\textsuperscript{+}/K\textsuperscript{+} exchanger, perhaps in coordination with NCX1 and/or H’K\textsuperscript{-}ATPase, to maintain low Ca\textsuperscript{2+}. Gap junctions in the blastoderm (Levin and Mercola, 1999) could serve to coordinate and stabilize the Ca\textsuperscript{2+} and ATPase signals across the anterior node. Bifurcation of the anterior Ca\textsuperscript{2+} field by the
prechordal plate, along with the proposed barrier activity of the midline (Danos and Yost, 1996), could then allow LR Cai\textsuperscript{2+} levels to be regulated autonomously.

Cai\textsuperscript{2+} mediates LR asymmetry decisions in chick

Although Cai\textsuperscript{2+} asymmetry across the node is necessary for laterality in mouse (McGrath et al., 2003) and now chick, its origins and relationship to other laterality mediators has been unclear. The identification of CICR/RyR as the origin of Cai\textsuperscript{2+} enrichment and asymmetry at Hensen’s node is a novel function for RyRs in the early embryo. The requirement for CICR/RyR offers a second mechanism for left-side enriched extracellular calcium to govern avian LR identity, in addition to its regulation of Notch signals (Raya et al., 2004); any interaction between Cai\textsuperscript{2+} and Notch remains to be determined. Because RyR proteins are expressed symmetrically along the anterior node and the primitive streak, their activity is likely to be regulated post-translationally.

In mouse, movement by monocilia within the ventral node is proposed to generate a fluid current that creates the Cai\textsuperscript{2+} asymmetry across the node; mutation of left-right dynein heavy chain (\textit{Lrd}; \textit{Dnahc11} – Mouse Genome Informatics) leads to immotile cilia, the collapse of Cai\textsuperscript{2+} asymmetry and the randomization of lateral identity (McGrath et al., 2003). Although the avian node architecture is quite different from that of mouse and it may lack a ‘ventral node’ (Manner, 2001), HH4– chick node possesses monocilia that project ventrally from the epiblast and towards the ventral endoderm (Essner et al., 2002). Unfortunately, it was not possible to determine which cell layer at the node was Cai\textsuperscript{2+} enriched. However, the existence of Cai\textsuperscript{2+} enrichment at the avian node shows that at least some aspects of the mechanisms used to establish LR laterality are conserved among amniotes. In this light, it may be worth noting that polycystin-2, a calcium channel linked to LR identity in mouse and zebrafish (Pennekamp et al., 2002; Bisgrove et al., 2005; Obara et al., 2006; Schottenfeld et al., 2007) can initiate CICR/RyR-mediated calcium release (Nauli et al., 2003) in a protonation-dependent manner (Gonzalez-Perrett et al., 2002). The contribution of polycystins to avian lateral identity remains unexplored.

Fig. 9. Cai\textsuperscript{2+} levels and heart laterality after treatment with proton ATPase antagonists. (A-C) Fura-2-loaded HH6 embryos were treated with the indicated agents and imaged ventrally. The upper panel shows a representative Fura-2 image; the lower panel is the line-scan quantitation of fluorescent intensity versus left-right axis for that embryo, at the position indicated by the yellow line in the upper panel. Hensen’s node is indicated by an asterisk. (A) Untreated embryo. (B) The \textit{H}{\textsuperscript{+}}\textit{V}{\textsuperscript{-}}-ATPase antagonist concanamycin reduces LR Cai\textsuperscript{2+}. (C) The \textit{H}{\textsuperscript{+}}\textit{K}{\textsuperscript{+}}-ATPase antagonist lansoprazole elevates right Cai\textsuperscript{2+}. (D) Embryos were treated with the indicated proton ATPase antagonists at HH4-HH6 and heart laterality was scored 20 hours later. The percentage of embryos with a right-looped (R, situs solitus) or left-looped (L, situs inversus) heart is shown; the number of embryos per treatment is indicated in parentheses. Treatments were: DMSO, concanamycin (100 \textmu M) and lansoprazole (7 mM). Asterisk indicates situs inversus frequencies that significantly differ from controls by \chi\textsuperscript{2} analysis (\textit{P}<0.05).

Fig. 10. LR Cai\textsuperscript{2+} levels in VAS or VAD quail embryos. Fura-2-loaded HH6 embryos were imaged ventrally, anterior to the top. (A, B) The upper panel shows a representative Fura-2 image; the lower panel presents the line-scan quantitation of fluorescent intensity versus the right-left axis for that embryo, at the position indicated by the yellow line in the upper panel. Asterisk indicates Hensen’s node. (A) VAS, vitamin A sufficient. (B) VAD, vitamin A deficient.
Serotonin and proton ion channels mediate Ca^{2+} asymmetry

How serotonin and the proton ion channels affect lateral identity is unclear. Our data suggest that serotonin operates specifically on the right side of the node to reduce Ca^{2+} and maintain asymmetry; 5-HT_{3} and 5-HT_{1} inhibition rapidly elevated right-side Ca^{2+} and randomized heart looping. Because serotonin and its 5-HT_{3} and 5-HT_{1} receptors are bilaterally expressed at these stages (Fukumoto et al., 2005b), there must exist signals that preclude left-sided serotonin action. These could include the serotonin transporters, two of which facilitate serotonin activity and have right-biased expression (Zucchi and Roncha-Testoni, 1997). The 5-HT_{3} and H+K+ATPase reduces Ca^{2+} and makes a barrier (light blue) for autonomous LR regulation of Ca^{2+}. By HH6, left Ca^{2+} is substantially enriched. Factors including RA, RyR, extracellular calcium and the H+V-ATPase sustain left Ca^{2+} elevation and Nodal induction, whereas serotonin effectors and the H+K+ATPase suppress right Ca^{2+}. Extracellular calcium activates Notch and supports CICR/RyR. See text for details.

Fig. 11. Model of Ca^{2+}i action in laterality. (A) At HH3+/HH4– elevated Ca^{2+}i (green) appears along the anterior Hensen’s node (HN), overlapping with Cai^{2+} expression; its appearance requires RyR and proton ATPases. A transient depolarization is also observed to the left of Hensen’s node (HN) and the primitive streak (PS). Gap junctions (blue dots) may relay and stabilize these early signals. (B) Left-side Ca^{2+}i enrichment occurs at HH4+/HH5–, and the head process (HP) bifurcates the Ca^{2+}i field. Shh (dark blue) becomes left-side restricted in the node. Midline expression of serotonin, 5-HT_{3}, 5-HT_{1} and H+K+ATPase reduces Ca^{2+}i and makes a barrier (light blue) for autonomous LR regulation of Ca^{2+}. By HH6, left Ca^{2+}i is substantially enriched. Factors including RA, RyR, extracellular calcium and the H+V-ATPase sustain left Ca^{2+}i elevation and Nodal induction, whereas serotonin effectors and the H+K+ATPase suppress right Ca^{2+}. Extracellular calcium activates Notch and supports CICR/RyR. See text for details.


